# THE ROLE OF EXTRACELLULAR NUCLEAR MOLECULES IN THE PATHOGENESIS OF AUTOIMMUNE DISEASE

EDITED BY: David Stephen Pisetsky, Reinhard Edmund Voll and Edit I. Buzás

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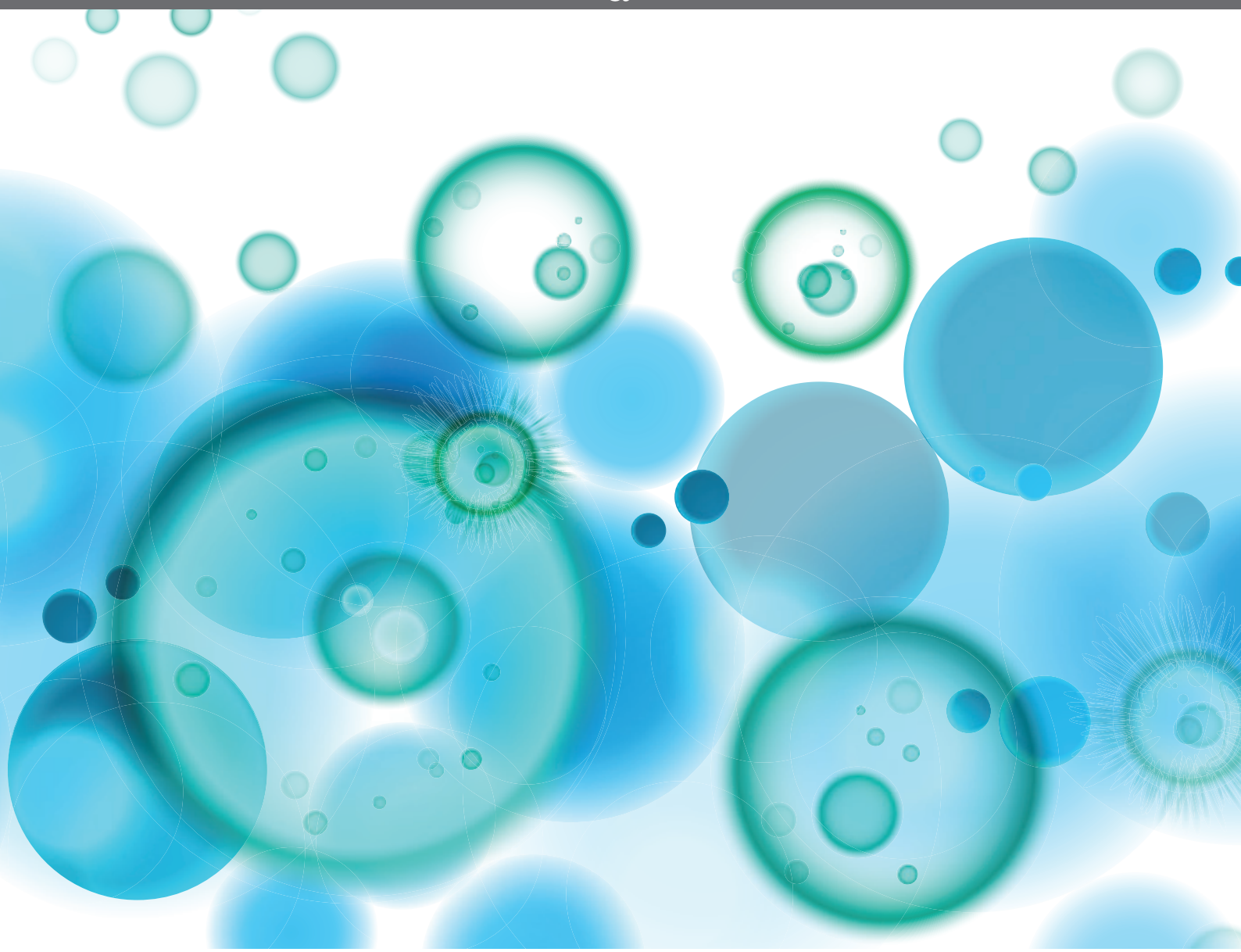

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# THE ROLE OF EXTRACELLULAR NUCLEAR MOLECULES IN THE PATHOGENESIS OF AUTOIMMUNE DISEASE

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EDITORIAL
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# Editorial: The Role of Nuclear Molecules in the Pathogenesis of Autoimmune Disease

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**Editorial on the Research Topic**

**The Role of Nuclear Molecules in the Pathogenesis of Autoimmune Disease**

OPEN ACCESS

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Nuclear molecules are a diverse set of macromolecules whose designation as nuclear derives from their location rather than chemistry or function. These molecules include DNA, RNA and proteins although, in general, nuclear molecules exist as complexes of proteins and nucleic acids inside the cell. The focus on the nucleus as a defining feature of these molecules, while clearly correct, may nevertheless underestimate the importance of the translocation events that nuclear molecules undergo to serve their function. Thus, while DNA, RNA and proteins are major chemical constituents of the nucleus, their location is neither uniform nor fixed. As a group, these molecules can all migrate around the cell and variably appear in the nucleus and cytoplasm. Furthermore, they can exit the cell to enter the blood where they can display powerful immunological activities, seemingly unrelated to their nuclear function, and can drive the pathogenesis of autoimmunity.

The articles in this Research Topic, "The Role of Nuclear Molecules in the Pathogenesis of Autoimmune Disease," provide an exciting perspective on molecules for which the nucleus is just one site of action. In this editorial, we will provide an overview of the field and indicate the areas in which the articles provide new understanding. While the field is rapidly evolving, nevertheless, the articles stand as important contributions that will help guide future directions. The excitement about this series in many respects reflects the early stage of the research. This is all new territory and key questions about the origin of this movement remain largely unanswered: is translocation to the extracellular space a normal physiological process for information transfer or signaling? Or, is this movement evidence for pathophysiology, the end-product of cellular damage and death, with nuclear molecules going rogue once outside the confines of the cell? Have extracellular activities shaped the evolution of these chemical structures or has the host adapted to the structures encountered?

Among nuclear molecules with prominent extracellular expression in autoimmune and inflammatory disease, DNA was identified in the blood many years ago with the study of systemic lupus erythematosus (SLE) providing compelling evidence for the critical role of extracellular DNA in disease pathogenesis (Duvvuri and Lood; Mustelin et al.; Soni and Reizis).

SLE is a prototypic autoimmune disease characterized by antibodies to nuclear molecules (antinuclear antibodies or ANAs). These target antigens include DNA, RNA and protein-nucleic acid complexes. Of ANAs, anti-DNA antibodies are unique in that they are markers for diagnosis, classification and disease activity.

In the pathogenesis of SLE, immune complexes containing nuclear molecules play a key role in the inflammation characteristic of this disease, especially in the kidney (Rekvig). As early studies showed, levels of anti-DNA antibodies can rise with disease activity, often in association with depression of complement, indicative of immune complex formation. While the formation of immune complexes consisting of DNA and anti-DNA does not necessitate a change in levels of extracellular DNA, in fact, DNA levels also rise concomitant with the increases in anti-DNA. The basis of this increase has long been a source of fascination and, indeed, is one of the impetuses to study extracellular DNA, truly an epicenter of SLE (Soni and Reizis).

The term extracellular specifies a locale, but it does not specify a chemical structure. In view of its interaction with histones and non-nuclear histone proteins in the cell, extracellular DNA is likely to be bound with histones in the form of nucleosomes or chromatin. The physical-chemical properties of nucleosomal DNA differ from that of free DNA including susceptibility to different extracellular nucleases as shown in studies on the biology of DNase 1L3 (1 like 3) (Soni and Reizis). Extracellular chromatin may be a more apt terminology for extracellular DNA but, in the development of novel biomarkers, the focus is DNA and not the histones since DNA can be interrogated in much greater depth to identify its source (e.g., malignant cells).

In addition to its association with proteins, DNA in the extracellular space can exist as a free or soluble form as well as a particulate. Extracellular vesicles, which include microvesicles (also termed microparticles) and other vesicle types, can be generated during the steady state, cell activation or cell death (Burbano et al.; Rasmussen et al.). Extracellular vesicles may contain DNA, histones and HMGB1 (high mobility group box 1) among other nuclear and cytoplasmic molecules and have potent activities that can promote inflammation as well as thrombosis. Apoptosis is an important source of extracellular nuclear molecules in a particle form. It is unknown, however, whether the formation of vesicles is a simple physical-chemical consequence of certain forms of cell death or if vesicle formation has evolved to promote phagocytic uptake or clearance. The transmission of danger signals is another potential action of particles that may have evolved.

NETosis is an additional process that can generate extracellular DNA that includes mitochondrial DNA which differs structurally from nuclear DNA in its lack of methylation of CpG motifs. Mitochondrial DNA also has the propensity for oxidation which can further increase its immunostimulatory potential. NETs (neutrophil extracellular traps) are an elaborate form of extracellular DNA which can be extruded from neutrophils and other immune cells as a defense strategy. Given its dense, mesh-like structure, a NET can trap bacteria, viruses or fungi, with killing accomplished by anti-bacterial proteins such as histones and enzymes contributed by neutrophils. Among extracellular forms of DNA, NETs can form large aggregates that bind other molecules and modulate their activities (Knopf et al.), with degradation of these structures also important in determining pathological states such as thrombosis, immune complex formation and autoimmunity. The assay of NETs is important in the study of immunopathogenesis although quantitation remains a challenge (van Brenda et al.).

Along with the recognition of the movement of nuclear molecules to the extracellular space came studies that DNA, RNA, histones and HMGB1 all have intrinsic immunological activity and could stimulate both toll-like receptors (TLRs) and non-TLR sensors. In particular, HMGB1 has diverse activities that mediate events in many autoimmune and inflammatory diseases (Gorgulho et al.; Sowinska et al.). These molecules may act alone or in concert as complexes such as chromatin which contain DNA and proteins (Ribon et al.). In this regard, bacterial DNA as well as host mammalian DNA can appear in the blood reflecting the presence of bacterial organisms and their growth as biofilms (Qiu et al.).

While DNA and RNA have immune activity, the location for the contact of nucleic acid sensors is critical. For stimulation of immunity, the contacts with nucleic acid sensors occur in the cytoplasm, including the endosomal compartment. In the context of diseases like SLE, the uptake of nucleic acids into cells of the innate immune system provides a source of either DNA or RNA to interact with the sensors and stimulate production of cytokines such as type 1 interferon. In this activation, the immune complexes provide a conduit of nucleic acids into different cell compartments.

In SLE, internal nucleic acid sensors can be activated by either extracellular DNA or RNA that enters the cell as immune complexes. An important function of these sensors is to promote host defense against intracellular infection whether virus, bacteria or fungi. Indeed, while models of host defense often focus on the extracellular space and generation of protective antibodies, inhibition of pathogen proliferation occurs intracellularly, with foreign nucleic acids as key signals to activate host defense. These sensors can also be triggered by nucleic acids that are aberrantly present in the cytoplasm because of cell stress as well as abnormalities in the DNase and RNase enzymes that keep the cytoplasm free from nucleic acids.

For DNA, the cytoplasm along with the nucleus is an important location for DNA, with mitochondria having their own DNA to encode certain mitochondrial molecules. Unlike nuclear DNA, mitochondrial DNA does not exist in a nucleosomal structure and histones are not present. Although mitochondrial DNA can be safely ensconced in a membrane-bound structure, it can be released into the cytoplasm with mitochondrial damage; mitochondrial DNA can also appear in the blood along with nuclear DNA during cell death. Importantly, mitochondrial DNA has immune activity because of its resemblance to bacterial DNA. It is of interest that patients with SLE produce antibodies to mitochondrial RNA while antibodies to other sources of RNA appear uncommon (Becker et al.).

Perhaps, mitochondrial RNA may differ in immunogenicity because of its structure or association with other immunostimulatory molecules present in the mitochondria.

The immune properties of extracellular DNA and RNA and their sensing systems have been one of the most exciting and productive areas of immunology research in the past few years as the papers in this series indicate. These studies have also revealed the importance of new forms of RNA such as lncRNA (long non-coding RNA) (Zhang et al.). Along with intriguing new data have come conceptual advances to understand host defense. To name a few, we would note the following ideas that have changed the paradigms in immunology: immune complexes provide a mechanism to transmit DNA and RNA to internal nucleic acid sensors; cell death is a key element of host defense; and defects in DNase and RNase enzymes can lead to autoimmunity. Another conceptual as well as technical advance relates to the use of proteomics to find other cell constituents that can serve as biomarkers in the blood (Idborg et al.).

While this Research Topic focuses on the extracellular location of nuclear molecules, the mechanisms described all relate to the mobility of nuclear molecules and the translocation events they can undergo. These events start with a translocation from the inside of a cell to its outside. A subsequent translocation event moves RNA and DNA from the outside of one cell back inside of another one. Once inside another cell, the DNA and RNA can access sensing systems that drive inflammation and cell death. From this perspective comes a host of novel ideas for developing new therapies: scavenging, degrading of binding extracellular nuclear molecules; inhibiting their sensors; and blocking the downstream mediators induced.

The story on extracellular nuclear molecules is just beginning and, as these reviews and original papers illustrate so insightfully, the nucleus is just one location where these molecules can act. Future studies will hopefully discover new ways to block their action once they have left the cell and provide new approaches to treat the broad range of autoimmune and inflammatory diseases in more effective and safer ways.

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REVIEW
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# Cell-Free DNA as a Biomarker in Autoimmune Rheumatic Diseases

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Endogenous DNA is primarily found intracellularly in nuclei and mitochondria. However, extracellular, cell-free (cf) DNA, has been observed in several pathological conditions, including autoimmune diseases, prompting the interest of developing cfDNA as a potential biomarker. There is an upsurge in studies considering cfDNA to stratify patients, monitor the treatment response and predict disease progression, thus evaluating the prognostic potential of cfDNA for autoimmune diseases. Since the discovery of elevated cfDNA levels in lupus patients in the 1960s, cfDNA research in autoimmune diseases has mainly focused on the overall quantification of cfDNA and the association with disease activity. However, with recent technological advancements, including genomic and methylomic sequencing, qualitative changes in cfDNA are being explored in autoimmune diseases, similar to the ones used in molecular profiling of cfDNA in cancer patients. Further, the intracellular origin, e.g., if derived from mitochondrial or nuclear source, as well as the complexing with carrier molecules, including LL-37 and HMGB1, has emerged as important factors to consider when analyzing the quality and inflammatory potential of cfDNA. The clinical relevance of cfDNA in autoimmune rheumatic diseases is strengthened by mechanistic insights into the biological processes that result in an enhanced release of DNA into the circulation during autoimmune and inflammatory conditions. Prior work have established an important role of accelerated apoptosis and impaired clearance in leakage of nucleic acids into the extracellular environment. Findings from more recent studies, including our own investigations, have demonstrated that NETosis, a neutrophil cell death process, can result in a selective extrusion of inflammatory mitochondrial DNA; a process which is enhanced in patients with lupus and rheumatoid arthritis. In this review, we will summarize the evolution of cfDNA, both nuclear and mitochondrial DNA, as biomarkers for autoimmune rheumatic diseases and discuss limitations, challenges and implications to establish cfDNA as a biomarker for clinical use. This review will also highlight recent advancements in mechanistic studies demonstrating mitochondrial DNA as a central component of cfDNA in autoimmune rheumatic diseases.

**Keywords: cell-free DNA, biomarker, autoimmune rheumatic diseases, systemic lupus erythematosus, rheumatoid arthritis**

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## INTRODUCTION

In 1948, Mandel and Metais were the first to report on the presence of cfDNA in human plasma (1). However, it was not until the discovery of high levels of cfDNA in systemic lupus erythematosus (SLE) patients in 1966 (2), that the interest in cfDNA as a potential biomarker for autoimmune diseases started. Early reports were flawed by leukocyte release of DNA during coagulation, with a major breakthrough in the field upon recognizing plasma as a better source of pathological cfDNA, with undetectable levels in healthy volunteers (3–5). These early observations were followed by the demonstration of cfDNA in other autoimmune diseases including rheumatoid arthritis (RA) (6). However, with the advent of more sensitive methods, cfDNA was also detected in plasma of healthy individuals, albeit at very low levels. This observation, together with the temporal association of the increased levels of cfDNA with disease activity in patients with SLE and RA, led to the proposition that cfDNA can be a potential biomarker for autoimmune diseases. Subsequent, technological advancements such as fluorometric assays and real-time PCR led to the simple and rapid quantification of cfDNA, also providing information on the intracellular origin of cfDNA, such as the mitochondria. Despite the initial excitement and technological advancements in cfDNA quantification, there was not a substantial interest in cfDNA as a biomarker for autoimmune diseases, until more recently with the increasing understanding into the role of DNA-sensing receptors in inflammation and autoimmunity, especially in SLE and RA. In this review, we will give an overview of basic biology of cfDNA, followed by evolution of cfDNA in SLE and RA as a biomarker of diagnosis, disease activity and progression, and as a prognostic marker of treatment response.

## MECHANISMS OF cfDNA RELEASE

Though our understanding of mechanisms contributing to the generation of cfDNA is evolving with several novel pathways described in recent years (7), it is still unclear which, if any, of the current models account for the elevated levels of cfDNA observed in patients with rheumatic disease. Beneath we will highlight key cell death processes and active release mechanisms, and their potential implication in rheumatic disease.

## APOPTOSIS

Apoptosis, also known as programmed cell death, is an essential part of physiological maintenance of cellular homeostasis that eliminates unwanted and damaged cells. Apoptosis is executed by effector caspases that are activated in extrinsic and intrinsic-pathways triggered by death-receptors and intracellular stimuli such as oxidative stress and DNA damage, respectively. Activation of caspases, a mark of an irreversible commitment to apoptosis, results in a proteolytic cascade leading to several characteristic morphological and biochemical changes in apoptotic cells that include cell and nuclear shrinkage, DNA fragmentation and lipid re-distribution (8, 9). Taking cues from these alterations as find-me signals, especially upon the exposure of phosphatidylserine onto the cell surface, apoptotic debris under normal conditions, is promptly recognized and cleared by phagocytes in a non-inflammatory process called efferocytosis (10). The clearance of apoptotic cells exerts powerful anti-inflammatory and immunosuppressive effects (11). In contrast, impaired clearance of apoptotic material and/or increased cell death process lead to an accumulation of intracellular antigens and DNA extracellularly, which long-term can lead to autoinflammatory responses (12–14). Inter-nucleosomal fragmentation of DNA into double-stranded DNA fragments of 180–200 bp by calcium-dependent endonucleases is the biochemical hallmark of apoptosis. The fragmented DNA is detectable as a ladder pattern when subjected to gel electrophoresis (15). Multiple characteristics of cfDNA suggest that it is reminiscent of apoptotic DNA. cfDNA, like apoptotic DNA, is a highly fragmented, low molecular weight double stranded DNA with an average size of ∼150–200 bp in length, a size corresponding to nucleosomal DNA, and exhibits a ladder pattern on gel electrophoresis as multiples of nucleosomal units (16). SLE, a disease characterized by increased apoptosis and impaired clearance of apoptotic cells (17, 18), shows evidence of low molecular weight cfDNA with an apoptosis-like size distribution pattern. DNA purified from SLE plasma formed discrete bands, predominantly with a unit size of ∼200 bp, characteristic of DNA found in oligonucleosomes (19). In another study that isolated DNA from the DNA-anti-DNA antibody immune complexes in sera of SLE patients, a strong correlation was observed between low molecular weight DNA sizes (both 30–50 and 150–200 bp), disease activity, and the frequency of renal disease (20). Genome-wide sequencing identified that plasma DNA in SLE patients exhibit size shortening (≤115 bp in length) that correlated with SLEDAI and anti-double stranded DNA (dsDNA) antibody level. In addition, IgG-bound DNA fragments of SLE patients are shorter (≤115 bp) than non-IgG bound DNA (19).

## NECROSIS

The presence of also high molecular weight cfDNA led to the proposition that necrosis could be the release mechanism (21, 22). Necrosis is an accidental form of cell death in response to physical or chemical injury characterized by cell swelling and rapid loss of plasma membrane integrity, leading to the release of intracellular contents. Necrosis results in non-specific digestion of chromatin, thus enabling release of high molecular weight DNA of many kilo base pairs (23). Necrotic release of cfDNA could be relevant in conditions including trauma, injury and sepsis, where levels of cfDNA were associated with the severity of trauma and post-traumatic complications (24, 25), injury (26, 27), and mortality in patients with sepsis (28, 29). Although the role of necrosis in the elevated levels of cfDNA observed in patients with rheumatic disease has not been carefully investigated, studies in SLE patients suggest that necrotic cell death can be a major source of cfDNA. Intracellular ATP concentration is one of the factors that determines the cell's fate to undergo cell death via apoptosis or necrosis. Interestingly,

CD4+ T cells from SLE patients are characterized by ATP depletion due to persistent mitochondrial hyperpolarization, which subsequently results in the uncoupling of oxidative phosphorylation i.e., continued production of reactive oxygen intermediates in the absence of ATP synthesis. This ATP depletion results in a diminished activation-induced apoptosis and sensitization of CD4+ T cells to undergo necrosis, thus enabling the release of cellular contents, including cfDNA, into the extracellular space (30, 31).

## NETosis

In response to microbes, as well as sterile inflammation, neutrophils can undergo a unique form of programmed cell death known as NETosis. It results in the extrusion of a web-like structure of nuclear-derived decondensed DNA coated with histones, granular proteins, and cytoplasmic proteins into the extracellular space. Since the extracellular chromatin fibrils could entangle microbes, the structures were named neutrophil extracellular traps (NETs) (32). Unlike suicidal NETosis described above, which results in the death of neutrophils, neutrophils may undergo vital NETosis, a process in which they only extrude a small amount of DNA, preferentially mitochondrial DNA (mtDNA), allowing for the neutrophil to remain alive and continue to exert antimicrobial actions (32, 33). Following the discovery of NETosis, and extrusion of DNA by neutrophils, several other cell types have been identified to release extracellular traps (34–37), a process termed ETosis. Considering dsDNA being the structural backbone of NETs, in the conditions of excessive NETosis (38) or impaired clearance (39, 40), remnants of NETs could account for elevated levels of circulating cfDNA. Consistent with this proposition, NET deposition was found to be associated with levels of cfDNA in various pathological conditions including SLE, rheumatoid arthritis, cancer, and transfusion-related acute lung injury (38, 41–44).

## PYROPTOSIS

Pyroptosis is a lytic form of inflammatory cell death induced by inflammasome activation in response to diverse pathogen and host-derived danger signals (45). Inflammasome activation leads to the processing and activation of inflammatory caspases, which in turn mediate the downstream inflammatory processes that include the processing of pro-inflammatory cytokines, IL-1β and IL-18, and lytic events associated with pyroptosis (46–48). Early during this cell death, pores are formed in the cell membrane in a caspase-dependent manner ultimately resulting in cell lysis and the release of intracellular inflammatory contents, including IL-1β and IL-18 (49). An unidentified-caspase1-activated nuclease leads to DNA fragmentation during pyroptosis (49). Hence, in conditions associated with caspase-mediated induction of IL-1β and IL-18, the disruption of cell membrane can contribute to release of cfDNA. Tan et al. (50) demonstrated that HIV patients who develop tuberculosis-associated immune reconstitution inflammatory syndrome following cART therapy exhibit increased inflammasome activation, represented by the increased plasma levels of IL-18 that correlate significantly with plasma levels of cell-free mtDNA (cf-mtDNA), a finding suggestive of pyroptosis in cfDNA release (51).

## ACTIVE SECRETION

Other than cell death, cells could actively secrete DNA in the form of extracellular vehicles (EVs), including exosomes and microparticles (microparticles). This cfDNA, present in membrane-bound EVs, may be protected from degradation by nucleases and can be released through the breakdown of EVs. Exosomes are small 30–100 nm vesicles released from the fusion of multivesicular bodies of endosomal origin with plasma membranes. The composition of exosomes includes nucleic acids, proteins, lipids and other metabolites (20). Through their biologically active components, including DNA, exosomes were shown to modulate various physiological and pathological processes (52, 53). A recent study by Fernando et al. (54), demonstrated that more than 93% of amplifiable plasma cfDNA is present in exosomes and the size of the majority of exosomal DNA is <200 bp, consistent with the size of cfDNA reported in plasma from patients with rheumatic disease. Alternatively, a study by Kahlert et al. (55) demonstrated that exosomes could also be the source of high molecular weight DNA (>10 kb). Interestingly, they could not detect any PCR amplifiable products in serum depleted of exosomes; also suggesting that the majority of cfDNA is present in exosomes. These findings suggest that the content of exosomes could vary depending on their cellular origin and the stimuli modulating their release from cells. Microparticles (MPs) or shedding vesicles, a small membrane-bound 100–1,000 nm vesicles can be released from apoptotic cells as blebs or can be actively secreted from living cells. DNA from MPs shows laddering pattern, resembling nucleolytic cleavage of apoptotic cells (56). In general, SLE patients have a higher frequency of pro-inflammatory MPs in the circulation (57, 58). MPs, in particular if released from activated platelets, could also be the source of cf-mtDNA (57, 59, 60). Importantly, upon platelet activation, mitochondria (either naked or localized within MPs) are extruded together with the bactericidal enzyme phospholipase A2, enabling digestion of membranes, allowing the pro-inflammatory mtDNA to escape unto the extracellular space (59). Indeed, levels of mtDNA increase concomitantly with levels of phospholipase A2 in platelet storage bags, with mtDNA levels being associated with adverse transfusion reactions (59).

## CLEARANCE OF cfDNA

We have so far only considered the extrusion of cfDNA. Another important component in generating elevated levels of circulating cfDNA is impaired clearance mechanisms. A rapid clearance of cfDNA is critical to prevent not only inflammation, but also the potential development of autoimmunity toward DNA, as seen in SLE. Early studies on cfDNA clearance revealed that under physiological conditions, cfDNA is rapidly degraded by endonucleases and eliminated from the circulation through

several organ systems, including the spleen, liver, and kidney (51, 61–63). Many factors can influence the ability of DNases to clear cfDNA, including whether the cfDNA is complexed with proteins, nucleosomes and/or antibodies, as well as whether the cfDNA is in free circulating form or encapsulated within membrane-enclosed particles, including exosomes, MPs and apoptotic bodies. Further, based on its intracellular origin, e.g., either nuclear or mitochondrial, cfDNA can exhibit different structural characteristics and stability (64–66).

Efficient degradation of free and protein-bound DNA i.e., nucleosomal DNA in plasma/serum is carried out by extracellular nuclease homologs, DNase I and DNase I-like III (DNase I L3), respectively (67, 68). While, DNase I efficiently cleaves free DNA, the digestion of nucleosomal DNA present in extracellular space and/or sequestered in MPs is majorly performed by DNase I L3 (69, 70). These specific activities of DNase I L3 are attributed to the presence of short, positively charged peptide in the carboxyl-terminal of DNase I L3 (71). Given the importance of DNase I and DNase I L3 in the degradation of circulating DNA, several studies have investigated the role of these nucleases in the context of SLE, a disease characterized by reduced ability to clear cellular debris. Abnormalities of DNase I activity reported in lupus include low serum DNase I activity particularly in patients with renal disease (72, 73), increased serum levels of DNase I inhibitors like G-actin that associated with the high titers of antinuclear antibodies (74), and novel mutations in the enzyme accompanied by elevated titers of anti-dsDNA antibodies (75, 76). Autoantibodies, including anti-dsDNA antibodies, can protect DNA from DNase I digestion (40, 77). Further, anti-DNase antibodies as observed in SLE, were shown to interfere with the enzyme activity, leading to low serum DNase activity (78). As briefly mentioned above, molecules interacting with the DNA may also affect the ability of the DNA to be recognized and degraded by DNase I. Cationic proteins like cathelicidin LL37, human neutrophil peptides and IL-26, protect cfDNA by forming insoluble aggregates through their charge interactions (79, 80) and mitochondrial transcription factor A (TFAM), a mitochondrial packaging protein is involved in the protection of mtDNA from nuclease degradation (81). In addition, defects in the cofactors that promote the DNase I activity (82–85) can also cause or perpetuate the decreased DNase I activity. For instance, complement component C1q, a deficiency of which is strongly associated with the genetic susceptibility of SLE, was shown to promote DNase I activity in degrading necrotic cell-derived chromatin (82). These cofactors likely promote the DNase I activity either by displacing DNA binding proteins from chromatin thus allowing the access to cleavage sites on DNA or by stabilizing DNase I on the target. With regards to DNase I L3, a homozygous loss-of-function variant mutation in *DNASE1L3* gene, identified in several families of pediatric-onset SLE patients, was found to be associated with a higher frequency of anti-dsDNA antibodies and lupus nephritis (86). Another study reported two unique *DNASE1L3* gene mutations in families with autosomal-recessive hypocomplementemic urticarial vasculitis syndrome (HUVS) (87). Incidentally, HUVS is more often associated with SLE, with >50% of HUVS patients often developing SLE (87). In this particular study, 3 of 5 children with HUVS carrying a homozygous frame-shift mutation in *DNASE1L3* gene developed severe symptoms of SLE accompanied by anti-dsDNA antibodies (87). In addition to extracellular nucleases, TREX1, a major mammalian intracellular DNase with a preference for single-stranded DNA (ssDNA) substrates, can be involved in the degradation of cfDNA that translocate to the cytosol through carrier proteins. TREX1 is defective in the degradation of oxidized substrates such as oxidized mtDNA, which are preferentially from SLE neutrophils (38, 88). Hence, in conditions like lupus, the persistent presence of oxidized cf-mtDNA in the cytosol of immune cells can potentially activate inflammatory pathways. TREX1 variant mutations are also reported in SLE (89, 90). Finally, complement C1q, as well as other complement components also play an important role in opsonizing dead cells or extracellular debris for phagocytosis, thus efficiently removing cfDNA from the circulation (82, 91). Other opsonins, including serum amyloid *P* component (92), IgM (93, 94), C-reactive protein (CRP) (95, 96), and Mannan Binding Lectin (97) serve similar functions in clearance of dying cells, with deficiencies in either one of the opsonins commonly leading to accumulation of cfDNA (98).

## INFLAMMATORY POTENTIAL OF cfDNA

Under physiological conditions, cfDNA is normally not inflammatory due to its rapid degradation as well as its inability to access intracellular DNA sensors. Consistent with this proposition, cfDNA failed to induce immune responses from plasmacytoid dendritic cells (pDCs), which are otherwise potent responders to microbial nucleic acids (79, 99, 100). Initially, this tolerance to self-DNA was thought to be due to the sequence composition differences between self- and microbial DNA. However, numerous studies have shown that self-DNA can be immunostimulatory provided it has access to intracellular DNA sensors (101–103). These carrier proteins, often elevated in inflammatory conditions (79, 104), can facilitate the uptake of DNA and also protect the DNA from degradation, thus promoting the induction of pro-inflammatory responses.

## BASED ON COMPLEXATION WITH CARRIER PROTEINS

In SLE, anti-dsDNA autoantibodies are one of the prominent carrier molecules of cfDNA into cells. Among others, anti-dsDNA antibodies, through their interaction with Fcγ receptor II (FcγRII) facilitate the receptor-mediated endocytosis of DNA into the TLR9-containing endosomal compartments of pDCs, eliciting a robust induction of interferon (IFN)-α (IFN-α), a cytokine markedly elevated in SLE and associated with disease activity (105). In an attempt to understand the role of the anti-dsDNA antibodies in promoting DNA-immune complex (IC)-mediated inflammation, Means et al. (104) undertook a series of experiment to dissect the role of the autoantibodies. Whereas, neutralization of FcgRII abrogated the immune reactivity of ICs, this could be rescued through a liposomal carrier. In all,

these elegant experiments suggested that the primary role of IgG autoantibodies is to facilitate the entry of DNA into cells and are not obligatory for the immunogenicity of DNA (104). Later, a study by Lande et al. (79), provided evidence that cationic microbial peptides that are released in the context of NETosis, confer the immunogenicity to DNA-ICs by protecting DNA from nuclease degradation and facilitating uptake. Further, their data suggest that complexes of self-DNA-antimicrobial peptides (i.e., LL37) constitute the immunogenic core of the DNA-ICs in SLE, since anti-DNA antibodies alone were not sufficient to confer immunogenicity to DNA. LL37 is highly expressed in the circulation of SLE patients (106). LL37 stably binds to DNA through charge interactions between the unique cationic α-helical residues of LL37 and anionic phosphate backbone of DNA, thus forming insoluble DNA aggregates that are resistant to nuclease degradation (107). Antimicrobial peptides, including human neutrophil peptides, seem to function synergistically with LL37 to promote pDC activation by DNA-ICs (79). IL-26, a cationic amphipathic cytokine secreted by Th17 cells seems to stabilize and thereby promote the immunogenicity of extracellular DNA (80). IL-26, through its clusters of cationic charges, binds, and aggregates human DNA, thus forming insoluble particles that are resistant to extracellular degradation. Further, Meller et al. (80) showed that IL-26-DNA complexes could induce IFN-α secretions from pDCs in TLR9-dependent manner, and the internalization of complexes is mediated by the attachment of IL-26 cationic residues to heparan-sulfate proteoglycan on the cell membrane. Later, Poli et al. (108) showed that IL-26 shuttles different forms of extracellular DNA (genomic DNA, mitochondrial DNA, and NETs) into the cytosol of monocytes and promotes cyclic GMP-AMP synthase (cGAS)-STING- and inflammasome-dependent pro-inflammatory responses. Further, high levels of IL-26-DNA complexes have been found in patients with anti-neutrophil cytoplasmic antibody-associated vasculitis (108), RA (109), psoriasis (110), and Crohn's disease (111). cfDNA can also be translocated into cell when bound to DNA-binding high mobility group box proteins, like high mobility group 1 (HMGB1) and TFAM that are released as DAMPs into extracellular space from damaged and dying cells and also during inflammation (112, 113). These molecules can specifically engage receptor for advanced glycation end products (RAGE) on pDCs to elicit TLR9-dependent IFN-α secretions for DNA ligands (114, 115). Further, DNA bound by these molecules can presumably undergo conformational changes that allow them to bind and activate a cytosolic DNA sensor, cGAS to initiate STING-mediated type-I IFNs (116).

## BASED ON OXIDATIVE STATUS OF cfDNA

Another important aspect that can render cfDNA inflammatory is the presence of oxidized nucleotides, as DNA oxidation, whether bound to cationic peptides or not, is recognized as a danger associated molecular pattern. Further, the immunogenicity of DNA seems to depend on the degree of oxidation (117). Elevated levels of oxidized cfDNA is observed in various inflammatory conditions (118), supporting the role of oxidative status in promoting the inflammatory potential of DNA. DNA can be oxidized by free oxygen radicals or reactive oxygen species (ROS) that are generated during cell death processes (119), thus resulting in the release of oxidized cfDNA. We have shown that neutrophil cell death i.e., NETosis in response to ribonucleoprotein-containing ICs (RNP ICs), is dependent on mitochondrial ROS and the released NETs are enriched in oxidized interferogenic mtDNA (38). Alternatively, cfDNA can be oxidized by free oxygen radicals or ROS that are released by activated phagocytic cells at sites of inflammation (120). Oxidized DNA undergo structural changes due to the base modifications introduced by free radicals. As a result, the oxidized DNA is more resistant to nuclease degradation, as compared to unmodified DNA, thus making the oxidized DNA available to initiate pro-inflammatory responses (117). Although ROS can cause diverse arrays of DNA base modifications, a lesion to guanine residue identified as 8-hydroxy-2′-deoxyguanosine (8-OHdG) remains the most abundant and well-characterized DNA lesion (121). In fact, 8-OHdG was identified as a marker to determine the oxidative stress in various pathological conditions (122). Oxidized DNA was shown to activate various inflammatory pathways including, cGAS-STING, TLR9, and NLRP3 inflammasome pathways (38, 117, 123, 124). Further, oxidation by ROS increases the antigenicity of DNA, as demonstrated by the enhanced reactivity of SLE sera with oxidized DNA compared to native DNA (88, 125, 126). Consistent with the above observation, DNA contained within SLE immune complexes show an accumulation of 8-OHdG, indicating that oxygen radicals play a key role in the SLE pathology by modulating the antigenic nature of DNA in circulation (125, 126).

## BASED ON INTRACELLULAR ORIGIN

The intracellular origin of cfDNA, e.g., either from nucleus or mitochondria, can also influence the inflammatory potential of cfDNA. MtDNA, unlike nuclear DNA, is a potent inflammatory trigger (26, 38, 123, 127–130). MtDNA, due to its prokaryotic origin, holds many features that are similar to bacterial DNA, including the presence of a relatively high content of unmethylated CpG motifs, which are rarely observed in nuclear DNA (131). The unmethylated CpG motifs are of particular importance as TLR9, the only endolysosomal DNA-sensing receptor, has a unique specificity for unmethylated CpG DNA. As such, mtDNA was shown to activate neutrophils through TLR9 engagement (26, 103). Thus, unless coupled to carrier proteins, mtDNA, but not nuclear DNA, can be recognized as a danger-associated molecular pattern inducing pro-inflammation through TLR9. Collins et al. (129), reported that intra-articular injection of mtDNA induces arthritis *in vivo*, proposing a direct role of mtDNA extrusion in the disease pathogenesis of RA. Also, the oxidative status of mtDNA makes it highly inflammatory (123, 127). MtDNA, in contrast to nuclear DNA, is characterized by elevated basal levels of 8-OHdG, a marker of oxidative

damage (66). The high content of oxidative damage in mtDNA is primarily attributed to the close proximity of mtDNA to ROS and relatively inefficient DNA repair mechanisms that can lead to the accumulation of DNA lesions (132, 133). We, as well as others, have shown that oxidative burst during NETosis can oxidize mtDNA and the released oxidized mtDNA by itself, or in complex with TFAM, can generate prominent induction of type I IFNs (38, 88, 117). Oxidative status of mtDNA renders its resistant to degradation by DNases such as TREX1, enabling it to activate multiple pro-inflammatory pathways (95). Oxidized mtDNA generated during programmed cell death is not limited to activate TLR9, but was shown to also engage the NRLP3 inflammasome, leading to the production of pro-inflammatory cytokines, IL-1β, and IL-18 (123, 127, 128, 130). MtDNA can also be recognized by cyclic GMP-AMP synthase (cGAS), a cytosolic dsDNA sensor to initiate a STING-IRF3-dependent pathway that in turn orchestrates the production of type I IFNs (134).

## DNASE I AS A THERAPEUTIC AGENT

Considering the role of DNase I in promoting the clearance of autoantigenic DNA and perhaps even the destruction of DNA in ICs, the therapeutic potential of DNase I has been explored. Initial studies by Johnson and colleagues in the 1950s demonstrated that bovine pancreatic DNase I is harmless to humans and is a very poor antigen (135). It was used as a liquefaction agent to treat disease conditions associated with exudative responses to inflammation and infection (136). These studies by Johnson et al., (135, 136) laid basis for the first therapeutic application of bovine DNase I to treat SLE where patients injected with DNase I showed improvement in clinical symptoms, including a rapid fall in the ESR and also in the levels of autoantibodies specific to DNA-containing antigens but not in other autoantibodies. However, contrary to the initial reports, bovine DNase I was found to be antigenic and patients developed antibodies toward it 4–6 weeks following the administration, thus precluding the therapeutic usage of DNase I (137). Meanwhile, animal studies with recombinant mouse DNase I to treat SLE in NZB/W F1 hybrid mice prompted further interest in DNase as therapeutic agent for SLE (138). Mice treated with DNase I from the early phases of disease development (4 months of age) had a prolonged survival of about a month that was paralleled by the delay in the selective formation of anti-DNA antibodies but not in other autoantibodies or total IgG levels. Further, a concomitant rise in anti-DNA antibodies was observed in urine suggesting the destruction of DNA-containing ICs in kidney by DNase treatment. The DNase-treated mice developed less severe glomerulonephritis compared to control mice. Anti-DNase antibodies were formed in all DNase-treated mice, although they did not rise and remained low throughout the treatment period. The effect of these anti-DNase antibodies on the DNase function was not clear. In addition, it was not clear why sustained effect of DNase could not be seen and if more enzyme could have been beneficial given the rise in DNase inhibitors with inflammation. Nevertheless, much more dramatic changes in clinical course were observed in a group of mice treated with DNase for 3 weeks at the peak of their disease activity (7 months of age). DNase-treated mice displayed significantly lower levels of serum creatinine and less proteinuria compared to controls accompanied by remarkably less severe histopathological changes in the kidney, suggesting that DNA-containing ICs might still be involved in the advanced stage of disease course. Since animals were killed at the end of experiment, the long-term effect of DNase treatment is not clear. The study also suggests that destruction of established ICs might be an effective therapeutic option for SLE, thus preventing the stimulation of autoreactive B cells. Investigations into the human usage of DNase I in SLE were prompted by the discovery of recombinant human DNase I (rhDNase) as a potential agent to attenuate the sputum viscosity in cystic fibrosis patients (139). RhDNase administered by an inhalation method was found to be safe and well-tolerated in patients with cystic fibrosis (140–142). Subsequently, a 40-day, phase 1b placebo-controlled clinical trial conducted in SLE patients with lupus nephritis showed that rhDNase I is safe in SLE patients, and that the treatment was not associated with the development of anti-rhDNase antibodies. Patients were given an initial single dose (25 or 125 μg/kg) of intravenous injection followed by 10 subcutaneous injections of rhDNAse I. However, the treatment did not result in a significant improvement in markers of disease activity including the levels of serum dsDNA antibodies, levels of complement components C3 and C4, levels of circulating immune complexes, serum cytokines levels (IL-6, IL-10, and TNF-α) and there was no change in the immune complex deposition in skin biopsies pre- and post-treatment. This lack of clinical efficacy could partly be explained by the observation that rhDNase at bioactive serum concentrations was maintained for only a few hours at both doses, and thus future studies should investigate the effect of dose or regimen that allows the maintenance of rhDNase at concentrations capable of serum hydrolytic activity for prolonged time periods. The absence of neutralizing antibodies to rhDNase unlike to bovine DNase I suggests the potential possibility for long-term therapy (143).

## CELL-FREE DNA IN RHEUMATIC DISEASES

### Systemic Lupus Erythematosus

SLE is a prototype autoimmune disease that effects multiple tissues and organs systems, including skin, joints, and kidney. Though known to have both environmental and genetic components, the etiology of the disease is not fully understood. Among several markers implicated in the SLE pathogenesis, the role of dsDNA is especially interesting. In the majority of the SLE literature, the pathological role of dsDNA in the disease pathogenesis is discussed from the perspective of dsDNA-containing immune complexes, frequently found in SLE patients, partaking in the development of lupus nephritis. The prevailing idea is that these immune complexes activate complement factors and Fc gamma receptor-bearing cells to initiate pathological

inflammatory responses. In fact, anti-dsDNA antibodies are listed among the classification criteria for diagnosing SLE in accordance with the American College of Rheumatology, and the Systemic Lupus International Collaborating Clinics classification criteria (144, 145). Other than the diagnostic utility, increasing research into the potential role of DNA sensing receptors (146) to initiate multiple pro-inflammatory pathways resulting in the secretion of SLE-associated type I interferons, brought dsDNA back into the center stage of the SLE pathogenesis. Subsequently, the interest to detect, quantify and/or characterize cfDNA in plasma/sera of SLE patients emerged. In the section below, we will present a glimpse of how the research into the field of cfDNA has evolved in SLE, with initial studies mainly focusing on the detection and quantification of cfDNA followed by studies to associate cfDNA levels to disease activity, progression and/or for monitoring treatment response. Finally, we will touch upon the application of advanced sequencing techniques to determine characteristics of plasma DNA, all with a common goal to explore the diagnostic and prognostic potential of cfDNA for SLE. Major findings of cfDNA in SLE as discussed in the following sections are summarized and listed in **Table 1**.

## EARLY REPORTS OF cfDNA DETECTION IN SLE

In 1948, Mandel and Metais were the first to report on the presence of cfDNA in human plasma (1). However, it was not until the discovery of high levels of cfDNA in SLE patients in 1966 by Tan et al. (2), that the interest in cfDNA as a potential biomarker for autoimmune diseases started. Tan et al. (2) used SLE sera with a precipitating antibody to DNA in a gel diffusion method to detect the presence of DNA in pathological sera. By this method, native (ds) DNA could be detected in sera of some SLE patients (11/95) and in patients with other disease conditions but not in the sera of healthy controls. Gel precipitation has a detection limit of 1 μg/ml; hence it is possible that samples with lower levels of cfDNA, especially of healthy controls could have gone undetected. Later, in 1968 Barnett used complement-fixation, to demonstrate the presence of cfDNA in normal and pathologic human sera and synovial fluids (3). Sera of patients positive for precipitating antibodies to DNA and having complement-fixing antibodies to DNA were used to detect DNA in human samples. Unlike Tan et al. (2), small but detectable amounts of DNA could be measured in normal sera. However, the levels of cfDNA were markedly elevated in pathologic sera and synovial fluid. In 1973, Koffler et al. (4), by hemagglutinin inhibition test, reported that about 50% of SLE sera are positive for the presence of single-stranded DNA (ssDNA) as an antigen. In contrast, ssDNA appeared infrequently in the sera of healthy controls (4% incidence). Almost at the same time, Davis and Davis (147), used a counterimmunoelectrophoresis (CIE) method to detect cfDNA in the plasma of patients with various illnesses including SLE. The number of positive samples in SLE patients (2/47, 4.2%) were not significantly different from healthy controls (1/83, 1.2%).

## THE CONTROVERSY OF DETECTION METHODS AND SERUM VS. PLASMA UTILITY FOR cfDNA MEASUREMENT

At the time when the clinical importance of cfDNA was being actively pursued, conflicting data on the detection of cfDNA in the circulation of healthy individuals led to controversies on the detection methods and on the significance of serum and plasma cfDNA levels for disease. In addition, a consensus was yet to be reached on the levels of cfDNA in plasma for normal vs. pathological scenarios. Davis and Davis (147), with their method of CIE, were the first to hint that cfDNA in sera, at least in part, could be an artifact of the method, i.e., DNA that is sporadically released into sera during blood clotting. This observation was confirmed by Steinman (149) in an elegant study that employed four different techniques with improved sensitivities and/or specificities to detect cfDNA in normal serum and plasma samples. In that study, plasma cfDNA remained undetectable by all four methods including a highly sensitive CIE method. Further, it was reported that the cfDNA measurements in plasma by ethidium bromide and diphenylamine assays, are mainly due to interfering substances (non-DNase sensitive substances) in plasma rather than true DNA. In contrast, cfDNA could be detected in serum samples by a CIE method. Based on these findings, it was concluded that cfDNA detection in plasma is pathological and levels >50 ng/ml for dsDNA and 100 ng/ml for ssDNA in plasma are abnormal. These findings were later confirmed by Dennin (167), who, by employing CsCl-buoyant density centrifugation found that in healthy adults the concentration of plasma cfDNA ranged from 3 to 11 ng/ml (167). In more recent times, Chen et al. (5), showed that serum samples from lupus patients had higher levels of cfDNA than the corresponding plasma samples by using a fluorochrome PicoGreen assay on purified cfDNA. A higher sera-to-plasma cfDNA ratio suggests that white blood cells from SLE patients are fragile and/or damaged and thus are prone to undergo disruption during coagulation releasing DNA. These studies strengthened the view that cfDNA levels from serum samples should be interpreted with caution especially when employing sensitive detection methods and if possible, should be replaced by carefully collected plasma samples.

## cfDNA AND DISEASE ACTIVITY IN SLE

The majority of studies reported an association between cfDNA and disease activity in lupus. However, there are a few studies with conflicting data on the link between cfDNA and disease activity (152, 154, 160, 168). Early reports by serial sampling of cfDNA from sera demonstrated that an increased appearance of cfDNA in SLE patients is associated with the exacerbation of disease and interestingly becomes undetectable following the clinical improvement (2, 4, 153). Koffler et al. (4) in a

**TABLE 1 |** Cell-free DNA research in Systemic lupus erythematosus and Rheumatoid arthritis.

| References | Year of publication | Patients (*n*) | Healthy controls, HC (*n*) | Method | cfDNA source | Observation |
|---|---|---|---|---|---|---|
| Tan and Kunkel (2) | 1966 | SLE (95) | 30 | Gel diffusion precipitin test | Serum | Frequency of positive test for cfDNA: SLE 11.5%, liver disease 15%, lymphosarcoma 3%, and none were observed in HC |
| | | Leukemia, lymphosarcoma, and lymphoma (29) | | | | cfDNA positivity fluctuate with disease activity |
| | | Multiple myeloma (15) | | | | |
| | | Acute rheumatic fever (9) | | | | |
| | | RA (17) | | | | |
| | | Liver disease (40) | | | | |
| | | Miscellaneous (myocardial infarction, renal disease, lung disease, infection, carcinoma) (72) | | | | |
| Barnett (3) | 1968 | Serum tested | 14 | Quantitative complement-fixation test | SF and Serum | ↑ cfDNA in the sera of patients with SLE, RA, systemic mastocytosis, uremia with chronic glomerulonephritis compared to HC |
| | | RA (6) | | | | ↑ cfDNA in SFs of patients with SLE, RA, Gout |
| | | SLE+Nephritis (6) | | | | |
| | | SLE-Nephritis (7) | | | | |
| | | Systemic Mastocytosis (1) | | | | |
| | | Uremia with Chronic Glomerulonephritis (1) | | | | |
| | | Vasculitis, Local (1) | | | | |
| | | Rheumatic Heart (1) | | | | |
| | | Psoriasis+RA (2) | | | | |
| | | Gout (1) | | | | |
| | | Scleroderma (1) | | | | |
| | | SF tested | | | | |
| | | SLE (1) | | | | |
| | | RA (3) | | | | |
| | | Gout (2) | | | | |
| | | Reiter's (1) | | | | |
| | | Infection (1) | | | | |
| Koffler et al. (4) | 1973 | SLE (60) | 56 | Hemagglutination inhibition test | Serum | ↑ cfDNA in sera of patients with SLE and RA compared to HC and other diseases |
| | | RA (54) | | | | Association with disease severity |
| | | Chronic glomerulonephritis (40) | | | | |
| | | Leukemia (19) | | | | |
| | | Malignant tumors (20) | | | | |
| | | Hospital diseases (99) | | | | |
| Davis and Davis (147) | 1973 | SLE (44) | 83 | Counterimmuno-electrophoresis | Plasma | Frequency of positive test for cfDNA: 1.2% in HC, 3.2% in RA, 4.2% in SLE, 1.4% preoperative, 44.0% post-operative |
| | | RA (28) | | | | |
| | | Newborn cord bloods (36) | | | | |
| | | Surgery | | | | |
| | | Preoperative (71) | | | | |
| | | Post-operative (50) | | | | |
| | | Nonsurgical (278) | | | | |
| | | Miscellaneous (60) | | | | |

*(Continued)*

**TABLE 1** | Continued

| References | Year of publication | Patients (*n*) | Healthy controls, HC (*n*) | Method | cfDNA source | Observation |
|---|---|---|---|---|---|---|
| Leon et al. (148) | 1977 | RA (70) | 61 | Radioimmunoassay | Serum | ↑ cfDNA in RA patients compared to HC |
| | | | | | | ↑ cfDNA levels in RA patients with active disease for <10 years, seronegative for rheumatoid factor |
| Steinman (149) | 1979 | SLE (43) | None | Modified counterimmuno-electrophoresis | Plasma | Frequency of positive test for cfDNA: 80% in SLE with CNS involvement and vasculitis, 20% in SLE with dermal vasculitis |
| | | SLE+CNS involvement (12) | | | | 5.5% in active SLE, none in other rheumatological disorders and SLE patients on treatment |
| | | SLE+Systemic Vasculitis (8) | | | | |
| | | SLE+Dermal Vasculitis (5) | | | | |
| | | Other rheumatological disorders and SLE on treatment (53) | | | | |
| Raptis and Menard (150) | 1980 | inactive SLE (5) | 3 | Nick translation on purified cfDNA | Plasma | ↑ cfDNA levels in active SLE patients compared to steroid-inactive SLE and HC |
| | | active SLE (2) | | | | cfDNA positivity fluctuate with disease activity |
| | | RA (2) | | | | |
| | | DM (1) | | | | |
| Leon et al. (151) | 1981 | Seropositive RA (26) | 95 | Radioimmunoassay | Serum and SF | ↑ cfDNA in SF and serum of RA, gout and pseudogout |
| | | Seronegative RA and variants (21) | | | | Temporal correlation between serum and SF cfDNA during active disease in RA patients |
| | | Non-classified and mono- and oligoarticular RA (6) | | | | |
| | | Gout and pseudogout (6) | | | | |
| | | OA (29) | | | | |
| | | OA+chonrocalcinosis (4) | | | | |
| | | Post-traumatic arthropathy (14) | | | | |
| Klemp et al. (152) | 1981 | Clinically active SLE (43) with specific organ or system involved | 58 | PAGE and fluorimetric scan on purified cfDNA | Plasma | Frequency of subjects with cfDNA <10 ng/ml: Clinically active SLE: 88%; Clinically inactive SLE: 82%; HC: 81% |
| | | Skin (8) | | | | Frequency of subjects with cfDNA >10 ng/ml: Clinically active SLE: 11.6%; Clinically inactive SLE: 17% |
| | | Musculoskeletal system (20) | | | | |
| | | Kidney (5) | | | | |
| | | Nervous system (3) | | | | |
| | | Cardiovascular system (1) | | | | |
| | | Nonspecific (constitutional symptoms) (6) | | | | |
| | | Clinically inactive (53) | | | | |
| Morimoto et al. (153) | 1982 | SLE (28) | 5 | $^{32}$P-phosphate incorporation into 5′ ends of DNA | Serum | ↑ concentrations of DNA in DNA/anti-DNA immune complexes of SLE patients |
| | | RA (4) | | | | DNA in DNA/anti-DNA immune complexes of SLE patients correlate with disease activity |

*(Continued)*

**TABLE 1 |** Continued

| References | Year of publication | Patients (*n*) | Healthy controls, HC (*n*) | Method | cfDNA source | Observation |
|---|---|---|---|---|---|---|
| McCoubrey-Hoyer et al. (154) | 1984 | SLE+Nephritis at the time of blood sampling (10) | 20 | Counterimmuno-electrophoresis on purified cfDNA | Plasma | ↑ concentrations of cfDNA in SLE patients compared to HC |
| | | SLE+Nephritis in the past that was inactive at the time of blood sampling (9) | | | | cfDNA levels in the plasma of SLE patients did not correlate with nephritis. |
| | | SLE-Nephritis (12) | | | | |
| Hajizadeh et al. (155) | 2003 | RA (54) | 30 and 22 | PCR and SDS–polyacrylamide-gel electrophoresis | SF and Plasma | PCR-amplifiable mtDNA fragments detected in SF of RA patients but not in HCs |
| | | | | | | ↑ of cfDNA in plasma of RA patients compared to HCs |
| | | | | | | mtDNA presence in SF correlated significantly with rheumatoid factor positivity |
| Collins et al. (129) | 2004 | RA (54) | 17 | PCR and SDS–polyacrylamide-gel electrophoresis | SF | PCR-amplifiable mtDNA fragments detected in SF of RA patients but not in HCs |
| Zhong et al. (156) | 2007 | RA (54) | 44 | qPCR on purified cfDNA | Serum and plasma | ↑ concentrations of cfDNA in RA patients compared to HC |
| | | | | | | ↑ serum-to-plasma cfDNA ratio in RA patients compared to HC |
| | | | | | | ↑ antibody-bound plasma cfDNA in RA patients compared to HC |
| Chen et al. (5) | 2007 | SLE (12) | 8 | PicoGreen assay (fluorescence detection) on purified cfDNA | Serum and Plasma | ↑ concentrations of cfDNA in SLE patients compared to HC |
| | | | | | | ↑ concentrations of cfDNA in the serum compared to plasma |
| Bartoloni et al. (157) | 2011 | SLE (44) | 66 | qPCR on purified cfDNA | Plasma | ↑ cfDNA in SLE, RA and SS |
| | | RA (20) | | | | Correlation of cfDNA with disease activity in SS |
| | | SS (48) | | | | |
| Cepika et al. (158) | 2012 | SLE (15) | 11 | qPCR on purified cfDNA | Serum | ↑ concentrations of cfDNA in SLE patients compared to HC |
| | | | | | | ↑ concentrations of cfDNA in SLE patients compared to HC following treatment. |
| | | | | | | ↓ cfDNA levels in chloroquine treated patients compared to untreated patients |
| Tug et al. (159) | 2014 | SLE (59) | 59 | qPCR on unpurified cfDNA | Plasma | ↑ concentrations of cfDNA in SLE patients compared to HC |
| | | | | | | No difference in the DNA integrity between SLE and HC |
| | | | | | | cfDNA levels fluctuate with disease activity |
| Zhang et al. (42) | 2014 | SLE (54) | 43 | PicoGreen assay (fluorescence detection) on unpurified cfDNA | Plasma | ↑ concentrations of cfDNA in SLE patients compared to HC |
| | | | | | | ↑ concentrations of cfDNA in SLE patients with LN compared to patients without LN |
| | | | | | | ↑ concentrations of cfDNA in SLE patients with active LN compared to patients with inactive LN |

*(Continued)*

**TABLE 1 |** Continued

| References | Year of publication | Patients (*n*) | Healthy controls, HC (*n*) | Method | cfDNA source | Observation |
|---|---|---|---|---|---|---|
| Chan et al. (19) | 2014 | SLE (24) | 11 | qPCR on purified plasma DNA | Plasma | ↑ aberrant genomic representation, size shortening and hypomethylation of plasma DNA |
| | | | | Plasma DNA sequencing and methylation analysis | | Correlation with SLEDAI and anti-dsDNA antibodies |
| Hendy et al. (160) | 2015 | SLE (52) | 25 | qPCR on purified cfDNA | Serum | ↑ concentrations of cfDNA in SLE patients compared to HC |
| | | | | | | cfDNA levels fluctuate with treatment |
| Dunaeva et al. (6) | 2015 | RA | 29 | qPCR on purified cfDNA | Serum | ↓ cfDNA levels in esRA compared to eRA, RRMS and HC |
| | | eRA (39) | | | | cfDNA levels in eRA, RRMS comparable to HC |
| | | esRA (26) | | | | |
| | | RRMS | | | | |
| Abdelal et al. (161) | 2016 | SLE (35) | 25 | qPCR on purified cfDNA | Plasma | ↑ concentrations of cfDNA in SLE patients compared to HC |
| | | RA (30) | | | | Correlation with ESR, anti-dsDNA, C3, C4 and SLEDAI-2000 |
| Rykova et al. (162) | 2017 | RA (74) | 63 | qPCR on purified cfDNA | Plasma and cell-surface bound | ↑ concentrations of plasma nuclear DNA in RA patients compared to HC. |
| | | | | | | No differences in plasma mtDNA levels between RA patients and HC |
| | | | | | | ↑ concentrations of cell-surface bound mtDNA and ↓ levels of cell-surface bound nuclear DNA in RA compared to HC |
| Hashimoto et al. (163) | 2017 | RA on bDMARD (30) | 21 | qPCR on purified cfDNA | Plasma and SF | ↑ concentrations of plasma cfDNA in RA patients compared to HC |
| | | OA (12) | | | | Compared to baseline ↑ concentrations of plasma cfDNA in RA patients with the introduction of biological DMARDs until 8 weeks |
| | | | | | | and is associated with improvement in disease activity |
| | | | | | | ↑ concentrations of SF cfDNA in RA patients compared to OA patients. |
| Laukova et al. (164) | 2018 | RA on bDMARD (37) | none | qPCR on purified cfDNA | Plasma | ↓ in total cfDNA, nuclear and mt DNA 6 months post-bDMARD treatment and association with clinical and laboratory parameters |
| Eldosoky. et al. (165) | 2018 | RA (35) | 22 | qPCR on purified cfDNA | Plasma | |
| Xu et al. (166) | 2018 | Pregnant women with SLE (36) | 199 | Fluorometric Qubit® dsDNA BR Assay Kit Qubit assay | Plasma | ↑ levels of cfDNA in non-pregnant and pregnant women with SLE compared to HC |
| | | Non-pregnant women with SLE (22) | 60 | | | ↑ levels of cfDNA in patients with active SLE compared to patients with inactive disease |
| | | | | | | Correlation of SLEDAI scores with higher cfDNA levels in entire patient cohort, non-pregnant and pregnant patients |

*Anti-dsDNA, Anti-double stranded DNA; bDMARD, Biological disease-modifying antirheumatic drugs; C3, Complement factor C3; C4, Complement factor C4; DM, Dermatomyositis; eRA, Early RA; ESR, Erythrocyte sedimentation rate; esRA, Established RA; HC, Healthy control; MtDNA, Mitochondrial DNA; OA, Osteoarthritis; qPCR, Quantitative real-time PCR; RA, Rheumatoid arthritis; RRMS, Relapsing-remitting multiple sclerosis; SF, Synovial fluid; SLE, Systemic lupus erythematosus; and SLEDAI, SLE disease activity index.*

serial study of 18 SLE patients, followed for periods of 6–51 months observed that in certain patients, cfDNA (ssDNA) can reach extreme high levels in the range of 125–250 μg/ml. Similar to Tan et al. (2), ssDNA antigen appearance was associated with episodes of clinical exacerbations and patients with prolonged presence of ssDNA (4–8 months) reported progressive renal disease (4). Evidence for *in vivo* antigen-antibody formation was demonstrated where ssDNA antigen appearance alternated or occurred simultaneously with anti-ssDNA antibodies. Morimoto et al. (153) observed a highly significant correlation between DNA derived from circulating immune complexes and disease activity index in SLE patients. A serial study of two patients demonstrated that the levels of cfDNA remain elevated (52 ng/ml) during the episodes of active disease and glomerulonephritis (100 ng/ml) and return to lower levels during clinical remission (10 ng/ml) and further becoming non-detectable with treatment (<1 ng/ml). Steinman (149), observed that majority of SLE patients (80%) with central nervous system involvement and/or systemic vasculitis have a persistent presence of cfDNA in plasma. Longitudinal studies in four SLE patients also confirmed this association with CNS and/or vasculitis, where only episodes associated with these manifestations are characterized by cfDNA appearance. Raptis et al. (150), showed that cfDNA exists in much higher levels in the plasma of untreated SLE patients with active disease compared to plasma of patients with corticosteroid-induced disease remission and healthy controls. A serial determination of plasma in SLE demonstrated that cfDNA levels are elevated at disease onset and diminished considerably when disease has stabilized accompanied by a concomitant decrease in the serum dsDNA binding activity. Tug et al. (159), though not observing a clear link between SLE disease activity index (SLEDAI) and cfDNA concentrations, found a significant correlation between fluctuations in cfDNA levels and transition from remission to deteriorating status. This study suggested that changes in disease state, in particular deterioration status, could be reflected by fluctuations in cfDNA (159). Zhang et al., (42), investigated the proposition that elevated levels of plasma cfDNA in SLE are related to lupus nephritis (LN). cfDNA concentrations were significantly higher in SLE patients with LN than in patients without LN and further subgrouping analysis revealed that patients with active LN had significantly elevated cfDNA concentrations compared to patients with inactive LN. Studies in SLE patients also reported that cfDNA levels correlate significantly with SLEDAI (53, 166). A recent study by Xu et al. (166), showed that median cfDNA levels are significantly higher in SLE patients with active disease compared to patients with inactive disease.

## cfDNA AND TREATMENT RESPONSE IN SLE

Considering that inflammation can cause the release of cfDNA and the fact that cfDNA itself can perpetuate ongoing inflammation leading to a vicious feedback loop, a drug treatment that reduces systemic inflammation or specifically antagonizes receptors that recognize DNA, would likely affect cfDNA levels as well. Currently, literature demonstrating the dynamics of cfDNA levels in SLE patients with treatment is sparse, but promising. Hendy et al. (160) observed that SLE patients following specific therapy with cytotoxic drugs show a significant reduction in serum cfDNA levels compared to pre-treatment and this was accompanied by a concomitant reduction in anti-dsDNA levels and anti-nucleosome antibodies. Cepika et al. (158) showed that sera cfDNA levels in SLE patients decrease significantly following treatment with chloroquine, a drug known to block the DNA-sensing TLR9 pro-inflammatory pathway. Although not significant, corticosteroid (CS) treatment also decreased serum cfDNA levels, suggesting that reduction in systemic inflammation decreased cfDNA levels. In contrast, in another study (157), the type of treatment, CS and/or immunosuppressive (IS) did not seem to affect the levels of plasma cfDNA in SLE patients.

## cfDNA AND ASSOCIATION WITH INFLAMMATORY MARKERS IN SLE

cfDNA has been receiving increasing attention as an inflammatory marker with the advent of new mechanistic studies highlighting the role of DNA sensing receptors in SLE pathogenesis (146). Hence, attempts were made to evaluate the relationship of cfDNA with other known markers of inflammation. Overall, although limited by the number of studies, it can be concluded that cfDNA levels in SLE associate well with several markers of inflammation. One study found a significant positive correlation between serum cfDNA levels and a generic marker of inflammation, CRP (149). NETs released from neutrophils and low-density granules (LDGs), could also be the source of cfDNA in SLE. Consistent with this assumption, Zhang et al. (42) found a highly significant positive correlation between levels of plasma cfDNA and the percentage of LDGs and neutrophil levels, suggesting that LDGs and neutrophils, through NET formation, can contribute to cfDNA in SLE patients. However, the authors never confirmed whether cfDNA levels correlated with presence of circulating NETs in these patients. In addition to the abnormal production of NETs, impaired clearance of NETs can also lead to elevated levels of cfDNA in SLE. Further analysis demonstrated a significantly lower DNase I activity in SLE patients compared to healthy controls, although no significant correlation could be observed between DNase I activity and cfDNA levels (42). Studies found contrasting associations for the levels of complement factors and cfDNA in SLE patients. Tug et al. (159) and Abdelal et al. (161) found a positive correlation between the levels of complement factors and plasma cfDNA levels in SLE patients. This observation was surprising given that complement consumption is commonly seen in active SLE patients. However, the authors speculated that this positive relationship between complement levels and cfDNA might be due to the increase in complement levels as a part of an acute phase response that can obscure the complement consumption. In contrast, a study by Hendy et al. (160) found a

significant negative correlation between levels of serum cfDNA and C3 in SLE patients.

Based on the central role of anti-nuclear antibodies in SLE, and the possibility that high levels of cfDNA in circulation might initiate and/or perpetuate the production of anti-dsDNA antibodies, a positive correlation between cfDNA levels and anti-dsDNA antibodies is expected. However, in contrast, studies reported either a lack of or negative correlation between cfDNA and anti-dsDNA antibody levels. McCoubrey et al. (154) and Hendy et al. (160) observed an inverse correlation between levels of plasma DNA in SLE patients and titers of antibody for DNA. In another study (157), cfDNA in plasma did not correlate with titers of anti-dsDNA antibodies. This situation can likely arise due to the accelerated tissue deposition of immune complexes during active disease. A serial sampling might provide a better picture of the association between levels of cfDNA and anti-dsDNA antibodies.

## QUALITATIVE FEATURES OF PLASMA cfDNA IN SLE

The SLE genome exhibits distinct qualitative features such as, a higher frequency of CpG dinucleotides (169), increased hypomethylation (170, 171), and increased oxidation (172). Interestingly, all these qualitative features promote the immune stimulatory properties of DNA to be recognized by DNA sensing receptors and subsequently induction of pro-inflammatory responses. Hence, it appears that cfDNA released from SLE patients is inherently proinflammatory and, therefore analyzing the qualitative changes of cfDNA might provide more in-depth understanding on the association of cfDNA with SLE pathogenesis and eventually it's utility as a biomarker for SLE. However, in SLE literature only one study has been reported investigating the qualitative changes of plasma cfDNA (19), warranting the need for more studies in this area of cfDNA research. Chan et al. (19) by a parallel genomic and methylomic sequencing observed various abnormalities in plasma DNA from SLE patients including aberrant measured genomic representations (MGRs), size shortening, fragments of <115 bp in size, and hypomethylation. Very interestingly all these plasma DNA abnormalities, as discussed below, were seemed to be modulated by anti-dsDNA antibodies, pathological circulating markers of SLE. It was observed that the frequency of aberrant MGRs correlated with the levels of serum anti-dsDNA antibodies. Subsequent experiments demonstrated that aberrant MGRs DNAs had increased binding affinity to anti-dsDNA antibodies. Thus, given the ability of IgG to protect DNA from subsequent degradation, DNA molecules with increased dsDNA antibody binding, such as aberrant MGR DNA, may have increased representation in cfDNA analyses due to their reduced clearance. The percentage of plasma DNA shortening in SLE correlated positively with SLEDAI and the anti-dsDNA antibody, suggesting that there is either an increased release or decreased clearance of short fragments in SLE. This observation aligns well with the evidence of increased apoptosis as well as defective clearance in SLE patients (17, 173). Further, IgG-bound DNA was enriched in short fragments (<115 bp), strengthening the proposition that IgG antibody has a preferential binding to short DNA fragments, that in turn protect them from degradation. Plasma DNA molecules from active SLE patients were more hypomethylated compared to inactive SLE and healthy controls. In addition, the degree of hypomethylation correlated with SLEDAI and anti-dsDNA antibody levels. Based on size distribution profiles and methylation density, it was suggested that plasma DNA in SLE patients exhibit size shortening with hypomethylation and are protected from degradation by antibody-binding.

## SUMMARY OF cfDNA RESEARCH IN SLE

Overall, SLE patients show elevated levels of cfDNA that fluctuate concomitantly with disease activity, inflammatory markers and to some extent with therapeutic interventions. The association of cfDNA with existing SLE diagnostic marker, anti-dsDNA antibodies, is unclear with conflicting data, suggesting that the dynamics of cfDNA in SLE is independent of anti-dsDNA antibodies. cfDNA also seems to reflect the genomic modifications characteristic of SLE disease. However, there are many factors that needs to be addressed in order to establish cfDNA as a biomarker for SLE from a clinical standpoint. cfDNA quantification as a diagnostic marker for SLE is promising but lacks clinical specificity since it is detected in other diseases albeit at lower levels. Findings from epigenomic research on cfDNA are critical in establishing the clinical biomarker specificity of cfDNA for SLE. Mechanistic studies of SLE pathogenesis have demonstrated an enhanced and preferential release of proinflammatory oxidized mitochondrial DNA into circulation by SLE neutrophils (38). Interestingly, none of the SLE-cfDNA studies have analyzed mitochondrial cfDNA in SLE patients. Given the preferential release of mitochondrial DNA in SLE, the quantification and characterization of mitochondrial DNA, and its relative abundance to nuclear DNA, will likely provide disease specific information on cfDNA in SLE. Apart from being a diagnostic marker, cfDNA might also function as a broad disease management marker for SLE, such as a marker of prognosis for remission, flare and/or treatment response. However, it requires a rigorous evaluation of cfDNA in longitudinal studies with large cohorts of patients and careful comparison with existing inflammatory and clinical makers of disease.

### Rheumatoid Arthritis

RA is, similar to SLE, an autoimmune rheumatic disease primarily affecting joints, with severe and disabling erosion (174). Though not as frequent as in SLE, also RA patients have been reported to develop anti-DNA antibodies (175). This has significance given the ability of DNA immune complexes to engage both antigen receptor and TLR9 simultaneously, inducing B cell proliferation and antibody secretion as seen in rheumatoid factor (RF) expressing B cells (176). Further, mtDNA and/or oxidized nucleic acid material was able to induce arthritis in mice (129). In addition to the above-mentioned evidence of DNA in RA, the potential release of cfDNA in general during inflammation, have led to many studies exploring the potential

of cfDNA as a biomarker of diagnosis, disease activity and progression, and/or treatment response in RA. Major findings of cfDNA studies in RA are summarized and listed in **Table 1**.

## cfDNA AND RA

Similar to SLE, the majority of studies reported elevated levels of circulating cfDNA in RA patients compared to controls (3, 4, 155, 156, 161–165). Cell-free nuclear and mtDNA content of synovial fluid in RA patients was reported to be many folds higher than corresponding plasma levels and was exclusive to patients, suggesting that cfDNA release in RA patients is mainly localized to the joints and is pathologically relevant (151, 155, 163). Previous investigations of Rykova et al. (162) in cancer, demonstrated that cfDNA levels in whole blood are a result of continuous exchange between free DNA and cell-surface bound (csb) DNA, and that free DNA binds to cells via direct binding to cell surface proteins and through plasma proteins. In their study on RA patients, Rykova et al. (162) found contrasting dynamics of free- and csb-DNA forms for nuclear and mtDNA compared to controls. While plasma mtDNA levels were not significantly different between RA and healthy controls, csb-mtDNA levels were significantly elevated in RA patients. In contrast, plasma nuclear DNA levels were significantly elevated in RA patients, with a significant decrease in the csb-nuclear DNA levels. The finding on csb-mtDNA is interesting given its role in inflammation, and the decrease in the levels of csb-nuclear DNA could be due to disease-induced changes in the composition of circulating nuclear DNA-protein complexes in RA patients, that might have influenced the nuclear DNA binding to cells (162). In contrast to prior studies, Dunaeva et al. (6) reported that serum cfDNA levels were comparable between patients with early RA (eRA) and healthy controls. Furthermore, patients with established disease (esRA) have significantly lower levels of serum cfDNA compared to patients with eRA and healthy controls. Levels of cfDNA in serum did not correlate with serum DNase activities, suggesting that lower cfDNA levels in esRA is not due to elevated DNase activities. Lower levels of cfDNA in esRA patients could be due to treatment with disease-modifying drugs that are known to reduce systemic inflammation and subsequent cfDNA release. Nevertheless, this study suggested that serum cfDNA can be used as a disease progression marker in RA patients (177).

## HIGH SERUM-TO-PLASMA cfDNA RATIO IN RA

Consistent with the prior observations in SLE, RA patients also exhibited a higher serum-to-plasma cfDNA ratio compared to healthy controls (156), implying that leukocytes in pathological conditions, in general, have an altered susceptibility to undergo cellular death and cfDNA release upon clotting. In an independent study authors evaluated if NETs are the source of higher cfDNA levels in sera of RA patients, based on their *in vitro* findings that neutrophils from RA patients were prone to undergo excessive NETosis. Accordingly, analysis of sera and corresponding plasma samples revealed that, sera from RA patients and not the plasma, have increased concentrations of cfDNA and NET-derived components compared to healthy controls. This observation suggested that coagulation during serum preparation triggers an extensive NETosis in RA neutrophils releasing NETs that in turn contribute to the elevated levels of cfDNA in serum (41).

## cfDNA AND SEROLOGICAL PARAMETERS IN RA

Evidence for the association of cfDNA with serological parameters of RA, including RF and anti-citrullinated protein antibodies (ACPA), is sparse, with conflicting data. Leon et al. demonstrated that higher levels of serum cfDNA in RA patients correlate with seronegativity (148). Consistent with the susceptibility of mtDNA to undergo oxidation, Hajizadeh et al. (155) found that patients that were positive for mtDNA in SF also had high levels of 8-OHdG, a marker of oxidative status. In contrast to Leon et al. (148) both cell-free mtDNA positivity and levels of 8-OHdG correlated significantly with rheumatoid factor positivity. Rykova et al. (162) found a negative correlation between ACPA and plasma mtDNA levels in RA patients. These data suggest that cfDNA and ACPA/RF could be independent circulating makers of RA development and their combination might result in an improved diagnostic tool for RA.

## cfDNA AND DISEASE ACTIVITY IN RA

In a majority of studies, cfDNA levels in RA patients were reported to be associated with disease activity and markers of inflammation (148, 151, 156, 161). DNA levels measured in paired samples of serum and synovial fluid (SF) from patients with arthritides [seropositive RA, seronegative RA variants including psoriatic arthropathy, ankylosing spondylitis and juvenile RA, gout, pseudogout, osteoarthritis (OA), and post-traumatic arthritis (TRA)], demonstrated that the RA patients, as well as patients with gout and pseudogout had the highest levels of cfDNA in SF and serum. Very low levels of cfDNA were seen in patients with OA and TRA. A serial determination of cfDNA revealed a temporal correlation between the elevated levels of DNA in serum and SF and parameters of disease activity and inflammation in some RA patients (151). Abdelal et al. (161) showed that elevated levels of plasma cfDNA in RA patients correlated significantly with erythrocyte sedimentation rate (ESR), CRP and Disease Activity Score-28 (DAS28, suggesting that plasma cfDNA can be a potential marker of disease activity in RA patients. In contrast, Hajizadeh et al. (155) did not find any obvious association between cell-free mtDNA in SF and markers of disease activity and severity, including extra-articular manifestations, erosion, leukocyte counts, CRP levels, or disease duration. Similar to SLE, the majority of plasma cfDNA in RA patients was found to be associated with antibody (156), suggesting the role of DNA-anti-dsDNA immune complexes in RA pathogenesis. More recently, Eldosky et al. (165) found that in RA patients, cfDNA levels are significantly higher in active

disease group compared to control group, while the levels are comparable between remission and control groups. ROC curve analysis revealed a sensitivity and specificity of 86 and 84%, to differentiate active and remission states of RA. Correlation analysis in all RA patients showed that cfDNA levels correlate significantly with DAS28-ESR, a marker of disease activity. In addition, cfDNA showed an inverse correlation with absolute lymphocyte count, suggesting a possible role of enhanced lymphocyte death in RA patients (178) including NETosis, in the formation of cfDNA. Overall, the study suggested that cfDNA can be potential marker of disease activity progression in RA (165).

## cfDNA AND TREATMENT RESPONSE IN RA

In RA patients, changes in cfDNA levels following the treatment with biological disease-modifying anti-rheumatic drugs (bDMARDs), were associated with the improvement in disease activity. Hashimoto et al. (163) reported that in a subset of RA patients, plasma cfDNA can be a predictor of early therapeutic response of bDMARDs. Specifically, a cfDNA elevation at 8 weeks can predict the therapeutic response of biological bDMARDs from 12 to 24 weeks. At baseline, plasma cfDNA levels in RA patients were significantly higher than the healthy controls. After the introduction of DMARDs, the cfDNA increase at 8 weeks was associated with a concomitant improvement in average SDAI score and disease activity. A study by Laukova et al. (164), further investigated the effect of bDMARDs on plasma cfDNA with regards to it subcellular origin, nuclear, and mitochondrial, respectively. This study, in contrast to Hashimoto et al. (163) demonstrated that plasma cfDNA decreases following dDMARDs therapy. Plasma samples were analyzed for cfDNA content from RA patients at baseline as well as 3- and 6-months post-bDMARD treatment. There was a clear improvement in clinical (DAS28, swollen and tender joints) and laboratory parameters (ESR, CRP) in all patients by 3 months following bDMARD therapy. Although the levels of total cfDNA, nuclear, and mitochondrial cfDNA started to decrease by 3 months, significant differences from baseline were observed only 6 months post-treatment, where the concentrations decreased by half. These observations were in contrast to Hashimoto et al. (163), where an increase in plasma cfDNA was observed until 8 weeks post-bDMARD treatment. However, in the absence of additional time points between baseline and 3 months, it is not possible to rule out the dynamics of cfDNA levels in the Laukova et al (164), study as well. Laukova et al. (164) further observed that in RA patients, lower concentration of total cfDNA, correlated positively with DAS28, ESR and CRP. No differences were found between good responders and moderate responders in the levels of total cfDNA, nuclear and mitochondrial cfDNA pre- and post-bDMARDs treatment. In good responders, the concentration of total cfDNA and nuclear cfDNA decreased significantly 6 months from the baseline. Since the decrease in cfDNA levels following bDMARDs therapy is much slower in comparison to other routinely measured laboratory and clinical parameters, the decrease in cfDNA was interpreted as a consequence of reduced inflammation due to treatment. On similar lines, Zhong et al. (156), observed that plasma cfDNA levels were markedly changed (either increase or decrease) in 7 out of 10 patients, 1 h after infusion of infliximab. In another study, a group of RA patients treated with rituximab showed a tendency of lower cell-surface bound mtDNA levels than a group treated with methotrexate and etoricoxib, although the difference did not reach statistical significance (156).

## SUMMARY OF cfDNA RESEARCH IN RA

To summarize, RA patients in general have elevated levels of circulating cfDNA and in SF, cfDNA is found at concentrations many times higher than in circulation, suggesting the role of localized inflammation in the release of cfDNA. Further, the detection of cfDNA exclusively in the joints (SF) of RA, strengthens the arthritic potential of cfDNA. Association of cfDNA with seropositivity is unclear with conflicting results and, the quantitative changes in cfDNA seem to reflect the disease progression and treatment response in RA. Data suggest that dynamics of cfDNA in RA patients is independent of existing diagnostic markers, ACPA and RF, and cfDNA in combination with ACPA/RF might form an improved diagnostic tool. Although, the role of mtDNA in arthritis is demonstrated (129), only two studies have quantified the levels of mtDNA in the circulation of RA patients, highlighting the need for more research to explore the role of circulating mtDNA as a biomarker for RA. While, the studies demonstrate the biomarker potential of cfDNA in RA, longitudinal studies with large cohorts of patients are needed to capture the dynamics of cfDNA in RA with disease progression and drug effects.

## COMPARISON OF cfDNA OBSERVATIONS BETWEEN SLE AND RA

Considering the many technical and sampling variations in different studies, direct comparisons of cfDNA levels between SLE and RA patients can only be made from studies where both patient cohorts were analyzed simultaneously. In general, SLE patients have higher concentrations of sera cfDNA (ssDNA) along with higher positivity for anti-ssDNA antibodies as compared to patients with RA (4). The association of cfDNA levels with serological parameters in both diseases, e.g., anti-dsDNA in SLE (154, 160) and ACPA and RF in RA (148, 155, 162), suggest that cfDNA may reflect common processes involved in both diseases, including inflammation and cell death. RA patients exhibit increased levels of mtDNA in plasma and SF (155, 162). Given recent studies implicating mitochondrial extrusion in the SLE pathogenesis (38, 88), we expect upcoming studies to demonstrate increased mtDNA levels also in SLE patients. cfDNA from both RA and SLE exhibit increased oxidation (125, 155), possibly a consequence of inflammation. Contrasting dynamics of cfDNA was observed with treatment for SLE and RA with cfDNA levels decreasing in SLE patients upon treatment (158, 160). In RA, however, cfDNA levels initially increase, where after they decrease at later time-points (163, 164). Finally, cfDNA levels associate with markers of disease activity

and inflammation in both RA and SLE (2, 4, 42, 148–151, 153, 156–158, 161).

## RECENT ADVANCES IN ANALYSIS OF cfDNA IN OTHER MEDICAL FIELDS

cfDNA is not only used for chronic inflammatory rheumatic diseases, but also in several other conditions, including non-invasive prenatal testing to detect fetal aneuploidies (179), cancers (180), as an early marker of allograft rejection and graft damage (181) and for tracking microbial infection, including the detection of oncogenic viral DNA (182). Methodological advancements in the analysis of cfDNA are comprehensively addressed in recent reviews (183, 184). In brief, emerging targeted approaches to screen for specific mutations of cfDNA include; digital droplet PCR (ddPCR), a highly sensitive method that allows the identification of rare targets based on the partitioning of samples into water-into-oil droplets; BEAMing (beads, emulsification, amplification, and magnetics) a combination of emulsion PCR and flow cytometry to achieve higher sensitivity; next generation sequencing (NGS), a powerful technique that allows the screening of both targeted and untargeted mutations; and methylated CpG tandem amplification and sequencing (MCTA-Seq) to identify genome-wide hypermethylated CpG regions. Other cutting-edge technologies that allow personalized therapies include targeted plasma re-sequencing (TAm-Seq) and personalized analysis of rearranged ends (PARE) which is based on the identification of disease-specific somatic rearrangements. Fluorescence correlation spectroscopy (FCS), a method based on the fluctuations of fluorescence due to the Brownian movement of fluorescence molecules, allows a rapid and sensitive detection of single molecules, including the size determination of DNA (185). FCS can effectively complement existing methods of cfDNA detection in autoimmune diseases.

## CLOSING REMARKS AND FUTURE DIRECTIONS

The relevance of DNA to the disease pathology of lupus is undisputed and, there is an increasing attention in RA as well. In contrast to non-specific markers of inflammation, cfDNA can be pathologically relevant to autoimmune rheumatic diseases given the role of DNA-sensing receptors in inflammation and autoimmunity. cfDNA allows a rapid, easy, non-invasive and repetitive method of sampling. A combination of these biological features and technical feasibility of sampling, position cfDNA as a potential biomarker of enormous utility for autoimmune rheumatic diseases. However, there are many issues that needs to be addressed toward this goal. It should be acknowledged that the underlying heterogeneity of autoimmune disease by itself, can contribute to a considerable amount of variation in the levels of cfDNA, and hence adequate measures must be taken to minimize the variations at the level of cfDNA sampling. Notably, there is a lack of uniformity on the type of sample (plasma/serum/synovial fluid), methods of sample collection/processing, free or cell-surface bound DNA, cfDNA extraction and cfDNA quantification, and also in the presentation and interpretation of quantitative cfDNA findings. Additional, complexity is brought by the advent of qualitative research of cfDNA, which needs to be standardized as well. Given this lack of homogeneity, it is not surprising that consensus is yet to be reached on cfDNA levels in healthy individuals. Further, the majority of studies have been cross-sectional, and were limited by sample sizes. However, in order to fully understand the biomarker potential of cfDNA in autoimmune rheumatic diseases, a systematic scientific framework with collaborative efforts is needed to conduct large, multicenter trials with prospective analyses.

## AUTHOR CONTRIBUTIONS

BD and CL conceptualized the manuscript idea. BD conducted the literature search and drafted the manuscript. CL provided critical revisions. Both BD and CL edited the manuscript.

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REVIEW
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# Sources of Pathogenic Nucleic Acids in Systemic Lupus Erythematosus

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A hallmark of systemic lupus erythematosus (SLE), and several related autoimmune diseases, is the presence of autoantibodies against nucleic acids and nucleic acid-binding proteins, as well as elevated type I interferons (IFNs), which appear to be instrumental in disease pathogenesis. Here we discuss the sources and proposed mechanisms by which a range of cellular RNA and DNA species can become pathogenic and trigger the nucleic acid sensors that drive type I interferon production. Potentially SLE-promoting DNA may originate from pieces of chromatin, from mitochondria, or from reverse-transcribed cellular RNA, while pathogenic RNA may arise from mis-localized, mis-processed, ancient retroviral, or transposable element-derived transcripts. These nucleic acids may leak out from dying cells to be internalized and reacted to by immune cells or they may be generated and remain to be sensed intracellularly in immune or non-immune cells. The presence of aberrant DNA or RNA is normally counteracted by effective counter-mechanisms, the loss of which result in a serious type I IFN-driven disease called Aicardi-Goutières Syndrome. However, in SLE it remains unclear which mechanisms are most critical in precipitating disease: aberrant RNA or DNA, overly sensitive sensor mechanisms, or faulty counter-acting defenses. We propose that the clinical heterogeneity of SLE may be reflected, in part, by heterogeneity in which pathogenic nucleic acid molecules are present and which sensors and pathways they trigger in individual patients. Elucidation of these events may result in the recognition of distinct "endotypes" of SLE, each with its distinct therapeutic choices.

**Keywords: lupus, interferon, nucleic acid sensors, mitochondria, reverse transcriptase**

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## INTRODUCTION

Systemic lupus erythematosus (SLE) is a serious autoimmune disease characterized by autoantibodies against nucleic acids and nucleic acid-binding proteins combined with immune complex deposition and inflammatory manifestations in multiple organ systems. The unpredictable course of the disease with its sudden exacerbations, often with new organ manifestations or symptoms, make it particularly difficult to manage (1, 2), not the least because the currently available drugs have limited efficacy and/or serious side-effects. Efforts to develop more selective and more efficacious therapies that address the core pathobiology of SLE, ideally with limited general immune suppression, continue to be hampered by our limited understanding of the underlying molecular drivers and mechanisms (3). To vividly illustrate this, the two newest therapeutics for SLE are hydroxychloroquine (4, 5) and belimumab (6, 7), approved by the FDA in 1966 (sic!) and 2011, respectively. Moreover, the latter had barely significant efficacy, only 9.8% SLE Responder Index improvement over placebo at 52 weeks at the highest 10 mg/kg dose (6).

Furthermore, while the presence of autoantibodies in SLE has been recognized for decades and their role in driving disease is considered well established, B cell depletion by anti-CD20 antibodies have failed to generate statistically significant efficacy in clinical trials in SLE (8). There is, however, a trend toward a benefit for patients in agreement with the ability of belimumab to reduce B cell numbers. It should also be noted that belimumab may affect plasma cells more than the depletion of CD20-positive B cells. T cell-directed therapies, such as calcineurin inhibitors (9) or CD28 blockade with CTLA4-Ig (10), have also yielded limited disease impact. These outcomes suggest that many of the well-documented immune abnormalities in SLE may be consequences, rather than drivers, of this disease.

## AUTOANTIBODIES AGAINST NUCLEIC ACIDS AND NUCLEIC ACID-BINDING PROTEINS

In SLE, the majority of patients develop autoimmunity toward nuclear antigens, conveniently measured as anti-nuclear autoantibodies (ANA). Though not selective for SLE, detecting ANA is a common test used to screen patients, and may, together with clinical presentation and other immunological features, suffice for SLE diagnosis. ANA contains a broad range of autoantibodies targeting among others chromatin, histones, double-stranded (ds) DNA, as well as the RNA-binding proteins Ro, La, Sm, and RNP. Anti-dsDNA antibodies are of particular interest in SLE, given their high diagnostic potential, with about 70–80% of the patients being positive for these antibodies, and titers commonly correlating with disease activity. Indeed, anti-dsDNA antibodies have been included in the classification criteria (11), as well as a serological component of the disease activity index SLEDAI (12). Further, anti-dsDNA antibodies are often associated with severe disease manifestations, including nephritis. Other than the diagnostic value, including associations with distinct disease features, these autoantibodies may be pathogenic through immune complex-mediated inflammation, complement activation and tissue destruction, and antibody-directed cellular cytotoxicity. In this review, we will limit our discussion of autoantibodies to their ability to transport nucleic acids, shielding them from external nucleases, and efficiently mediating their uptake into immune cells through Fc receptors, complement receptors, scavenger receptors, and others.

## THE "IFN SIGNATURE" IN SLE PATIENTS

In 2003, Tim Behrens' group (13), the team of Virginia Pascual and Jacques Banchereau (14), and Mary Crow (15) published their discovery that SLE patient blood contain active type I interferon (IFN) and a high expression level of IFN-stimulated genes (ISGs), now referred to as the "IFN signature." Although indications that IFNα may be important in the lupus pathogenesis had been published earlier (16–18), this still was a surprising finding because the principal function of type I IFN is in host defense against viral infection, while SLE is not an infectious disease. Nevertheless, the IFN signature is now a well-established observation in 70–90% of SLE patient populations world-wide (19–22). Individual IFNs are technically difficult to measure (23) due to their very low concentrations and presumed rapid consumption, but it seems that many of the 17 different type I IFNs, which includes 13 IFNα isoforms, IFNβ, and the three less explored members, IFNε, IFNκ, and IFNω are elevated in SLE patients, as well as in patients with Sjögren's syndrome (24, 25), systemic sclerosis (26, 27), polymyositis, dermatomyositis (28, 29), rheumatoid arthritis (30, 31), and other related diseases. Importantly, there seems to be differences between patients in which specific members of the type I IFN family are elevated (see sections Patient heterogeneity with regard to nucleic acids and their sensors? and Can SLE be divided into clinically meaningful subpopulations based on "endotype"?). In addition, patients may have increased type II IFN (IFNγ) (25) and/or type III IFNs (IFNλ1, IFNλ2, and IFNλ3, also known as IL-29, IL-28A, and IL-28B) (32). While the type I and III IFNs are functionally overlapping (all genes induced by type III IFNs are also induced by type I IFNs), IFNγ is instrumental in a distinct aspect of the immune system, namely the activation of CD4 Th1 and CD8 T cells, natural killer (NK) cells, and other elements of a general immune response. Nevertheless, over 900 of the 1,300 ISGs induced by IFNγ are also induced by type I IFNs, which induces a total of over 1,500 ISGs, suggesting significant overlap in downstream consequences.

Type I IFNs have a spectrum of effects on the immune system and beyond, particularly upregulating numerous mechanisms of on anti-viral defense. They stimulate emergency myelopoiesis (33), monocyte differentiation into myeloid dendritic cells (34, 35), antigen presentation, cytotoxic T cell differentiation (36), and B cell differentiation into plasma cells (37). The 1,500 ISGs encode many immune-modulating as well as direct antiviral proteins (38), including many components of the pathways that lead to type I IFN production in what constitutes a rapid positive feedback loop to augment the response.

While an extensive literature illuminates the close association of type I IFNs with SLE pathogenesis and disease activity (21, 39), perhaps the most conclusive evidence for a causal role in the disease was the statistically significant efficacy in phase 2 clinical trials (40) of an antibody that blocks the type I IFN receptor used by all type I IFNs. In contrast, an antibody that blocks IFNα alone (41) was efficacious only in a small subset of patients. It should also be noted that blocking the type I receptor did not bring clinical improvement to all SLE patients even if the IFN signature declined by over 90% in the treated patient population. Nevertheless, elevated type I IFNs are the closest we have to a smoking gun in SLE and a set of related autoimmune diseases. This, in turn, begs the question: why are type I IFNs elevated in SLE patients?

## NUCLEIC ACID SENSORS COUPLED TO INTERFERON PRODUCTION

Given that the best recognized role of type I IFN is in defense against viral infection (38), it seems that one could find important

clues about the upstream mechanisms of SLE from recent advances in viral immunity. The primary threat that a virus brings is its RNA or DNA genome, which will hijack the cellular biosynthetic machinery for its own replication and virion production, with detrimental consequences for the host cell. Even more alarming, retroviruses will reverse transcribe their RNA genome and insert the resulting DNA into the host genome as a permanent provirus. To combat these ancient foes, evolution has produced several cellular mechanisms for the detection of non-self RNA and DNA (**Figure 1**). Four principal pathways operate in the cytosol and on the surface of intracellular organelles: the DNA-sensor "cyclic GMP, AMP synthase" (cGAS) (42), the RNA sensors "retinoic acid-inducible gene I" (RIG-I) (43), "melanoma differentiation-associated gene 5" (MDA5) (43–45), and "RNA-activated protein kinase" (PKR) (46, 47), while a fifth pathway responds to extracellular DNA or RNA brought into the cell by receptor-mediated endocytosis and is initiated by Toll-like receptors (TLRs) 3, 7, 8, and 9 in the endosomal compartment. A mechanism to blend the extracellular and intracellular sensing pathways was recently reported (48): the transporter protein SIDT2 in the endosomal membrane functions to let dsRNA escape the endosome into the cytosol, where it can trigger MDA5. There are additional, more recently discovered nucleic acid sensors, such as DDX1, 21, 36, and 41, IFI16, and Aim2 (49). All of these pathways lead to type I IFN production through activation of IRF3 and related transcription factors. They also activate other signaling pathways that lead to the production of additional cytokines. The resulting type I IFNs are secreted, bind to the type I IFN receptor, and signal through the JAK/STAT pathways to upregulate ISG-encoded proteins with direct anti-viral activity, including nucleases, helicases, chaperones, and many of the sensors and their adapters and signaling proteins (38). Type I IFN can act in both autocrine and paracrine fashion and the response to them may differ between different responding cell types.

Whereas, nucleic acids are the main triggers of type I IFN production, the cell type producing them and the exact nature of the triggering nucleic acid will determine which type I IFNs are produced. For example, plasmacytoid dendritic cells (pDC) have a particularly high capacity to produce several isoforms of IFNα in response to viruses or immune complexes that contain nucleic acids (50–52), including those containing IgE (52), by a TLR7 or 9-dependent mechanism. Non-immune cells, on the other hand, tend to produce predominantly IFNβ in response to cytosolic RNA or DNA through the sensors MDA5 (dsRNA), RIG-I (RNA), and cGAS (dsDNA), with other sensors participating, particularly in neutrophils that do not express cGAS (53).

## TLRs in SLE

TLRs are central to the immune system's ability to recognize molecular structures associated with cellular damage or pathogens (54), including nucleic acids by TLRs 3, 7, 8, and 9 (**Figure 1A**). Since their discovery over 20 years ago, much of the early literature assumed that their role in SLE was certain (55–58), particularly since their ligation triggers type I IFN production and circulating immune complexes that contain nucleic acids are present in abundance in most SLE patients (59), as well as in the patients with related diseases like Sjögren's syndrome, polymyositis, dermatomyositis, mixed connective tissue disease, and others. It still seems very likely that these immune complexes drive production of IFNα by plasmacytoid dendritic cells (pDC) primarily through activation of TLR7 and maybe 9 (60). However, so far all tested antagonists of TLR 7 and/or 9 (61) have failed to provide any efficacy in placebo-controlled clinical trials in SLE patients. If type I IFNs indeed are important, but TLR7 and 9 inhibition does not produce a therapeutic benefit, then reality must be more complex. Indeed, the more recent discovery of other sensors for nucleic acid, such as cGAS, RIG-I, and MDA5 introduced other options for nucleic acid sensing leading to type I IFN. Nevertheless, it still seems likely that TLR7/9 drive IFN production in response to circulating immune complexes that contain nucleic acid and thereby contribute to the IFN signature seen in SLE patients. Unfortunately, clinical trials with TLR antagonists did not report what effects these drugs had on the IFN signature.

Recent advances in TLR research has revealed intriguing new details about the mechanisms of their ligand interactions, including their ability to bind self-nucleic acids (62–66). While TLR9 was originally proposed to only sense bacterial DNA with CpG sites, it is now clear that it can also recognize chromosomal and mitochondrial DNA (digested into small fragment by DNase II). Similarly, TLR3 responds to self-derived non-coding RNA, such as U1 RNA that might be released upon cellular stress, including exposure to UV radiation, while TLR7 and 8 can also recognize RNA and DNA degradation products (66). Another recent study found that phagocytosis of anti-dsDNA IgE antibodies (found to be increased in some SLE patients) via the high-affinity Fc$\varepsilon$RI receptor for IgE, mediates TLR9-mediated sensing of self-DNA in the phagosomes and potentiates IFN production by plasmacytoid dendritic cells (52).

Another potentially important aspect of the TLR pathways is that the *TLR7* and *TLR8* genes are located on the X-chromosome: there are indications that *TLR7* may escape the normal silencing of one of the two X chromosomes in females (67), resulting in higher levels of TLR7 expression and, hence, stronger responses to TLR7 simulation in immune cells in women, perhaps contributing to the 9:1 gender bias in SLE. In further support of a role of TLR7 quantity in the disease, copy number variations (68, 69) and single-gene polymorphisms (70) in *TLR7* are associated with SLE susceptibility.

## Activation of cGAS and RNA Sensors in SLE

A recent paper provided the first direct evidence that the cGAS pathway is activated in at least a subset of SLE patients: the second messenger cyclic-guanine, adenosine-2,3-phosphate (cGAMP), which is synthesized exclusively by cGAS upon DNA binding, was detected by mass spectrometry in 7 of 30 SLE patients (71). While it may seem that this represents a small portion of SLE patients, it is important to recognize that the data represent a single snap-shot in time and that cGAMP is a short-lived second messenger present in minute quantities. Thus, it may well be that cGAMP is elevated in more SLE patients.

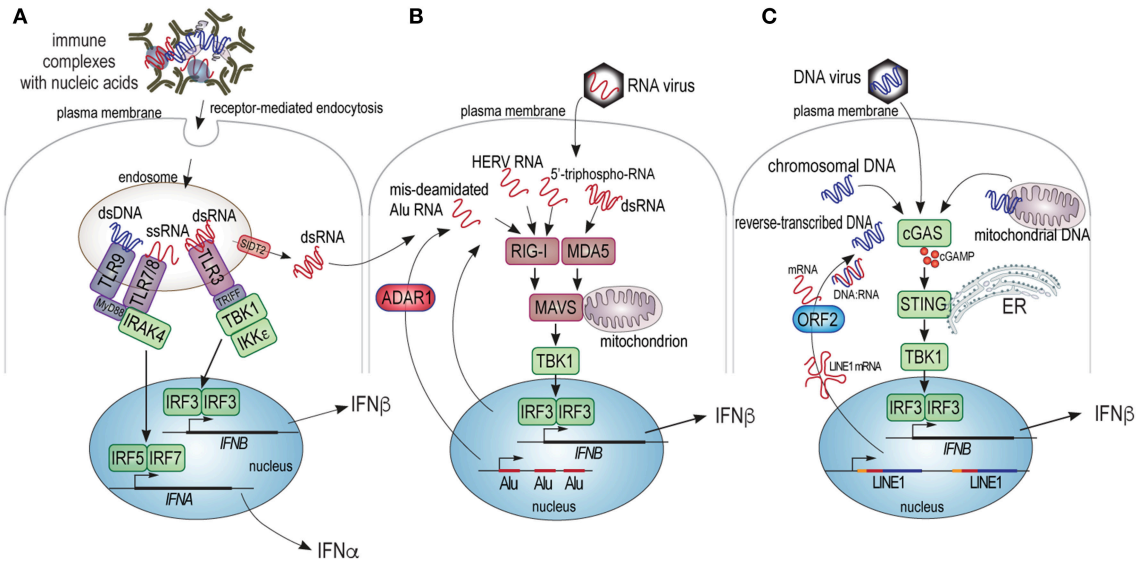


**FIGURE 1 |** Schematic illustration of the cellular sensors of pathogenic DNA and RNA and their signaling pathways leading to type I IFN production. **(A)** Extracellular nucleic acids present in immune complexes, free mitochondria, or other structures can be internalized into cells by receptor mediated endocytosis and trafficked to endosomes that contain TLR3, 7, 8, or 9, which recognize dsRNA, ssRNA, and dsDNA, respectively. TLR3 signals through the TRIFF adapter and the protein kinases TBK1 and IKKε, which phosphorylate and activate the IRF3 transcription factor, which (with co-factors) transactivates the gene for IFNβ. TLR7, 8, and 9 signal through the MyD88 adapter and the IRAK4 protein kinase to primarily phosphorylate and activate transcription factors IRF5 and IRF7, which participate in the transactivation of some or all of the 13 genes for isoforms of IFNα. Finally, the SIDT2 transporter in the endosome membrane can mediate the exit of dsRNA into the cytosol of the cell to be sensed by MDA5. **(B)** Cytosolic RNA from exogenous viruses, or endogenous transcripts improperly deaminated by ADAR1, or containing recognizable retroviral motifs (HERV RNA), or potentially other aberrant RNA species can trigger RIG-I or MDA5, which principally bind ssRNA and dsRNA, respectively. Upon ligand binding RIG-I or MDA5 trigger the oligomerization of the MAVS protein, which assembles a protein complex on the mitochondrial membrane, resulting in activation of the TBK1 protein kinase, which activates IRF3. **(C)** Cytosolic DNA from exogenous viruses, pieces of chromatin, mitochondrial DNA, or reverse-transcribed RNA, triggers dimerization and activation of cGAS leading to the synthesis of cGAMP, which activates the STING adapter on the surface of the endoplasmic reticulum (ER). STING, in turn, activates the TBK kinase, which activates IRF3.

Direct evidence for activation of RNA sensors in SLE patients was also reported recently (72). Twenty two of sixty-seven examined SLE patients had evidence of polymerization of the mitochondrial antiviral signaling protein MAVS, which is downstream of both RIG-I and MDA5 (**Figure 1B**) and acts by generating a protein complex that activates the kinases required for IRF3 activation and type I IFN production. This aggregation of MAVS indicates that either RNA sensor was triggered in 32% of the patients.

## AICARDI-GOUTIÈRES SYNDROME–A MONOGENIC DISEASE OF NUCLEIC ACID PROCESSING

Additional insights into the molecular mechanisms that can drive type I interferons and cause interferon-dependent human disease come from a monogenic inherited inflammatory syndrome called Aicardi-Goutières syndrome (AGS) (73–77), which, together with a few related diseases, is included in the concept of the "type I interferonopathies" (78). AGS usually presents neonatally as a suspected serious viral infection with fever, chills, and a failure to thrive, accompanied by high levels of type I IFNs. However, a virus is not detected and the symptoms continue unabated. Over time, AGS patients develop neurological deficits and brain calcifications, likely due to the neurotoxicity of IFNs, as well as systemic autoimmunity with autoantibodies against nucleic acids and nucleic acid-binding proteins very similar to those in SLE patients. In fact, many AGS patients meet the diagnostic criteria for SLE (73–77).

AGS is a caused by mutations in any one of eight genes: *TREX1*, *RNASEH2A, RNASEH2B, RNASEH2C*, *SAMHD1*, *ADAR1*, *IFIH1*, *TMEM173* (73–77). The first 5 of these genes are primarily involved in the defense against retroviruses and their endogenous remnants in our genome (79). In fact, many of these genes were first discovered as "restriction factors" by researchers studying how HIV replicates in certain cells, but not in others. The revelation that our genome contains many evolutionarily conserved genes that confer resistance to HIV suggested that HIV is not the first exogenous retrovirus to infect us, but, in fact, is just the latest in a very long series of retroviral infections resulting in germline integrations of numerus families of retroviruses that today constitute as much as 8% of our genome (or as much as ∼40% if other retroelements of ancient retroviral origin are also counted).

The three other genes that can induce AGS are homozygous loss-of-function mutations in the gene for "adenosine deaminase acting on RNA 1" (ADAR1), *IFIH1* which encodes MDA5, and the gain-of-function variant of *TMEM173* which encodes constitutively active STING (the direct effector protein for

cGAS, **Figure 1C**) and results in the constitutive activation of this pathway in the absence of aberrant DNA (80). The two latter genes demonstrate that chronic activation of RNA sensing (MDA5) or DNA (cGAS/STING) leads to an SLE-like condition in humans.

## SOURCES OF TYPE I IFN-TRIGGERING DNA AND RNA

Since type I IFNs are elevated in most SLE patients and appear to play an important role in SLE pathogenesis and since perturbations in nucleic sensing pathways lead to a disease (i.e., AGS) characterized by chronically elevated type I IFN and autoimmunity with many of the same autoantibodies as in SLE, it seems logical to ask if sensor-triggering nucleic acids might be present in SLE patients. Alternatively, the function or regulation of one or several sensors might be faulty. Although evidence exists for the association of genetic variants of DNA and RNA sensors with SLE, and mutations in them can cause type I interferonopathies (80), such mutations are present in a very small subset of SLE patients. Hence, it would be important to elucidate which nucleic acids are aberrantly present in SLE patient. What is their nature and origin?

Although viruses have long been suspected to play some role in triggering several different autoimmune diseases, there is little evidence for a persistent presence of viral RNA or DNA in SLE patients. If aberrant nucleic acids are present in SLE patients to trigger the DNA and/or RNA sensors discussed above, they likely are derived from endogenous sources, such as chromosomal DNA, mitochondrial DNA, DNA made by reverse-transcription from RNA templates, RNA transcripts from normally silent loci of ancient viral origin (that still somehow resemble viral RNA), mis-edited RNA, or otherwise altered or improperly processed RNA molecules (**Figure 1**). We will discuss these potential sources one by one.

### Chromosomal DNA

While chromosomal DNA normally is well protected by myriad binding proteins and a highly ordered packing into nucleosomes and higher order structures, DNA damage or faulty DNA replication can, in principle, dislodge smaller pieces of DNA, for example as nuclear blebs or micronuclei found in cancers (81). The existence of several effective DNA repair mechanisms indicate that DNA damage does occur in cells for a variety of reasons, including during normal aging. It is conceivable that DNA damage could produce pieces of DNA that trigger cGAS and subsequent type I IFN production (82, 83). Loss of DNA degradation by DNase1L3 causes an autosomal recessive form of SLE with early life onset and high prevalence of nephritis (84), and loss of the Trex1 DNase (85, 86) also leads to constitutive type I production and SLE or AGS, indicating that rapid elimination of aberrant DNA is important for the maintenance of health.

Cell death, whether by physiological programmed cell death mechanisms, such as apoptosis, or, more likely, by more pathological, inflammatory, or toxic mechanisms like necrosis, pyroptosis, or necroptosis, can result in the release of chromosomal DNA (and RNA) into the extracellular milieu (87, 88). Many protective processes have evolved to minimize this exposure to chromatin and the highly toxic histones (89). Apoptotic cells are recognized by specific receptors for phosphatidylserine, annexin V, and other molecules that serve to mark apoptotic cells to facilitate their rapid removal by tissue macrophages (90) and the reticuloendothelial system. When these mechanisms are faulty or overwhelmed by massive numbers of dying cells, anti-nuclear and nucleic acid-directed autoantibodies and autoimmune disease may develop (87). For example, severe viral infections that are accompanied by immune-mediated killing of large numbers of infected cells typically result in measurable titers of anti-nucleic acid autoantibodies in otherwise healthy individuals. However, these titers tend to be relatively modest and they decline after the infections is cleared. The complement system (91), particularly C1q, also participates in the non-inflammatory removal of dying cells, perhaps explaining why complement deficiencies predispose to SLE (92). C1q also influences type I IFN production by a more direct mechanism (93, 94).

### Mitochondrial DNA

Another source of nucleic acids are the mitochondria (95, 96), which serve many functions besides oxidative phosphorylation and production of ATP, such as metabolism, inflammation and cell death. Though mainly found intracellularly, we recently discovered that neutrophils can extrude mitochondria together with chromosomal DNA during the formation of neutrophil extracellular traps (NETs) (95, 97). The externalization of mitochondria depended on the generation of reactive oxygen species (ROS) and the extruded mitochondria contained highly oxidized (8-OHdG) mitochondrial DNA, inducing IFNβ generation in a process requiring the intracellular DNA sensor adaptor protein, STING (95). Blocking mitochondrial ROS generation *in vivo* ameliorated lupus-like disease in MRL/*lpr* mice (95). These observations appear to be clinically relevant as *ex vivo* neutrophils from SLE patients displayed ongoing mitochondrial ROS production and spontaneous extrusion of oxidized inflammatory mitochondrial DNA (95). Similar to NET formation, as described above, other forms of cell death, such as TNF-mediated necroptosis, have been shown to involve the release of intact mitochondria into the extracellular environment (98, 99). Though the intracellular source(s) of extruded DNA has yet to be verified in other forms of cell death, e.g., pyroptosis, we find it likely that any form of cell death that includes breakdown of the plasma membrane will result in the release of mitochondria or their components, such as mitochondrial DNA.

Mitochondria and mitochondrial DNA may also be released from live cells upon their activation, as shown in neutrophils, eosinophils, mast cells and platelets (100–103). In neutrophils, this process has been coined "vital" NETosis, as the neutrophil remains alive after the extrusion event. Circulating platelets are thought to be the primary source of cell-free mitochondria given the large abundance of platelets in blood. Work from the laboratory of Eric Boilard has demonstrated that platelets, upon activation, may extrude naked mitochondria able to undergo respiratory burst (103). Unless these mitochondria

are rapidly cleared, secreted phospholipase A2 will hydrolyze and weaken the mitochondrial membrane, causing disruption and the release of inflammatory mitochondrial DNA and other damage-associated molecules (103). Platelet-mediated extrusion of mitochondria can occur in concentrated platelet preparations and is associated with adverse reactions upon transfusions (103, 104). Mitochondria can also be released as part of microparticles from many different cells, including platelets, neuronal and glial cells, as well as hepatocytes (103, 105, 106). The role of platelet-mediated release of mitochondrial DNA is of particular interest in rheumatic disease, including SLE, given the marked platelet activation and subsequent development of cardiovascular morbidity and mortality observed in these patients (107–110).

While the role of mitochondrial extrusion and mitochondrial DNA (oxidized or not) in SLE remains to be clarified, it is clear that they can be derived from many different cells and involve either the activation or death of these cells. It also appears that the DNA sensors TLR9 (105, 111–113) and cGAS (95, 114, 115) can be triggered by mitochondrial DNA, presumably depending on its subcellular location or pathway of receptor-mediated internalization (**Figure 1**). Elevated amounts of free mitochondrial DNA have been observed in several conditions, including chronic inflammatory diseases, trauma, cardiovascular disease and rheumatoid arthritis, perhaps promoting inflammation and even mortality (103, 116–118). Further studies to elucidate the mechanisms by which extruded mitochondria and/or mitochondrial DNA are cleared will be important for our understanding of this biology and for the design of therapeutic regimens to prevent the contribution of mitochondria and/or their DNA to human autoimmunity.

## Reverse-Transcribed RNA

The third source of DNA that may trigger type I IFN synthesis is intracellular DNA made by the reverse transcription of cellular RNA. Our genome encodes three different families of reverse transcriptases (RTs): telomerase (*TERT*), the *pol* genes of many endogenous retroviruses, and the second open-reading frame (ORF2) of the long interspersed nuclear element-1 (LINE1). Of these enzymes, telomerase is highly specialized to synthesize TTAGGG repeats in the 194 telomeres of our diploid chromosomes using the *TERC* RNA template (119, 120), while retroviral RTs only function to convert the RNA genome of an incoming retrovirus to a DNA provirus and to insert it into the genome. Although our genome contains thousands of endogenous retroviral provirus loci, none of them appear to be infectious anymore (with the possible exception of HERV-K113). This leaves only the LINE1 ORF2 enzyme as a candidate RT capable of generating aberrant DNA that could trigger type I IFN production through cGAS activation. It has been demonstrated to have robust RT activity (121–123), which is key for retrotransposition (124) and which is sensitive to some clinically used RT inhibitors (125, 126).

The LINE1 element represents a remnant of an ancient retrovirus that retained, or later acquired, a degree of autonomy through the conservation of a primordial RT, which endows it with the ability to transpose by a "copy and paste" mechanism. The LINE1 RNA transcript is 6 kb long and contains two open-reading frames (79): ORF1, which encodes a 40-kDa RNA-binding protein that co-localizes with LINE1 mRNA in stress granules together with other RNA-binding proteins (127), such as Ro60, La, and U1 Small nuclear ribonucleoprotein of 70 kDa (127), and ORF2, which encodes a 150-kDa RT and endonuclease. Attesting to the effectiveness of the LINE1 element's ability to transpose, there are over half a million copies of it throughout our genome. However, due to defense mechanisms (including those encoded by the AGS genes) and mutational rate, the vast majority of these copies are truncated and mutated and no longer have the ability to transpose. It has been estimated that < 180 LINE1 copies are seemingly intact, but that only 5 or 6 of them are active ("hot") today (128). The LINE1 ORF2-encoded RT is also involved in generating and transposing Alu element copies (129) and was, over evolutionary time, responsible for generating all of our processed pseudogenes (130). In other words, the LINE1-encoded RT has had a profound impact on our genome and our health.

The study of AGS revealed that transcription of retroelement loci is very low in healthy individuals, but that AGS patients have elevated levels of retroelement mRNAs and proteins, including enzymatically active LINE1 RT (79). In fact, LINE1 RT may be the main producer of pathogenic DNA that triggers type I IFN production (131) in AGS patients with *TREX1*, *RNASEH2A, RNASEH2B, RNASEH2C*, and *SAMHD1* mutations. TREX1 is the DNase that degrades intracellular DNA made by reverse transcription (86, 132), including DNA in complex with RNA as occurs during reverse transcription, while RNaseH2 preferentially acts on the RNA in such heteroduplexes (77). Finally, SAMHD1 is a phosphohydrolase specific for the deoxynucleotide triphosphates (dATP, dTTP, dGTP, and dCTP) required for reverse transcription (133). In a mouse model of AGS, the *Trex1*$^{-/-}$ mouse (86), the animals develop a systemic inflammation with immune cell infiltrates in many organs and they die early from a severe carditis. These animals can be rescued from death by treatment with the RT inhibitors tenofovir plus nevirapine (134), indicating that reverse transcription is a key step in the pathogenesis of systemic inflammation in this model. However, there is also a published paper refuting these data (135). A human clinical trial with RT inhibitors in AGS is under way.

There is some evidence that LINE1 retroelements are activated also in SLE patients (136–138). This appears to correlate with a global decrease in DNA methylation, which is well documented in SLE (139, 140) and likely relates to the decreased expression of DNA methylases DNMT1 and DNMT3a (141, 142). Demethylating agents like 5-aza-2′deoxycytidine (143) also cause a dramatic upregulation of LINE1 and Alu element transcription in lymphocytes (144). In addition, transfer of 5-aza-2′deoxycytidine-treated T cells into healthy mice results in an SLE-like disease (145). The drugs that can induce "drug-induced lupus," notably hydralazine and procainamide, are demethylating agents (146). Other known triggers of lupus flares, like UV light, oxidative stress, inflammation and exogenous viruses also induce genomic hypomethylation (147, 148).

We are aware of only two papers that report the detection of LINE1-encoded ORF1 and ORF2 proteins in samples from patients with SLE or related diseases. Mavgrani and co-workers

(136) showed by immunoblotting and immunohistochemistry that p40/ORF1 was readily detectable in kidney samples from lupus nephritis patients and in salivary gland biopsies of Sjögren's patients. Staining correlated with IFNβ in somatic cells and IFNα in infiltrating plasmacytoid dendritic cells. As activation of LINE1 elements in autoimmune patients (136–138) appears to involve demethylation of the LINE1 promoter (136, 138, 140), these authors also analyzed the methylation of CpG sites in the LINE1 promoter and found it to be reduced in patients with elevated LINE1 expression. In the second paper, Kalogirou et al. demonstrated that ORF2 is upregulated in the ductal cells of salivary gland biopsies from patients with Sjögren's syndrome (149).

## Cellular RNAs and RNA Editing by ADAR1

Many viruses have an RNA genome and do not (unlike the retroviruses) generate any DNA. A set of cellular RNA sensors have evolved to detect these viruses (150) (**Figure 1B**), a challenging task given the abundance of cellular RNA species. It remains incompletely understood how these sensors can distinguish between self and foreign RNA molecules, but the length of double-stranded RNAs (150) and capping modifications of the 5′ and 3′ ends of RNA molecules (151), as well as the presence or absence of other types of RNA processing, appear to matter. The delicate balance between the recognition of self- vs. foreign RNA is well illustrated by the *IFIH1*-A947T allele, which encodes a variant of MDA5 that enhances anti-viral immunity, but increases the risk of autoimmunity (152, 153).

Extracellular RNA, for example in immune complexes with the Ro protein (154), can also enter immune cells via receptor-mediated endocytosis followed by trafficking to the endosomal compartment where TLR3 will react to dsRNA and TLR7 and 8 with single-stranded RNA (as well as other ligands, see section TLRs in SLE). From this compartment, dsRNA may also exit into the cytosol through the SIDT2 channel (48) to trigger cytosolic MDA5. This pathway likely exists to aid in the detection of RNA viruses that are captured by antibodies, complement, or scavenger receptors.

The role of ADAR1 is also very interesting as this enzyme is involved in the post-transcriptional editing of mRNAs by converting adenosine to inosine, which is read as a guanosine during translation. Interestingly, in humans (unlike other organisms) the majority of the deaminated adenosines are non-coding and located in RNA molecules derived from Alu elements and other transposable sequences (155). The induction of type I IFN by mutated ADAR1 is dependent on MDA5, but not on RIG-I, suggesting that RNA editing is important and that its absence triggers the MDA5 pathway as if viral dsRNA was present.

## Endogenous Retroviral RNA

While exogenous viruses introduce RNA (or DNA) molecules that can be recognized as foreign by cellular sensors, it remains doubtful that RNA transcripts from endogenous proviruses (which constitute as much as 8% of our genome) would be seen as foreign as they are transcribed and processed by the normal cellular machinery. Nevertheless, since these sequences are of viral origin, it is possible that some of them still contain sequence motifs that allow cellular RNA sensors to recognize them as non-self. If so, one would expect the relevant loci to be among the most recently incorporated ones, which may not yet have accumulated domesticating mutations. Furthermore, since most people do not develop autoimmunity, one would also assume that they normally are effectively silenced in healthy individuals, but perhaps aberrantly expressed in patients with SLE or related diseases.

## Transposable Element RNA

A much more interesting category of RNAs in autoimmunity research are those encoded by Alu elements and other short transposable elements, not perhaps because of their origin, but because they have been experimentally implicated in several settings. An important paper in this respect reported that a large portion of all RNA present in circulating anti-Ro autoantibody immunocomplexes was Alu element RNA (154). In fact, other SLE autoantigens, such as La (156), also bind Alu RNA. Furthermore, $Ro^{-/-}$ mice (157) develop autoimmunity resembling SLE, suggesting that the normal function of Ro is important for preventing the Alu element RNA, and perhaps other cellular RNA molecules (158, 159), from triggering RNA sensors. The discovery that the RNA-editing enzyme ADAR1 primarily edits Alu transcripts in humans (155) and that the LINE-1 encoded RT has catalyzed the reverse transcription and genomic insertion of over a million copies of the Alu element in our genome, as well as the co-localization of LINE1 proteins with Ro and La, hints at a central, but still enigmatic, role of this RNA biology in SLE pathogenesis. It also remains unknown how Ro-Alu RNA complexes end up in the extracellular compartment, but one can suspect that cell death by several programmed mechanisms must be involved.

# PATIENT HETEROGENEITY WITH REGARD TO NUCLEIC ACIDS AND THEIR SENSORS?

While it seems likely that type I IFNs play an important role in the pathogenesis of SLE and related diseases, it also becoming clear that their inhibition is not a cure for all patients. For example, 10–30% of SLE patients do not have an IFN signature, suggesting that their disease does not involve elevated type I IFNs and may therefore be molecularly altogether different. Furthermore, within the subpopulation of SLE patients with an IFN signature, therapeutic antibodies that neutralize IFNα (41, 160, 160, 161) or all type I IFNs (by blocking their receptor) (40) have been clinically efficacious in some patients, but not in others. The reasons for this heterogeneity are not understood, but may be related to the complexity of the interferon system, the coverage of different interferons by the therapeutics, the upstream drivers of type I IFN production, which depend not only on the cells that produce them but also the ligands that drive type I IFN. It should also be noted that even if pathogenic nucleic acid species induce much of their downstream effect through type I IFNs, there are also some IFN-independent consequences (e.g., through NF-κB

activation) that may contribute to SLE, but not be blocked by IFN antibodies.

At present, it is not known whether SLE patients with an elevated type I IFN gene signature always have the same nucleic acid sensor(s) activated or if each individual patient has a unique pattern that may include any or all of them. It is also unknown if the same pathogenic nucleic acid species are present in all patients, or if they too are different from patient to patient. Furthermore, we cannot entirely exclude the possibility that the nucleic acid sensors are sufficiently dysfunctional (for any number of reasons) to trigger type I IFN production even in the absence of any aberrant DNA or RNA. However, based on the heterogeneity of SLE and the heterogeneity in response to therapeutic IFN blocking antibodies, we find it most likely that there is also heterogeneity in the presence of pathogenic DNA and RNA species resulting in the activation of a different set of sensors in each patient.

Based on GWAS and other genetic data, it also seems that a great deal of patient heterogeneity is conferred by the presence of disease-predisposing or -protective alleles in many genes, most of which are immune-related. While pathogenic nucleic acids may be instrumental in initiating and perpetuating SLE, the overall sensitivity of the immune system, as determined by all these gene variants in immune-related genes (e.g., *MHC* and *PTPN22*), will affect how readily such nucleic acids tip the balance between transient responses vs. frank autoimmune disease. Interestingly, the SLE-predisposing variant of PTPN22 not only affects T and B cell signaling, but also type I IFN production (162).

## CAN SLE BE DIVIDED INTO CLINICALLY MEANINGFUL SUBPOPULATIONS BASED ON "ENDOTYPE"?

The unpredictable response of patients with SLE to standard of care medication is a significant challenge in rheumatology. The current paradigm is to treat patients with escalating doses of increasingly potent immunosuppressive drugs until the clinical response is deemed sufficient and then taper off the strongest immunosuppressants, particularly steroids. Even so, many patients never achieve complete remission but continue to suffer various degrees of symptoms that compromise their health and quality of life, not to mention the threat of sudden exacerbations.

In contrast, the treatment of rheumatoid arthritis made an important advance with the introduction of etanercept in 1999 (163–165). However, this drug also gave rise to the concept of "TNF-non-responders." While most patients at least initially respond clinically to etanercept and other TNF blockers, 20–30% respond poorly, if at all. Other biologics have typically met with similar outcomes: good efficacy in many patients, but always a number of non-responders or initial responders who lose efficacy over time. It seems that non-responders represent individuals whose disease differs molecularly from the responders', such that the disease process does not involve, or readily circumvents, the specific target for the drug and therefore continues unabated.

We believe that this responder/non-responder dichotomy is also relevant in SLE and related diseases, where new drugs in recent clinical trials have generally yielded poor efficacy or a minority of (partial) responders and a majority of non-responders. As SLE is clinically highly variable, it is easy to believe that it is molecularly heterogenous as well. We propose here that SLE patients could be grouped into molecularly distinct categories ("endotypes") based on which nucleic acid sensors are active and the IFN species produced in response to them (**Figure 2**):

1) IFN-independent SLE, represented by the 10–30% of patients who do not have a type I IFN gene signature and, therefore, unlikely any nucleic acid sensor activation.
2) SLE patients whose elevated and disease-driving type I IFNs are restricted to isoforms of IFNα, which are predominantly made by immune cells via TLR7/9 in response to circulating immune complexes that contain nucleic acids.
3) SLE patients with predominantly IFNβ, which is typically made by epithelial and other cells via activation of cGAS,

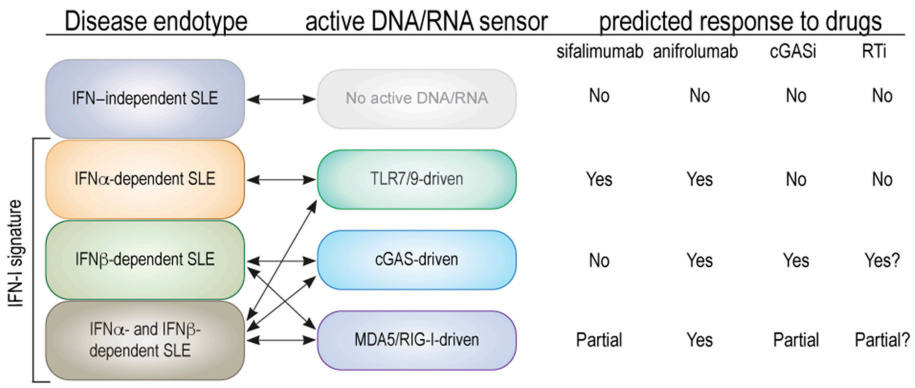


**FIGURE 2 |** Proposed endotypes of SLE based on type I IFN subtype and relevant nucleic acid sensors. The first column represents the four proposed endotypes of SLE with double arrows connecting them to the relevant nucleic acid sensors. The predicted (or known) effects on each SLE endotype of an antibody that neutralizes IFNα only (sifalimumab), an antibody that blocks all type I IFNs (anifrolumab), and hypothetical cGAS or RT inhibitors are indicated as "yes" for a substantial clinical benefit, "no" for none, and "partial" if only one of two parallel mechanisms are expected to be inhibited.

RIG-I, or MDA5. These patients may be at an early stage of SLE development (including preclinical disease), and have not yet developed circulating immune complexes with nucleic acids. Alternatively, their disease will never develop such immune complexes.

4) SLE patients who have numerous IFNαs and IFNβ (and perhaps IFNε, IFNκ, or IFNω) and both TLR7/9 and intracellular nucleic acid sensor pathways active. If the third category includes early disease, this fourth endotype may contain severe and late stage disease.

What we propose is the personalized medicine notion that patients suffering from a disease like SLE can be subdivided by patient endotype into subsets that share a specific molecular mechanism that originally initiated and continues to perpetuate their disease, and that the inhibition of this mechanism by a selective therapeutic approach will provide a strong clinical benefit specifically to patients within this subset, but perhaps not to others. Practical examples of this concept exist in medical practice today in oncology and respiratory medicine (166) but are still absent in rheumatology. Key to the utility of this concept is the development of practical tests ("biomarkers") that can determine which endotype individual patients belong to. In this particular case, such biomarkers would naturally quantitate nucleic acids, the activation of the sensors, and/or assess the spectrum of IFNs in patient blood or tissue.

To fully test our SLE endotype concept, future trials with new and more targeted therapeutics for SLE should include the relevant biomarkers to ask if therapeutic efficacy falls within one or another SLE endotype. In other examples of the endotype concept (166), this type of patient stratification approach has resulted in astonishing levels of efficacy within the relevant endotype, but marginal impact on patients of other endotypes. Oftentimes, these same clinical trials failed to meet their primary endpoint when the all-comers population was assessed.

## AUTHOR CONTRIBUTIONS

TM, CL, and NG contributed equally to the writing of this review and share accountability for its content.

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# Autoantibodies in Systemic Lupus Erythematosus Target Mitochondrial RNA

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The mitochondrion supplies energy to the cell and regulates apoptosis. Unlike other mammalian organelles, mitochondria are formed by binary fission and cannot be directly produced by the cell. They contain numerous copies of a compact circular genome that encodes RNA molecules and proteins involved in mitochondrial oxidative phosphorylation. Whereas, mitochondrial DNA (mtDNA) activates the innate immune system if present in the cytosol or the extracellular milieu, it is also the target of circulating autoantibodies in systemic lupus erythematosus (SLE). However, it is not known whether mitochondrial RNA is also recognized by autoantibodies in SLE. In the present study, we evaluated the presence of autoantibodies targeting mitochondrial RNA (AmtRNA) in SLE. We quantified AmtRNA in an inducible model of murine SLE. The AmtRNA were also determined in SLE patients and healthy volunteers. AmtRNA titers were measured in both our induced model of murine SLE and in human SLE, and biostatistical analyses were performed to determine whether the presence and/or levels of AmtRNA were associated with clinical features expressed by SLE patients. Both IgG and IgM classes of AmtRNA were increased in SLE patients ($n = 86$) compared to healthy controls ($n = 30$) ($p < 0.0001$ and $p = 0.0493$, respectively). AmtRNA IgG levels correlated with anti-mtDNA-IgG titers ($r_s = 0.54$, $p < 0.0001$) as well as with both IgG and IgM against β-2-glycoprotein I (anti-$\beta_2$GPI; $r_s = 0.22$, $p = 0.05$), and AmtRNA-IgG antibodies were present at higher levels when patients were positive for autoantibodies to double-stranded-genomic DNA ($p < 0.0001$). AmtRNA-IgG were able to specifically discriminate SLE patients from healthy controls, and were negatively associated with plaque formation ($p = 0.04$) and lupus nephritis ($p = 0.03$). Conversely, AmtRNA-IgM titers correlated with those of anti-$\beta_2$GPI-IgM ($r_s = 0.48$, $p < 0.0001$). AmtRNA-IgM were higher when patients were positive for anticardiolipin antibodies (aCL-IgG: $p = 0.01$; aCL-IgM: $p = 0.002$), but

AmtRNA-IgM were not associated with any of the clinical manifestations assessed. These findings identify mtRNA as a novel mitochondrial antigen target in SLE, and support the concept that mitochondria may provide an important source of circulating autoantigens in SLE.

**Keywords: autoantibodies-blood, mitochondria-RNA, antimitochondrial antibody (AMA), autoimmue disease, systemic lupus erythematosus (SLE), autoantigens, extranuclear nucleic acids**

## INTRODUCTION

The mitochondrion is an intracellular organelle involved in the regulation of numerous cellular functions, among which the best known are ATP production and programmed cell death (1, 2). Mitochondria are considered as deriving from the endosymbiosis of an α-synfular; proteobacterium (3, 4), providing the organelles many bacterial features (3, 5–9).

Different cellular lineages (10–18) may extrude their mitochondria upon activation. Extracellular mitochondria have been identified in damaged tissues (8, 18–20); diverse inflammatory conditions (11, 12, 14, 21–24); and in the blood of critical care patients (22). As mitochondria retained several characteristics of their ancestral prokaryotic origin, the release of mitochondrial components onto the extracellular milieu can activate the innate immune system (25, 26). The efflux of mtDNA is facilitated by megapores formed in the mitochondrial membrane during apoptosis, and detected by the cytosolic DNA sensors cGAS and stimulator of interferon genes (STING) pathway, thereby leading to type I interferon synthesis (27). Cardiolipin, N-formylated peptides, mtDNA, ATP and reactive oxygen species are known mitochondrial damage-associated molecular patterns (9, 28–30). They further activate cells through nuclear oligomerization domain-like receptors (28, 29, 31), toll-like receptors (TLR) (e.g., TLR9 for mtDNA), or formyl peptide receptors (9, 28–31).

Systemic lupus erythematosus is an autoimmune disease characterized by the presence of circulating immune complexes and inflammation in multiple organs and tissues. Recent evidence point to an involvement of mtDNA, liberated by neutrophils, in the activation of STING and type-I IFN production in SLE (11, 12). Moreover, extracellular mtDNA can enhance leukocyte migration and degranulation (32), and promotes the secretion of the pro-inflammatory cytokine TNF-α by plasmacytoid dendritic cells (33). Production of autoantibodies targeting several mitochondrial components was reported in SLE as well as in other diseases [e.g., primary biliary cirrhosis (PBC), antiphospholipid syndrome (APS), and cardiomyopathies] (**Figure 1**). Anti-mitochondrial autoantibodies recognize proteins, such as those involved in oxidative phosphorylation, phospholipids or unidentified epitopes present in the mitochondrial membrane. Despite the extensive literature regarding antibodies targeting the cardiolipin (also known as the mitochondrial antigen M1) in SLE, the anti-mitochondrial autoantibody repertoire and their antigenic targets remains mostly uncharacterized (12, 34–37). Using intact mitochondria and mtDNA as antigens to screen autoantibodies in SLE patients, we have shown that different sets of autoantibodies also target the mitochondrial outer membrane and mtDNA (36). Given the accumulating evidence for mitochondrial release during inflammatory pathogenesis, these observations point to a role for mitochondria both in the stimulation of the innate immune system and as a potential source of autoantigens.

Whereas, the mitochondrion has already been described as a source of mtDNA during inflammation (17, 21, 32), it is not known whether its important RNA content (mtRNA) can contribute to the autoantigenic load in SLE. Despite its presence at high copy numbers, the mitochondrial genome is very compact (38–40) During its translation into mitochondrial messenger RNA (38), a long polycistronic transcript is generated from each strand of mtDNA prior to undergoing processing into mtRNA molecules. This highly regulated process is thought to occur in a particular location in the mitochondrion, called mitochondrial RNA granules (41), and requires key RNA processing enzymes such as the members of the FASTK family of proteins (42). The human mitochondrial transcriptome comprises 16S ribosomal RNA molecules (78%), transfer (13%), messenger (8%) and small non-coding antisense (1%) mtRNA molecules. The complete mitochondria transcriptome is controlled by the cell's energy requirements, and therefore varies greatly depending on its tissue distribution. In the heart, 30% of the total messenger RNA molecules are of mitochondrial origin, whereas ∼5% of the total messenger RNA load in less metabolically active cells such as leukocytes is encoded by mitochondrial genes (39). The important quantity of mtRNA may thus represent a major antigenic load for the adaptive immune system upon release of mitochondria onto the extracellular milieu.

With the accumulating evidence supporting the liberation of mitochondrial components into the extracellular milieu in SLE (11, 12), it is crucial to identify the various mitochondrial antigens. In the present study, we examined whether the RNA molecules present in mitochondria are antigenic. The levels of anti-mtRNA (AmtRNA) were measured in SLE sera, and we determined whether AmtRNA were associated with antibodies against whole mitochondrial organelles (AwMA) and mtDNA (AmtDNA). We also investigated the occurrence of AmtRNA in an induced model of murine SLE. Finally, we determined whether

**Abbreviations:** $\beta_2$GPI, β-2-glycoprotein I; aCL, anticardiolipin antibodies; AMA, antimitochondrial antibodies; AwMA, anti-whole mitochondria antibodies; LA, lupus anticoagulant; mtDNA, mitochondrial DNA; mtRNA, mitochondrial RNA; SARD-BDB, systemic auto-immune rheumatic disease biobank and data repository.

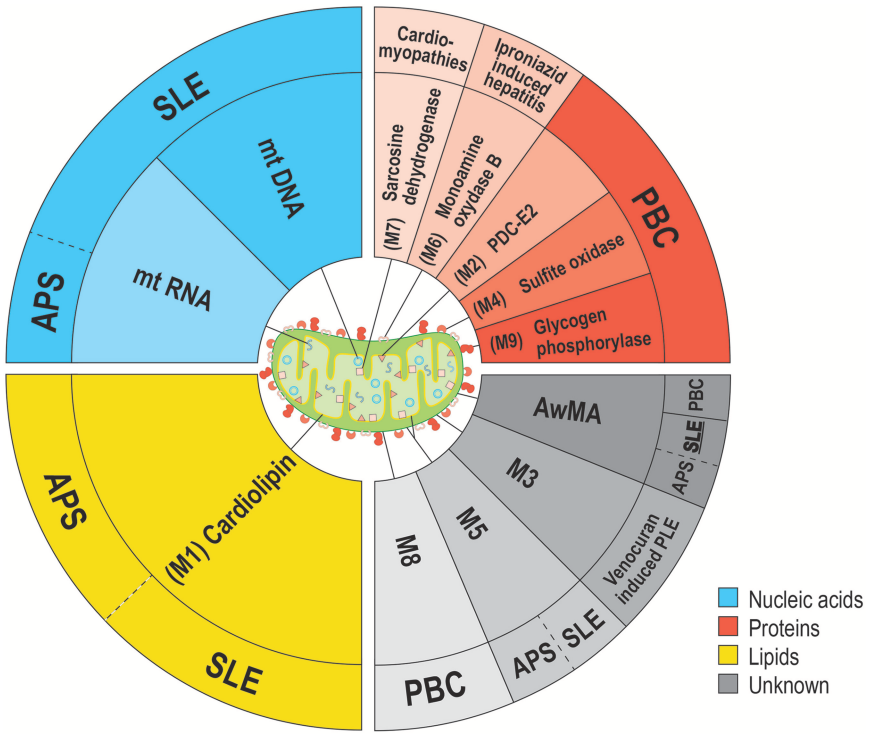


**FIGURE 1 |** Anti-mitochondrial antibodies and related diseases. Several types of anti-mitochondrial antibodies (AMA) have been reported in various diseases. The epitopes targeted by AMA cover all families of biomolecules: lipids (yellow background), proteins (red hues) or nucleic acids (blue hues). However, the precise nature of some mitochondrial epitopes targeted by AMA are still unclear. (gray hues). To date, the sole mitochondrion-specific phospholipid antigen reported in both APS and SLE is cardiolipin (M1). M1 is located within the mitochondrial inner membrane (MIM) in healthy organelles, but may be displayed on the outer membrane (MOM) upon damages to the organelle. Distinct AMA against an unknown antigen (M5) were also reported in both APS and SLE. Four antigens are associated with PBC; PDC-E2 (M2, MIM), sulfite oxidase (M4, MOM), M8 (MOM), and glycogen phosphorylase (M9, MOM). These mitochondrial antigens are peptidic, with the exception of M8, whose nature remains uncharacterized. Sarcosine dehydrogenase (M7) is another immunogenic protein that is targeted by autoantibodies in patients suffering from cardiac conditions (i.e., hypertrophic or idiopathic cardiomyopathies or acute myocarditis). Two types of AMA were reported as iatrogenically induced in human patients: AMA-M3 (unknown, MOM) and AMA-M6 (monoamine oxidase B, MOM). In addition to these autoantibodies, we have reported the presence of autoantibodies targeting whole mitochondria (AwMA) in patients with SLE, APS, and PBC (with higher titers found in SLE donors). Moreover, antibodies specific to the mtDNA were specific to SLE patients. In the present study, we describe autoantibodies against mtRNA in patients with SLE and APS.

AmtRNA were associated with disease manifestations in patients with SLE.

# MATERIALS AND METHODS

## Induced Model of Murine SLE

This study was carried out in accordance with the recommendations of the Canadian Council on Animal Care. The protocol was approved by McGill University Animal Care Committee. C57BL/6 mice were obtained from Harlan Sprague Dawley Inc. (Indianapolis, IN, USA) and housed in a specific-pathogen-free animal facility at the animal facility of the Research Institute of the McGill University Health Center. Female (10–12-weeks-old) mice were injected intravenously (i.v.) with 100 μL human β2-GPI (20 μg) (Crystal Chem Inc., Elk Grove Village, IL, USA), followed 24 h later by a 100 μL i.v. injection of lipopolysaccharide (LPS from *E.coli*, serotype O111:B4; 10 μg) (List Biological Laboratories, Campbell, CA, USA). β2-GPI and LPS injections were repeated every 2 weeks for a total of three rounds of immunizations, and then at 2-month intervals for the fourth and the fifth immunizations. C57BL/6 mice injected i.v. with PBS and LPS following the same schedule were used as controls. Mice were bled 1 week after the fifth immunization and serum was kept frozen at −70°C until testing.

## Mitochondria Isolation

Mitochondria were isolated from the livers of C57BL/6 mice as previously described (43). In brief, cells and tissues were disrupted by grinding in a glass/Teflon tissue potter containing 12 mL ice-cold mitochondrial isolation buffer (10 mM Tris, 1 mM EGTA, 200 mM sucrose) for each gram of liver. Debris were pelleted twice at 700 *g*, for 10 min at 4°C and the supernatants were transferred to fresh tubes. Mitochondria were further separated from other cellular fractions by three centrifugation steps (twice at 7,000 *g* and once at 10,000 *g*, for 10 min at 4°C). Between each step, pelleted mitochondria were re-suspended in 12 mL isolation buffer. Samples were kept at −80°C until required for RNA isolation.

## Mitochondrial RNA Isolation

Mitochondrial RNA was isolated using the Aurum™ Total RNA Mini Kit (Bio-Rad, Hercules, CA, USA), following the manufacturer's instructions. Ribonucleic acid yields were quantified using a BioDrop μLITE and its proprietary software (BioDrop Ltd., Cambridge, UK). The absence of contamination by mitochondrial DNA was assessed by resolution of 1 μg untreated mtRNA and the same amount of RNAse A-treated (QIAgen, 100 μg/mL) mtRNA on a 1.5% (w/v) agarose gel (**Supplementary Figure 1A**). 15.09 ± 2.74 μg mtRNA were isolated for each mg of bicinchoninic acid assay (BCA)-dosed mitochondria used ($n = 3$).

## Enzyme-Linked Immunoassays for the Detection of Antibodies Targeting Mitochondrial Antigens

Clear 96-well High Bind half-area flat bottom ELISA *microplates* (Corning, New York, USA) were pre-coated with 100 μL per well of 1% protamine sulfate (Sigma-Aldrich) in double-distilled water for 1 h at RT. Plates were then washed thrice with PBS and loaded with mtRNA. Plates were coated overnight at 4°C, washed thrice and non-specific binding was blocked for 4 h at 37°C with 100 μL per well of ELISA blocking buffer (PBS−10% FBS−0.5% gelatin). Wells were rinsed three times with PBS and incubated in duplicate with serum diluted (1:150 for human and 1:50 for mice) in incubation buffer (PBS−10% FCS−0.3% gelatin). Plates were washed thrice with PBS and incubated for 90 min at RT with either γ or μ chain-specific-alkaline phosphatase-(AP) conjugated goat anti-human IgG or IgM (Sigma-Aldrich) for human serum, or γ chain-specific-horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Sigma-Aldrich) for mice (1:1000) in secondary antibody buffer (PBS−0.4% bovine serum albumin [BSA]). Unbound antibodies were washed thrice with PBS. Signals from AP-conjugated antibodies were developed with *para*-nitrophenol phosphate (*p*-NPP) for ∼30 min at 37°C, and HRP-conjugated antibodies were developed with 3,3′,5,5′-tetramethylbenzidine (TMB) at RT. The reaction was stopped with 2 N sulfuric acid ($H_2SO_4$). Optical densities (OD) were measured at 405 nm (*p*-NPP) or 450 nm (HRP) on a SpectraMax 190 microplate reader (Molecular Devices, Sunnyvale, CA, USA), using SoftMax Pro 5.4.1 (Molecular Devices). For each experiment, blank values (i.e., wells coated with mtRNA, but without sera) were subtracted from each measurement.

The quantity of purified mitochondrial RNA (mtRNA) required for coating half-area flat-bottom 96-well ELISA microplates (Corning, New York, USA) was optimized following the aforementioned protocol, by using increasing concentrations from 0 to 1,600 ng of coating mtRNA. Pooled sera (1:150) from 6 SLE patients, who had previously tested positive for AmtDNA and AwMA, were incubated after blocking non-specific binding. The peak signal for optical densities at 405 nm was obtained with 200 ng of coating mtRNA (**Supplementary Figure 1B**).

## Ethics and Study Approval

This study was carried out in accordance with the recommendations of the Research Ethics Board of the CHU de Québec—Université Laval with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by Research Ethics Board of the CHU de Québec—Université Laval.

## Human Serum Samples

The human sera tested in this study were obtained from the Systemic Autoimmune Rheumatic Disease (SARD) biobank and data repository (SARD-BDB) located at the *CHU de Québec-Université Laval* (UL). This SARD-BDB and the specific use of the sera for the present study were approved by the *CHU de Québec-UL* research ethics board (#B13-06-1243 and #B14-08-2108, respectively). Patients with SLE met the 1982 ACR classification criteria for SLE (revised in 1997) (44, 45). A peripheral blood sample was collected at the time of their first visit. Serum samples from 30 healthy donors and 87 SLE patients included in the SARD-DBD cohort were used in the present study. However, one patient had no clinical data available and was therefore excluded for bio-statistical comparisons (i.e., $n = 86$ SLE donors for these tests).

## Additional Serum Samples

Sera from a cohort of patients and controls from the University of Toronto Lupus Clinic, as well as patients with primary biliary cirrhosis (PBC) from Quebec City, were used in additional exploratory analyses to test the presence of AmtRNA in patients with the antiphospholipid syndrome (APS, $n = 12$) and PBC ($n = 12$). APS patients and healthy controls, distinct from those included in the SARD-BDB ($n = 43$), were originally recruited between August 2010 and October 2011, and gave consent to allow remaining biospecimens to be used for future studies on lupus biomarkers. This study has been reviewed and approved by the Research Ethics Board of the University Health Network (#10-0637-BE) and of the CHU de Québec—Université Laval (#B14-08-2108). APS patients met 1999 Sapporo criteria for the disease (revised in 2006) (46, 47), and healthy controls were recruited if they had no known illnesses and had no infectious symptoms at the time of the blood draw. Donors gave a single blood sample that was linked to their anonymized clinical data. PBC patients were positive for anti-mitochondrial antibodies and presented clinical criteria for the disease (47, 48).

## Clinical Variables Collected in SLE Patients

### Sociodemographic Variables

Information was collected concerning patient's age, gender, marital status, and ethnicity at the first visit in the SARD-BDB.

### Patient Characteristics Including Exposures to Cardiovascular Risk Factors

A body mass index (BMI) was calculated and reported as underweight, normal, overweight and obese. Hypertension and diabetes mellitus were documented as present or absent. Smoking history was reported as non-smokers, ex-smokers or current smokers. Female patients were considered post-menopausal in the absence of menstruations for more than 12 continuous months.

### Disease Specific Characteristics

ACR classification criteria (44, 45) were documented for each of the 11 categories and a total score calculated (5 ± 1.28). Disease duration, lupus disease activity using the SLE Disease Activity Index–2000 (SLEDAI-2K) (49, 50) and lupus damage using the Systemic Lupus International Collaborating Clinics (SLICC)/ACR damage index (SDI) (51, 52) were collected during the clinical visit matched to the blood specimen draw. Both the SLEDAI-2K and the SDI are reported as continuous variables and they both have proven validity, reliability, and perform well in observational studies.

### Medication Variables

Antimalarial use was defined as use of hydroxychloroquine or chloroquine at the current visit. Steroid use was defined as prednisone use in the past year.

### Clinical Outcomes

Clinically relevant lupus disease activity and damage were used as clinical outcome in our analyses and were defined as a SLEDAI-2K of 4 or more to capture clinically active lupus and a SDI of 1 or more to capture clinically significant damage. Other outcome variables included arterial and venous thrombotic event ever in the past and presence of lupus nephritis according to the presence or absence of the renal item of the SLICC Classification criteria for SLE (53). Presence or absence of carotid plaques, as well as average carotid-intima media thickness (CIMT) was also documented by carotid ultrasound following a standard examination of both carotids (standard carotid ultrasound research protocol using an Esaote MyLab Five ultrasound machine with digital images sent for blind reading at the IMT Core Laboratory of the Montreal Heart Institute).

### Information From Clinical Laboratories

For SLE patients, an automated complete blood count was documented. The anti-dsDNA, anticardiolipin antibodies (aCL) (IgG and IgM—laboratory cut-offs of 40 GPL or MPL units) and anti-β2-GPI (IgG and IgM—laboratory cut-offs above the 99th percentile of controls) were measured by ELISA. The lupus anticoagulant assay (LA) followed international guidelines for the performance of this functional assay (54). The above tests were performed in a clinical laboratory at CHU de Quebec-Universite Laval as part of routine care.

### Information From Research Laboratories

In addition to the measurements provided by the clinical laboratories, our research laboratory performed antibody assays to detect AwMA and AmtDNA, following previously described methods (36).

## Statistical Analyses

Descriptive statistics are presented as mean with standard deviation or frequency with percentage without missing values for continuous and categorical variables, respectively. Comparisons between groups were performed using the Student's, Wilcoxon or Kruskal-Wallis tests depending on the nature of the variables and their distribution. Spearman correlations were calculated to assess association between continuous variables. Associations between AmtRNA and clinical outcomes were studied by bivariate and multivariate logistic regressions, for dichotomous and continuous outcomes, respectively. The latter were adjusted for gender, disease duration, age, BMI, antimalarial medication and prednisone use. ROC curves were generated to assess the predictive ability of AmtRNA to discriminate between SLE and controls, and their area under the curve (AUC) was calculated. Participants' results were considered positive for AmtRNA when their value was above the cut-off value identified after maximizing Youden's Index. A 95% confidence interval was obtained for the cut-off using 10,000 bootstrap samples. Performance measures are presented with their 95% exact confidence interval. Statistical analyses were performed with Prism 7 software (GraphPad Software Inc., La Jolla, CA, USA) and SAS version 9.4 (SAS Institute Inc., Cary, NC, USA) and Figures were assembled with Photoshop CS6 13.0 (Adobe Systems Inc., Mountain View, CA, USA).

# RESULTS

We used our quantitative ELISA to assess whether AmtRNA from the IgG subclass (AmtRNA-IgG) could be detected in an induced model of murine SLE in which the production of circulating IgG against whole mitochondria (AwMA) and mitochondrial DNA (AmtDNA) was previously reported (36). Antibodies against mtRNA were significantly increased ($p = 0.0005$) in the sera of SLE mice compared with control mice (**Figure 2**).

A cohort of 86 SLE patients (**Tables 1–5**) and 30 healthy controls (19 females [63.3 %], 11 males [36.7%], age: 49.33 ± 7.68 years) was studied to determine the occurrence of AmtRNA-IgG and AmtRNA-IgM in human SLE. The proportion of male donors in the healthy group was higher than in the SLE cohort (i.e., 36.7% vs. 16.3% of male donors, respectively) as well as than the 1:10 male-to-female sex bias reported in the disease. We thus verified that the anti-mitochondrial antibody titers measured were not influenced by sex, using Wilcoxon test and found no significant differences ($p$-values between 0.14 and 0.97). Both AmtRNA-IgG and -IgM were significantly increased in SLE patients, compared with healthy individuals ($p = 0.0002$ and $p = 0.0493$, respectively) (**Figure 3**; **Supplementary Figure 1**). In healthy donors, AmtRNA-IgM were higher than AmtRNA-IgG levels (0.32 ± 0.24 vs. 0.16 ± 0.12), suggesting that antibodies targeting mitochondrial epitopes may be present in healthy individuals even in the absence of any detectable pathology.

In a separate exploratory analysis using donors distinct from those included in the SARD-BDB, AmtRNA-IgG were also significantly increased in patients with APS, an autoimmune condition often associated with SLE ($p < 0.001$ vs. healthy controls). However, no differences in AmtRNA-IgG were observed between patients with PBC, a disease known for an adaptive immune response against mitochondrial autoantigens, and healthy controls ($p = 0.31$) (**Figure 4**).

Autoantibodies to genomic dsDNA (anti-dsDNA) and to β-2-glycoprotein I (anti-$\beta_2$GPI, IgG, and IgM) were evaluated during the clinical work-up of a patient with an increased

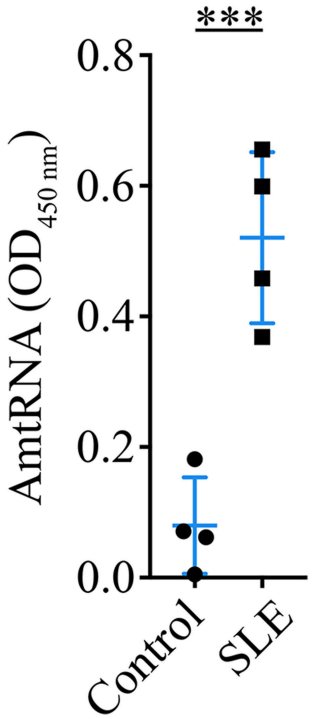


**FIGURE 2 |** Circulating anti-mitochondrial RNA autoantibodies are detectable in sera from mice with induced SLE. Sera (1:50) from mice with induced SLE were incubated on ELISA plates coated with 200 ng murine mtRNA per well. Mice with induced SLE displayed a significant increase in serum antibodies against mtRNA in comparison to control mice. $N = 4$ mice per group. Data show the mean ± SD. Student's *t*-test. ***$p < 0.001$.

**TABLE 1 |** Sociodemographic characteristics in the SARD-BDB.

| Variable | *n* | Mean ± SD [or *n* (%)] |
|---|---|---|
| Female | 86 | 72 (83.7) |
| Age (years) | 86 | 49.41 ± 14.60 |
| Marital status | | |
| Single | 82 | 14 (17.1) |
| Married | | 55 (67.1) |
| Tobacco intake | | |
| Non-smokers | 83 | 48 (57.8) |
| Smokers | | 14 (16.9) |
| Ex-smokers | | 21 (25.3) |

**TABLE 2 |** Clinical characteristics in the SARD-BDB.

| Variable | *n* | Mean ± SD [or *n* (%)] |
|---|---|---|
| Disease duration | 86 | 10.43 ± 10.69 |
| Body mass index | 86 | 25.55 ± 4.97 |
| Post-menopausal | 64 | 38 (59.4) |
| Hypertension | 86 | 11 (12.8) |
| Diabetes | 84 | 2 (2.4) |
| Malar rash | 85 | 19 (22.4) |
| Discoid rash | 85 | 12 (14.1) |
| Photosensitivity | 85 | 36 (42.4) |
| Oral ulcers | 85 | 26 (30.6) |
| Arthritis (≥2 peripheral joints) | 85 | 69 (81.2) |
| Serositis | 85 | 22 (25.9) |
| Renal disorders | 85 | 22 (25.9) |
| Neurological disorders | 85 | 4 (4.7) |
| Hematological disorders | 85 | 68 (80.0) |
| Immunological disorders | 85 | 62 (72.9) |
| Anti-nuclear antibodies (ANA) | 85 | 85 (100.0) |

**TABLE 3 |** Outcome variables of the study.

| Variable | *n* | Mean ± SD [or *n* (%)] |
|---|---|---|
| SLEDAI-2K (Score) | | 3.24 ± 3.96 |
| SLEDAI-2K ≥ 4 | 86 | 36 (41.9) |
| SDI (score) | | 3.24 ± 3.96 |
| SDI ≥ 0 | 86 | 36 (41.9) |
| Thrombosis | | 10 (11.6) |
| Arterial events | 86 | 3 (3.5) |
| Venous events | | 4 (4.7) |
| Presence of plaque in the carotid | 63 | 24 (38.1) |
| Carotid intima-media thickness (CIMT, μm) | 34 | 0.63 ± 0.13 |
| Nephritis | 61 | 14 (23.0) |

*SDI, lupus severity disease index; SLEDAI-2K, systemic lupus erythematosus disease activity index−2000.*

likelihood of SLE. We examined whether titers in AmtRNA (IgG and IgM) and levels of anti-dsDNA or anti-$\beta_2$GPI were associated with each other in the patients, and found correlations between levels of AmtRNA-IgG and those of both anti-$\beta_2$GPI-IgG and IgM ($r_s = 0.22$, $p = 0.05$). AmtRNA-IgM titers only displayed a strong correlation with anti-$\beta_2$GPI-IgM ($r_s = 0.48$, $p < 0.0001$). Conversely, no correlations were observed between AmtRNA and concentrations of anti-dsDNA (**Table 6**). We also determined whether the levels of AmtRNA correlated with IgG and IgM antibodies targeting mitochondrial epitopes localized in diverse sub-compartments of the organelle (36). Specifically, we measured antibodies recognizing intact whole mitochondria (AwMA), which most likely bind epitopes found on the outer mitochondrial membrane; aCL, which target cardiolipin, a phospholipid located mainly within the mitochondrial inner membrane; and AmtDNA, which recognize mitochondrial DNA. We found that AmtRNA-IgG levels correlated with AmtDNA-IgG ($r_s = 0.54$, $p < 0.0001$) and with AwMA-IgG ($r_s = 0.24$, $p = 0.03$), but not with aCL (IgG and IgM). AmtRNA-IgM concentrations correlated with AmtDNA-IgM ($r_s = 0.83$, $p < 0.0001$), AwMA-IgM ($r_s = 0.71$, $p < 0.0001$), aCL-IgG ($r_s = 0.27$, $p = 0.02$), and aCL-IgM ($r_s = 0.57$, $p < 0.0001$). Thus, in addition to the newly described AmtRNA, different sets of anti-mitochondrial antibodies occur conjointly in SLE.

One of the main features of SLE is the expression of numerous autoantibodies in patients (55), some of which are known to be associated with the clinical expression of the disease (56). We assessed whether AmtRNA are qualitatively associated with

**TABLE 4 |** Information about medications taken by SLE patients ($n = 86$) in the SARD-BDB.

| Variable | *n*(%) |
|---|---|
| Anticoagulation/anti-platelets | 13 (15.1) |
| Antimalarial | 70 (81.4) |
| Prednisone | 18 (20.9) |
| Lipid lowering | 14 (16.3) |
| Diabetes medication | 2 (2.3) |
| **LUPUS TREATMENTS** | |
| Hydroxychloroquine | 65 (76) |
| Chloroquine | 6 (7) |
| Azathioprine | 15 (17) |
| Methotrexate | 15 (17) |
| Leflunomide | 1 (1) |
| Mycophenolate mofetil | 11 (13) |
| Mycophenolic acid | 1 (1) |
| Cyclophosphamide (PO or IV) | 3 (4) |

*IV: intravenous injection; PO: per os.*

**TABLE 5 |** Laboratory measurements.

| Variable | *n* | Mean ± SD [or *n* (%)] |
|---|---|---|
| Platelets (0.10^9/L) | 86 | 221.63 ± 72.68 |
| White blood cells (0.10^9/L) | 86 | 5.80 ± 2.06 |
| Creatinine clearance | 26 | 91.88 ± 21.31 |
| AmtDNA (OD 405 nm) | | |
| IgG | 86 | 0.49 ± 0.53 |
| IgM | 86 | 0.45 ± 0.38 |
| AwMA (OD 405 nm) | | |
| IgG | 86 | 0.34 ± 0.37 |
| IgM | 86 | 0.56 ± 0.58 |
| AmtRNA (OD 405 nm) | | |
| IgG | 86 | 0.42 ± 0.38 |
| IgM | 86 | 0.52 ± 0.47 |
| Lupus anticoagulant (LA) | 61 | 8.69 ± 22.76 |
| Anticardiolipin antibodies (aCL) | | |
| IgG | 79 | 11.33 ± 12.39 |
| IgM | 79 | 6.92 ± 13.92 |
| Anti-β2GPI antibodies | | |
| IgG | 79 | 2.78 ± 6.59 |
| IgM | 79 | 3.36 ± 3.89 |
| Anti-dsDNA antibodies | 22 | 31.01 ± 80.40 |

*$\beta_2$GPI, β-2-glycoprotein I; AmtDNA, anti-mitochondrial DNA antibodies; AmtRNA, anti-mitochondrial RNA antibodies; AwMA, anti-whole mitochondria antibodies; OD, optical density.*

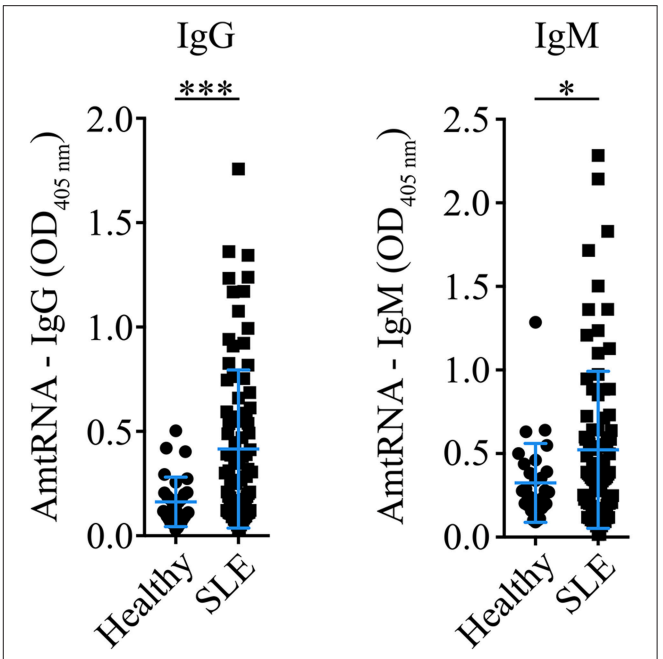


**FIGURE 3 |** Antibodies targeting mitochondrial RNA (AmtRNA) are elevated in SLE patients. Two different isotypes of antibodies against mtRNA, IgG (**left** panel) and IgM (**right** panel), were assessed in SLE patients and healthy individuals included in the SARD-BDB. Both AmtRNA IgG and IgM were significantly increased in SLE patients, compared to healthy individuals ($p = 0.0002$ and $p = 0.0493$, respectively). SLE: $N = 86$; Healthy controls: $N = 30$. Data show the mean ± SD. Student's *t*-test. $^*p < 0.05$; $^{***}p < 0.001$.

positivity to several autoantibodies commonly found in SLE, including anti-dsDNA, aCL, and LA. AmtRNA-IgG levels were higher in presence of anti-dsDNA antibodies ($p < 0.0001$), whereas AmtRNA-IgM titers were elevated in presence of aCL-IgG and -IgM ($p = 0.01$ and $p = 0.002$, respectively) (**Table 7**). Of note, circulating AmtRNA-IgM tended ($p = 0.06$) to be increased in the presence of LA in SLE patients.

We examined whether AmtRNA were associated with disease manifestations in 86 SLE patients for whom detailed clinical information were available (**Table 8**). Higher levels of AmtRNA-IgG were associated with a lower occurrence of plaque in the carotid using a bivariate analysis [OR(95% CI) = 0.14 (0.02–0.91); $p = 0.04$], but this significance was lost in the multivariate logistic regression [OR(95% CI) = 0.16 (0.01–1.81); $p = 0.14$]. We found no association between AmtRNA-IgG and two clinical indices; one measuring SLE disease activity (SLEDAI-2K ≥ 4) and the other indicating damages (SDI > 0), both by bi- and multivariate analyses. However, higher concentrations of AmtRNA-IgG were positively associated with elevated anti-dsDNA antibodies in both models. AmtRNA-IgG were not associated with lupus nephritis in a bivariate analysis [OR(95% CI) = 0.17 (0.02–1.71); $p = 0.13$], but this association became significant in the multivariate model [OR(95% CI) = 0.02 (0.00–0.68); $p = 0.03$]. In contrast, AmtRNA-IgM were not significantly associated with any of these clinical outcomes by either the bi- or multi-variate analysis.

Furthermore, we assessed if our conclusions were identical in patients with higher disease activity by repeating our calculations with patients having a SLEDAI-2K score > 6 (i.e., for 15 patients, compared to 36 with a cut-off value at a SLEDAI-2K score ≥ 4). The associations between AmtRNA-IgG with SLEDAI-2K > 6 were [OR(95% CI) = 2.71 (0.71–10.31)] for the bivariate logistic regression and [OR(95% CI) = 1.99 (0.40–10.00)] for the multivariate regression model. Values for the associations between AmtRNA-IgM and SLEDAI-2K > 6 for

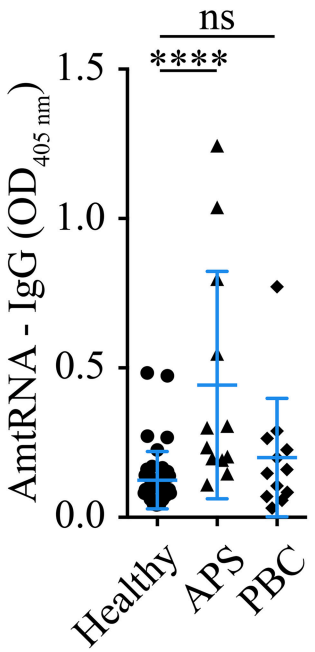


**FIGURE 4 |** Detection of AmtRNA in two different diseases with anti-mitochondrial antibodies. Antiphospholipid syndrome (APS) and primary biliary cirrhosis (PBS) are two diseases with antibodies targeting mitochondrial antigens; cardiolipin (M1) in APS and PDC-E2 (M2), sulfite oxidase (M4), M8 (whose target is still unclear) and sarcosine dehydrogenase (M9) in PBC. Sera (1:150) from patients with APS presented a significant increase in circulating autoantibodies against mtRNA, compared to healthy individuals, whereas PBC patients had levels similar to the controls. Healthy: $N = 43$, APS: $N = 12$, PBC $N = 12$. Data are Mean ± SD. Kruskal-Wallis test with multiple comparisons to controls/healthy donors; Dunn's correction. ****$p < 0.001$.

**TABLE 6 |** Correlations of anti-mtRNA levels, with titers of other auto-antibodies in SLE patients.

| | | AmtRNA | |
|---|---|---|---|
| | | **IgG** | **IgM** |
| AmtDNA | IgG | $\mathbf{r_S = 0.54}$ | $r_S = 0.19$ |
| | | $\mathbf{p < 0.0001}$ | $p = 0.08$ |
| | IgM | $r_S = -0.01$ | $\mathbf{r_S = 0.83}$ |
| | | $p = 0.92$ | $\mathbf{p < 0.0001}$ |
| AwMA | IgG | $\mathbf{r_S = 0.24}$ | $r_S = 0.14$ |
| | | $\mathbf{p = 0.03}$ | $p = 0.21$ |
| | IgM | $r_S = -0.03$ | $\mathbf{r_S = 0.71}$ |
| | | $p = 0.78$ | $\mathbf{p < 0.0001}$ |
| AmtRNA | IgG | / | $r_S = 0.16$ $p = 0.15$ |
| | IgM | $r_S = 0.16$ $p = 0.15$ | / |
| Anti-$\beta_2$GPI antibodies | IgG | $\mathbf{r_S = 0.22}$ | $r_S = 0.18$ |
| | | $\mathbf{p = 0.05}$ | $p = 0.11$ |
| | IgM | $\mathbf{r_S = 0.22}$ | $\mathbf{r_S = 0.48}$ |
| | | $\mathbf{p = 0.05}$ | $\mathbf{p < 0.0001}$ |
| Anti-dsDNA antibodies | | $r_S = 0.13$ | $r_S = 0.11$ |
| | | $p = 0.56$ | $p = 0.62$ |

*Values are presented as Spearman correlation coefficient ($r_S$) and p-value. $\beta_2$GPI, β-2-glycoprotein I; aCL, anti-cardiolipin antibodies; AwMA, anti-whole mitochondria antibodies. AmtDNA, anti-mitochondrial DNA antibodies; Anti-dsDNA, antibodies against double-stranded DNA. Data in bold are statistically significant ($p < 0.05$).*

bi- and multivariate analyses were [OR(95% CI) = 0.53 (0.12–2.25)] and [OR(95% CI) = 0.37 (0.07–1.89)], respectively. Thus, the conclusions remain the same using either SLEDAI-2K cut-off value.

To determine whether AmtRNAs might qualify as efficient predictors of SLE, we optimized cut-off values by Youden's method (**Table 9**). Calculated cut-off values were 0.30 for AmtRNA-IgG and 0.52 for AmtRNA-IgM. Both parameters were very specific for SLE (0.90 for IgG and 0.87 for IgM). Even though both Ig isotypes displayed a certain lack of sensitivity [43 SLE patients (49%) positive for AmtRNA-IgG and 33 (38%) for IgM], their positive predictive values (0.93 and 0.89) suggest that AmtRNAs may be considered as biomarkers of interest. Importantly, of all of the anti-mitochondrial autoantibodies measured, AmtRNA-IgG was the most potent at discriminating SLE patients from healthy donors. In this regard, AmtRNA-IgG was closely followed by AmtDNA-IgM. In contrast, AwMA (IgG and IgM) and AmtDNA-IgG failed to efficiently discriminate SLE patients from healthy controls.

## DISCUSSION

Although the interplay between extracellular mitochondria and innate immunity has been well-described, the interactions between mitochondria and the adaptive immune system are less appreciated. Mitochondrial components are generally seen as potential damage-associated molecular pattern (DAMP) if released by cells, but their inflammatory potential may be different if they are also recognized by autoantibodies. Herein, we propose mtRNA as a novel source of mitochondrial autoantigens with high relevance to SLE.

Mitochondrial RNA is not the only mitochondrial sub-component with antigenic potential in SLE. The first descriptions of anti-mitochondrial antibodies (AMA) were published in the 1980's. However, the actual epitope(s) of some AMA remain unidentified (57). Thus, AmtRNA add to the more recently appreciated AmtDNA and AwMA (36). Adaptive autoimmunity targeting mitochondrial motifs is not unique to SLE: a humoral immune response against mitochondrial autoantigens was reported in various diseases, and described as 9 different types of AMA targeting distinct epitopes (namely, M1 to M9) (36, 57). While AMA have been observed in different contexts such as in cardiovascular diseases, iatrogenic disorders, secondary syphilis, APS and SLE, they are best characterized in PBC (57, 58). The latter is characterized by progressive infiltration of autoreactive lymphocytes through the hepatic portal system (48, 59, 60). These cells display targeted autoreactivities directed against different mitochondrial antigens specifically expressed by bile ducts (60) such as the E2 subunit of the pyruvate dehydrogenase complex (PDC-E2, also known as mitochondrial antigen M2) (61–64), sulfite oxidase (M4) (65), glycogen oxidase (M9) (47, 66, 67) as

**TABLE 7 |** Association of AmtRNA with clinically relevant SLE autoantibodies.

| | | AmtRNA | |
|---|---|---|---|
| | | **IgG** | **IgM** |
| aCL | IgG | (–) 0.26 ± 0.43 | **(–) 0.34 ± 0.40** |
| | | (+) 0.42 ± 0.91 | **(+) 0.56 ± 0.90** |
| | | $p = 0.14$ | $\mathbf{p = 0.01}$ |
| | IgM | (–) 0.26 ± 0.43 | **(–) 0.33 ± 0.38** |
| | | (+) 0.53 ± 0.95 | **(+) 0.86 ± 1.43** |
| | | $p = 0.19$ | $\mathbf{p = 0.002}$ |
| Lupus anticoagulant | | (–) 0.25 ± 0.45 | (–) 0.35 ± 0.40 |
| | | (+) 0.40 ± 0.67 | (+) 0.57 ± 0.98 |
| | | $p = 0.20$ | $p = 0.06$ |
| Anti-dsDNA antibodies | | **(–) 0.19 ± 0.28** | (–) 0.34 ± 0.41 |
| | | **(+) 0.70 ± 0.71** | (+) 0.55 ± 0.27 |
| | | $\mathbf{p < 0.0001}$ | $p = 0.10$ |

*Values presented as median ± IQR and Wilcoxon test p-value for patient positives (+) or negatives (–) for each variable.*
*aCL, anti-cardiolipin antibodies; AmtRNA, anti-mitochondrial RNA antibodies; Anti-dsDNA, antibodies against double-stranded DNA. Data in bold are statistically significant ($p < 0.05$).*

well as other antigens that have not yet been described (M8). Detection of these AMA in PBC by ELISA have a prognostic value: patients positive for AMA-M4 and -M8 suffer from active and/or progressive forms of the disease (68), whereas patients with only AMA-M2 and -M9 display diseases with delayed evolutions (69) (recapitulated in **Figure 1**).

How exactly the mitochondrial antibodies are produced is not completely understood, but mitochondrial antigens can be generated through the degradation of old or damaged mitochondria by a specific form of autophagy known as mitophagy. Autophagosomes containing mitochondria travel through the endolysosomal system, leading to the degradation of its cargo and allowing the production of mitochondrial peptides that can be processed and expressed by the major histocompatibility complex (MHC). Both MHC-I and MHC-II have been implicated (70), an involvement for the latter being suggested in the surveillance of mitochondrial mutations occurring in cancer (71, 72). However, a recent study revealed that mitochondrial antigen processing can also occur independently of mitophagy. In this case, mitochondrial antigens are carried to endosomes by mitochondrial-derived vesicles formed by a mechanism regulated by the proteins PINK1 and Parkin (73, 74). Whether these mechanisms are involved in the processing of mtRNA molecules remains to be established.

Mitochondrial RNA is a recognized trigger of TLR8, which similarly to bacterial RNA, stimulates peripheral blood mononuclear cells (75). As the most metabolically active cells express more mtRNA, they are more likely to contribute to mtRNA antigenic load (39). Our study demonstrates that mtRNA is also recognized by antibodies, suggesting that Fc receptors may be implicated in the internalization of mtRNA-IgG complexes by endosomes, thereby favoring interactions with TLR8. Mitochondria express various RNA species, the main one being ribosomal 16S RNA molecules (39). However, the respective antigenicity of each mtRNA species was not assessed in the present study. Moreover, the presence of certain nuclear messenger RNA has been described within mitochondria (39), which could also account for the antigenicity potential of the mitochondria. Considering the evidence for mitochondrial release in different pathogeneses, our demonstration of the presence of antibodies directed against mitochondrial RNA further confirms the role of mitochondria as a source of autoantigens in autoimmunity.

We observed associations between the three sets of mitochondrial antibodies (AwMA, AmtDNA, and AmtRNA), pointing to their common source. Moreover, both AmtDNA-IgG and AmtRNA-IgG were associated with positivity for anti-dsDNA antibodies, suggesting close relationships between auto-antibodies targeting distinct nucleic acids.

While the IgG targeting mtRNA were significantly elevated in SLE patients, the IgG recognizing mtDNA and whole mitochondria were not increased in these patients. These observations contrast with our previous findings, which involved a different cohort of patients and showed that AmtDNA and AwMA were significantly increased in SLE (36). The patients included in our previous work were recruited by the University of Toronto Lupus Clinic. The patients recruited in the present study (SARD-BDB) are characterized by a shorter median duration of the disease (10 vs. 6 years) that may account for reduced organ damage as indicated by the SDI score (median: Toronto = 1; SARD-BDB = 0) and the frequency of patients with lupus nephritis (Toronto: 38.5%; SARD-BDB: 16.3%). These differences may reflect the course of the disease with earlier titers of autoantibodies clearing detrimental circulating autoantigens (i.e., in the SARD-BDB cohort) until other pathophysiological processes such as epitope spread occur, eliciting immune complex-mediated organ damage. Another interesting aspect is the discrepancy between the representation of the various ethnicities included in both cohorts. The SARD-BDB is almost exclusively composed of Caucasians (Caucasian: 97.7%, Black: 1.2%, Other ethnicities: 1.2%), whereas the Toronto cohort includes a more diverse ethnic panel (Caucasian: 57 %, Black: 18 %, Asian: 21%. Other: 5%). Such differences between two groups of patients may also impact results such as the incidence, prevalence and mortality rates (76–79). Together, these differences may reflect upon the protective effects of AmtRNA-IgG reported in the present study. Moreover, these elements suggest that the spectrum of anti-mitochondrial antibodies may shift during the course of the disease.

The heterogeneity of the disease duration for the SLE patients included in the SARD-BDB allows the optimization of cut-offs by Youden's method that discriminate positive from negative samples. However, calculation of a universal cut-off requires detection of AmtRNA in newly-diagnosed SLE patients. Additionally, associations between AmtRNA and clinical features of the disease should be interpreted with caution, as clinical outcomes identified might have occurred before or at the same time than the blood draw. Verification of the temporal relationship between the production of AmtRNA and clinical

**TABLE 8 |** Association of AmtRNA with clinical manifestations in SLE.

| | AmtRNA | | | |
|---|---|---|---|---|
| | IgG | | IgM | |
| | OR (CI) | *p* | OR (CI) | *p* |
| Thrombotic events | 1.28 (0.24;6.77) | 0.77 | 0.93 (0.22;3.93) | 0.92 |
| | [1.15 (0.17;7.87)] | [0.88] | [1.00 (0.18;5.61)] | [1.00] |
| Presence of plaque | **0.14 (0.02–0.91)** | **0.04** | 0.83 (0.25–2.76) | 0.76 |
| | [0.16 (0.01–1.81)] | [0.14] | [0.82 (0.23–2.91)] | [0.76] |
| SLEDAI-2K ≥ 4 | 2.30 (0.73–7.26) | 0.16 | 0.86 (0.34–2.17) | 0.75 |
| | [3.04 (0.78–11.77)] | [0.11] | [0.68 (0.25–1.88)] | [0.46] |
| SDI ≥ 0 | 0.95 (0.28–3.21) | 0.94 | 0.50 (0.16–1.58) | 0.24 |
| | [0.85 (0.15–4.92)] | [0.85] | [0.46 (0.11–1.86)] | [0.28] |
| Positivity to anti-dsDNA antibodies | **34.97 (6.26–195.55)** | **<0.0001** | 1.92 (0.67–5.50) | 0.23 |
| | **[70.60 (6.31–789.47)]** | **[0.0005]** | [2.01 (0.50–8.11)] | [0.33] |
| Lupus nephritis | 0.17 (0.02–1.71) | 0.13 | 0.43 (0.08–2.30) | 0.33 |
| | **[0.02 (0.00–0.68)]** | **[0.03]** | [0.25 (0.04–1.48)] | [0.12] |

*Values presented as odds ratios (95% Wald Confidence Interval) and p-value from logistic regressions. In each instance, bivariate results are followed by multivariate analysis (between square brackets). Values in bold have a p-value ≤ 0.05.*

*AmtRNA, anti-mitochondrial RNA antibodies; CI: 95% Wald Confidence Interval; OR, odds ratio; SDI, lupus severity disease index; SLEDAI-2K, SLE disease activity index−2000. Data in bold are statistically significant (p< 0.05).*

**TABLE 9 |** Performance of cut-off values for AmtRNA, AwMA, and AmtDNA (OD $405_{nm}$).

| | | Cutpoint (95% BCI) | Sensitivity (95% ECI) | Specificity (95% ECI) | PPV (95% ECI) | NPV (95% ECI) | AUC (95% ECI) |
|---|---|---|---|---|---|---|---|
| AmtRNA | IgG | **0.30 (0.11–0.54)** | **0.49 (0.38–0.60)** | **0.90 (0.73–0.98)** | **0.93 (0.82–0.99)** | **0.38 (0.27–0.50)** | **0.72 (0.62–0.82)** |
| | IgM | **0.52 (0.24–0.64)** | **0.38 (0.28–0.49)** | **0.87 (0.69–0.96)** | **0.89 (0.75–0.97)** | **0.33 (0.23–0.44)** | **0.62 (0.51–0.72)** |
| AwMA | IgG | 0.30 (0.12–0.44) | 0.36 (0.26–0.47) | 0.80 (0.61–0.92) | 0.84 (0.68–0.94) | 0.30 (0.21–0.42) | 0.57 (0.45–0.69) |
| | IgM | 0.68 (0.19–1.37) | 0.24 (0.16–0.35) | 0.87 (0.69–0.96) | 0.84 (0.64–0.96) | 0.29 (0.20–0.39) | 0.48 (0.37–0.60) |
| AmtDNA | IgG | 0.44 (0.22–1.25) | 0.35 (0.25–0.46) | 0.77 (0.58–0.90) | 0.81 (0.65–0.92) | 0.29 (0.19–0.40) | 0.51 (0.40–0.62) |
| | IgM | **0.36 (0.24–0.57)** | **0.51 (0.40–0.62)** | **0.83 (0.65–0.94)** | **0.90 (0.78–0.97)** | **0.37 (0.26–0.50)** | **0.65 (0.55–0.75)** |

*Values in bold have an AUC significantly different than 50%.*

*AmtDNA, anti-mitochondrial DNA antibodies. AmtRNA, anti-mitochondrial RNA antibodies. AUC, area under the curve. AwMA, anti-whole mitochondria antibodies. BCI, Bootstrap Confidence Interval. ECI, Exact Confidence Interval. NPV, Negative Predictive Value. OD, optical density. PPV, Positive Predictive Value. Data in bold are statistically significant (p< 0.05)*

outcomes would require a study of the variation in anti-mitochondrial antibodies titers over time and their levels at the onset of a clinical outcome in a large prospective inception cohort.

Systemic lupus erythematosus is a highly complex disease, many aspects of which still elude researchers (80). To date, only a limited number of biomarkers are available (81, 82). There is an intense effort to discover new biomarkers that would allow specific discrimination of SLE patients from both healthy individuals and those with diseases that have clinical features close to those of SLE (83). From this perspective, we present AmtRNA-IgG as antibodies present in SLE and APS, two diseases that are often associated with each other. Interestingly, AmtRNA-IgG appeared to be associated with less lupus nephritis and plaque formation in the carotid. Together, these elements indicate that AmtRNA may have prognostic value and help to identify patients with specific clinical profiles. Moreover, the different associations of AmtDNA and AmtRNA with lupus nephritis (AmtDNA are positively associated with nephritis, while AmtRNA display a negative association) may help predict SLE patients at risk of kidney damage.

Our study highlights that expression of a broad repertoire of anti-mitochondrial antibody subtypes (AMA; AMA-M1, AMA-M5, AwMA, AmtDNA, AmtRNA) is a major feature of SLE, with specific targets being associated with different clinical features. Future studies dedicated to the characterization of the mitochondrial autoantigens

recognized in SLE and their outcome on disease progression may provide useful information that will ultimately help to improve diagnosis, prognosis, and stratification of SLE patients.

## ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Research Ethics Board of the CHU de Québec—Université Laval with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by Research Ethics Board of the CHU de Québec—Université Laval.

## AUTHOR CONTRIBUTIONS

Experiments were conceived and designed by YB, IA, PF, and EB. JR contributed critical reagent, resources, and expertise. Experiments were performed by YB, GM, IA, and HB-F. Data were processed and analyzed by YB, GM, IA, R-CL, A-SJ, and supervised by R-CL, A-SJ, PF, and EB. The manuscript was written by YB, GM, PF, and EB, and critically reviewed by all authors.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.01026/full#supplementary-material

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HYPOTHESIS AND THEORY
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# The dsDNA, Anti-dsDNA Antibody, and Lupus Nephritis: What We Agree on, What Must Be Done, and What the Best Strategy Forward Could Be

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This study aims to understand what lupus nephritis is, its origin, clinical context, and its pathogenesis. Truly, we encounter many conceptual and immanent tribulations in our attempts to search for the pathogenesis of this disease—and how to explain its assumed link to SLE. Central in the present landscape stay a short history of the early studies that substantiated the structures of isolated or chromatin-assembled mammalian dsDNA, and its assumed, highly controversial role in induction of anti-dsDNA antibodies. Arguments discussed here may provoke the view that anti-dsDNA antibodies are not what we think they are, as they may be antibodies operational in quite different biological contexts, although they bind dsDNA by chance. This may not mean that these antibodies are not pathogenic but they do not inform how they are so. This theoretical study centers the content around the origin and impact of extra-cellular DNA, and if dsDNA has an effect on the adaptive immune system. The pathogenic potential of chromatin-anti-dsDNA antibody interactions is limited to incite lupus nephritis and dermatitis which may be linked in a common pathogenic process. These are major criteria in SLE classification systems but are not shared with other defined manifestations in SLE, which may mean that they are their own disease entities, and not integrated in SLE. Today, the models thought to explain lupus nephritis are divergent and inconsistent. We miss a comprehensive perspective to try the different models against each other. To do this, we need to take all elements of the syndrome SLE into account. This can only be achieved by concentrating on the interactions between autoimmunity, immunopathology, deviant cell death and necrotic chromatin in context of elements of system science. System science provides a framework where data generated by experts can be compared, and tested against each other. This approach open for consensus on central elements making up "lupus nephritis" to separate what we agree on and how to understand the basis for conflicting models. This has not been done yet in a systematic context.

**Keywords: chromatin, dsDNA, autoimmunity, lupus nephritis, enigma, controversies**

## INTRODUCTION

In this critical review, different aspects of pathogenic processes suspected or proven to be involved in lupus nephritis are discussed; (*i)* The exposure of dsDNA, and the impact of its surface structure and net charge exposed in pure dsDNA vs. DNA in chromatin; (*ii)* Anti-dsDNA antibodies, whether homologous or heterologous depending on whether instigated by DNA or non-DNA structures, and what they recognize in glomeruli; (*iii)* If lupus nephritis in a critical sense is an intrinsic part of SLE; and as a direct consequence of the last question; (*iv)* Whether SLE is an abstraction without a clear definition, which may allow us to regard lupus nephritis as a single disease entity; and (*v)* Whether production of anti-dsDNA antibodies induce the same pathogenic processes in non-SLE (like in cancer) patients as they do in SLE. In other words, can lupus nephritis etiologically be regarded as an integrated part of SLE—or can it stand alone? These dilemmas may not center around a clinical diagnosis, but around processes that may describe the molecular and cellular events that in sum define lupus nephritis. In this context, it is important to discuss factors that prime the inflammatory processes in lupus nephritis, and not secondary inflammatory mediators like complement activation, cytokines or their receptors, because the initiators of lupus nephritis inherit the principle, while inflammatory pathways are secondary responses instigated by the principal inducers of lupus nephritis—like type II or type III immune mediated tissue inflammation. In fact, if we summarize data over the last decades, both type II and type III have been claimed to account for lupus nephritis. One tribulation is whether type II immune mediated nephritis is more like Goodpasture syndrome (1, 2) than like lupus nephritis. However, there are many more problems that need to be solved before we can develop a true pathogenic model of lupus nephritis (see below). These problems represent the focus of this study.

## THE dsDNA: STRUCTURE, AUTOIMMUNE INDUCER, AND TARGET—STATUS AND A SHORT SCIENTIFIC HISTORY

In two foregoing studies, an historical and contemporary overview of anti-dsDNA antibodies (3) and a condensed history of the evolution of our contemporary opinions on SLE (4) have been published. These two studies aimed at a central understanding of the role of dsDNA and how it is involved in lupus nephritis. On the other hand, it is possible that dsDNA plays a bystander role in the disease, if e.g., anti-dsDNA antibodies recognize different obligate glomerular structures (see below). In that sense it is essential to approach historical and contemporary studies and hypotheses as backdrops to understand how paradigms related to SLE and anti-dsDNA antibodies have evolved over time. In other words, history is also in this context important to consider in order to understand contemporary paradigms. Ludvik Fleck once said*: "For the current state of knowledge remains vague when history is not considered, just as history remains vague without substantive knowledge of the current state*" [(5), cited in (4)].

Whether the antibodies described in 1957 in SLE (6–9) were specific for dsDNA and not for other DNA structures like ssDNA can be discussed in terms of history of science on dsDNA. The scientific history of DNA originates from studies performed during the 19th century. DNA was first identified as a unique substance in the late 1860s by the Swiss chemist Friedrich Miescher [(10), see also the biographical presentation of Miescher by (11)]. In the aftermath of Miescher's discovery, studies revealed fundamental details about the DNA molecule. This resulted in important discoveries describing the chemical composition of DNA, including its primary chemical components and the ways in which chains joined with one each other. Central scientists were Phoebus Levene, who provided evidence that different forms of nucleic acids existed—DNA and RNA, and he also determined that DNA contained adenine, guanine, thymine, cytosine, deoxyribose, and a phosphate group (12); and Erwin Chargaff, who was the first to present evidence that the DNA structure exists as a double helix constituted by two complementary single-strand DNA molecules (13, 14) (see below). With these important and pioneering studies, the enigma of inheritance started to be revealed.

A central researcher aiming to solve this scientific puzzle was Rosalind Elsie Franklin, a British chemist [see the comprehensive biography on Rosalind Franklin by Brenda Maddox, (15)]. Franklin contributed significantly to the discovery of the three-dimensional structure of dsDNA by X-ray crystallography (16). In these studies, she precisely described the double helix of DNA, a discovery that placed her in the first row of those days biochemical scientists aimed to describe the nature, structure and function, basically of Miescher's "nuclein" (11) and transformed it into dsDNA! Watson and Crick and their studies that were published in 1953 stated that the DNA molecule exists in the form of a three-dimensional double helix (17). Their conclusions were based particularly on Franklin's analyses and her interpretations, but also on results of the studies performed by Levene and Chargaff. One may consider if Watson and Crick at all should be in the first line of candidates to receive the Nobel price. Levene, Chargaff and Franklin presented all the elements to describe dsDNA as a double helix three-dimensional DNA structure.

The central work of Chargaff, Levene, and Franklin were remodeled into the paradigms now called The Chargaff's rules. These paradigms state that any DNA from cells of any species have a 1:1 ratio (base Pair Rule) of pyrimidine and purine bases. They stated that the amount of guanine equals the amount of cytosine, and that the amount of adenine equals the amount of thymine. This double helix pattern of DNA is equal in DNA from all species and provides evidence that we all evolve from the same genetical principle (see **Figure 1** and **Table 1**).

### Chargaffs Rules (13, 14)

Chargaff demonstrated that the double helix was created and stabilized by A–T and C–G interactions. The data of his experiments were organized and summarized as

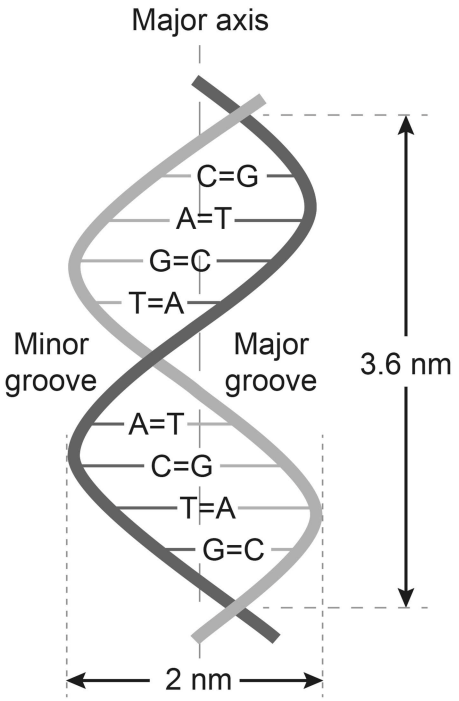


**FIGURE 1 |** Structure of dsDNA and Chargaffs rules for a double-helix dsDNA. In this figure Chargaff's first rule demonstrates that DNA from any cell of all organisms have a 1:1 ratio (base Pair Rule) of pyrimidine and purine bases and, more specifically, that the amount of guanine is equal to cytosine and the amount of adenine is equal to thymine. This pattern is found in both strands of the DNA. The figure also demonstrates Chargaffs second rule saying that the proportion of A/T and C/G holds true for both strands.

Chargaff's Two Rules (see **Table 1** for examples including human dsDNA):

1. The number of Adenine bases is equal to the number of Thymine bases and the number of Cytosine is equal to Guanine bases: (nA = nT; A/T = 1; nC = nG; C/G = 1), and the sum of A, T, C, G, is always 100% in the DNA double helix molecule isolated from a cell.
2. The proportion of A/T and C/G holds true for both strands.
   In sum: A/T=G/C=1.

All antibodies that bind nucleic acids characterized by the ratio in the formula 1 given above must consequently bee defined as anti-dsDNA or anti-native dsDNA antibodies.

Considering the rough methods Chargaff, co-workers and successors used to purify nuclein (DNA), the double helix must have been very robust. We know that the DNA purified for the purpose of detecting anti-dsDNA antibodies was in fact dominantly dsDNA also without further active elimination of ssDNA [(19, 20), Rekvig, unpublished observations]. Thus, the dsDNA structure described above turned out to be the target for anti-dsDNA antibodies in SLE, a statement that also may be valid for the early 1938 detection of anti-DNA antibodies in context of infections (21–23), and that complexes between them had the potential to induce inflammation in SLE-related lupus nephritis [for review see e.g., (4) and below].

**TABLE 1 |** This table is a representative sample of Chargaff's et al. (13) data, taken with slightly modified table published by Bansal (18), listing the base composition of DNA from various organisms.

| Organism | Taxon | %G | %C | G/C | %A | %T | A/T | %GC | %AT |
|---|---|---|---|---|---|---|---|---|---|
| Maize | *Zea* | 22.8 | 23.2 | 0.98 | 26.8 | 27.2 | 0.99 | 46.1 | 54.0 |
| Octopus | *Octopus* | 17.6 | 17.6 | 1.00 | 33.2 | 31.6 | 1.05 | 35.2 | 64.8 |
| Chicken | *Gallus* | 22.0 | 21.6 | 1.02 | 28.0 | 28.4 | 0.99 | 43.7 | 56.4 |
| Rat | *Rattus* | 21.4 | 20.5 | 1.00 | 28.6 | 28.4 | 1.01 | 42.9 | 57.0 |
| Human | *Homo* | 20.7 | 20.0 | 1.04 | 29.3 | 30.0 | 0.98 | 40.7 | 59.3 |
| Grasshopper | Orthoptera | 20.5 | 20.7 | 0.99 | 29.3 | 29.3 | 1.00 | 41.2 | 58.6 |
| Sea Urchin | Echinacea | 17.7 | 17.3 | 1.02 | 32.8 | 32.1 | 1.02 | 35.0 | 64.9 |
| Wheat | *Triticum* | 22.7 | 22.8 | 1.00 | 27.3 | 27.1 | 1.01 | 45.5 | 54.4 |
| Yeast | *Saccharomyces* | 18.7 | 17.1 | 1.09 | 31.3 | 32.9 | 0.95 | 35.8 | 64.4 |
| *E. coli* | *Escherichia* | 26.0 | 25.7 | 1.01 | 24.7 | 23.6 | 1.05 | 51.7 | 48.3 |
| φX174 | *PhiX174* | 23.3 | 21.5 | 1.08 | 24.0 | 31.2 | 0.77 | 44.8 | 55.2 |

*The data support both of Chargaff's rules (13, 14). An organism such as φX174 with significant variation from A/T and G/C equal to one, is indicative of single stranded DNA.*

## ANTI-dsDNA ANTIBODIES: HOW ARE THEY FORMED—AND IN WHICH PRINCIPAL CLINICAL CONTEXTS

To answer these questions, we have to rigorously define whether an anti-dsDNA antibody represents a response to exposed dsDNA or to a non-dsDNA/non-DNA structure by molecular mimicry (see below).

### Anti-dsDNA Antibodies: Is dsDNA a Stable Structure That May Be Immunogenic *in vivo*?

Interpretation of the structure originally called nuclein, as a derivation from Chargaffs rules, the DNA was most probably used in the first assays in the form of the canonical double helix DNA. Since the A/T and G/C ratios were stable [**Table 1**, and **Figure 1** (13, 17)] and since they in sum did not deviate toward an overrepresentation of any of the bases that could indicate presence of ssDNA domains (**Table 1**), we can in retrospect conclude that the autoantibodies observed in 1957 in SLE (6–9) recognized dsDNA as a stable structure—and that they cross-reacted with dsDNA from as different species as like viral, bacteria, and mammals. DNA is present in all nucleated cells. If exposed chromatin is potentially dangerous to the body, as discussed by e.g., Darrah and Andrade (24) and by others (25–29), this may illuminate how important it is to remove chromatin from dying cells in an abrupt and silent way.

However, in individuals with anti-dsDNA antibodies and impaired clearance of cell debris including necrotic chromatin, like in SLE (30–37), this may change the situation from a controlled removal of chromatin into a condition where chromatin debris remains exposed and may be a contributor to produce and or amplify anti-dsDNA antibodies by interaction with TLR9 and to promote inflammation. This may be caused by slow removal of extra-cellular dsDNA by e.g., silencing of DNase I or blocking of DNase I activity since binding of anti-dsDNA

antibodies to DNA may inhibit the effect of the endonucleases [discussed in (29)]. In this situation, externalized DNA is further targeted by anti-dsDNA/anti-chromatin antibodies, and immune complexes may be formed both *in situ* and in circulation (3, 38–40) and, as a consequence, induce serious inflammation (see below).

Furthermore, once anti-dsDNA antibody production is initiated (irrespective mechanism), an anti-dsDNA antibody may bind dsDNA in the extra-cellular compartment. In a normal situation, *in vivo* autologous dsDNA is rapidly and completely digested by DNases. On the other hand, anti-dsDNA antibodies may be produced and form immune complexes with the consequence that autologous DNA-containing fragments are resistant to DNases, then they may bind the DNA-specific B cell receptor and is transported into endosomes/lysosomes where TLR9 is sensing unmethylated CpG dinucleotides (41). Stimulated TLR9 acts via MYD88 and TRAF6, leading to NF-kappa-B activation, cytokine secretion and the inflammatory response (42, 43). TLR9 promote in this way increased inflammation and amplification of anti-dsDNA antibodies [(44, 45), see a model in **Figure 2**].

In sum, the pioneers that described nuclein (11) as dsDNA (12, 13, 17), had a substantial influence on the discovery of anti-dsDNA antibodies in an autoimmune context in 1957 (6–9) and on the potential of dsDNA and anti-dsDNA antibody complexes to drive inflammation, as we see in e.g., lupus nephritis.

## SLE: A Disease With High Rates of Infectivity and DNA-Specific Autoimmunity—Is the Latter Depending on the First?

Does the immune system need infection as a sine qua non-contributor to incite anti-chromatin/anti-dsDNA antibodies (see main hypothesis in **Figure 3A**)—and is this hypothesis a factual substantiation of the hapten-carrier system in which T cells recognize the infectious-derived chromatin-bound protein (exemplified by polyomavirus T antigen in **Figure 3A**) while B cells recognize hapten-like autologous chromatin structures as dsDNA, histones, transcription factors and other chromatin-associated proteins [**Figure 3A** (46–50), reviewed in (3, 51)].

Over so many years, we have not succeeded in understanding what the immune system recognize and act upon in context of spontaneous production of anti-dsDNA antibodies *in vivo*. Since the 1980s, studies were concerned around the following problems; what instigated anti-dsDNA antibodies, what were their targets, dsDNA or non-DNA structures (52–54), why did they correlate with disease (SLE) [(55), reviewed in (4)], how to detect them in the most appropriate way, and what make them pathogenic (56–58)? This is a concentrate of the problems being in focus over the last 50 years—*and still is*. Do cell death in context of infection, and consequent release of hetero-complexes between host chromatin and infectious-derived ligands explain the whole repertoire of chromatin antibodies in SLE (see **Figure 3A**), known to be overrepresented with respect to infections and to factual production of anti-dsDNA antibodies [see e.g., references (59–72)]? For example, the Epstein-Barr nuclear antigen 1 (EBNA1) (50); the C-terminal DNA-binding domain of the human papillomavirus E2 protein (46); the Fus 1 peptide derived from Trypanosoma cruzi (73); or polyomavirus large T antigen (47, 48) have all the evident and predictive potential to render dsDNA/chromatin immunogenic *in vivo* upon complex formation. Infection and autoimmunity may therefore be linked together in many situations where infections tend to be chronic or recurrent, and cell death rates are high (see **Figure 3A** as a model to explain linked production of different chromatin antibodies when infectious-derived proteins bind chromatin fragments). This model is not restricted to chromatin autoimmunity, but also to other autologous proteins. For example, Dong et al. demonstrated that complexes of T antigen and the tumor suppressor protein p53 terminated tolerance to p53 (74, 75).

In this sense, one idea is that infectious DNA-binding proteins and DNA/chromatin fragments are walking hand in hand in their successful promotion of chromatin autoimmunity. In this picture B cells represent the autoimmune hand while infectious protein-specific T cells represent the immune hand—and upon contact they stimulate each other and transform the B cells to be autoantibody-producing plasma cells. This has been directly demonstrated in studies were T antigen-expressing plasmids were injected in experimental animals under control of eukaryotic transcription factors (47, 48). In this context, exposed chromatin and its different molecular structures can all be *targeted* by anti-dsDNA and anti-chromatin antibodies if induced by chromatin fragment-viral DNA-binding proteins (See **Figures 3A**,**B** for a model thinking). This model says that specters of chromatin antibodies which are induced by chromatin-peptide complexes all can target exposed chromatin *in situ* and provoke serious inflammation.

## Cancer: A Group of Malignant Diseases With High Rates of Infectivity and DNA-Specific Autoimmunity—Is the Latter Depending on the First?

In this sense, cancer may represent a mirror image of autoimmunity in SLE with respect to infectivity rates and termination of tolerance for dsDNA and chromatin constituents. In line with this, anti-dsDNA antibodies are frequently detected in cancers [reviewed in (3, 4)]. In 1991, the Nobel prize winner zur Hausen suggested that most of all of human cancers worldwide are linked to viral infections, including human papillomaviruses, human T-cell leukemia viruses, hepatitis B virus, Epstein-Barr virus and polyomaviruses (76, 77). At the same time, several virus-associated cancer forms are connected with the production of autoantibodies against dsDNA [see e.g., (47, 48) reviewed in (3)]. The impact of viruses in different cancer forms, and if or how viruses influence the malignancy of tumor cells may, according to zur Hausen, need to be revised in light of new viruses that has been discovered in cancer forms since zur Hausen's data were discussed in his 1991 Science paper (76). Since cancer and SLE are largely segregated, the slight over-representation of cancer in SLE (78) does not reduce the arguments for the view that anti-dsDNA

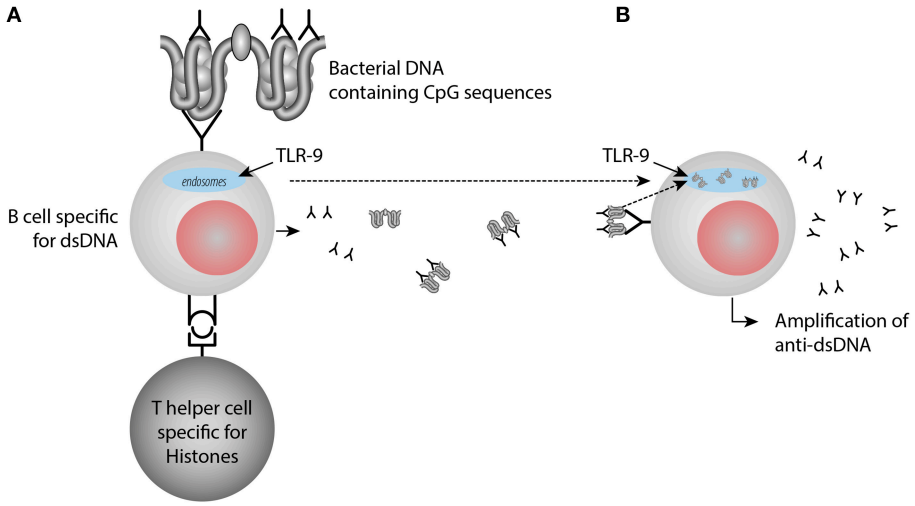


**FIGURE 2 |** Amplification of anti-dsDNA antibody responses through activation of TLR9 by immune-complexes containing DNA-anti-DNA. In **(A)** anti-dsDNA antibodies are induced by a classical hapten-carrier complex, in which dsDNA in form of small chromatin complexes represent the hapten and histone peptides represent the carrier protein. These interactions transform B cells into anti-dsDNA antibody producing plasma cells and enter the extracellular space. Upon cell death, chromatin is degraded and removed in a fast and silent way by DNases. Anti-dsDNA antibodies bind these small chromatin fragments, make them resistant to DNase. Then they bind dsDNA through the dsDNA-specific B cell receptor and the dsDNA fragments enter into the endosomes, where TLR9 is sensing unmethylated CpG dinucleotides **(B)**. Stimulated TLR9 promotes cytokine secretion and the inflammatory response and amplification of anti-dsDNA antibodies. TLR9 promote in this way increased inflammation and amplification of anti-dsDNA antibodies upon TLR9 sensing of CpG9.

antibodies are generated independently, although possibly by similar molecular and cellular mechanisms (3) in the two different types of conditions.

In SLE, the antibodies may crossreact with and bind inherent renal antigens or chromatin fragments exposed in kidneys [(79), present study] and initiate nephritis, although the two binding profiles are principally different as one is type II and the other is type III immune mediated inflammation. On the other hand, inflammation in juxtaposition to a tumor may indicate that autoantibodies may target chromatin released from necrotic tumor cells and promote local inflammation in analogy to kidney inflammation caused by antibody-binding to chromatin exposed in glomeruli (80, 81). Implication of anti-dsDNA antibodies in tumor-associated tissue has not been directly investigated. Also in cancers, anti-dsDNA antibodies are from a principal concern not clinical epiphenomenons, although their genesis is still poorly understood (if not categorized as local infectious-driven autoantibodies as principally outlined in **Figures 3A,B**). One potent hypothesis may therefore be the impact in cancers of infections and anti-dsDNA antibodies that are induced by complexes of tumor-derived chromatin and DNA-binding infectious-derived peptides.

## Genesis of the Anti-dsDNA Antibody *in vivo*: Closely Linked to Infections

The role of light chain editing to abolish and control anti-dsDNA reactivity is recently discussed in SLE [see reference (4) for a brief discussion]. This type of regulation can be impaired by SLE susceptibility factors, thereby allowing DNA-specific B cells to expand in SLE [see (4) and references herein].

Till now, no clear evidence have been presented that convincingly state that anti-dsDNA antibodies are *initiated* by sole exposed autologous DNA/chromatin [(3, 51, 73), discussed in a highly relevant way back in 1994 by (82)], irrespective whether they are exposed as native or cell death-associated modified chromatin structures [discussed in (3, 29, 51)]. However, infectious-derived DNA/chromatin-binding proteins in complex with chromatin fragments can provide strong T helper cell stimuli and promote transformation of autoimmune B cells into autoantibody-producing plasma cells (3). This brings to light that the *infectivity* state characteristic of SLE or of cancers is in intimate context with (auto-)immune competent cells both physically and functionally (47, 48). This was directly hinted on already at the time of the first discovery of anti-dsDNA antibodies in 1938–1939 in patients suffering from bacterial infections (21–23, 83). Other up today examples of the link between polyomavirus infection and anti-dsDNA antibodies was shown in small children with primary polyomavirus BK infections (84). These infected children produced antibodies to polyomavirus T antigen and transiently to mammalian dsDNA. T antigen is the BK virus' transcription factor and is therefore a DNA-binding protein that in a native situation binds both viral and host cell DNA [see above, reviewed in (85)]. In this situation T antigen was assumed to serve as a T helper cell-stimulating protein presented by DNA-specific B cells, once the T cells had been primed by dendritic cells presenting T antigen-derived peptides [discussed in **Figure 3**, reviewed in (3)]. Thus, both along the spontaneous BK virus infection (48, 84) and as a consequence of experimental expression of T antigen *in vivo* or other infectious agents, appearance of anti-dsDNA and other anti-chromatin antibodies is a predictive outcome [(46, 47),

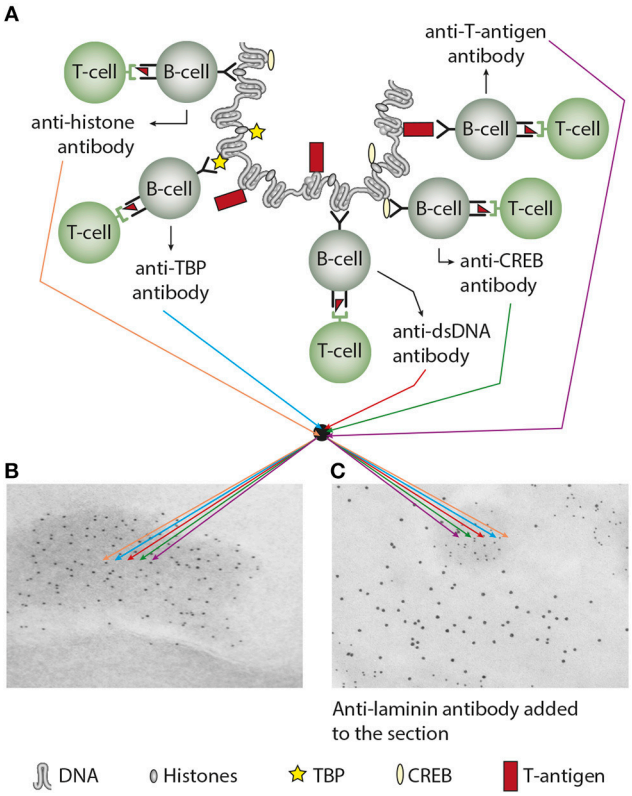


**FIGURE 3 |** Experimental induction of anti-dsDNA antibodies and other chromatin autoantibodies by *in vivo* expression of a single viral dsDNA-binding protein. In **(A)**, Injection of normal mice with plasmids encoding wild type polyomavirus DNA-binding T antigen in context of eukaryotic promoters predictively induced production of antibodies to T antigen and significant production of antibodies to mammalian dsDNA, histones, and to certain transcription factors like TATA-binding protein (TBP) and cAMP- responsive element-binding protein (CREB). All autologous chromatin-derived ligands physically linked to T antigen can therefore be rendered immunogenic to autoimmune B cells that present peptides derived from T antigen. Therefore, concerted production of autoantibodies specific for chromatin antigens, including dsDNA and histones, is not depending on a systemic lupus erythematosus background, but may appear also in quite healthy individuals. In **(B)** the group of chromatin autoantibodies notably including anti-dsDNA antibodies target exposed chromatin in kidneys. As demonstrated by immune electron microscopy, it is evident that the autoantibodies target electron dense structure (EDS), convincingly demonstrated to constitute chromatin fragments (the left immune electron microscopy in **(B)**. These autoantibodies did not bind GBM structures or in the mesangial matrix (seen as clean membranes). However, anti-laminin antibodies added to the sections *in vitro* bound GBM (sees as 10 nm gold particle-labeled antibodies), and they did not co-localize with *in vivo*-bound anti-chromatin antibodies (seen as 5 nm gold particles) **(C)**. These data argue for the fact that anti-dsDNA/anti-chromatin antibodies bound chromatin fragments, and they did *not* bind inherent, regular membrane components. **(A)** Is copied from Rekvig (4).

reviewed in (51)]. Then, why do children with primary BKV that produce anti-dsDNA antibodies not develop lupus-like nephritis or dermatitis? This may be explained by absence of exposed chromatin in glomeruli and in the dermal basement membrane zone of the skin due to the transient nature of the infection. This will be further discussed below.

## Deviant Cell Death Events Promote Exposure of DNA/Chromatin—Immunogen or Target?

If exposed dsDNA in form of chromatin has the potential to induce anti-dsDNA antibodies remain as an attractive, although yet an unproven model (29). Chromatin released by cell death may be linked to aseptic inflammation, and to the role of disordered cell death processes like exposure of DNA-containing neutrophil extracellular traps (NETs), secondary necrotic chromatin, microparticles, and may be linked to reduced elimination of dead cell debris (whether of apoptotic or necrotic origin) (27, 33, 86). NETs were first observed by Brinkmann et al. (87). Still, however, their function as an assumed complex defense structure (88) is not fully resolved [see a thoughtful discussion by (89)]. On the other hand, NETS and secondary necrotic chromatin have in several studies been suspected to be involved in inflammatory processes (28, 35, 90–92), and is assumed to account for increased levels of anti-chromatin antibodies. The latter association does not, according to my understanding of the relevant literature, mean that NETS or apoptotic chromatin *induce* anti-dsDNA antibodies. This is thoroughly discussed by Gupta and Kaplan in their review (25) who reached the conclusion that "*….many of the molecules externalized through NET formation are considered to be key autoantigens and might be involved in the generation or enhancement of autoimmune responses in predisposed individuals…..*" However, they did not state that NETS had the potential to *induce* anti-dsDNA antibodies. Similarly, Pieterse and van der Vlag conclude in their study "*…it can be concluded that increased apoptosis or NETosis on its own is not sufficient to break immunological tolerance to nuclear autoantigens in SLE, and additional factors are required to turn apoptotic material or NETs into danger triggers of autoimmunity.*" (29). Still, we do not understand whether NETs, necrotic chromatin or microparticles have the potential to induce antibodies to dsDNA or to native histones, although it has been demonstrated that they may initiate antibodies against cell death-modified histones [discussed in (25)].

In support of these considerations, Radic and Dwivedi have recently published a comprehensive and critical review on controversies related to NETs, cell death and autoimmunity (93). They came to the same conclusion as presented here as they hesitate to accept that NETs promote humoral autoimmunity against native chromatin components, inclusive dsDNA. The autoimmune consequence of perturbed order of cell death and the impact on adaptive immunity is hard to comprehend. It is probably an abstraction and not proven by evidence that these processes have the potential to promote production of anti-dsDNA antibodies, although the same structures may drive innate immune-dependent inflammation in SLE (36, 90, 94). However, diminished removal of nuclear debris has been demonstrated to correlate with production of antibodies to cell death-induced structural changes of proteins in chromatin. This is in harmony with earlier observations that while histone H4 is non-immunogenic, triacetylated histone H4 is (95). Recently, Dieker et al.

observed that autoantibodies against modified histone peptides in SLE patients were associated with disease activity and lupus nephritis (91).

Similarly, T cell responses to analogous modified structures do not allow us to interprete such (helper) T cells as activator of B cells and thus induce true, anti-native dsDNA autoantibodies [(26, 91, 94, 96–104), reviewed by Pieterse and van der Vlag (29)]. Data that demonstrate that deranged cell death debris can activate T and B cells specific for altered self chromatin are settled by solid experiments (26, 35, 91, 91, 92, 105). Whether antibodies or T cells against death-associated chromatin modifications have the potential to induce inflammation has not been thoroughly studied, but their recognition and binding to modified (homologous) chromatin structures exposed as NETs might well promote *in situ* formed immunocomplexes, and consequently inflammation. In harmony with these critical comments, Gordon et al. (96) demonstrated that NETs inhibition by different approaches, like genetically manipulated Nox-deficient mice, or by deletion of PADi4 or pharmacological inhibition of PAD4 activity hardly had any influence on nephritis, and NETs inhibition did not affect any aspects of nephritis, did not lead to loss of tolerance, nor to immune activation (96). Pharmacological inhibition of PAD activity did not affect the progression of nephritis into end-organ disease in inducible models of glomerulonephritis. The authors conclude that the data oppose the concept that NETs promote autoimmunity and target organ injury in SLE (96) in agreement with earlier observations by Campbell et al. (97).

Nevertheless, NETs may serve as *in situ* targets for the autoimmune responses and participate in evolution of organ injury in SLE. Thus, true anti-dsDNA antibodies may have the ability to sensitize NETs by forming immune complexes and to initiate inflammation since dsDNA in NETs may remain in their native state, and not modified during deviant cell death, as opposed to immunity to chromatin in secondary necrotic cells in which apoptotically modified autoantigens (dsDNA, high mobility group box 1 protein, apoptosis-associated chromatin modifications, e.g., histones H3-K27-me3; H2A/H4 AcK8,12, 16; and H2B-AcK12) are present (106).

Autoimmunity to dsDNA and native chromatin exists, but till now, their spontaneous appearance in a native context is still enigmatic. There is no solid evidence to say that native chromatin has immunogenic potential. However, native chromatin in complex with a DNA-binding viral protein (see above) is immunogenic because T cell tolerance, as is operative for native chromatin, is circumvented by the immunogenic infectious-derived carrier protein. There are yet no firm evidence stating that antibodies to native dsDNA are induced by perturbed cell death, although disorganized cell death may induce and *enhance* production of antibodies to chromatin-associated proteins modified in context of cell death (29, 93). Thus, although anti-dsDNA antibodies are easily detected in SLE, it is hard to explain why the antibodies materialize themselves and how they harm organs like the kidneys and skin in context of SLE (see below).

## PATHOGENIC POTENTIAL OF ANTI-dsDNA ANTIBODIES

Isolated dsDNA is negatively charged due to solvent phase exposed phosphate groups that makes up every nucleotide that consists of pentose, nitrogenous bases, and phosphate groups (see above). This makes it unlikely that isolated dsDNA binds directly to glomerulus basement membranes (GBM) in context of lupus nephritis because GBM is overall anionic and would therefore repel dsDNA. Rather, since mammalian dsDNA is part of chromatin, consisting of histone octamers, histone H1, and a large array of other non-histone proteins with various charges, dsDNA may indirectly bind to GBM through interaction of solvent phase cationic protein tails with anionic GBM structures. This forms the basis for formation of immune complexes between anti-dsDNA antibodies and dsDNA, and deposition of the complexes *in situ* along the GBM, and in the mesangial matrix of circulating dsDNA-containing immune complexes [reviewed in (3) and (81)]. By using surface plasmon resonance, we demonstrated that isolated dsDNA did not bind collagen or laminin, while chromatin fragments bound with relatively high avidity, irrespective presence or absence of complex-bound anti-dsDNA antibodies (107, 108). These data harmonize nicely with experiments performed in the Berden laboratory, where they demonstrated that immune complexes of anti-dsDNA antibodies and nucleosomes bound in glomeruli of perfused kidneys, while highly pure anti-dsDNA antibodies did not bind (109–111). Nevertheless, these data open for two ways how chromatin may promote inflammation; either by binding anti-dsDNA antibodies to chromatin exposed *in situ*, or by binding preformed chromatin-IgG complexes to GBM.

### Anti-dsDNA Antibodies: Are They Induced by dsDNA or Non-dsDNA Structures *in vivo*?

On the other hand, anti-dsDNA antibodies have in many studies been proven to be instigated by non-DNA structures [discussed in e.g., (3, 112–114), see **Table 2** for examples]. Therefore, anti-dsDNA antibodies may represent two principally different antibody populations; real anti-dsDNA antibodies induced by dsDNA, or (quasi) antibodies with potential to bind dsDNA although instigated by non-dsDNA structures. We are today not able to distinguish which is which. In context of the question if anti-dsDNA antibodies are induced by dsDNA or non-dsDNA structures *in vivo*, a logic issue would be if anti-dsDNA antibodies are *pathogenic* because they recognize dsDNA (homologous interaction) or non-dsDNA (heterologous interaction) in the kidneys.

Thus, anti-dsDNA antibodies may exert a pathogenic process by direct binding to inherent cross-reactive renal structures. This demonstrates that anti-dsDNA antibodies may promote two principally opposite pathogenic processes; They either bind chromatin fragments that are exposed and associated with GBM structures [denoted in this context "the chromatin model" see models in **Figures 3**, **4** (80)] or, they bind directly to glomerular antigens like laminin,

**TABLE 2 |** Examples of anti-dsDNA antibodies that cross-react with non-DNA structures.

| Anti-dsDNA antibody crossreact with | References |
|---|---|
| α-actinin | (113) |
| α-actinin | (115, 116) |
| Laminin | (117) |
| C1q | (118) |
| Laminin | (119) |
| Nucleosomes/laminin* | (120) |
| Platelet integrin GPIIIa 49–66 | (121) |
| Toll like receptor 4 | (122) |
| NR2 glutamate receptor | (123) |
| Cell surface proteins | (124) |
| Ribosomal P protein | (125) |
| Cross-reactive anti-dsDNA antibodies (2002) | (126) |
| Phosphorylcholine/phospholipids | (127) |
| EBNA 1 | (128) |
| Entactin | (114) |
| Entactin** | (129) |

*Renal eluates in this study contained several antibodies, notably with nucleosome antigens and laminin. Definitive prove for cross-reaction between laminin and nucleosomes-dsDNA was not provided.*

***Mono-specific anti-Entactin antibodies is included to be suggested as a control of non-cross-reactive, non-dsDNA antibodies to determine if they still have nephritogenic potential (see reference (43) for details).*

collagen, entactin, and others by cross-reaction (denoted "the cross-reaction model," see relevant data in **Table 2**, and **Figure 4**). Two variants of the chromatin model exist. In one; chromatin fragments are exposed in membranes and matrices due to the fact that chromatin fragments bind membranes and matrices at high affinity. If anti-dsDNA antibodies bind this form of chromatin, the immune complexes are formed *in situ* (107). In the other variant, formation of IgG-chromatin immune complexes occurs in circulation. Such pre-formed IgG- chromatin fragment immune complexes may bind primarily in the mesangial matrix and in GBM [reviewed in (3) and (4)]. The second variant is experimentally demonstrated. Injection of immunologically normal mice with highly pure anti-dsDNA monoclonal antibodies (mAbs) resulted in deposition of chromatin-fragment–IgG complexes in the mesangium and GBM (131, 132). Concentration of circulating chromatin fragments was significantly reduced after infusion of the antibodies. Similarly, if (NZB × NZW) nephritic mice were injected with pure biotinylated mAbs, these antibodies were observed in immune complex deposits observed as electron dense structures (EDS) in the mesangial matrix and in GBM (132). These data demonstrate that the experimental mAbs bound chromatin fragments *in vivo*. However, these experiments did not allow us to conclude whether they formed immune complexes *in situ* or in circulation. In line with this, circulating DNA–anti-dsDNA antibody complexes have been described (133) and discussed (134) in the context of SLE. Whether circulating complexes were associated to a certain stage of nephritis was not determined in those studies.

Combining data discussed above, mesangial nephritis, representing the early phase of lupus nephritis, may be instigated by circulating immune complexes (132), while progression of lupus nephritis into end stage organ disease is associated with silencing of the major renal endonuclease DNase I (see below). Loss of renal DNase I leaves chromatin from apoptotic cells undigested, and being retained in GBM. *In situ* formation of immune complexes by binding of circulating anti-dsDNA antibodies to the exposed chromatin fragments forms the basis for severe lupus nephritis (135). Thus, the chromatin model is in clear opposition to the cross-reactive model, and reflects the real pathogenic process of lupus nephritis (see below). It seems that we are far from reaching consensus on pathogenesis of lupus nephritis. Importantly, a proven cross-reactivity of an anti-dsDNA antibody will not provide information about which of the target antigens that binds the antibody *in vivo*.

## Why Are Anti-dsDNA Antibodies Pathogenic—and Are They All Pathogenic?

A central question is if the pure existence of anti-dsDNA antibodies is pathogenic through binding cross-reactive, obligate renal structures in SLE, or if they are epiphenomenons in absence of exposed chromatin structures (3, 4, 114, 136, 137). Thus, we have to evaluate how and why they are harmful, and in which contexts (36, 90, 94, 114, 138). This dilemma relates to the pathogenic effect irrespective whether in SLE or in other diseases like different cancer forms (see above). There is no reason to assume that an anti-dsDNA antibody produced in context of SLE differ in pathogenic impact from those produced in context of infection or cancer. There are many reasons to argue for and against these paradigms. However, these antibodies are pathogenic, but only in presence of relevant target structures. In other words; all have pathogenic potential, but they do not always transform potentiality into activity—i.e., transformation depends on whether the targets are exposed and accessible *in vivo*.

# LUPUS NEPHRITIS: CONTEXTS AND PATHOGENESES

While in end 1930s, DNA without further structural distinction or knowledge was determined to be an acceptor for anti-dsDNA antibodies (21–23, 83, 139). Shortly after the presence of anti-dsDNA antibodies were confirmed, they were in 1957 described in SLE (6–9, 140). In the following text, exposed dsDNA, like in NETs, chromatin or microparticles will not be further discussed. Rather, *specificity* of nephritogenic antibodies will be the focus.

In context of the discovery of SLE-related anti-dsDNA antibodies, it was soon clear that these antibodies were associated with SLE and with lupus nephritis. This perception represented a conceptual advantage in our understanding of pathogenic processes in SLE, although Fu et al. (136) proposed that anti-dsDNA antibodies are not crucial nor necessary to cause lupus nephritis. Nevertheless, this concept derives from 5 facts: (1) DNA has been reported to bind collagen, a component of GBM (141); (2) chromatin fragments bind laminin and collagen (107); (3) the nephritogenic antibodies bind DNA (chromatin

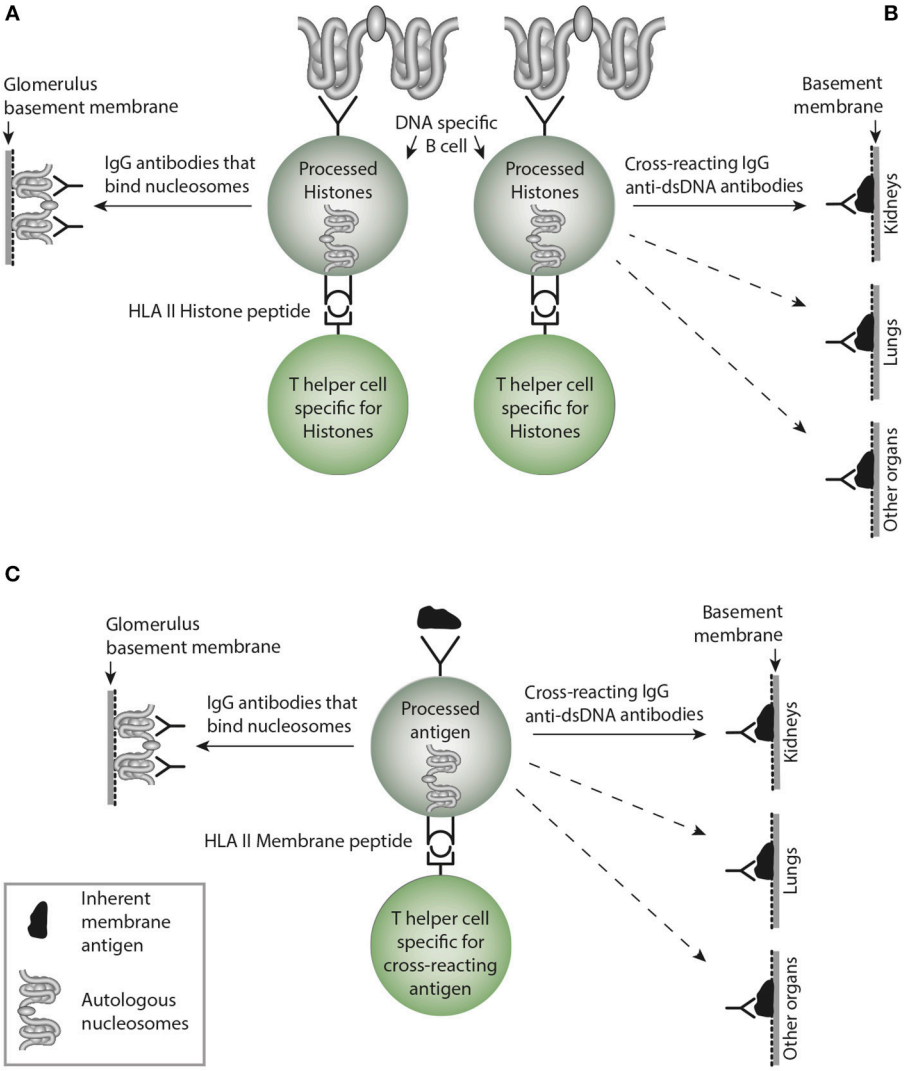


**FIGURE 4 |** Non-cross-reacting and crossreacting IgG immune responses induced by homologous or cross-reacting antigens. In **(A)** the B cells are specific for DNA as presented in chromatin. In the left side, B cells recognize and produce antibodies that bind (dsDNA) chromatin, i.e., homologous recognition. These may target exposed chromatin and initiate lupus nephritis by a Type III immune mediated tissue inflammation. In **(B)** the B cells are specific for dsDNA, but may, however secrete cross-reacting antibodies not targeting solely dsDNA. Instead they bind non-dsDNA cross-reacting inherent glomerular basement membranes, like entactin, laminin or collagen (see text). This has not been entirely investigated, but these cross-reactive antibodies might well initiate Goodpasture analog kidney, skin or lung diseases. This has not been considered in the literature (see text). In **(C)**, the B cells are specific for a (membrane)-component and cross-react with nucleosomes. Since the IgG antibodies may recognize membrane components in e.g., lung and other organs, they inherit the nature of collagen IV-like antibodies in Goodpasture syndrome. Although many such cross-reactions have been described, they have not drawn much attention in pathophysiological contexts. More studies are needed to explore these contexts. This figure is reprinted from Rekvig (130).

fragments) (38, 142); (4) anti-dsDNA antibodies can be purified from nephritic kidneys (38, 143, 144); (5) infusion of anti-dsDNA antibodies promote nephritis by binding glomerular structures (either GBM or exposed chromatin) in non-autoimmune mice (114, 122, 131, 132, 145, 146).

In a strict context, these facts involve recognition of DNA by antibodies linked to autoimmune inflammation in SLE, but do not necessarily provide information about which of the structures represent glomerular targets for the SLE-associated antibodies [chromatin or inherent glomerular structures, see e.g., the divergent interpretations by (130) and (40)]. The data only demonstrate that the pathogenic antibodies recognize *at least* dsDNA. As we will see, anti-dsDNA antibodies may even not by definition be denoted anti-dsDNA antibodies due to the vast number of cross-reactions/cross-stimulations with non-DNA/non-chromatin ligands or complex structures—like those in matrices and membranes (see the following discussion of this problem). Traditionally, we call this antibody family "anti-dsDNA" and/or "cross-reacting anti-dsDNA" antibodies. But these are merely biological abstractions

as long as we are not able to explain their real initiators and targets *in vivo*.

## LUPUS NEPHRITIS PATHOGENESIS: THE NEED TO DISTINGUISH AND VALIDATE PATHOGENIC MODELS

From what we know today, we may be forced to define a hierarchy of antibody specificities that are involved in the genesis of lupus nephritis. This may, surprisingly, not be a concise distinction: Maybe monospecific anti-dsDNA antibody is a fiction—indicating that all antibodies are oligospecific (multiple specificities)—or at least cross-reactive (dual specificity)? We have simply not yet sufficient information about this problem [see e.g., (147, 148)]. These somewhat naïve statements cannot rule out other non-DNA lupus nephritis-associated inflammatory factors, like antibodies that are dominantly specific for glomerular structures, as collagen (2, 149), laminin (115, 150), entactin (114), or the role of T cells [see e.g., discussions in (151, 152, 152–156)]. These may be relevant candidates to understand the inflammatory genesis of lupus nephritis.

### A Hierarchy of Disparate Anti-dsDNA Antibodies Are Pathogenic in Lupus Nephritis

In this context, there is an imperative need to understand the biological and pathogenic meaning of these factual observations. Therefore, we have to dissect *in vivo*-bound antibodies and antibodies with potential to bind *in vivo*, into four categories:

- Antibodies specifically binding chromatin and DNA (51), and anti-dsDNA antibodies that may be formed as a consequence of somatic mutation, even though the reverted germ-line V regions did not show any measurable autoreactivity in the elegant study of Wellman et al. (157). Their results indicate that anti-dsDNA autoantibodies may even develop from non-autoreactive B-cells by somatic hypermutation (157);
- Antibodies that cross-react with DNA and non-DNA glomerular structures (see **Table 2**, for examples and corresponding references);
- Antibodies that bind native chromatin fragments but not dsDNA;
- Antibodies bound *in vivo* but have no specificity for chromatin structures, but for glomerular non-DNA structures exposed in the membranes, like entactin, laminin, and collagen [see **Table 2** with relevant references, and the extensive review by (158)].

### One Pathogenic Model Implies That Anti-dsDNA Antibodies Bind Glomerulus Membrane-Associated Chromatin Fragments

Co-localization immune electron microscopy (IEM) analyses demonstrated that the electron-dense structures in mesangial matrix and in GBM were targeted *in vitro* by antibodies to dsDNA, histones and transcription factors, whereas co-localization terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) IEM demonstrated that these structures contained high concentrations of *in vivo*-bound IgG and TUNEL-positive DNA in both murine (159) and human (159, 160) lupus nephritis. These and similar data (135) indicate that anti-dsDNA antibodies exert their nephritogenic effect by binding to exposed chromatin fragments in glomerular membranes and the mesangial matrices (143, 160, 161), which is consistent with the fact that antibodies eluted from nephritic kidneys show specificity for chromatin fragments, histones and DNA as common denominators, although several other specificities have been detected in such eluates [see above (38, 143, 144)]. The early phase of mesangial nephritis might indeed be initiated by circulating immune complexes consisting of chromatin fragments complexed with IgG (132). Notably, by high resolution IEM we never observed antibodies bound *in vivo* to native GBM itself, nor to the mesangial matrix surrounding EDS (see **Figure 3B** as example, where antibodies are confined to EDS leaving GBM unstained).

#### The Role of Renal DNase I in Progressive Lupus Nephritis

We have demonstrated that progressive lupus nephritis correlates with loss of the central renal Dnase I endonuclease mRNA, and DNase I endonuclease activity. This event coincided with significantly reduced fragmentation of chromatin, leaving large chromatin fragments that accumulate *in situ* in glomeruli. If this happens in glomeruli of a person that produce anti-dsDNA antibodies, complexes of these partners (IgG anti-dsDNA antibodies and retained chromatin fragments) exert deleterious inflammatory effects on the integrity and function of the kidneys. Although not proven by solid evidence, chromatin fragments in kidneys with selectively silenced DNase I gene expression may derive from kidneys themselves, at least in progressive disease (86, 135, 162, 163).

#### Chromatin Metabolism in Kidneys in the Course of Lupus Nephritis

From both theoretical considerations and the comprehensive sets of coherent data discussed above, it is fair to conclude that glomerular extracellular chromatin fragments play a direct role in lupus nephritis, where they serve as homologous targets for anti-dsDNA/anti-chromatin antibodies. This conclusion also implies that the antibodies do not have an *a priori* nephritogenic potential in absence of chromatin. However, when chromatin is exposed in glomeruli, the antibodies are rendered nephritic (132). That is, isolated presence of either of the factors—the antibody or chromatin—remain in the body as clinical epiphenomenons! Therefore, the core nature of both murine and human lupus nephritis is pointing at an *acquired error of renal chromatin metabolism* due to silenced DNase I gene expression as a key event in disease progression [reviewed in (3, 4)].

## Another Pathogenic Model to Describe Lupus Nephritis Implies That Cross-Reactive Anti-dsDNA Antibodies Interact Directly With Glomerular Non-DNA Structures

The cross-reactive model inherits several problems that need to be described by experiments and analyses in lupus-prone mice and patients. The following questions need considerations.

### Is the Cross-Reacting Immune Response Sustained Over Time—The Problem of Affinity Maturation?

This is a central problem in this model. Sustained immune B cell stimulation may open for a successive loss of the cross-reactive specificity while the homologous response may remain with increased avidity and titer. Considering a sustained stimulus of the dsDNA-specific B cells by dsDNA, they will increase avidity for dsDNA as a consequence of affinity mutations, and since these mutations are random, they will/may by chance mutate away from the cross-reactive specificity. Thus, over time the cross-reactive specificity may slowly die out due to sense mutations for the immunogen (dsDNA), and non-sense mutation for the crossreacting specificity that inevitably will die out (see model thinking as presented in **Figure 5**).

### Is the Avidity for a Cross-Reactive Antibody Similar for Both Ligands or Will the Highest Avidity Direct the Antibody to That Antigen?

When we started our studies on the pathogenesis on lupus nephritis, we foresaw this problem. Therefore, we developed highly sensitive electron microscopy (EM) variants that with relatively high precision could determine the nature of the glomerular targets for nephritogenic antibodies. This included transmission EM and IEM to trace binding of antibodies *in vivo*, co-localization IEM, where we added different experimental antibodies to the renal sections, like antibodies to dsDNA, histones, transcription factors and GBM ligands like laminin, in order to analyze which of the added antibodies co-localized with *in vivo*-bound IgG. In addition, we analyzed loci of *in vivo* bound antibodies by TUNEL-colocalized IEM where we observed that TUNEL-positive DNA co-localized with *in vivo*-bound IgG. All our results were consistent and demonstrated that IgGs that bound *in vivo* were exclusively seen in EM as part of electron dense structures (see details in **Figure 3B**). No binding to podocytes or to regular GBM structures were observed (79). The same was true when we translated these experimental analyses to human lupus nephritis (79, 159).

### Will Antibodies Mono-Specific for a Non-DNA Cross-Reacting Antigen Bind in Glomeruli?

This question—and obvious deviating experiments—is in fact neglected in the literature in this context. We know that many anti-dsDNA antibodies cross-react with a large panel of non-dsDNA structures [See **Table 2**, and e.g., (112)]. By injecting cross-reacting (dsDNA-X) and non-cross-reacting non-dsDNA (X) antibodies into mice, may solve if one—or both specificities contribute to lupus nephritis.

### Similarly, If Crossreaction of Anti-dsDNA Antibodies With Renal Antigens Is Instrumental in Initiating Lupus Nephritis, Then Why Does the Disease Start in the Mesangial Matrix?

This is exactly what we observe in the BW mouse after injection of purified anti-dsDNA antibodies (135), and linked to loss of renal DNaseI endonuclease, the disease expanded from mesangial nephritis to membranoproliferative nephritis with deposits of the antibodies in GBM where they co-localize with chromatin fragments. If the antibodies bound *in vivo* crossreacted with e.g., laminin or entactin, we expected they should bind simultaneously in the mesangium and GBM.

### Are Cross-Reactive Antibodies Eluted From Nephritic Kidneys?

In search of the biological meaning of cross-reacting antibodies as essential in lupus nephritis, there are so far too few systematic analyses addressed to solve this problem. One clear exemption is the study of Deocharan et al. They analyzed anti-dsDNA antibodies that crossreacted with α-actinin and observed strong antibody activity in renal eluates (113). However, it is difficult from such observations to determine if the antibodies bound exposed chromatin or exposed α-actinin. More important, it would have been of strong interest if control injection with a non-cross-reacting (non-dsDNA) counterpart was performed. If they could be rescued from kidneys by elution, it would have been easier to make stronger conclusion on the impact of assumed pathogenic cross-reactive antibodies.

## CONCLUDING REMARKS

This study discusses two central problems: Are antibodies binding dsDNA really anti-dsDNA antibodies, or do they recognize dsDNA after being instigated by a non-dsDNA (cross-reacting) antigen. Secondly, and in line with the first problem, are these antibodies nephritic because they bind chromatin exposed on glomerular membranes or are they nephritic because they recognize inherent glomerular membrane structures. These two models—the chromatin model and the cross-reactive model—are still not fully understood, have not been agreed on, and are still promoting controversies. Yet, the discussions and contradictions aimed to describe the pathogenesis of lupus nephritis characterize the contemporary situation. Thus, we still lack a coordinated and open minded approach to obtain a general and evidence-based perspective by not taking all aspects of the syndrome SLE into account. This can only be achieved by concentrating on the biological and pathophysiological interactions between its different disease-promoting elements. We need a framework in which dissection of published data generated by experts in different fields like immunology, pathology, immunopathology, and experimental animal research can be combined and confronted with each other simply in order to determine what we agree on (is the anti-dsDNA antibody important?), what must be done (study the impact of the other side of the cross-reacting anti-dsDNA antibody), and what the best strategy forward must be (to collaborate between the different schools of hypotheses). Whatever its nature

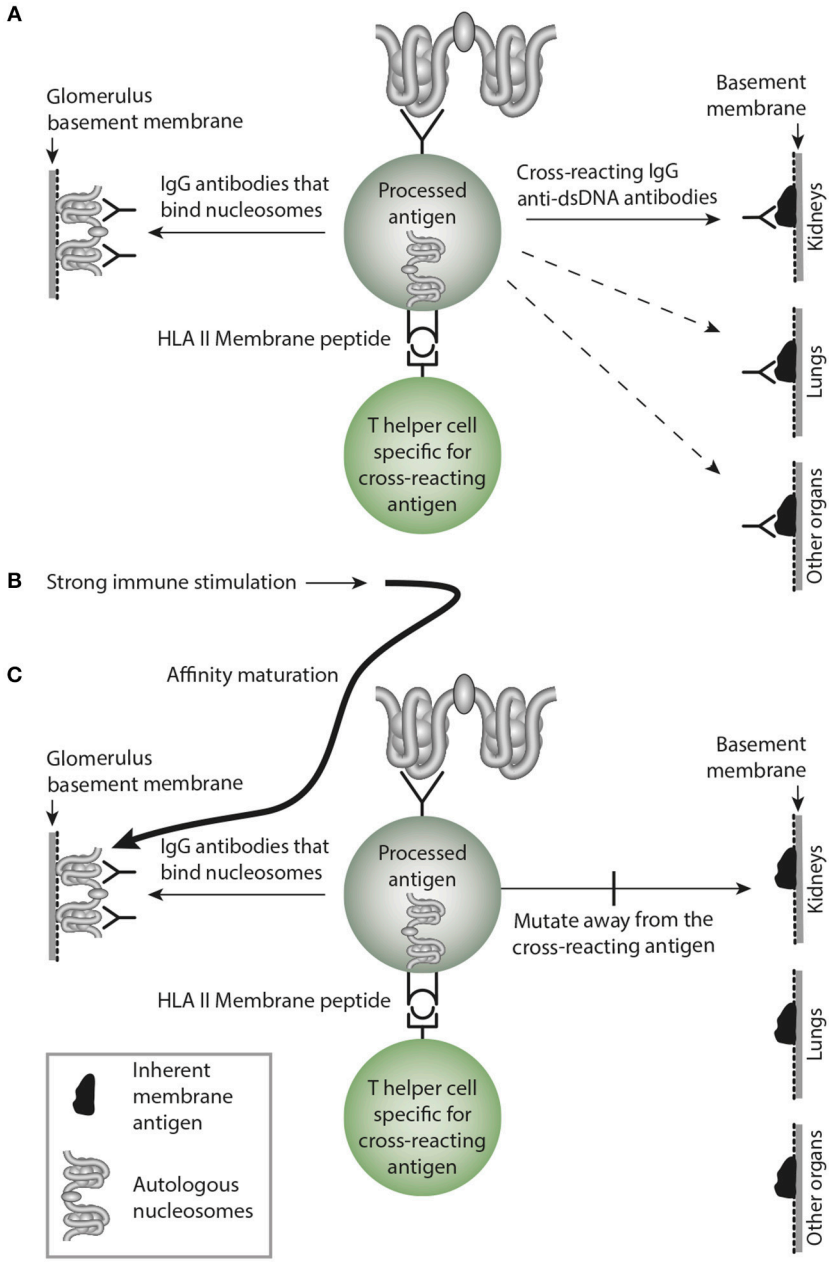


**FIGURE 5 |** Affinity maturation may transform a cross-reacting antibody into a monospecific antibody. In **(A)** a B cell bind nucleosomes by its antigen receptor, process them and present nucleosomes-derived peptides in context of HLA class II to peptide-specific T helper cells. In this example, the B cell transform into antibody-secreting plasma cells, and the emerging cross-reacting IgG antibody recognize nucleosomes, and they cross-react with an inherent glomerular membrane antigen like e.g., laminin. Since laminin is part of membranes in different organs, the cross-reactive antibody may bind in glomeruli, lungs and in other organs, similar to the anti-collagen IV in Goodpasture Syndrome. In **(B)** a strong and possibly sustained stimulus recruits more somatically mutated IgG molecules. In **(C)** mutations are selected that promote increased affinity for the B cell antigen, while mutations diminish affinity for the cross-reacting antigen, since these antigens are not selected by nucleosomes. This is a mechanism that may transform oligospecific into mono-specific IgG antibodies. This model therefore indicates that the effect of the cross-reactive specificity may over time faint or die out.

and origin might be, anti-dsDNA antibodies are a strange and challenging phenomenon—so is lupus nephritis and SLE also. And do not forget the role of T cells in lupus nephritis! As a conclusion for now, we are producing increasing numbers of puzzle pieces connected to the eponym SLE. We are not, however, halting and concentrating on organizing the picture that may tell us why the puzzle pieces belong to each other. New phenomenons are not needed if we do not put them into a context leading to our understanding of SLE and how to treat it.

## DISCLOSURE

The author declares he has no competing interests, or other interests that might be perceived to influence the results and discussions reported in this manuscript.

## ETHICS STATEMENT

The present manuscript is a review on murine and human SLE and lupus nephritis. All data are taken from original studies approved by relevant ethical committees.

## AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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# Circulating Levels of Interferon Regulatory Factor-5 Associates With Subgroups of Systemic Lupus Erythematosus Patients

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Systemic Lupus Erythematosus (SLE) is a heterogeneous autoimmune disease, which currently lacks specific diagnostic biomarkers. The diversity within the patients obstructs clinical trials but may also reflect differences in underlying pathogenesis. Our objective was to obtain protein profiles to identify potential general biomarkers of SLE and to determine molecular subgroups within SLE for patient stratification. Plasma samples from a cross-sectional study of well-characterized SLE patients ($n = 379$) and matched population controls ($n = 316$) were analyzed by antibody suspension bead array targeting 281 proteins. To investigate the differences between SLE and controls, Mann–Whitney *U*-test with Bonferroni correction, generalized linear modeling and receiver operating characteristics (ROC) analysis were performed. K-means clustering was used to identify molecular SLE subgroups. We identified Interferon regulating factor 5 (IRF5), solute carrier family 22 member 2 (SLC22A2) and S100 calcium binding protein A12 (S100A12) as the three proteins with the largest fold change between SLE patients and controls (SLE/Control = 1.4, 1.4, and 1.2 respectively). The lowest *p*-values comparing SLE patients and controls were obtained for S100A12, Matrix metalloproteinase-1 (MMP1) and SLC22A2 ($p_{adjusted} = 3 \times 10^{-9}$, $3 \times 10^{-6}$, and $5 \times 10^{-6}$ respectively). In a set of 15 potential biomarkers differentiating SLE patients and controls, two of the proteins were transcription factors, i.e., IRF5 and SAM pointed domain containing ETS transcription factor (SPDEF). IRF5 was up-regulated while SPDEF was found to be

down-regulated in SLE patients. Unsupervised clustering of all investigated proteins identified three molecular subgroups among SLE patients, characterized by (1) high levels of rheumatoid factor-IgM, (2) low IRF5, and (3) high IRF5. IRF5 expressing microparticles were analyzed by flow cytometry in a subset of patients to confirm the presence of IRF5 in plasma and detection of extracellular IRF5 was further confirmed by immunoprecipitation-mass spectrometry (IP-MS). Interestingly IRF5, a known genetic risk factor for SLE, was detected extracellularly and suggested by unsupervised clustering analysis to differentiate between SLE subgroups. Our results imply a set of circulating molecules as markers of possible pathogenic importance in SLE. We believe that these findings could be of relevance for understanding the pathogenesis and diversity of SLE, as well as for selection of patients in clinical trials.

**Keywords: Interferon regulating factor 5 (IRF5), antibody suspension bead arrays, subgroups, biomarker discovery, plasma proteomics, unsupervised clustering, hierarchical clustering, SLE - Systemic Lupus Erythematous**

## INTRODUCTION

Systemic Lupus Erythematosus (SLE) is a heterogeneous systemic autoimmune disorder with a plethora of clinical manifestations. Clinical and immunological criteria, defined by the American College of Rheumatology (ACR) (1), are used to classify the disease for research purposes, but reliable diagnostic biomarkers are lacking. The diversity of the disease is a great obstacle and might reflect differences in pathogenesis between different subgroups. Several recent reviews highlight the importance of defining subgroups of SLE to better treat patients with tailored medicine, and in order to increase efficacy in clinical trials (2–5). Accordingly, there is a great need for exploring subgrouping and novel diagnostic biomarkers in SLE.

Few biomarkers have been implemented in clinical routine reflecting the difficulties of biomarker research in lupus (6). Screening of a large number of proteins (>50) but in a limited number (<50) of SLE patients have been performed to identify biomarkers in SLE (7–10). In this study we analyzed 281 proteins using a suspension bead affinity proteomics approach (11), in plasma samples from a total of 695 individuals comprising SLE and matched controls. Selection of proteins is crucial to obtain representative protein profiles. However, the current knowledge of protein functions is far from complete and transcription factors and other nuclear molecules could have unknown functions in the circulation or may, regardless of function, constitute novel biomarkers. The intra- and extracellular functions of a protein might be different and unconventional secretion is also possible (12). Therefore, both nuclear and cytoplasmic molecules are relevant to study in the circulation with the aim to identify potential biomarkers and possible pathogenic pathways.

In a previous study we presented protein profiles for two predefined SLE subgroups, delineated based exclusively on the autoantibody profiles, but also corresponding to clinical observations and experience (13). In the present study we used a different approach and performed unsupervised clustering of the obtained protein profiles to investigate an unprejudiced division of SLE patients. In addition, experimental validation of biomarker candidates discriminating between SLE and control was performed. Our main focus was to identify molecular subgroups in SLE since these, despite similar clinical phenotypes, may benefit from different treatment perspectives.

## MATERIALS AND METHODS

Plasma protein profiles were obtained for SLE patients and controls utilizing antibody suspension bead arrays for protein profiling. An overview of the study design can be found in **Figure 1**.

### Patient Cohort and Controls

Fasting plasma samples were obtained from patients in the Karolinska SLE cohort consisting of 379 SLE patients and 316 population-based controls with matching age, gender and residential area. All SLE patients included in this cross-sectional study, were adults and diagnosed according to the ACR SLE criteria (1). Both patients and controls underwent a structured interview and physical examination as previously described (14). Clinical and serological data for the SLE patients are summarized in **Table 1** and in previous work (13). Medication is reported in **Supplementary Table S-1**, and demographic data for the controls are shown in **Supplementary Table S-2**.

### Protein Profiling by Antibody Suspension Bead Arrays

A number of 281 proteins were selected as previously described (13), i.e., based on published data on suggested biomarkers in inflammation/SLE/myositis, microarray data comparing SLE and controls and an untargeted mass spectrometry-based proteomic analysis suggesting additional biomarker candidates. A customized set of 367 antibodies (**Supplementary Table S-3**) was utilized to target unique epitopes of these proteins in a screening experiment (**Figure 1B**) (13). The antibodies were selected from the Human Protein Atlas (HPA, www.proteinatlas.org) project and are affinity-purified polyclonal antibodies that

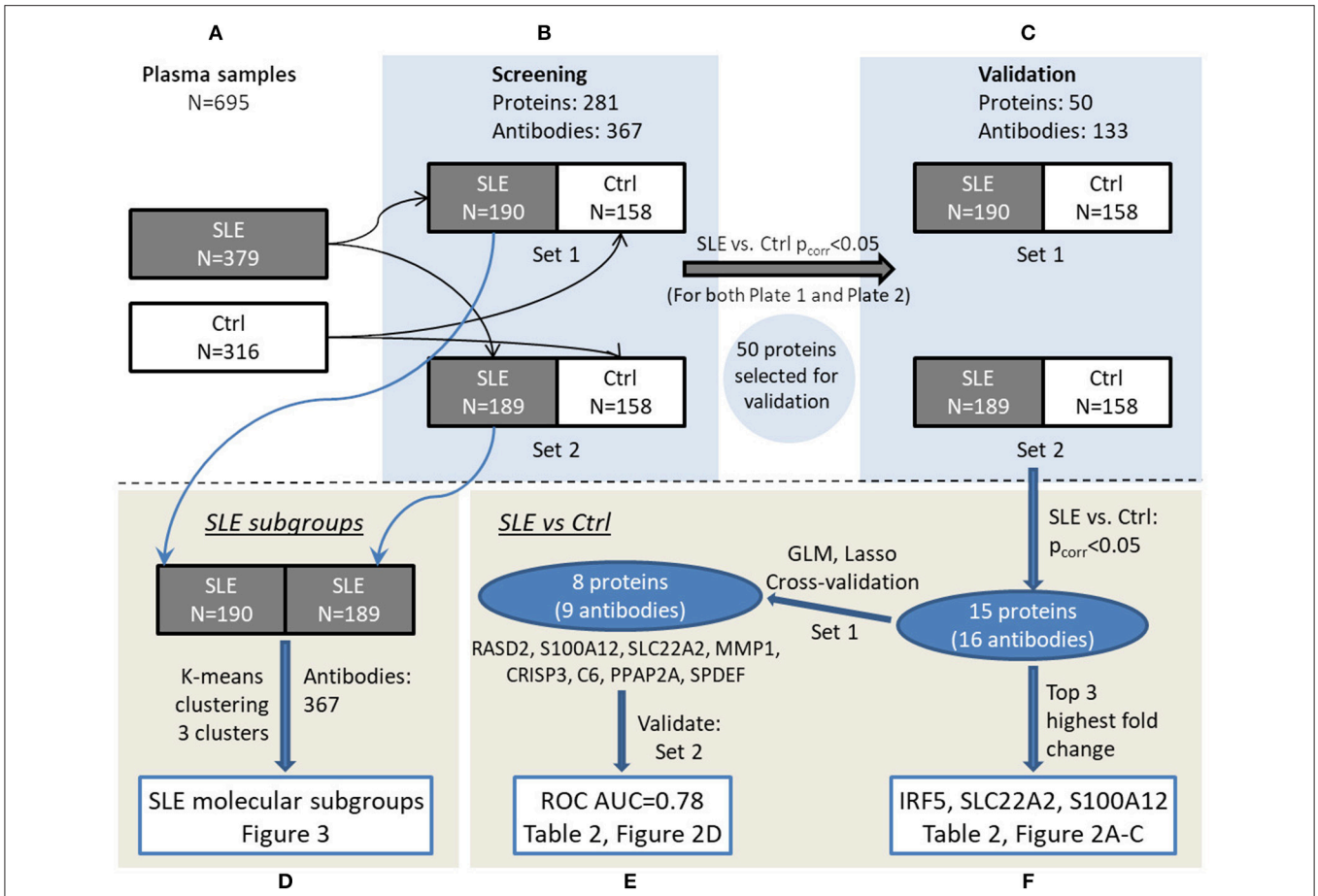


**FIGURE 1 |** Overview of the experimental workflow. Plasma samples **(A)** were randomized in set 1 and set 2 for screening phase **(B)** followed by validation phase **(C)**. Data were analyzed to investigate SLE subgroups **(D)** as well as comparing SLE and control in a multivariate **(E)** and univariate **(F)** manner, respectively, and main results can be viewed in the referred figures.

have been extensively validated (17). Protein profiles were generated using antibody suspension bead array (18). In brief, the 367 HPA antibodies were attached to color-coded magnetic beads, then incubated with 45 µl diluted and biotinylated EDTA-plasma, followed by an addition of streptavidin-conjugated R-phycoerythrin (Invitrogen), and finally analyzed using a FlexMap3D instrument (Luminex Corp.). Data was evaluated as described below and 50 proteins (53 antibodies) were selected for further validation experiments (**Figure 1C**). In the validation experiment, additional HPA antibodies ($n = 80$) targeting other antigenic regions of these proteins were coupled to beads resulting in a validation assay of 133 antibodies toward the selected 50 proteins (**Supplementary Table S-4**).

## Data Analysis of Antibody Suspension Bead Array Data

The measured signals, reported as median fluorescent intensities (MFI) from FlexMap3D were imported into R (19). As previously described (20), outliers were identified in the raw data by robust principal component analysis (R package: rrcov) and excluded from further analysis. Subsequently, probabilistic quotient normalization (PQN) was performed on the MFIs to compensate for dilution errors and/or total amount of plasma proteins of the samples (21), followed by LOESS normalization on MA coordinates, per antibody, based on the MFIs to minimize the batch effects (22). Data quality was assessed by comparing replicates per 96-well plate, in combined 384-well plates and inter 384-well plates. Thereafter the data was split into two separate but comparable datasets (**Figure 1B**) with similar age and gender distribution and equal number of SLE patients and controls (**Supplementary Table S-2**). Set 1 consisted of 190 SLE patients and 158 controls, and set 2 of 189 SLE patients and 158 controls. This data is referred to as the data from the screening phase. Proteins reaching significance (after Bonferroni correction) comparing SLE and control, with the same direction in fold change between SLE/control, in both sample set 1 and set 2 in screening phase, were selected for validation (**Figure 1C**, $n = 50$). The validated proteins that were significantly different comparing SLE and controls ($n = 15$), were used for further interpretation. A generalized linear model with lasso regularization (R package: glmnet)

**TABLE 1 |** Clinical and serological data are reported for the three molecular subgroups as well as for the entire cohort of SLE patients.

| | Entire SLE cohort[a] | Molecular SLE subgroups[a] | | | Comparing SLE subgroups[b] | | |
|---|---|---|---|---|---|---|---|
| | ***n* = 357** | **RF-IgM/SSA/SSB subgroup *N* = 51** | **IRF5 low subgroup *N* = 129** | **IRF5 high subgroup *N* = 177** | **RF-IgM/SSA/SSB vs. IRF5 low** | **RF-IgM/SSA/SSB vs. IRF5 high** | **IRF5 low vs. IRF5 high** |
| Age (years) | 47.2 (34.3–58.1) | 45.4 (33.6–56.8) | 41.4 (31.1–54.2) | 51.0 (37.8–60.3) | $P = 0.50$ | $P = 0.07$ | $\mathbf{p = 0.0003}$ |
| Gender %F | 87% | 90% | 87% | 86% | 0.62 | 0.65 | 0.92 |
| Disease duration (years) | 11.5 (4.4–21.7) | 6.9 (1.5–14.4) | 11.6 (4.5–20.6) | 12.9 (5.3–23.2) | $P = 0.06$ | $P = 0.005$ | $P = 0.19$ |
| SLE ACR criteria | 6 (5–7) | 6 (5–7) | 6 (5–7) | 6 (5–7) | $P = 0.81$ | $P = 0.43$ | $P = 0.18$ |
| SLAM | 6 (4–10) | 8 (5–12) | 6 (3.5–9.5) | 6 (3.5–9.5) | $P = 0.02$ | $P = 0.02$ | $P = 0.90$ |
| SLEDAI-2k | 4 (0–7) | 4 (1–7) | 3 (0.5–7.5) | 4 (0–7) | $P = 0.74$ | $P = 0.70$ | $P = 0.96$ |
| C3a *Kruskal–Wallis test p < 0.0001* | 268.4 (192.7–537.1) | 351.8 (243.2–991.4) | 434.8 (181.7–3092) | 250.9 (191.3–324.2) | $P = 0.83$ | $\mathbf{P < 0.0001}$ | $\mathbf{P < 0.0001}$ |
| RF IgA (IU/ml) *Kruskal–Wallis test p < 0.0001* | 5.3 (3.4–12) | 16.6 (5.6–66.9) | 4.1 (2.8–7.3) | 5.7 (3.7–11.2) | $\mathbf{P < 0.0001}$ | $\mathbf{P = 0.0001}$ | $\mathbf{P = 0.0005}$ |
| RF IgG (μg/ml) *Kruskal–Wallis test p = 0.0001* | 11 (6.9–23) | 20 (8.9–54.6) | 10 (6.5–17.5) | 11 (6.8–19.2) | $\mathbf{P < 0.0001}$ | $\mathbf{P = 0.0004}$ | $P = 0.23$ |
| RF IgM (IU/ml) *Kruskal–Wallis test p < 0.0001* | 1.3 (0.63–4.7) | 28 (13.5–44.6) | 1.1 (0.6–2.4) | 1.1 (0.5–2.1) | $\mathbf{P < 0.0001}$ | $\mathbf{P < 0.0001}$ | $P = 0.67$ |
| IgA total (mg/ml) | 2.8 (2–3.9) | 3.1 (2.1–4.2) | 2.7 (1.9–3.6) | 2.9 (2–3.9) | $P = 0.08$ | 0.38 | $P = 0.15$ |
| IgG total (mg/ml) | 12.8 (10.4–16.6) | 16.7 (12.7–20.6) | 11.7 (9.5–14.7) | 12.8 (10.3–16.1) | $\mathbf{P < 0.0001}$ | $\mathbf{P < 0.0001}$ | $P = 0.05$ |
| IgM total (mg/ml) | 0.92 (0.58–1.4) | 1.2 (0.92–2.1) | 0.96 (0.62–1.40) | 0.8 (0.49–1.3) | $P = 0.0017$ | $\mathbf{P < 0.0001}$ | $P = 0.03$ |
| ESR (mm/hour) | 19 (11–33) | 30 (16.5–46) | 14 (9–27) | 21 (12–36) | $\mathbf{P < 0.0001}$ | $P = 0.04$ | $\mathbf{P = 0.0004}$ |
| hsCRP (mg/l) | 1.7 (0.68–5.3) | 1.4 (0.51–5.7) | 1.1 (0.48–4.7) | 2.2 (0.83–5.8) | $P = 0.28$ | $P = 0.18$ | $\mathbf{P = 0.0003}$ |
| Fibrinogen (g/l) | 4.1 (3.4–5.0) | 3.9 (3.1–4.6) | 3.8 (3.2–4.8) | 4.4 (3.6–5.2) | $P = 0.95$ | $P = 0.006$ | $\mathbf{P = 0.0005}$ |
| TNF-α (pg/ml) | 4.5 (3.3–6.2) | 4.8 (3.5–6.7) | 4.0 (2.8–5.7) | 5.1 (3.6–6.4) | $P = 0.015$ | $P = 0.77$ | $\mathbf{P = 0.0005}$ |
| Fibronectin (mg/ml) *Kruskal–Wallis test p = 0.0008* | 0.38 (0.25–0.46) | 0.40 (0.29–0.50) *N = 33* | 0.41 (0.32–0.48) *N = 80* | 0.31 (0.19–0.44) *N = 95* | $P = 0.78$ | $P = 0.03$ | $\mathbf{P = 0.0002}$ |
| Leptin (mg/ml) *Kruskal–Wallis test p = 0.0002* | 14294 (4776–27938) | 13321 (5026–21162) | 7878 (2240–20389) | 19502 (8474–48617) | $P = 0.23$ | $P = 0.05$ | $\mathbf{P < 0.0001}$ |

*SLE American College of Rheumatology (ACR) classification criteria; SLAM, SLE Activity Measure (15); SLEDAI-2K, SLE Disease Activity Index (16). C3, Complement factor 3, RF IgA/G/M, Rheumatoid factor immunoglobulin A/G/M; ERS, erythrocyte sedimentation rate; hsCRP, high sensitivity C-reactive protein,*

[a] *Median (25% quantile - 75% quantile), NR, not reported. Serology data obtained as described in previous work.*

[b] *Mann–Whitney U-test for pairwise comparison of subgroups was used to characterize subgroups. P-values <0.001 without adjustment for multiple testing are highlighted in bold. Kruskal–Wallist test, i.e., comparing more than two groups and compensating for multiple testing, highlighted only RF-IgM, RF-IgG, RF-IgA, Leptin, Fibronectin and C3a as significantly different (names highlighted in italic).*

was used to find panels of proteins to predict SLE patients and controls where the sample set 1 and set 2 corresponded to test set and training set, respectively. This was followed by analysis and visualization by performing receiver operating characteristic (ROC) analysis (R package: pROC) and confidence intervals (CI) for the area under the curve (AUC) were calculated (23).

In order to identify molecular SLE subgroups, unsupervised clustering was applied on the screening data. To prepare the data for principal component analysis (PCA), the data for each dataset (190 SLE patients in set 1 and 189 SLE patients in set 2) was log2-transformed and centered on the mean of each antibody. In set 1 PC1 and PC2 explained 14 and 12% respectively of the variance, and in set 2 the explained variances by PC1 and PC2 were 18 and 16% respectively. Clustering of samples was done on the first two principal components by using K-means clustering, emphasizing on the variables with greatest variance and the Calinski-Harabasz criterion was used to find the number of clusters in the data.

## Production of Recombinant IRF5 Protein

Multiple constructs of IRF5 (Uniprot ID Q13568) were sub-cloned into the expression vectors pNIC28-Bsa4 and pNIC-Bio3 (Genbank acc. No EF198106, JN792439). After performing small-scale screening for soluble recombinant protein expression as previously described (24), clones corresponding to constructs covering the regions M1-V120 and E232-L434 were selected for generation of single-chain fragment variable (scFv) binders. Expression and purification of selected clones and full-length IRF5 was performed essentially as previously described (25, 26), and a detailed protocol can be

found in **Supplementary Methods and Results**. Final protein batches were analyzed by SDS-PAGE and subsequently flash frozen in liquid nitrogen and stored at −80°C until use.

## Generation of Antibody Fragments Against IRF5

Single-chain fragment variable (scFv) clone J-IRF5-5 was generated by phage display technology using a human synthetic library denoted SciLifeLib. The phage selection procedure was performed basically as described earlier (27), but the first round of selection, including the steps of antigen-phage incubation to trypsin elution, was carried out in 1.5 ml tubes on a rotator with no automation. The number of washing steps was modified and increased with succeeding selection rounds; five in round one and seven in round four. Also, the recovered phages were propagated in XL1-Blue *E. coli* between the selection rounds. Re-cloning of the selected material in pool followed by transformation into TOP10 *E. coli*, small-scale expression of 94 randomly picked scFv and subsequent enzyme-linked immunosorbent assay (ELISA) for detection of recombinant full-length IRF5, i.e., verifying binding to target, and sequencing experiments were performed equivalent to previously reported (27). Affinity measurements were performed using a Biacore T200 biosensor instrument (GE Healthcare) as described in **Supplementary Figure S-1**. The top candidate (J-IRF5-5), binding the construct region E232-L434 of IRF5, obtained a measured affinity of 5 nM. Validation by ELISA and Biacore was extended by using IP-MS performed on cell lysate from HEK293 cells (300 µl) as previously described (27). The lysate was spiked with a small amount of recombinant IRF5 full-length protein (0.7 µg), as IRF5 is normally expressed at very low levels in HEK293 cells, and MS data was acquired in data dependent mode using a top 10 method. IRF5 was identified as the highest ranked protein in the obtained list of proteins (**Supplementary Table S-5**). This verifies that the antibody can capture its target in a complex mixture. The top candidate, J-IRF5-5, was then used in IP-MS on plasma samples as described below.

## Immunoprecipitation Followed by Mass Spectrometry (IP-MS) of IRF5 in Plasma

Heparin-plasma from a myositis patient and two SLE patients recruited at Karolinska University Hospital were analyzed by IP-MS as previously described (28) with a few adjustments. In brief, to an aliquot of 100 µl plasma, 400 µl of lysis buffer (1 mM Tris-HCl, 42 mM NaCl, and 0.01% NP-40 in water, pH 7.9) containing protease inhibitor cocktail (Roche) was added. Recombinant IRF5 protein (0.7 µg) was added to plasma and used as a positive control. An aliquot of 4 µg J-IRF5-5 was added to plasma samples and incubated overnight at 4°C. As negative controls a scFv antibody, generated in the same way as J-IRF5-5 but targeting an unrelated antigen, was added to plasma and to another vial, J-IRF5-5 was added to a sample without plasma (lysis buffer only). Forty microliters of anti-FLAG M2 magnetic beads was added and incubated 2–5 h at 4°C. The beads were washed three times (5–10 min in 4°C) with low salt buffer (1 mM Tris-HCl, 10 mM NaCl, and 0.01% NP-40 in water, pH 7.9) and two times with low salt buffer without NP-40. Elution was performed by 2 × 100 µl 0.5 M ammonium hydroxide and evaporated in Speedvac.

Samples were reconstituted in 50 mM ammonium bicarbonate and subsequently reduced by 1 µl of 100 mM TCEP-HCl at 37°C for 1 h, alkylated by 1 µl of 500 mM iodoacetamide in dark for 45 min and digested using 1 µg trypsin at 37°C. Sample clean-up was performed in 50 mM ammonium bicarbonate using HiPPR™ Detergent Removal Spin Column Kit according to manufacturer's instructions. Obtained peptide samples were desalted using ZipTip® pipette tips or Pierce C18 Tips (Thermo Scientific) prior mass spectrometry analysis. A standard of IRF5 peptides were generated using full length IRF5 recombinant protein applying the same digestion protocol.

Peptides were separated using an Ultimate 3000 RSLCnano system. Samples were trapped on an Acclaim PepMap nanotrap column (C18, 3 µm, 100 Å, 75 µm × 20 mm), separated on an NanoEase™ M/Z HSS column (C18, 1.8 µm, 100Å, 75 µm × 250 mm), (Thermo Scientific) and analyzed on a Q Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Fisher Scientific, San Jose, CA, USA). Peptides were separated using a gradient of A (3% ACN, 0.1% FA) and B (95% ACN, 0.1% FA), ranging from 3 to 40% B in 50 min with a flow of 0.25 µl/min. The Q Exactive was operated in a data dependent manner utilizing targeted SIM/ddMS$^2$ method with an inclusion list containing masses corresponding to four unique IRF5 peptides. The survey scan was performed at 70.000 resolution from 400 to 1,200 m/z, with a max injection time of 100 ms and target of 1 × $10^6$ ions. For generation of HCD fragmentation spectra, a max ion injection time of 250 ms and Automated Gain Control (AGC) of 3 × $10^6$ were used before fragmentation at 30% normalized collision energy.

## Detection of IRF5 Positive Microparticles in Plasma

In another set of SLE patients ($n = 63$), citrate plasma was analyzed for detection of microparticles (MPs) expressing IRF5. Characteristics of these SLE patients and details about the sample collection can be found in **Supplementary Methods and Results**. Healthy controls ($n = 20$) matched for age and gender to the SLE patients were also included in this study. Platelet-poor plasma were centrifuged (2,000 g for 20 min followed by 13,000 g for 2 min) and the supernatants were then incubated with polyclonal anti-IRF5-Fluorescein isothiocyanate (FITC) (Biorbyt, UK) as described in **Supplementary Methods and Results**. MPs were measured by flow cytometry on a Beckman Gallios instrument (Beckman Coulter, Bream CA, USA) and were defined as particles between ∼0.3 µm and 0.9 µm in size.

## Detection of IRF5 in Plasma by Sandwich ELISA

Nunc immobilizer amino plates (Thermo scientific) were coated with commercial mouse anti-human IRF5, antibody targeting

aa 176–240 (Antibodies-online.com, ABIN121152). A standard curve was obtained using recombinant IRF5 protein at 0.156, 0.312, 0.625, 1.25, 2.5, 5.0, and 10 ng/ml (50 μl/well). Plasma samples from 25 SLE patients and 14 controls were diluted 1:2 in 0.1% BSA/PBS before adding 50 μl per well. As a secondary antibody, rabbit anti-human IRF5 (HPA046700, i.e., the antibody used in the antibody suspension bead array) was used. Donkey anti-rabbit IgG HRP-conjugated antibody was added for detection using TMB substrate and optical density was read at 450 nm.

## Lipid Mediators and Cytokines Data Extracted From Related Projects

In a previous study, sphingolipids were measured by LC-MS/MS in a selection of patients from our SLE cohort (29). Since one of the proteins characterizing the RF-IgM/SSA/SSB subgroup was ceramide synthase 5 (CERS5), which catalyzes the formation of $C_{16:0}$-ceramide, we utilized data from this study where $C_{16:0}$-ceramide was quantified. Among the analyzed patients (with data available from both $C_{16:0}$-ceramide and antibody suspension bead array data), 16 patients were found to belong to the RF-IgM/SSA/SSB subgroup, 39 to IRF5 low subgroup and 44 patients belonged to the IRF5 high subgroup.

Previously, 20 cytokines were analyzed in plasma from the entire Karolinska SLE cohort (14), and data from cytokines relevant in inflammation, i.e., TNF-α, IL-6, IL-8, Il-10, IL-16, and IP-10, were analyzed with respect to the identified molecular subgroups in this work.

Interferon α (IFN-α) was measured by ELISA in the Karolinska SLE cohort in another study (30), and data was utilized in this work to study levels of IFN-α in IRF5 high and low subgroups. Data on IFN-α was obtained for 66% of the patients in the IRF5 low subgroup and in 70% of the patients in IRF5 high subgroup. Values below limit of quantification (LOQ) was set to LOQ/2.

## Genetic Data on IRF5

The Karolinska SLE cohort had previously been genotyped using the Immunochip Illumina Infinium Assay (31, 32). Two previously reported independent *IRF5* SLE risk variants rs4728142 and rs10488631 (a proxy to rs35000415) were selected from this data for association with IRF5 protein levels in quantitative trait locus (QTL) analyses. Genotype data was available for 253 SLE patients and 280 controls.

## Statistics

For comparison between SLE and controls Mann-Whitney U-test was used. Bonferroni-corrected *p*-value at a threshold of 0.05 was used as a measure of significance unless otherwise stated. When comparing three or more groups, i.e., when comparing the three molecular subgroups, Kruskal Wallis test or Fisher's exact test (for categorical data) was used. In addition, in **Table 1**, Mann–Whitney *U*-test have been used for independent comparisons between molecular subgroups in favor for scientific reasoning of selected variables with cautious interpretation of *p*-values (33, 34). Spearman rank correlation was used to investigate correlations between variables. Additional details about analysis of antibody suspension bead array data, linear modeling and K-means clustering can be found in section Data Analysis of Antibody Suspension Bead Array Data. Calculations were performed using R (19), GraphPad Prism 7 and Excel 2016. IRF5 protein QTL analysis was performed on $\log_{10}$ transformed IRF5 protein levels using linear regression in R assuming an additive genetic model.

# RESULTS

## General Biomarker Candidates of SLE

Fifty-three antibodies, targeting 50 proteins, showed significant differences between SLE patients and controls in both sample set 1 and set 2, i.e., in two separate experiments performed in parallel containing samples from different patients/controls. In the following validation experiment, the plasma samples ($n$ = 695) were analyzed using 133 antibodies targeting the 50 selected proteins. Protein profiles with low correlation (Spearman's rho $< 0.40$) to the screening data were removed. The remaining 15 proteins, targeted by 16 antibodies (**Table 2**, **Figure 1F**), showed a median correlation to the screening data of rho = 0.78 with a minimum correlation of rho = 0.46. Antibody target sequence for all 15 proteins can be found in **Supplementary Table S-6**.

The proteins yielding the largest fold change between SLE patients and controls ($p < 0.05$), were interferon regulatory factor 5 (IRF5), solute carrier family 22 member 2 (SLC22A2, organic cation transporter 2, OCT2) and S100 calcium binding protein A12 (S100A12, Calgranulin-C) (**Figure 2**). Of the 15 proteins in **Table 2**, three were found to be decreased, i.e., sterile alpha motif (SAM) pointed domain containing E26 transformation-specific (ETS) transcription factor (SPDEF), Apolipoprotein L6 (APOL6), and Cysteine-rich secretory protein 3 (CRISP3), and twelve were found to be increased in the SLE patients compared to controls. Seven of the proteins were classified as plasma proteins and two proteins, IRF5 and SPDEF, were transcription factors (**Supplementary Table S-7**). Levels of IRF5 were up-regulated and SPDEF were down-regulated in SLE compared to controls.

Proteins that showed significant differences between SLE patients and controls (**Table 2**), were used to create a linear model (**Figure 1E**). Obtained model suggested a biomarker panel of 9 antibodies, targeting 8 proteins, i.e., GTP-binding protein Rhes (RASD2), S100A12, SLC22A2, Matrix metalloproteinase-1 (MMP1), CRISP3, complement component C6 (C6), Phospholipid phosphatase 1 (PPAP2A), SPDEF, achieving a ROC AUC of 0.78 (95% CI: 0.73–0.83) for prediction of SLE patients and controls (**Figure 2**). In comparison, the highest achieved AUC from a single protein was 0.73 (95% CI: 0.67–0.78) for MMP1 and 0.72 (95% CI: 0.66–0.77) for S100A12, and a panel of three proteins (S100A12, SLC22A2, and PPAP2A) yielding an AUC of 0.74 (95% CI: 0.69–0.80). This panel of 8 proteins is suggested as general biomarker candidates to differentiate between SLE and controls, independently of SLE subgroups.

Applying strict statistical univariate analysis (Bonferroni correction) only two associations were identified between proteins and clinical data (i.e., serological data, clinical symptoms, disease activity scores). Lower levels of S100A12 were

**TABLE 2 |** The 15 proteins (16 antibodies) differentially expressed comparing SLE and control.

| Protein name short | Full protein name | UniProt[a] | Correlation screening vs. validation[b] | Fold change (SLE/Ctrl) | *p*-value (SLE vs. Ctrl)[c] |
|---|---|---|---|---|---|
| IRF5 | Interferon regulatory factor 5 | Q13568 | 0.96 | 0.48 | 4.5E-02 |
| SLC22A2* | Solute carrier family 22 (organic cation transporter), member 2 | O15244 | 0.8 | 0.44 | 4.6E-06 |
| S100A12* | S100 calcium binding protein A12 | P80511 | 0.77 | 0.28 | 3.3E-09 |
| RASD2* | GTP-binding protein Rhes | Q96D21 | 0.86 | 0.26 | 1.7E-05 |
| NOS3 | Nitric oxide synthase 3 (endothelial) | P29474 | 0.93 | 0.26 | 4.1E-02 |
| MMP1* | Matrix metallopeptidase 1 (or interstitial collagenase) | P03956 | 0.63 | 0.17 | 3.2E-06 |
| SPDEF* | SAM pointed domain containing ETS transcription factor | O95238 | 0.87 | −0.14 | 1.3E-02 |
| UBAC1 | UBA domain containing 1 | Q9BSL1 | 0.71 | 0.13 | 1.4E-04 |
| TRIM33 | Tripartite motif containing 33 | Q9UPN9 | 0.84 | 0.13 | 3.0E-03 |
| CFI | Complement factor I | P05156 | 0.65 | 0.13 | 2.9E-02 |
| APOL6 | Apolipoprotein L, 6 | Q9BWW8 | 0.84 | −0.13 | 4.5E-02 |
| PPAP2A* | Phosphatidic acid phosphatase type 2A (or Phospholipid phosphatase 1) | O14494 | 0.82 | 0.12 | 9.9E-03 |
| GRAP2 | GRB2-related adaptor protein 2 | O75791 | 0.69 | 0.11 | 3.4E-03 |
| CRISP3* | Cysteine-rich secretory protein 3 | P54108 | 0.75 | −0.10 | 5.5E-04 |
| CRISP3* | Cysteine-rich secretory protein 3 | P54108 | 0.46 | −0.10 | 1.8E-03 |
| C6* | Complement component 6 | P13671 | 0.68 | 0.10 | 3.7E-03 |

*Proteins are sorted based on log-fold change between SLE samples and controls. Proteins included in suggested biomarker panel are indicated by an asterisk ("*").*

[a] *Protein ID in UniProt (35).*

[b] *The Speaman's rho correlation coefficients for screening and validation data are reported.*

[c] *The highest Bonferroni-corrected p-value among set 1 and set 2 comparing SLE and Controls is reported.*

associated in patients with a history of lupus nephritis, i.e., "nephritis ever" defined by ACR criteria (median signal of 1591 vs. 1409, with IQR of 613 vs. 583, and Bonferroni-adjusted Mann-Whitney U-test p-value of 0.008) and IRF5 protein levels showed a weak negative correlation to C3a plasma concentration in SLE patients (Spearman's rho $= -0.32$, $p < 0.0001$).

## SLE Molecular Subgroups

Unsupervised clustering of the 281 analyzed proteins (screening data, K-means clustering) was performed to find potential molecular subgroups among the SLE patients (**Figure 1D**). Three distinct clusters were obtained in both experimental set 1 (**Figure 3A**) and set 2 (**Figure 3B**) and nine of the 10 proteins with the highest absolute PCA loadings were identical between the two sets. These 9 proteins were evaluated by analyzing the median protein levels and revealed concordant protein profiles between the sets (**Figure 3C**). This panel of biomarker candidates can be used to differentiate between suggested molecular subgroups. Molecular subgroup 1 (red, $n = 51$) showed higher levels of E-selectin (SELE), solute carrier family 22 (SLC22A2), Ceramide synthase 5 (CERS5) and Integrin subunit beta 1 (ITGB1, Glycoprotein IIA, CD29). Molecular subgroup 2 (green, $n = 129$) showed lower levels of IRF5, Ubiquitin-like protein ISG15 (ISG15), endothelial nitric oxide synthase (NOS3) and SLC22A2, and is further referred to as the IRF5 low subgroup. This subgroup was found to be similar to the control group (gray) as shown in **Figure 3C**. Molecular subgroup 3 (blue, $n = 177$) showed higher levels of IRF5, ISG15, NOS3, and interleukin-2 receptor subunit alpha (IL2RA) and is referred to as the IRF5 high subgroup. Levels of IRF5 in the three subgroups as well as in the two sample sets are shown in **Figure 3D**.

Including all available clinical and serological data, considering associations comparing all three molecular subgroups (Kruskal–Wallis test), only rheumatoid factor (RF)-IgM, RF-IgG, RF-IgA, Leptin, Fibronectin, and C3a were found to be significantly different ($p < 0.05$) in at least one subgroup after correction for multiple testing (**Supplementary Figure S-2**). Molecular subgroup 1 was found to have high RF-IgM levels (**Figure 4**) as well as high levels of autoantibodies toward Sjögren's Syndrom antigen A/B (SSA/SSB) (**Supplementary Table S-8**). This subgroup is further referred to as the RF-IgM/SSA/SSB subgroup. We also observed higher levels of RF-IgG and RF-IgA as well as higher levels of total IgG and IgM in this subgroup (**Table 1**, **Supplementary Figure S-2**). In addition, this subgroup showed higher frequency (45%) of patients with secondary Sjögren's syndrome (sSS), as defined according to the American-European Consensus criteria (36), compared to the IRF5 high and low subgroup (both 19%) (**Supplementary Table S-9**). Patients in the RF-IgM/SSA/SSB subgroup showed lower frequency of nephritis (20%) compared to IRF5 low (43%) and IRF5 high (48%) subgroups. Lower ESR were reported for the IRF5 low subgroup (**Table 1**). The IRF5 high subgroup was slightly older compared to other subgroups, showed lower levels of C3a and increased levels of inflammatory markers e.g., TNF-α, fibrinogen and hsCRP (**Table 1**).

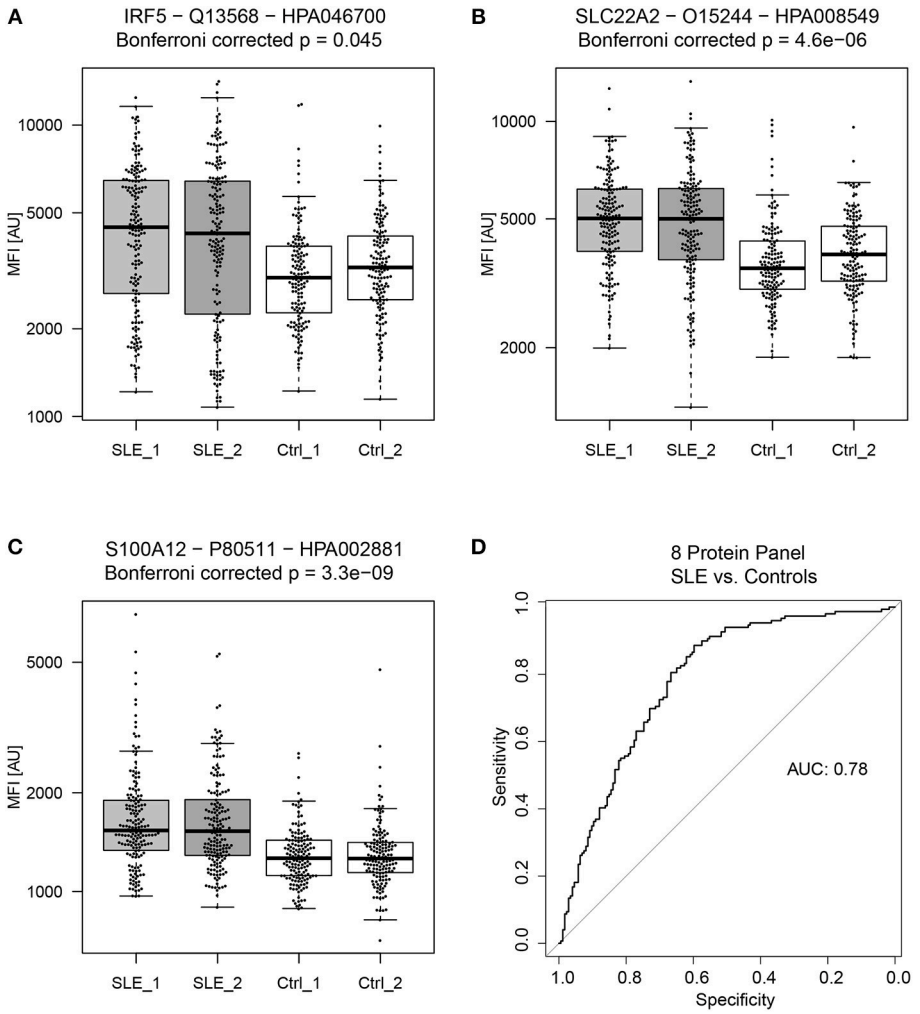


**FIGURE 2 |** General biomarker candidates of SLE. Proteins showing the highest absolute fold change between SLE patients and controls (in both sample set 1 and 2) were **(A)** Interferon regulatory factor 5 (IRF5), **(B)** Solute carrier family 22 member 2 (SLC22A2) and **(C)** S100 calcium binding protein A12 (S100A12). A panel of 8 proteins, consisting of 9 antibodies proved to be the best panel for classifying SLE patients from controls. The panel of 8 proteins consist of RASD2, S100A12, SLC22A2, MMP1, CRISP3, C6, PPAP2A, and SPDEF and achieved an ROC AUC of 0.78 for the prediction of SLE patients and controls **(D)**.

CERS5 was, as mentioned, increased in the RF-IgM/SSA/SSB subgroup, and is an enzyme catalyzing the formation of $C_{16:0}$-ceramide. We have previously quantified levels of sphingolipids in SLE (29) and data was available for a selection of patients. $C_{16:0}$-ceramide levels were 415 ± 143 nM (mean ± SD) in RF-IgM/SSA/SSB subgroup ($n = 16$), 305 ± 79 nM in IRF5 low subgroup ($n = 39$) and 331 ± 84 nM in IRF5 high subgroup 3 ($n = 44$) (**Supplementary Figure S-3**) (Kruskal–Wallis test $p = 0.02$) supporting our finding of higher levels of CERS5 in RF-IgM/SSA/SSB subgroup.

## IRF5 in Plasma

To confirm the presence of IRF5 in plasma immunoprecipitation tandem mass spectrometry (IP-MS/MS) was used. The targeted MS/MS method was optimized for four unique tryptic IRF5 peptides using recombinant IRF5 protein as a standard. No peaks corresponding to IRF5 were detected in the blank samples and no carry-over was observed between runs. MS/MS spectra of two of the peptides detected in plasma from a SLE patient is shown in **Figure 5**. IRF5 could repeatedly be detected in plasma aliquots from a myositis patient using IP-MS utilizing peptide exact mass (high-resolution m/z) and retention time. Levels were close to detection limit and fragment spectra of IRF5 peptides could not always be obtained although aliquots from the same sample were analyzed. Adding the criteria of reporting fragmentation spectra of the unique peptides, IRF5 was detected in two out of three separate experiments, not detected in one SLE patient and for the second SLE patient fragment spectra could be obtained in one out of two experiments. IP-MS, as used here, is not a quantitative method and the capture of IRF5 might slightly differ between experiments and not reach detection limit. Therefore, this is not the method

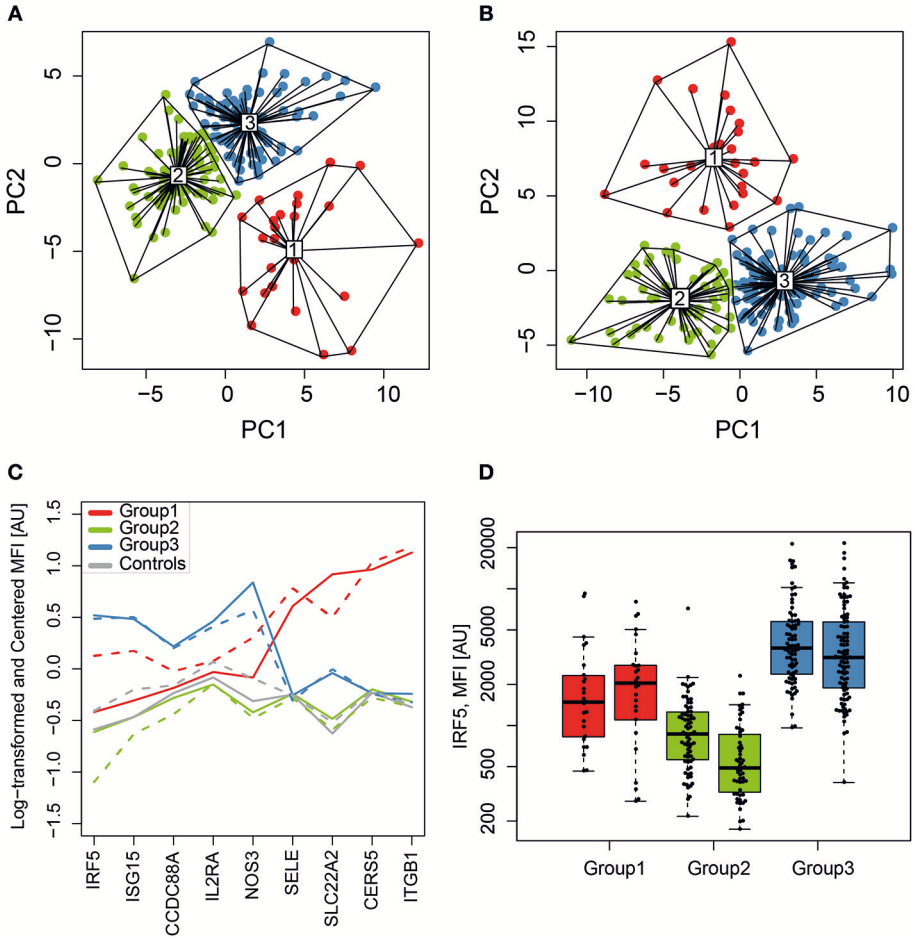


**FIGURE 3 |** SLE molecular subgroups. K-means clustering, visualized on the two first principal components (PC1 and PC2), identified three subgroups (1-red, 2-green and 3-blue) in sample set 1 **(A)** and set 2 **(B)** with a similar clustering pattern. The relative protein profiles **(C)** of the 9 proteins with the highest loadings in both sample sets for the RF-IgM/SSA/SSB (red, $n = 51$), the IRF5 low (green, $n = 129$) and the IRF5 high (blue, $n = 177$) molecular subgroups are shown and both sample set 1 (solid line) and set 2 (dashed line) shows concordant protein profiles. It is evident that the IRF5 high subgroup discriminate from the IRF5 low subgroup based on levels of IRF5, ISG15, and NOS3, while it is evident that the RF-IgM/SSA/SSB subgroup differentiate from the other two in levels of SELE, SLC22A2, CERS5, and ITGB1. Controls are included in gray for comparison but was not included in the clustering. Levels of IRF5 **(D)** are compared between the three molecular SLE subgroups RF-IgM/SSA/SSB (red), IRF5 low (green), and IRF5 high (blue) subgroup.

of choice in a screening of the entire cohort comparing SLE and controls. Nevertheless, in cases where IRF5 was detected there is no doubt about the identity of IRF5 and that IRF5 is present in plasma. The IP, accurate retention times (RT) and high-resolution accurate-mass of unique IRF5 peptides and their fragment spectra, confirm the presence of IRF5 in the circulation.

To further investigate the presence of IRF5 in plasma we analyzed IRF5 positive microparticles (MPs). The number of circulating MPs exposing IRF5 were significantly higher in SLE ($n = 63$) compared to healthy controls ($n = 20$) ($130.5 \pm 88$ vs. $36.5 \pm 14$ MPs/μl, $p < 0.0001$) (**Figure 6A**). IRF5 positive MPs were more frequently exposed on endothelial derived MPs (CD62E+ MPs) compared to platelet and leukocyte derived MPs ($p < 0.0001$) (**Figure 6B**). Furthermore, total IRF5+ MPs (regardless of origin) were significantly higher in patients with higher disease activity ($p < 0.05$) (SLE activity measure (SLAM) (15) equal or above 6) (**Supplementary Figure S-4**).

In addition, we developed a sandwich ELISA for detection of IRF5 in plasma. IRF5 levels were significantly higher ($p = 0.014$) in SLE ($n = 25$) compared to controls ($n = 25$) (**Supplementary Figure S-5**). However, the sensitivity of this assay was not sufficient for screening of the entire cohort since the majority of the SLE patients analyzed (56%, $n = 14$) report levels below quantification limit. Within this data we aimed to correlate our results with the results obtained by the suspension bead array. Excluding data outside the quantitative range of the ELISA, Spearman's rank correlation analysis was performed on data from 11 SLE patients. A strong correlation (Spearman's rho = 0.63, $p < 0.05$) and a moderate $R^2$ of 0.36 was obtained (**Supplementary Figure S-6**). However, the three samples resulting in levels above the quantification range of the

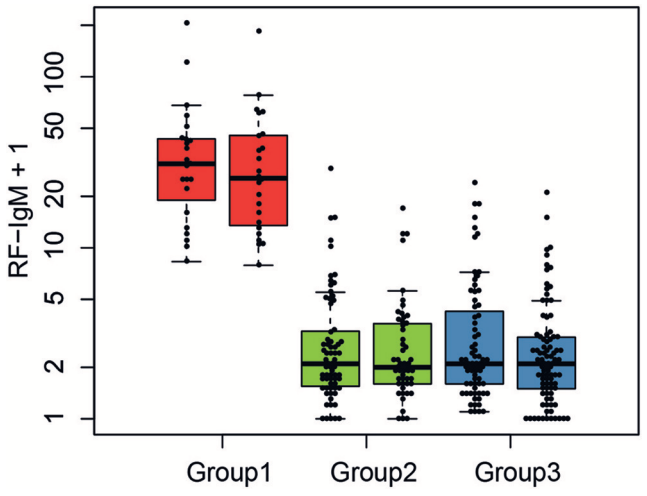


**FIGURE 4 |** Serological characteristics of SLE molecular subgroups. The levels of RF-IgM are compared between the three molecular SLE subgroups RF-IgM/SSA/SSB (red), IRF5 low (green), and IRF5 high (blue) subgroup.

ELISA, showed low or medium levels of IRF5 as measured by the suspension bead array.

*IRF5* gene polymorphism is an established risk factor in SLE (32, 37). To investigate whether IRF5 levels in plasma were regulated by known SLE genetic risk variants in *IRF5* we performed a protein quantitative trait locus analysis for two previously reported SNPs (32). We identified a weak additive association between IRF5 protein levels and the *IRF5* SLE risk variant rs4728142 ($p = 0.003$, beta = 0.07) in SLE patients and controls combined, but this effect was not apparent in either group alone. There was no association between rs10488631 and IRF5 plasma protein levels (**Supplementary Figures S-7, 8**).

Serum levels of IFN-α in the IRF5 high subgroup was not significantly different compared to the IRF5 low subgroup. In both subgroups 40% were defined as having detectable levels of IFN-α and the concentration (average ± SD) was 78 ± 122 pg/ml and 67 ± 149 pg/ml for the IRF5 low and IRF5 high subgroup, respectively. The number of IFN-α high patients, defined as a concentration of >100 pg/ml) was 15 in both subgroups.

## DISCUSSION

IRF5, a transcription factor involved in regulation of interferon and cytokine production, showed the largest fold change among the differentially expressed proteins between SLE patients and controls. We also observed large variations in IRF5 levels between subgroups of SLE patients. *IRF5* gene polymorphism is a well-known risk factor in SLE (38, 39) and in several other rheumatic diseases (40). IRF5 is an intracellular protein, nevertheless we detected IRF5 in plasma using affinity-based proteomics and the extracellular location was confirmed by IP-MS in a selection of plasma samples. To further illustrate the presence of IRF5 in the circulation we report that IRF5 expressing microparticles (detected by a different antibody) are increased in SLE compared to controls.

We identified a weak positive association between IRF5 protein levels and the number of SLE risk alleles at one of two SNP representing the *IRF5* SLE genetic association. However, this effect was not apparent when separating the data from SLE patients and control individuals, thus it could be driven by the allele frequency and protein level differences between these two groups. This indicates that the *IRF5* SLE risk variants are not the sole drivers for the differences in IRF5 plasma levels that we observe. As recently discussed elsewhere (41), the contribution of IRF5 genetic risk to disease susceptibility is not known, and it is possible that *IRF5* may have both a genetic and non-genetic contribution.

It is an intriguing and novel finding that the IRF5 protein occurs in the circulation and that it stands out as a potential biomarker for SLE. The high IRF5 levels in the circulation may reflect increased cell death in SLE patients. However, the IRF5 levels also vary to a large extent within the group of SLE patients. In addition, SPDEF, another transcription factor, showed the opposite regulation in SLE plasma (10% decrease) and unless SPDEF is strongly down-regulated in SLE, the difference in IRF5 cannot solely be explained by increased cell death/loss during apoptotic clearance in patients. Reports of transcription factors in circulation are sparse (42, 43) and by our approach using antibodies designed to target a short linear sequence of the protein, it is not possible to determine if the protein is full-length or represents a splice variant or other modified product. There is no information about extracellular function of IRF5. However, the fact that IRF5 may be found on microparticles, known to mediate cell-cell signaling, merits further investigations. In addition, further studies are needed to investigate if the IRF5 protein is actively secreted and to study possible extracellular functions of IRF5.

Interestingly our unsupervised clustering of SLE patients demonstrate that IRF5 is characteristic for two different SLE subgroups. The IRF5 low subgroup also showed lower levels of ISG15, an ubiquitin-like protein that is conjugated to intracellular target proteins upon activation by IFN-α and IFN-β (44, 45), suggesting that this subgroup might be described as a less interferon dependent subgroup. On the other hand, the IRF5 high subgroup seems to be an interferon-driven subgroup with higher levels of IRF5 and ISG15 and one might speculate that patients in these two subgroups could respond differently to IFN-α-inhibition. Serum levels of IFN-α did not differ between IRF5 high and low subgroup and might be explained by that IFN-α is regulated by several genes and not only by IRF5. Building on these observations, we suggest stratification of patients based on plasma levels of IRF5 prior to clinical trials targeting the IFN pathway. These subgroups need to be further investigated, e.g., in the light of type I IFN blockers (46) not reaching primary endpoint. Stratification based on IRF5 levels may be more efficient, definitely less expensive and more suitable to implement in clinical routine, than to measure interferon signature on a gene level.

In the IRF5 high subgroup, we detected higher levels of NOS3 (endothelial (e)NOS) as compared to the IRF5 low subgroup.

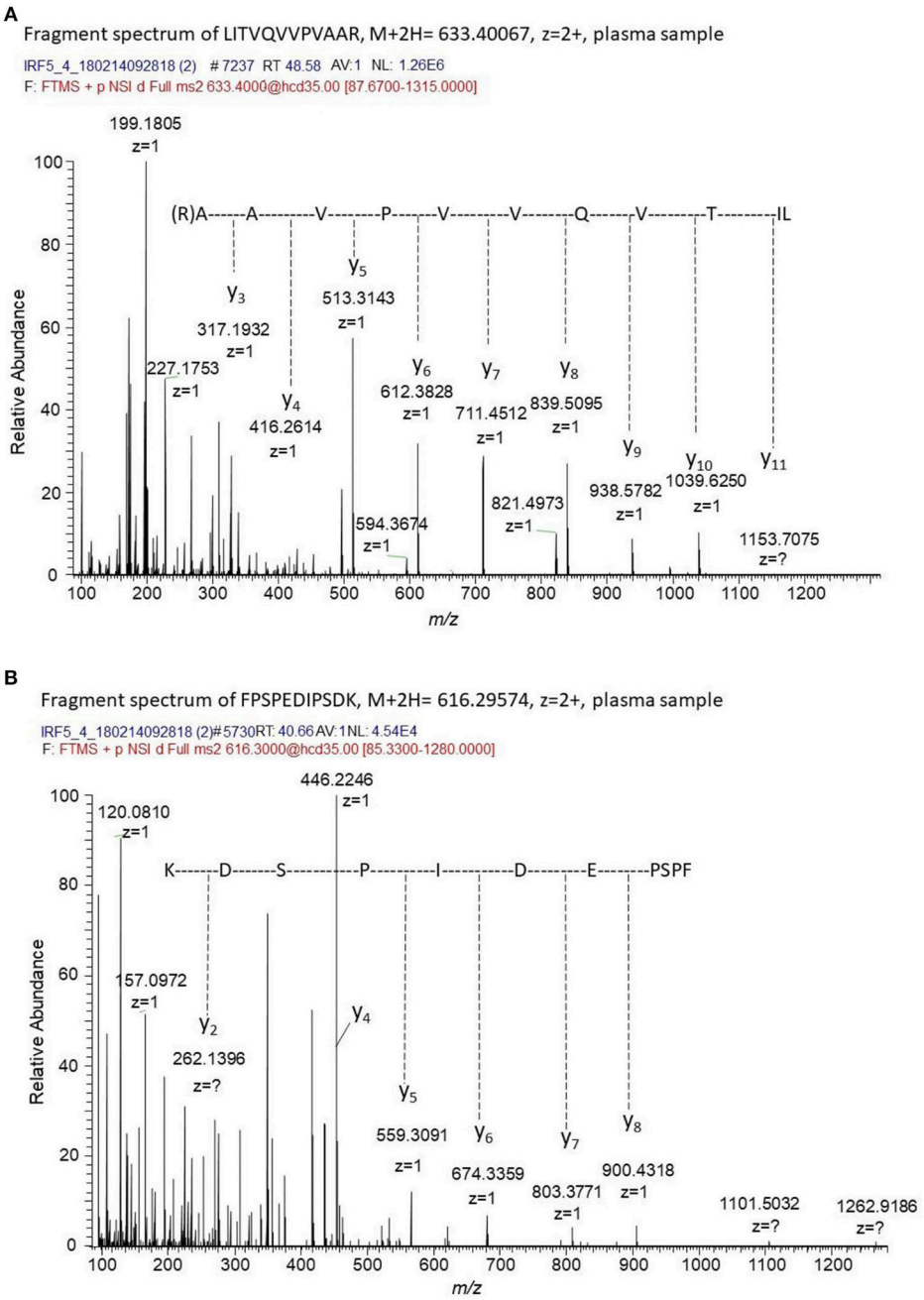


**FIGURE 5 |** Fragment spectra of endogenous IRF5 detected in plasma. The generated recombinant antibody (J-IRF5-5) was used to capture IRF5 by immunoprecipitation in a plasma sample from a SLE patient. We obtained fragment spectra of two unique peptides of IRF5, i.e., **(A)** LITVQVVPVAAR with $[M+2H]^{2+}$ m/z of 633.4007 eluting at a retention time of 48.6 min and **(B)** FPSPEDIPSDK with $[M+2H]^{2+}$ m/z of 616.29574 eluting at a retention time of 40.7 min. The retention times, the masses of the unique peptides and the fragment spectra of these peptides confirms the presence of IRF5 in this plasma sample.

NOS3, an important regulator of nitric oxide (NO) production, which is essential for cardiovascular and immune functions through regulation of vascular tone, leucocyte adhesion and platelet aggregation (47, 48). NOS3 is vasoprotective and low levels of NOS3 are related to endothelial dysfunction (49). In this context, it is difficult to dissect if the low levels of NOS3 indicate an increased risk of cardiovascular events in the IRF5 low subgroup. It is also possible that the high levels of NOS3 in the IRF5 high subgroup reflect damaged blood vessels since NOS3 is expressed in the endothelium and not expected to be increased in the circulation. In the microparticles, analyzed in another set of SLE patients, the IRF5 positive microparticles were mainly of

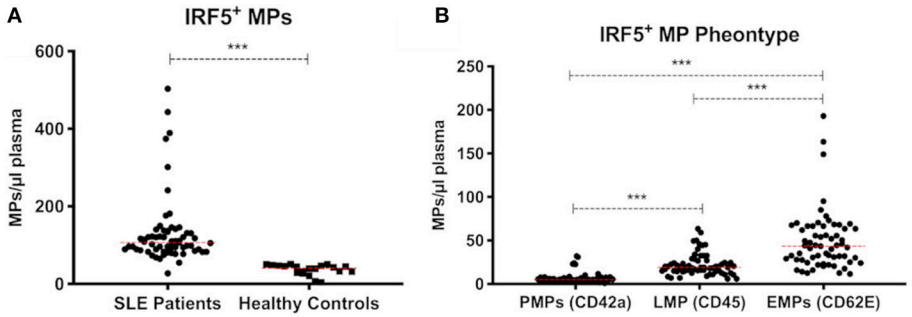


**FIGURE 6 |** IRF5+ microparticles. Total IRF5+ MPs in SLE patients ($n = 63$) and healthy controls ($n = 20$) are shown **(A)**. IRF5+ MPs in SLE patients were phenotyped based on cell origin **(B)**. PMPs, platelet derived MPs; LMPs, leukocyte derived MPs; EMPs, Endothelial derived MPs. *** $< 0.001$ (Mann–Whitney). Data is presented as MPs/µl plasma.

endothelial origin, suggestive of endothelial damage. CCDC88A (girdin, APE), a protein important for angiogenesis (50), was also increased in the IRF5 high subgroup and decreased in the IRF5 low subgroup. Inflammatory markers were increased in this subgroup indicating that the IRF5 high subgroup is characterized by more pronounced inflammation and one may speculate that anti-inflammatory treatment is more likely to be beneficial for this subgroup of SLE patients.

The RF-IgM/SSA/SSB subgroup is characterized by increased levels of SELE (endothelial cell adhesion molecule, E-selectin, CD62E, ICAM-1), ITGB1, SLC22A2, and CERS5. SELE is a cell adhesion glycoprotein on endothelium that can be stimulated by e.g., TNF-α (35, 51). ITGB1 is a cell surface receptor, which is part of the integrin family and it is important for cell adhesion (52). CERS5 synthesizes C16-ceramide and the increase in CERS5 was supported by the increase of C16-ceramide in this subgroup. Ceramides are signaling lipids involved in cell adhesion, inflammation as well as in a variety of other physiological functions (53, 54). This subgroup was also associated with higher levels of rheumatoid factor (RF) as well as higher levels of SSA/SSB antibodies. We previously reported high levels of RF-IgM (13), as well as higher levels of total IgG (55), in SLE patients with SSA/SSB antibodies. The RF-IgM/SSA/SSB subgroup share features with Sjögren's syndrome.

In a parallel study the same proteins were investigated but the subgroups were predefined by autoantibody profile, building on previous studies and own clinical experiences (13). The SSA/SSB+ subgroup in that study consisted of 63 patients and the largest fraction (43%) was assigned to the RF-IgM/SSA/SSB subgroup in this study, while the second largest portion (32%) was found in the IRF5 high subgroup which is in line with a pronounced interferon signaling in the SSA/SSB+ subgroup. The frequency of nephritis was similar and relatively low in both RF-IgM/SSA/SSB and SSA/SSB+ subgroups (20 and 21% respectively) while higher in other subgroups (>40%). CERS5 and ITGB1 were proteins characteristic for both RF-IgM/SSA/SSB and SSA/SSB+ subgroups. The second subgroup in our previous work, i.e., an aPL+ subgroup ($n = 66$), was to the largest extent (58%) found in the IRF5 high subgroup in this work and only 5% overlapped with the RF-IgM/SSA/SSB subgroups. Both the IRF5 high and the aPL+ subgroups were characterized by pronounced inflammation. Our conclusions are based on analysis of a large number of samples. However, validation in additional SLE cohorts and in other disease cohorts is needed.

Although validated HPA antibodies, targeting unique peptide sequences, were used, there is still a risk that these mono-specific polyclonal antibodies give rise to unspecific signals. Adding additional antibodies to the same protein enhance the probability of detecting the correct protein (56). However, the different epitopes targeted by the additional antibodies might be subject to differences in post translational modifications or differ in affinity and might not confirm the detection although the correct protein is present. In this work we confirmed the identity of one protein (IRF5) in plasma by IP-MS using a recombinant monoclonal antibody. We were also able to confirm the increased levels of IRF5 in SLE patients compared to controls in a subset of individuals utilizing a sandwich ELISA with a complementary capturing antibody. Although we did not validate the differences in IRF5 levels in subgroups of SLE in the entire cohort, we are confident of the detection of IRF5 in plasma.

Diagnostic biomarkers and novel insight into possible pathogenic pathways in SLE are of great importance and we here report a panel of biomarker candidates that could differentiate between SLE and controls. Utilizing unsupervised clustering of protein profiles, three molecular subgroups were revealed and could be characterized by another set of biomarker candidates. The RF-IgM/SSA/SSB subgroup essentially reflects the autoantibody defined SSA/SSB+ subgroup, which has previously been described (13, 55). The novel finding of circulating IRF5 protein is of importance for the other two subgroups. We suggest that stratification of patients based on circulating levels of IRF5 prior to e.g., IFN modulating treatments may be a valuable strategy. Furthermore, the IRF5 high subgroup expressed multiple signs of systemic inflammation, indicating that these patients may benefit from anti-inflammatory treatment. This work adds new information to the emerging need to classify the heterogeneous sample groups within SLE. The extension of these observations indicate that subgroups might be subject to different treatment perspectives, despite similar clinical profile.

## ETHICS STATEMENT

This study was carried out in accordance with the recommendations of The Local Ethics Committee of the Karolinska University Hospital/Karolinska Institutet in Stockholm. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by The Ethical Review Board in Stockholm (EPN Stockholm).

## AUTHOR CONTRIBUTIONS

HI substantial contributions to the design of the work, data interpretation, performed IP of IRF5, and drafted the manuscript. AZ substantial contributions to the design of the work, proteomic analysis, data interpretation, and drafted the manuscript. EO performed and analyzed IP-MS experiments, and wrote sections of the manuscript. EW produced recombinant IRF5 and wrote sections of the manuscript. CP performed phage display selections of recombinant antibodies and their subsequent validation and wrote sections of the manuscript. FM analysis and interpretation of IRF5 positive MPs and wrote sections of the manuscript. AC performed analysis of lipid mediators and critically revised the manuscript. AS performed analysis of RF and critically revised the manuscript. PP and JS performed genetic data analysis and wrote sections and critically revised the manuscript. CF-C developed and supervised the ELISA analysis and critically revised the manuscript. JR interpretation of RF data and design of RF experiments and critically revised the manuscript. VO performed analysis of IFN-α and critically revised the manuscript. GG clinical evaluation of sAPS patients and critically revised the manuscript. MK clinical evaluation of sSS patients and critically revised the manuscript. AL supervision of leptin and fibronectin measurements and critically revised the manuscript. CW responsible for analysis of lipid mediators and critically revised the manuscript. AS and LR provided genetic data and critically revised the manuscript. KK responsible for MS-analysis of IRF5 and critically revised the manuscript. HP made the phage display library, supervised phage display selections and antibody validation, and wrote sections of the manuscript. SG supervision of antigen and antibody production and validation of recombinant antibodies and wrote sections of the manuscript. IG responsible for SLE cohort and clinical data and critically revised the manuscript. PN substantial contributions to the design of the work and responsible for affinity proteomic platform and drafting the manuscript. ES substantial contributions to the design of the work, responsible for SLE cohort and clinical data, and drafting the manuscript. PJ substantial contributions to the design of the work, responsible for biomarker study, and drafting the manuscript. All authors contributed to manuscript revision, read and approved the final version.

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# Extracellular Chromatin Triggers Release of Soluble CEACAM8 Upon Activation of Neutrophils

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Increased concentrations of extracellular chromatin are observed in cancer, sepsis, and inflammatory autoimmune diseases like systemic lupus erythematosus (SLE) or rheumatoid arthritis (RA). In SLE and RA, extracellular chromatin may behave as a danger-associated molecular pattern (DAMP). Polymorphonuclear neutrophils (PMN) are described as typical pro-inflammatory cells but possess also immunoregulatory properties. They are activated in SLE and RA but surprisingly remain moderately studied in these diseases, and especially the disease-associated stimuli triggering PMN activation are still not completely characterized. PMN express plasma membrane carcinoembryonic antigen-related cell adhesion molecule (CEACAM) 8 (CD66b) and secrete a soluble form of CEACAM8 after activation. Soluble CEACAM8 has in turn immunoregulatory functions. However, few natural stimuli inducing soluble CEACAM8 secretion by PMN have been identified. Here we demonstrate for the first time that extracellular chromatin triggers secretion of soluble CEACAM8 by primary human PMN. Priming of PMN was not required. Secretion was associated with activation of PMN. Similar induction of soluble CEACAM8 release was observed with purified mono-nucleosomes as well as long chromatin fragments and occurred in a time-dependent and concentration-dependent manner. Results indicate that chromatin induces both neo-synthesis of soluble CEACAM8 and release of soluble CEACAM8 through degranulation. In addition, we report the presence of soluble CEACAM8 at high concentration in the synovial fluid of RA patients. Thus, we describe here a novel mechanism by which a natural DAMP, with inflammatory properties in SLE and RA, induces soluble CEACAM8 secretion by activated PMN with potential immunoregulatory consequences on other immune cells, including PMN.

**Keywords: extracellular chromatin, inflammation, neutrophils, autoimmune diseases, soluble CEACAM8 (soluble CD66b), immunomodulation**

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## INTRODUCTION

Systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) are two inflammatory autoimmune and rheumatic diseases of unknown etiology and triggered by a combination of genetic and environmental factors as well as immune dysregulation. In addition to sepsis and cancers, extracellular chromatin is present in SLE and RA. In SLE, chromatin and especially mono-nucleosomes (the fundamental DNA packing unit, a complex of 180 base pairs of DNA and one copy of histone H1 and two copies of histones H2A, H2B, H3, and H4) are detected in the circulation of patients (1) as a result of both increased apoptosis and decreased clearance of apoptotic cells and extracellular chromatin. Chromatin represents a major autoantigen in SLE. IgG3 anti-nucleosome autoantibodies are associated with active disease (2). In RA patients, cell-free chromatin is detected in the synovial fluid of inflamed joints (3) and deposits in affected joints where they form immune complexes (4). In these patients, chromatin might be released by polymorphonuclear neutrophils (PMN) recruited into inflamed tissues and dying after activation, or part of it might derive from neutrophil extracellular traps (NET) released (NETosis) upon activation (5). We have shown that extracellular chromatin activates several innate immune cells, may behave as a danger-associated molecular pattern (DAMP) and might be pathogenic in RA and SLE. Indeed, chromatin triggers activation of dendritic cells from healthy donors (HD) and SLE patients (6). Moreover, it activates PMN from HD, SLE, and RA patients (7) in a Toll-like receptor (TLR) 9-independent manner (8), leading to secretion of pro-inflammatory cytokines as well as interferon (IFN)-α, a key cytokine in SLE, and induction of NET (9). In the latter studies, we also observed that once recognized, chromatin is endocytosed by PMN.

Importantly, PMN are activated in RA and SLE patients. Originally described as short lived cells, they can actually survive more than five days *in vivo* in humans (10). They probably participate to the pathogenesis of these inflammatory diseases as they are described as typical pro-inflammatory cells and are able to interact with the pro-inflammatory Th17 lymphocytes (11). In RA patients, PMN differentiate into dendritic-like cells (12) and express RANK-L (13), suggesting a role in osteoclastogenesis and bone destruction. Surprisingly, PMN remain relatively poorly studied in this context. Recent data suggest that PMN also exert regulatory (14) or even anti-inflammatory functions (15) and can behave as B lymphocyte-helper cells (16), indicating that a tight regulation is required *in vivo*. Particularly, the triggers involved, and especially the disease-specific or -associated ones, have to be better characterized. Likewise, the cross-talk between PMN and other cell types, especially with both innate and adaptive immune cells, have to be examined.

PMN express plasma membrane carcinoembryonic antigen-related cell adhesion molecule (CEACAM) 8 or CD66b, a glycosylphosphatidylinositol (GPI)-anchored glycoprotein which is solely expressed by granulocytes (17), of which 95% are PMN. CD66b is thus specific to granulocytes and is used as a granulocyte and even a PMN marker. CEACAM8 is stored in specific (secondary) granules (18). Due to its increased expression in stimulated PMN, it is also an activation marker as sign of rapid degranulation. In addition, upon stimulation PMN secrete a soluble variant of CEACAM8 (19). Particularly, release of soluble CEACAM8 reflects degranulation of secondary granules. Soluble CEACAM8 has immunoregulatory functions. Indeed, soluble CEACAM8 has chemotactic activities for lymphocytes (20) and is known to bind to plasma membrane CEACAM1 (CD66a) (21). The latter is expressed on epithelia, endothelial cells, and various leukocytes subtypes as two major splice variants, CEACAM1-S, and CEACAM1-L (22). Only the L-variant contains immunoreceptor tyrosine-based inhibitory motifs (ITIM) in its cytoplasmic domain. Binding of soluble CEACAM8 to plasma membrane CEACAM1 triggers different functions like a co-stimulatory activity on B lymphocytes (20) or inhibition of TLR2 response in lung epithelial cells (23), and thus an anti-inflammatory signal through binding to plasma membrane CEACAM1 (24). As CEACAM are cell-cell communication molecules, soluble CEACAM8 may only be detected on other cells as a result of binding with other membrane CEACAM. Thus, the combination of stimuli encountered *in vivo* may dictate soluble CEACAM8 immunoregulatory effects. However, the natural stimuli inducing soluble CEACAM8 secretion need to be characterized. Therefore, we have investigated whether the cell-free DAMP chromatin can trigger soluble CEACAM8 secretion upon PMN activation. Moreover, we have tested whether soluble CEACAM8 is abnormally secreted in RA patients.

## METHODS

### Human Samples

EDTA-blood from random, healthy individuals (blood bank of Bobigny, contract 13/A/107, France), and RA patients (Rheumatology Department, Avicenne Hospital, Bobigny, France) was used. RA patients fulfilled the American College of Rheumatology-European League Against Rheumatism 2010 criteria. We focused on RA patients who were not treated with biologic therapy. All RA patients had a history of positivity for anti-citrullinated protein antibodies (ACPA). Blood was used to prepare leukocytes and plasma. Fresh cell-free synovial fluids were also collected from RA patients as well as patients with gout or osteoarthritis. Informed consents were collected. Experiments were approved by the local ethics committee CPP Paris Ile de France (NI-2016-11-01).

### Chromatin Purification

Chromatin and nucleosomes were prepared under sterile conditions from calf thymus as previously described (6, 25). Briefly, nuclei were isolated and then digested by micrococcal nuclease (Sigma-Aldrich). The reaction was stopped by EDTA and centrifuged. The pellet was harvested and nuclei were lyzed. After centrifugation, the supernatant containing chromatin was collected. This fraction is composed of chromatin fragments of different sizes, including high molecular weight complexes. When used in cell culture, the lysis buffer served as a negative control. In other experiments, mono-nucleosomes were used. In that case, chromatin was further purified by ultracentrifugation on 5–29% sucrose gradients. As a negative control in cell culture,

the purification buffer was used, i.e., an empty sucrose gradient loaded with lysis buffer only. All chromatin fractions were analyzed by agarose gel electrophoresis (1.5%) and SDS-PAGE (18%). Of note, free self DNA is not strongly immunogenic and histones are 100% conserved in human and calf.

## Cell Isolation and Culture

Polymorphonuclear neutrophils and peripheral blood mononuclear cells (PBMC) were freshly isolated by dextran sedimentation (Axis Shield) from peripheral blood as previously described (7). Contaminating red blood cells were lysed using cold ACK hypotonic buffer ($NH_4Cl$, $KHCO_3$, and EDTA). PMN purity was estimated by flow cytometry (**Supplementary Figures 1A,B**).

PMN (defined as $CD66b^+$, $CD11b^+$, $CD3^-$, $CD19^-$, $CD56^-$ cells, purity >95% of living cells) were cultured ($10^6$ cells/ml) in RPMI 1640 medium (Gibco) supplemented with 10% heat-inactivated fetal calf serum (FCS, biowest) in the presence/absence of 5 ng/ml phorbol myristate acetate (PMA), 5 ng/ml lipopolysaccharides (LPS, from *S. typhimurium*, a TLR4 agonist, all from Sigma-Aldrich), 2 μM synthetic oligonucleotide containing unmethylated CpG motifs (a TLR9 agonist, InvivoGen) or purified chromatin or its purification buffer as a control. In some cases, activation studies were performed with 25 μg/ml polymyxin B (an inhibitor of LPS, Sigma-Aldrich) or after pre-incubation at 56°C for 1 h to induce heat shock. Cell activation was estimated after 0.5–14 h by flow cytometry and by measuring cytokine secretion in cell culture supernatants by ELISA. Secretion of soluble CEACAM1, soluble CEACAM6, and soluble CEACAM8 was determined by ELISA.

## Flow Cytometry

Purity and phenotype of PMN were determined by staining with monoclonal antibodies (mAb) specific for CD66b (FITC-conjugated, clone G10F5) or CD11b (PE-conjugated, clone ICRF44), CD3 (PerCP-conjugated, clone UCHT-1), CD19 (APC-conjugated, clone LT19), CD56 (PE-conjugated, clone B159), or the corresponding isotype control, at 4°C in staining buffer (PBS containing 5% heat-inactivated FCS, 100 μg/ml human γ-globulin (Calbiochem), 0.02% sodium azide), and according to classical protocols. Cell viability was estimated by propidium iodide (PI) staining. To analyze PMN activation, plasma membrane CD11b expression levels were estimated on CD66b-positive cells after staining with mAb specific for CD66b and CD11b. All antibodies were purchased from BD Biosciences (except anti-CD3 and anti-CD19, ImmunoTools). PMN activation was confirmed by measuring oxidative burst and the phagocytic activity after incubation with dichlorofluorescin diacetate (DCFDA, Sigma, 25 μM) or phycoerythrin-labeled polystyrene microspheres (1 μm in diameter, Fluoresbrite Plain Microspheres PCRed, Polysciences, $5 \times 10^6$ microbeads for 2 $\times 10^5$ PMN), respectively, for 2 h at 37°C and then fixation in 1% paraformaldehyde. DCFDA becomes green fluorescent when oxidized in dichlorofluorescein (DCF). In some cases, fresh untouched cells were directly stained in whole blood after red blood cell lysis to compare PMN and PBMC using different gates. Cells were analyzed on a four-color FACSCalibur apparatus (Becton Dickinson). Data were evaluated with CellQuest Pro software (Becton Dickinson). Plasma membrane CD66b and CD11b expression levels (mean fluorescence intensity, MFI) are depicted as mean ± standard deviation (SD) of triplicates.

## ELISA

Detection of secreted soluble CEACAM1, soluble CEACAM6, and soluble CEACAM8 was performed using self-established sandwich ELISA as reported previously (23). Briefly, plates (Costar) were coated with polyclonal rabbit antibody against human CEA (DAKO, 5 μg/ml). Remaining binding sites were blocked with PBS-BSA (3%, Sigma). Plates were then incubated with culture supernatants. Standards were prepared using CEACAM1-Fc, CEACAM6-Fc, and CEACAM8-Fc proteins. Bound CEACAM were detected with 10 μg/ml anti-CEACAM1 (clone 18/20), anti-CEACAM6 (clone 1H7-4B), or anti-CEACAM8 (clone 6/40/c) mAb. Then, peroxidase-conjugated AffiniPure goat anti-mouse IgG antibody (Jackson ImmunoResearch, diluted 1:5000) was incubated. Enzyme reaction was visualized using TMB (Sigma) as substrate and stopped with $H_2SO_4$. Absorbance was measured at 450 nm.

Interleukin (IL)-8 and IL-10 secretion by human PMN was quantified by sandwich ELISA using OptEIA set (BD Biosciences) or mAb pair and streptavidin-peroxidase conjugate (eBioscience) and according to the manufacturer's instructions.

Soluble CEACAM and cytokine concentrations in cell culture supernatants are depicted as mean ± SD of triplicates. Soluble CEACAM8 concentrations in plasmas and synovial fluids are depicted as mean ± standard error of the mean (SEM).

## Statistical Analysis

For cell cultures, representative experiments are presented and the numbers of independent experiments using different donors and different chromatin preparations are indicated. Levels of plasma membrane CD66b/CD11b expression or soluble CEACAM/IL-8 secretion are depicted as mean and SD of triplicates of the representative culture. In addition, significance of differences between chromatin/nucleosomes and the purification buffer has been tested in individual experiments using a two-tailed unpaired *t*-test with or without Welch's correction. Correlations between soluble CEACAM concentrations and expression levels of plasma membrane CD66b and CD11b were assessed by using two-tailed Spearman tests. Soluble CEACAM8 concentrations in plasma from HD and RA patients were compared using a two-tailed Mann-Whitney test. Soluble CEACAM8 concentrations in all RA plasma and RA synovial fluids were compared using a two-tailed Mann-Whitney test. Soluble CEACAM8 concentrations in RA patients for whom plasma and synovial fluid were collected in parallel were compared using a two-tailed Wilcoxon signed rank test. Percentages of HD and RA patients with high concentrations of circulating soluble CEACAM8 were compared using Fisher's test. Data were analyzed using GraphPad Prism software ($p < 0.05$ was considered significant).

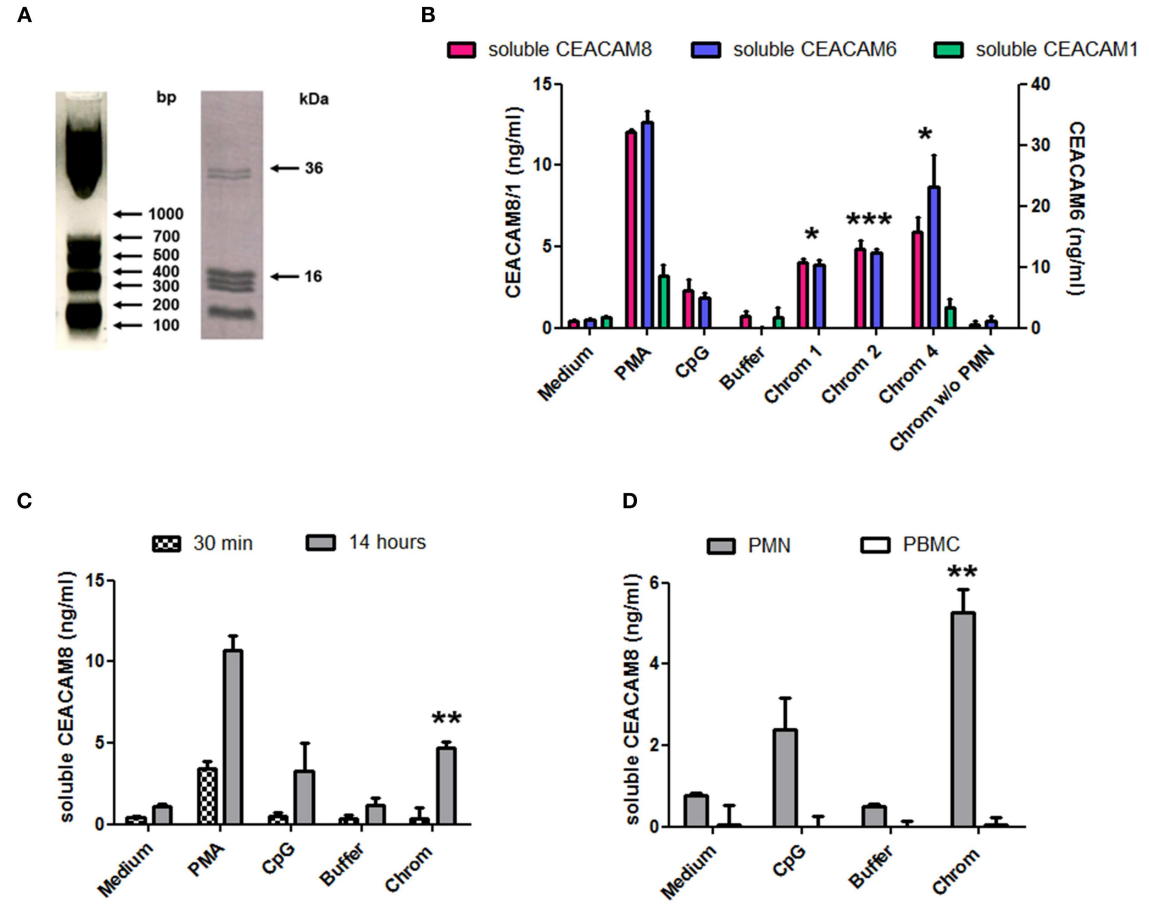


**FIGURE 1 |** Extracellular chromatin fragments of different sizes trigger the release of soluble CEACAM8 by activated PMN. **(A)** Chromatin was obtained after nuclease digestion and lysis of nuclei and was then analyzed by 1.5% agarose gel electrophoresis (left) and 18% SDS-PAGE (right). Molecular weight markers are indicated. bp, base pairs. The doublet at ~35 kDa represents histone H1. **(B)** Freshly isolated human PMN were cultured for 14 h at $10^6$ cells/ml with different stimuli and the release of soluble CEACAM1, CEACAM6, and CEACAM8 was estimated in the supernatants by ELISA. PMA, phorbol myristate acetate; CpG, oligonucleotide containing unmethylated CpG motifs (TLR9 agonist); buffer, nuclei lysis buffer; Chrom, chromatin (1, 2, 4 indicate concentrations in μg/ml). As a control, chromatin was incubated without (w/o) PMN. **(C)** PMN were activated as in **(B)** for 14 h or 30 min and secretion of soluble CEACAM8 was estimated (4 μg/ml chromatin was used). **(D)** PMN and autologous PBMC were cultured and activated (4 μg/ml chromatin) in parallel for 14 h and then secretion of soluble CEACAM8 was determined. Shown is one representative experiment of seven independent experiments using different donors and different chromatin preparations. Mean and SD of triplicates are shown. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ for soluble CEACAM8/6 concentrations after culture with chromatin vs. the purification buffer.

## RESULTS

### Cell-Free Large Extracellular Chromatin Fragments Trigger Secretion of Soluble CEACAM6 and CEACAM8 by PMN

The capacity of extracellular chromatin to trigger secretion of soluble CEACAM8 (CD66b) by PMN was investigated. We focused on chromatin and not free histones or DNA for several reasons. Free extracellular histones and DNA are usually not observed at high concentrations in RA and SLE patients. Most of extracellular histones or DNA is rather detected in chromatin, i.e., DNA complexed with histones, and eventually additional associated proteins. Moreover, we have previously reported that, in contrast to chromatin, histones do not activate PMN (7) or dendritic cells (6). On the other hand, it should also be noted that free mammalian DNA is usually poorly stimulatory. Free DNA can only efficiently trigger activation of innate immune cells when it is forced to enter cells or to reach endosomes or when it is present in immune complexes or when it is opsonized e.g., by histones, like in chromatin. Likewise, we have previously shown that extracellular chromatin triggers PMN activation but not free DNA, even DNA purified from chromatin (7). Thus, we first tested chromatin fragments of different sizes using nuclease-digested and lyzed nuclei, without further purification by ultracentrifugation. These preparations contain a mixture of nucleosomal oligomers and larger nucleosomal complexes, as evidenced by DNA size (with individual bands and not a smear), and the presence of the five histones (**Figure 1A**). Extracellular chromatin triggers the release of soluble CEACAM8 by human PMN *in vitro* (**Figure 1B**) in a

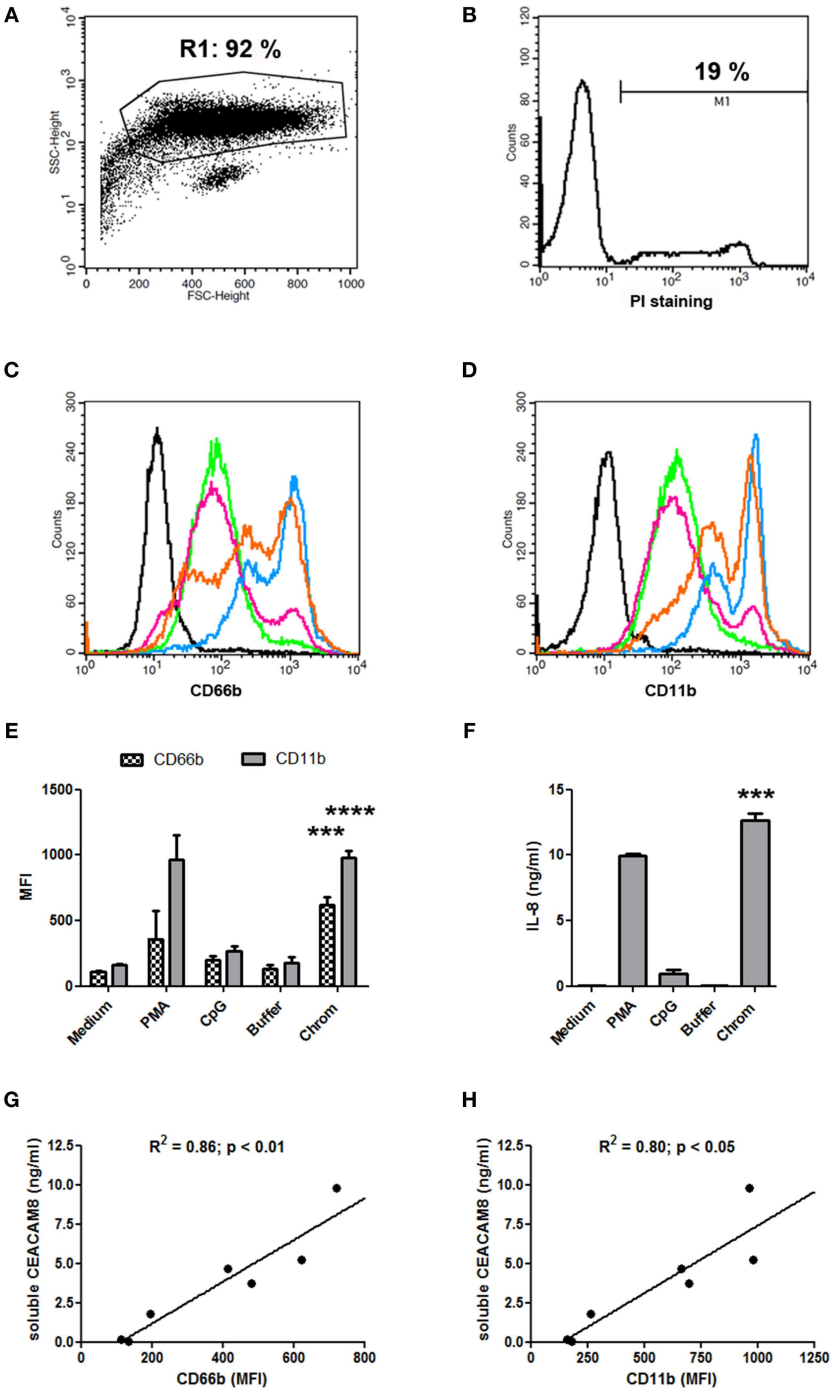


**FIGURE 2 |** Chromatin-induced soluble CEACAM8 secretion is associated with PMN activation. **(A,B)** Classical PMN shape **(A)** after 14 h of cell culture in medium only (non-activated PMN) and percentage of dead cells **(B)**. PMN were analyzed by flow cytometry after staining with propidium iodide (PI). Size (FSC) and granularity (SSC) are depicted. Numbers represent percentages of gated cells and dead (PI-positive) PMN. **(C–F)** After 14 h, cell activation was estimated by flow cytometry **(C–E)** or ELISA **(F)** with PMN from **Figure 1C**. **(C,D)** Representative plasma membrane CD66b (CEACAM8) **(C)** and plasma membrane CD11b **(D)** expressions after chromatin activation are represented. Black histogram, PMN in medium stained with isotype control. All other histograms represent PMN stained with CD66b- or CD11b-specific mAb; green, PMN in medium; pink, PMN with chromatin purification buffer; orange, PMN with 1 μg/ml chromatin; blue, PMN with 4 μg/ml chromatin. **(E)** CD66b (CEACAM8) and CD11b expression levels for all stimuli are summarized (chromatin, 4 μg/ml). MFI, mean fluorescence intensity. **(F)** IL-8 secretion was quantified (chromatin, 4 μg/ml). **(G,H)** Correlations between concentrations of secreted soluble CEACAM8 and levels of plasma membrane CD66b (CEACAM8) **(G)** and CD11b **(H)** expression by activated PMN were determined using two-tailed Spearman tests. Shown is one representative experiment of seven independent experiments using different donors and different chromatin preparations. Mean and SD of triplicates are shown. ***$p < 0.001$; ****$p < 0.0001$ for PMN cultured with chromatin vs. the purification buffer.

concentration-dependent manner, in contrast to the purification buffer (which is the true negative control). PMA and the TLR9 agonist also induce soluble CEACAM8 as previously reported (23). As a control, we verified that no signal was detected in the absence of PMN, excluding that CEACAM-specific antibodies cross-react with chromatin (**Figure 1B**). Interestingly, chromatin also induces soluble CEACAM6 (CD66c) release, but not soluble CEACAM1 (**Figure 1B**). Chromatin only induces strong soluble CEACAM8 secretion after 14 h and not after 30 min, in contrast to PMA which is known to induce degranulation, and thus vesicle release, within minutes (**Figure 1C**). These two time points were used to compare the fast release of pre-stored soluble CEACAM8 from intracellular granules (which takes just minutes) to the secretion of soluble CEACAM8 after several hours of stimulation, allowing neo-synthesis (thus transcription and translation) as previously described (23). Actually, secretion of neo-synthesized soluble CEACAM8 is already detectable after 6 h (**Supplementary Figure 2**) but differences between non-stimulated and stimulated PMN are amplified after 14 h, because soluble CEACAM8 is accumulated in supernatants over time after stimulation without increasing spontaneous secretion in non-stimulated PMN. Induction of some PMN functions requires priming, i.e., pre-activation e.g., by granulocyte-macrophage colony-stimulating factor (GM-CSF) before stimulation. Importantly, we observed that chromatin directly triggers soluble CEACAM8 secretion without requirement of PMN priming as no cytokine was used. GM-CSF pre-sensitizes PMN but did not trigger soluble CEACAM8 release (data not shown). Likewise, chromatin does not require immune complex formation to release soluble CEACAM8 as no autoantibody was used. We also confirmed that soluble CEACAM8 is specifically secreted by activated PMN, and not by autologous PBMC (**Figure 1D**). Furthermore, we confirm in our system published data showing that CD66b (CEACAM8) is specifically expressed by PMN in whole blood (**Supplementary Figures 1C–E**). Chromatin-induced soluble CEACAM8 secretion was associated with PMN activation, as shown by plasma membrane CD66b (CEACAM8) and CD11b up-regulation (**Figures 2A–E**) and IL-8 secretion (**Figure 2F**). Using optimized cell culture conditions, 92% of cells still have a typical PMN shape after 14 h (**Figure 2A**) with only 19% of dead cells (**Figure 2B**), as estimated by flow cytometry. It should be noted that PMN viability is even higher upon activation (**Supplementary Figure 3**), where cell activation (estimated by CD11b up-regulation) is inversely associated with cell death. Other groups have actually already reported similar culture conditions (26). Extracellular chromatin up-regulates both plasma membrane CD66b (CEACAM8) (**Figures 2C,E**) and CD11b (**Figures 2D,E**) in a concentration-dependent manner. At 14 h post PMN activation, levels of soluble CEACAM8 and plasma membrane CEACAM8 (**Figure 2G**) as well as plasma membrane CD11b (**Figure 2H**) were strongly positively correlated. Next, to exclude release of soluble CEACAM8 by dying PMN, cells were treated at 56°C to induce heat shock and subsequent death. No clear secretion of soluble CEACAM8 was observed when PMN were treated either for 1 h at 56°C, or when they were pre-treated at 56°C and then cultured for 14 h in medium (**Supplementary Figure 4**). On the contrary, secretion of soluble CEACAM8 was even reduced (−24%) when PMN were pre-treated at 56°C in the presence of PMA and then cultured for additional 14 h. All these controls prove that secretion of soluble CEACAM8 is not a consequence of PMN stress and death. Moreover, we have previously shown that chromatin is not toxic for PMN (7).

## Mono-Nucleosomes Activate PMN to Secrete Soluble CEACAM8

We next focused on purified mono-nucleosomes to refine activation-induced soluble CEACAM8 release. Mono-nucleosomes are indeed a main nucleosomal complex observed in the circulation of SLE patients and are deposited in joints of RA patients. We only analyzed soluble CEACAM8 because this CEACAM is specific to granulocytes. After digestion/lysis of nuclei, chromatin was further purified on sucrose gradients by ultracentrifugation. Fractions containing mono-nucleosomes were collected. These preparations essentially contain mono-nucleosomes (180 base pairs of DNA and the five histones, **Figure 3A**). Using purified nucleosomes, we confirmed induction of soluble CEACAM8 secretion by PMN (**Figure 3B**). Nucleosomes induced soluble CEACAM8 in a dose-dependent manner, as compared to the empty gradient (the purification buffer), which is the true negative control. No signal was observed in the absence of PMN, confirming the specificity of the CEACAM8 signal. Anew, soluble CEACAM8 secretion was detected after 14 h, but not after 30 min. Nucleosome-induced soluble CEACAM8 secretion was also associated with PMN activation, as shown by plasma membrane CD66b (CEACAM8) and CD11b up-regulation (**Figure 3C**) and IL-8 secretion (**Figure 3D**). We also excluded that nucleosome-induced PMN activation was due to endotoxin contamination as it was not inhibited by polymyxin B, in contrast to LPS (**Figure 3D**). PMN activation in response to extracellular mono-nucleosomes was also confirmed in some donors by showing both increased oxidative burst and increased phagocytic activity (**Figures 3E–G**). On the contrary, NETosis was not observed in response to chromatin (data not shown) and thus was not associated with soluble CEACAM8 release. We have recently shown that NET activate macrophages and PMN, especially in RA (27). However, different and optimized experimental settings were used for NET induction. Likewise, IL-10 secretion by chromatin-activated PMN was not observed (data not shown), suggesting that PMN with an immuno-modulatory activity were not triggered.

## Concentrations of Soluble CEACAM8 Are Elevated in Inflamed Joints of RA Patients

To support a potential role of soluble CEACAM8 in inflammatory autoimmune diseases, we measured and compared concentrations of soluble CEACAM8 in the plasma of HD and patients with RA, a disease with pathogenic involvement of PMN. Low levels of soluble CEACAM8 were detected in both HD (mean = 0.67 ng/ml) and RA patients (mean = 0.99 ng/ml) with no significant statistical difference (**Figure 4A**). Only two RA patients (4.4 vs. 0% in HD, not significant) showed high soluble

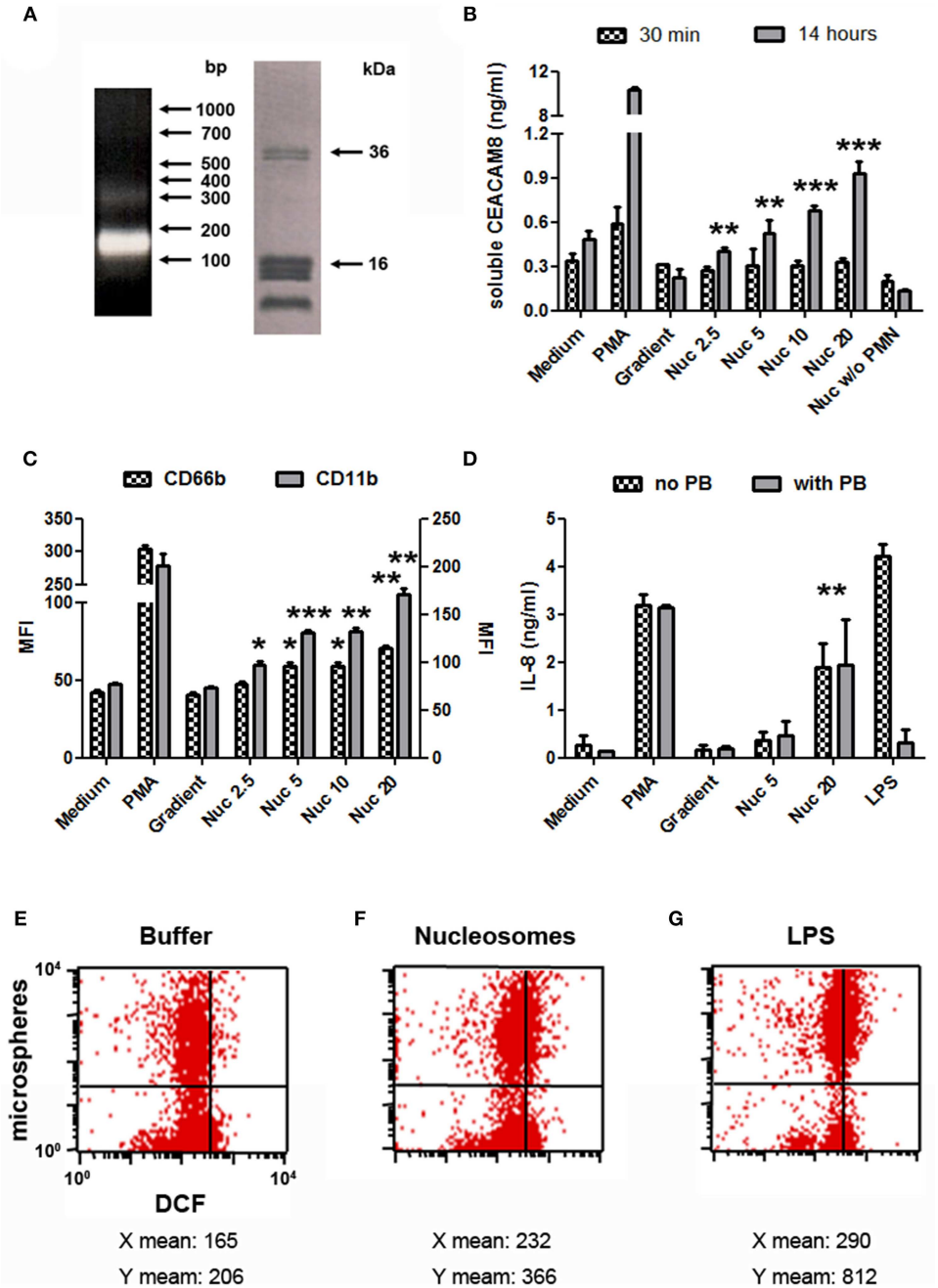


**FIGURE 3 |** Mono-nucleosome-induced PMN activation leads to secretion of soluble CEACAM8. **(A)** Chromatin was further purified by ultracentrifugation on 5–29% sucrose gradients to get mono-nucleosomes. Purified nucleosomes were analyzed by 1.5% agarose gel electrophoresis (left) and 18% SDS-PAGE (right). Molecular weight markers are indicated. bp, base pairs. The doublet at ~35 kDa represents histone H1. **(B)** Freshly isolated human PMN were cultured for 30 min or 14 h with different stimuli and the release of soluble CEACAM8 was estimated in the supernatants by ELISA. PMA, phorbol myristate acetate; Gradient, empty sucrose gradient loaded with nuclei lysis buffer instead of chromatin; Nuc, purified mono-nucleosomes (2.5, 5, 10, 20 indicate concentrations in μg/ml). As a control, nucleosomes were incubated without (w/o) PMN. **(C,D)** PMN were activated as in **(B)** for 14 h and cell activation was estimated by flow cytometry **(C)** or ELISA **(D)**. Plasma membrane CD66b (CEACAM8) and CD11b expression and IL-8 secretion were determined. MFI, mean fluorescence intensity. In **(D)**, PMN were cultured with or without polymyxin B (PB, a LPS antagonist). Shown is one representative experiment of three independent experiments using different donors and different purifications of nucleosomes. Mean and SD of triplicates are shown. **(E–G)** Chromatin-induced PMN activation leads to increased phagocytic activity and oxidative burst. Freshly isolated human PMN were cultured for 2 h in medium supplemented with the chromatin purification buffer **(E)** or stimulated with 20 μg/ml purified mono-nucleosomes **(F)** or 5 ng/ml LPS **(G)**, in the presence of dichlorofluorescin diacetate (which is oxidized in dichlorofluorescein (DCF), x axis) and phycoerythrin-conjugated microspheres (y axis) to measure oxidative burst and phagocytosis, respectively, by flow cytometry. Shown is one representative experiment of three independent experiments using different donors. Mean fluorescence intensities for both axes are depicted below each corresponding dot-plot. LPS, lipopolysaccharides. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ for PMN cultured with nucleosomes vs. the purification buffer.

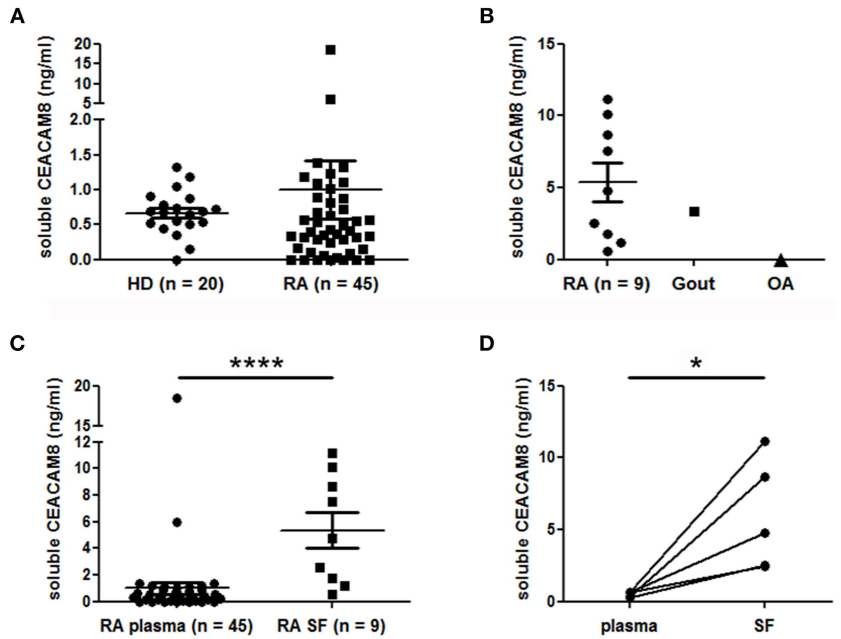


**FIGURE 4 |** Soluble CEACAM8 is enriched in inflamed tissue of patients suffering from rheumatoid arthritis. **(A)** Circulating soluble CEACAM8 concentrations were estimated by ELISA in the plasma of healthy donors (HD) and rheumatoid arthritis (RA) patients. **(B)** Concentrations of soluble CEACAM8 were determined in the synovial fluid of nine RA patients, one gout patient and one osteoarthritis (OA) patient. **(C)** Comparison of soluble CEACAM8 concentrations in all RA plasmas and all RA synovial fluids tested. **(D)** Comparison of soluble CEACAM8 concentrations in the plasma and the synovial fluid of five RA patients. Mean and SEM are presented. $^{*}p < 0.05$; $^{****}p < 0.0001$.

CEACAM8 concentrations that could not be explained by clinical data. To determine whether soluble CEACAM8 is produced locally in inflamed tissues rather than systemically, soluble CEACAM8 was measured in synovial fluid. Interestingly, we show for the first time that soluble CEACAM8 is present at high concentration in the synovial fluid of inflamed joints from RA patients (**Figure 4B**, mean = 5.4 ng/ml), suggesting that soluble CEACAM8 is enriched in affected tissues. Interestingly, elevated soluble CEACAM8 concentration was also observed in the synovial fluid of one patient with gout (an inflammatory disease with strong PMN influx), whereas no soluble CEACAM8 was detected in the synovial fluid of one patient with osteoarthritis (a non-inflammatory disease with low PMN influx). When all RA samples were compared, soluble CEACAM8 concentrations were significantly higher in the synovial fluid than in the plasma (**Figure 4C**, $p < 0.0001$). Particularly, in the five RA patients for whom we obtained simultaneously plasma and synovial fluid, concentrations of soluble CEACAM8 were significantly increased in all the synovial fluids when compared to plasmas (**Figure 4D**, $p < 0.05$).

## DISCUSSION

We demonstrate here for the first time that extracellular chromatin induces secretion of soluble CEACAM8, a molecule with immuno-modulatory functions, after activation of human PMN. Concentrations of soluble CEACAM8 measured *in vitro* are similar to concentrations measured in synovial fluids and are therefore physiologic concentrations. Thanks to its diverse activities and via its binding to plasma membrane CEACAM1, which is expressed on different cell types including PMN (28), soluble CEACAM8 is involved in the control of immune responses and PMN communication, not only in PMN-PMN communication but also in the cross-talk with other innate immune cells and even in PMN-adaptive immunity cross-talk. Because, extracellular chromatin is a major DAMP detected in RA and SLE patients and triggering sterile inflammation, this mechanism may participate to the pathogenesis of these inflammatory diseases.

Our kinetics study suggests that chromatin directly triggers both neo-synthesis and subsequent secretion of soluble CEACAM8 rather than the rapid release of CEACAM8 pre-stored in granules, as observed with PMA. Chromatin also triggers plasma membrane-bound CEACAM8 (CD66b) up-regulation. As expected, soluble CEACAM8 was only secreted by activated PMN, and not by autologous PBMC, as CEACAM8 (CD66b) is a granulocyte marker. Because soluble CEACAM8 release was positively correlated with plasma membrane-anchored CEACAM8 and CD11b expression, this process is associated with degranulation and PMN activation. PMN activation was confirmed by increased IL-8 secretion, phagocytic activity as well as oxidative burst, whereas IL-10 secretion and NETosis were not observed.

Importantly, we also demonstrate for the first time that soluble CEACAM8 is present at high concentrations in RA synovial fluids, whereas its concentration was low in the plasma and

comparable to that of HD. This suggests that soluble CEACAM8 is enriched in inflamed RA joints. These synovial fluids are also known to be enriched in PMN and extracellular chromatin. Soluble CEACAM8 might be released locally after activation of recruited PMN and may amplify the inflammatory process. Of note, membrane-bound CEACAM8 (CD66b) is up-regulated on PMN isolated from RA synovial fluid (29).

Further studies will be necessary to determine how chromatin triggers soluble CEACAM8 release. We have already reported that free mammalian DNA isolated from purified nucleosomes and purified histones do not activate PMN (7), suggesting that the nucleosomal structure is important to trigger activation. In addition, we have previously shown that TLR2/4, endosomal acidification, and TLR9 [including the cell surface TLR9 we described on PMN (30)] are not required for nucleosome-induced PMN activation (7, 8). Nevertheless, other intracellular DNA sensors might be involved (31), like AIM2 (32), DAI (33), or STING (34), the latter being up-regulated by extracellular chromatin (9) and potentially involved in lupus pathogenesis for example.

In conclusion, extracellular chromatin triggers a strong secretion of soluble CEACAM8, which may lead to an over-reaction of the immune system by interaction with a broad range of immune cells. Studies are currently performed to determine whether NET also trigger soluble CEACAM8 secretion. Indeed, we have recently reported that NET, and especially RA NET, are pro-inflammatory and activate PMN and macrophages (27), supporting the pathogenic role of PMN in RA. Finally, we are measuring concentrations of soluble CEACAM8 in samples from patients suffering from other autoimmune diseases.

## ETHICS STATEMENT

EDTA-blood from random, healthy individuals (blood bank of Bobigny, contract 13/A/107, France), and RA patients (Rheumatology Department, Avicenne Hospital, Bobigny, France) was used. Informed consents were collected. Experiments were approved by the local ethics committee CPP Paris Ile de France (NI-2016-11-01).

## AUTHOR CONTRIBUTIONS

All authors were involved in drafting the manuscript. PD designed the research, performed part of the experiments, analyzed, and interpreted data, and wrote the manuscript. MR performed the experiments, analyzed, and interpreted data. JM performed part of the experiments and analyzed data. LS selected patients and analyzed data. BS contributed reagents, analyzed, and interpreted data.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.01346/full#supplementary-material

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# Computational Methodologies for the *in vitro* and *in situ* Quantification of Neutrophil Extracellular Traps

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Neutrophil extracellular traps (NETs) are a neutrophil defensive mechanism where chromatin is expelled together with antimicrobial proteins in response to a number of stimuli. Even though beneficial in many cases, their dysfunction has been implicated in many diseases, such as rheumatoid arthritis and cancer. Accurate quantification of NETs is of utmost importance for correctly studying their role in various diseases, especially when considering them as therapeutic targets. Unfortunately, NET quantification has a number of limitations. However, recent developments in computational methodologies for quantifying NETs have vastly improved the ability to study NETs. Methods range from using ImageJ to user friendly applications and to more sophisticated machine-learning approaches. These various methods are reviewed and discussed in this review.

**Keywords: neutrophil extracellular traps, myeloperoxidase, neutrophil elastase, citrullinated histone, machine-learning**

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## INTRODUCTION

Publications describing the formation of neutrophil extracellular traps (NET) have increased exponentially since their initial description in 2004 (1). Formed as a response of neutrophils to microorganisms and a host of other stimuli, NETs consist of decondensed chromatin released from the nucleus through the cytoplasm into the extracellular space (1). Nuclear and cytoplasmic components are mingled in the NETs and include antimicrobial peptides, such as myeloperoxidase (MPO), neutrophil elastase (NE) and, in certain instances, citrullinated histones (H3Cit) (1–3). NETs are believed to prevent dissemination and propagation of various pathogens (4–6). However, even though NETs might be beneficial, inappropriate function and tissue damage have been implicated in multiple pathologies i.e., pre-eclampsia (2, 7), diabetes and gestational diabetes (8–11), rheumatoid arthritis (RA) (3, 12, 13), systemic lupus erythematous (SLE) (14), community acquired pneumonia (15), sepsis (16), thrombosis (17), acute respiratory distress syndrome (18), and cancer (19, 20).

Clearly, it is evident that NETs are of considerable importance when studying innate immunity, understanding disease mechanisms or when using them as biomarkers or therapeutic targets. Thus, accurate, reproducible, high throughput and objective quantification is paramount for the study of NETs. Unfortunately, quantification is still plagued by a number of issues, such as sampling bias, insufficient objectivity, low throughput, being tedious, labor-intensive, high in cost and difficult to compare across laboratories (21–25). Luckily, recent advancements in technology allow for computational methodologies to circumvent a number of these disadvantages; being either semi

or fully automated, with fully automated methods being more advantageous (25) i.e., higher in throughput, lower in cost, more sensitive and more reproducible across laboratories.

For this review we discuss the different methods for NET sample preparation followed by various computational solutions available for NET quantification. These solutions are only applicable for samples prepared for *in vitro* and *in situ* quantification of NETs. *In vivo* detection and quantification of NETs is important and it must be noted that quantification is usually done using *in situ* methods. NETs can also be detected and quantified *in vivo* by analysing serum or plasma for specific NET markers (12, 15, 26–29), however, since these do not involve computational methodologies for more automated quantification, they are not discussed in this review.

## *IN VITRO* AND *IN SITU* SAMPLE PREPARATION FOR AUTOMATED QUANTIFICATION OF NETS

All available techniques used to visualize NETs for quantification have comprehensibly been reviewed by de Buhr and Köckritz-Blickwede (30). **Table 1** provides a complete overview of these methods including their advantages and disadvantages. Methods include SYTOX/PicoGreen (fluorescence reader or fluorescence microscopy) (1, 31, 32, 40, 41), immunolabelling (immunofluorescence microscopy [IFM] (22, 31–39), microscopy imaging flow cytometry [MIFC] (21), flow cytometry [FACS] (42), and electron microscopy (SEM and TEM) (31, 43). The most widely published and accepted techniques are SYTOX and IFM (24, 30) and thus, are the easiest to implement and with the best quantitative computational methodologies available.

SYTOX does not pass through intact cell membranes and detects NETs by staining extracellular DNA (51, 52). Its use has a number of advantages i.e., low cost and easy implementation. However, a major disadvantage is the susceptibility to false positives due to apoptosis or necrosis of neutrophils (24, 30, 53). Thus, quantification of NETs by SYTOX should always be supplemented with IFM i.e., specific labelling for NET markers, such as MPO and H3Cit (24, 30, 53). This is standard practice for *in vitro* detection of NETs and for most computational methodologies developed for these techniques.

FACS and MIFC (immunolabelling for MPO, NE, or H3Cit) also allow for robust, rapid, specific and sensitive detection of NETs in suspension (21, 30, 42). However, detection of neutrophils that have already undergone NETosis is not possible and thus cannot completely replace IFM (30). In addition, MIFC has an advantage over FACS since the technique combines FACS data as well as imaging for single cells (21, 30). Both FACS and MIFC are more challenging to implement compared to SYTOX and IFM based methods, because they are slightly more technical in nature.

As pointed out by de Buhr and Köckritz-Blickwede (30), an important consideration is the detection of NETs in *in vivo* tissue sections i.e., *in situ* detection. Since NETs are mainly quantified *in vitro* using neutrophils from peripheral blood, or ELISA based methods using serum (12, 15, 26–29), detection of NETs in localised tissue holds great importance, as was determined in placenta (7), intestine (1), kidney (27), lung (48), intracoronary material (49), and skin (50). It is possible in certain conditions that NETosis might be completely missed if not investigated *in situ*. Immunolabelling for NET specific markers on tissue sections is well-published and automated methods for their detection exist.

No automated methods for detection of NETs using SEM and TEM are available to our knowledge.

## SEMI AND FULLY-AUTOMATED COMPUTATIONAL METHODS

**Table 2** compares the advantages and disadvantages of all the computational metholdogies discussed in this review for easy comparison.

### Computational Methodologies Available for SYTOX Stained NETs

Two methods for semi-automated quantification for NETs stained with SYTOX exist i.e., DNA Area NETosis Analysis (DANA) (23) and another using 3-dimensional confocal scanning laser microscopy (3D-CSLM) (46). DANA involves the use of a fluorescence microscope, ImageJ macros and a Java based programme with a batch processing option. Easy to follow YouTube tutorials for DANA also exist (45). Quantifying NETs by 3D-CSLM requires skilled confocal operators. No easy to follow protocols for quantification using ImageJ exist, which could make it more difficult to implement.

For 3D-CSLM, NETs are quantified based on SYTOX green area corrected to PKH26 area (binds to membranes indicating neutrophils). Using this approach, Kraaij et al. (46) successfully detected NETs in neutrophils exposed to RA and SLE serum using 3D-CSLM. Immune complexes produce lower and more subtle NETs (54) and 3D-CSLM together with ImageJ were successful in their quantification, making it a highly sensitive semi-automated technique (46). For DANA, NET-like structures are quantified on a per cell, per image and per sample basis. DANA can also sufficiently exclude overlapping cells and fragments, which might be recognized as false positives (23). These characteristics of DANA are not possible using 3D-CSLM and ImageJ. Rebernick et al. (23) were also successful in detecting spontaneous NETs in RA neutrophils using DANA.

Rebernick et al. (23) went further to show that DANA detected a similar amount of NETs compared between to two individual readers and reduced the time for analysis from 7–10 to 1.5 h. The authors were also able to detect NETs in DAPI stained murine cells, indicating robustness for the program.

Since only SYTOX is used, time required for pipetting is significantly reduced. However, in order to confirm results from the assay, IFM of specific NET markers is likely needed (24, 30, 53). Both methods do provide unintentional bias between sample quantification, and eliminate inter-individual variability. For DANA, reproducibility of results across laboratories is also likely achievable due to its robust nature. It must be noted that in our experience, DANA still requires a large amount of human

**TABLE 1 |** Summary of the main NET visualization techniques used for quantification of NETs and their advantages or disadvantages.

| Dye | Technique | Parameter | Advantages | Disadvantages | Selected references |
|---|---|---|---|---|---|
| SYTOX dye/PicoGreen | FM, eye | Percentage of NET formation | Visible differentiation between necrosis and NETosis | Occasionally biased by selection of field of view, staining of DNA in NETs by DNA-intercalating dye can be blocked by cationic peptides | (1, 31, 32) |
| Antibody against histone-DNA complexes + Dapi | IFM, eye | Percentage of NET formation | Visible differentiation between necrosis and NETosis | Occasionally biased by selection of field of view | (31–36) |
| Antibody against elastase and histone-DNA complexes + Hoechst 33342 | IFM, Image J | Percentage of NET formation | Unbiased software-based quantification | Clump of NETs derived from multiple cells count as one single event, occasionally biased by selection of field of view | (37) |
| Antibody against histone-DNA complexes + Dapi | IFM, Image J | Level of NET degradation | Unbiased software-based quantification | Occasionally biased by selection of field of view | (38, 39) |
| Antibody against histone-DNA complexes + Dapi | IFM, open source software | Level of NET degradation | Unbiased software-based quantification | Occasionally biased by selection of field of view | (22) |
| SYTOX dye/PicoGreen | FR | DNA release (μg/mL) | Unbiased | No differentiation between necrosis and NETosis, staining of DNA in NETs by DNA-intercalating dye can be blocked by cationic peptides | (31, 40, 41) |
| PicoGreen after nuclease digestion | FR | DNA release (μg/mL) | Unbiased | Staining of DNA in NETs by DNA-intercalating dye can be blocked by cationic peptides, less sensitive compared to antibody-mediated detection of NETs | (31, 36) |
| Antibody against MPO + Hoechst | MIFC | Percentage of NET formation | Unbiased, automated, enables differentiation between suicidal NETosis and vital NETosis | Imaging of cells currently undergoing NETosis and thus this method may miss those that have already lysed | (21) |
| Antibody against H3cit + MPO | Flow cytometry | Percentage of NET formation | Unbiased, automated, can be combined with sorting | Does not detect H3cit-independent events | (42) |
| Uranyl-acetate, osmium tetroxide, ruthenium red-osmium tetroxide, Cuprolinic Blue | TEM | Morphology of NET-releasing cells | Visible differentiation between necrosis and NETosis, can be used in combination with immunostaining of certain structures in NETs | Occasionally biased by selection of field of view | (31, 43, 44) |
| Osmium tetroxide/gold | SEM | Amount and structure of NETs-releasing cells | Visible differentiation between necrosis and NETosis, can be used in combination with immunostaining of certain structures in NETs | Occasionally biased by selection of field of view | (31, 43, 44) |

*Adopted from de Buhr and Köckritz-Blickwede (30). IFM, immunofluorescence microscopy; FM, fluorescence microscopy; FR, fluorescence reader; MIFC, microscopy imaging flow cytometry; MPO, myeloperoxidase; TEM, transmission electron microscopy; SEM, scanning electron microscopy; H3cit, histone citrullination.*

input for optimization of the program and large datasets with many different individual donors can still be time-consuming to analyse.

A more fully automated and high-throughput way to quantify NETs involves quantification of extracellular DNA using SYTOX green in a plate assay. However, this technique is known for being susceptible to false positives (24, 30) since NETs are not quantified based on morphology, but rather RFU. Even though this method is considered to be unbiased, non-visualization of NETs and non-specific staining of DNA prevents differentiation of necrosis and NETosis, and blocking of staining can occur due to the presence of cationic peptides (30).

## Computational Methods Available for IFM

For NET quantification using IFM, one semi-automated method (37) and two fully automated methods exist (22, 25). For the semi-automated method, NETs are quantified based on morphological and spatial distribution

**TABLE 2 |** Advantages and disadvantages of the main computational methodologies available to quantify NETs *in vitro* and *in situ*.

| NET staining technique | Compatible quantification method | Advantages | Disadvantages | Selected references |
|---|---|---|---|---|
| SYTOX | DANA | Easy to follow tutorials, individual cell analysis, exclusion of false positives, high reproducibility and robustness, reduced analysis time | Human optimisation required, confirmation with additional NET markers required | (23, 45) |
| | 3D-CSLM | Highly sensitive, robust | Skilled 3D-CSLM operator required, false positives, confirmation with additional NET markers required | (46) |
| | Plate assay | Fully automated, high-throughput, robust | False positives, non-visualization of NETs, confirmation with additional NET markers required | (24, 30) |
| IFM | ImageJ | Use of freeware, robust | Possible reproducibility problems across laboratories, possible sampling bias, difficult to implement, human input required, clumping cells quantified as one | (37) |
| | NETQUANT | Fully automated, easy to implement, reproducible and robust, individual cell analysis with multiple NET criteria, exclusion of false positives, high-throughput, advanced post-analysis data | MATLAB licence required | (25) |
| | Machine learning | Fully automated, high-throughput, sensitive, reproducible, exclusion of false positives | Informatics knowledge required, training for new conditions required, clumping cells quantified as one | (22, 47) |
| MIFC | Machine learning | Fully automated, high-throughput, sensitive, reproducible, exclusion of false positives | Informatics knowledge required, training for new conditions required | (46) |
| *In situ* sections | Machine learning | Fully automated, high-throughput, sensitive, reproducible, exclusion of false positives | Informatics knowledge required, training for new conditions required | (48) |
| | CSLM | Specific, easier to implement than machine learning protocols | Specific software required | (49) |
| | ImageJ | Use of freeware, robust | Additional NET markers required, subject to false positives | (50) |

using ImageJ (37). Fully automated methods for NET quantification include using a supervised machine-learning algorithm (regression model) trained on visually annotated images (22) or NETQUANT, a MATLAB application that quantifies NETs based on a number of criteria i.e., increases in cell surface area of single cells, deformation of DNA circularity, increase in DNA:NET bound protein ratio (25).

In our experience, NETQUANT is the most user friendly and easiest to implement with the user interface being extremely easy to use (25). The machine-learning method of Coelho et al. (22) is more technically challenging since knowledge in Python is required, even though a guide on GitHub exists (47). Furthermore, since the algorithm was trained using PMA stimulated neutrophils, new training would be required for new conditions to be investigated since NETs differ by stimuli (55), whereas for NETQUANT, metadata from images is used allowing the app to adapt to different conditions and thus be really robust. The semi-automated method requires multiple steps involving ImageJ, such as segmentation, thresholding, and particle analysis to quantify NETs, making it more difficult to implement compared to NETQUANT. These additional steps could also risk sampling bias or reduce reproducibility across laboratories.

Another advantage of NETQUANT is the inclusion of the watershed algorithm (56). This allows the app to differentiate NETs in contact with each other, a feature not available in other methods. Other methods would segment clumps of neutrophils or NETs as one and not individually. The batch processing option of NETQUANT also allows for image analysis of large datasets within minutes, providing detailed single-cell data and thus allowing for more advanced post-analysis of NET formation.

All methods were successful in NET detection in varying conditions, such as PMA stimulation, cytokine induction and even in the presence of pathogens. Coelho et al. (22) and Mohanty et al. (25) went one step further and showed that their methodologies correlated well to the detection of NETs comparing two individual experts.

Currently, NETQUANT appears to be the most unbiased and uses the most stringent, biologically relevant NET definition criteria that can be applied rapidly over many different datasets.

## Computational Methods for MIFC

Apart from using the software provided for MIFC (IDEAS, considered to be semi-automatic, with batch processing possible) (21), only one fully automated methodology for NET quantification using MIFC data exists (48). The method developed by Ginley et al. (48) is a supervised machine learning algorithm for NET detection (chromatin staining only) using MIFC data. With a support vector machine (SVM), it provided a more well-rounded performance than an alternative

convolutional neural network (CNN) approach. This was due to the amount of training data required. Since the algorithm only considered cells stimulated with PMA, additional training for different conditions would be necessary. Moreover, similar to Coelho et al. (22), the technical nature of the protocol can make it difficult to implement for persons lacking knowledge in informatics.

## Computational Methods Available for *in situ* Prepared Sections

The same authors as above (48) used an unsupervised learning method on confocal images obtained from thin sections of lung tissue in a murine fungal pneumonia model stained for DNA, MPO and histone H1. The percent pixels of H1, present in decondensed nuclei colocalised with MPO, was the classification criteria. Applying deep CNN to this co-localisation data, a supervised approach can be applied. The pixel wise sensitivity/specificity was 0.99/0.98 for NET detection on 14 images using the unsupervised learning method. Their supervised CNN method uses object patches that had an object-wise holdout sensitivity/specificity of 0.86/0.90 on 631 object patches (from two images). This is the most automated method for NET detection in tissue sections. Unfortunately, as with other machine-learning methodologies, it can be challenging to implement.

Santos et al. (49) developed a semi-automated method for NET detection in paraffin-embedded intracoronary thrombus aspirate samples. Using confocal microscopy, NETs in the sections are detected by staining for DNA, MPO, and H3Cit. Thus, the method is highly specific and easier to implement than that of machine-learning algorithms proposed by Ginley et al. (48). Naturally, analysis is slightly more tedious and slower than the fully automated methods of Ginley et al. (48). A disadvantage is the requirement for specific analysis software i.e., SF SOFTWARE VERSION 2.6.07266 (LEICA). Since the method is largely based on co-localisation, development of methods using Imaris might provide more robust methods for cross-laboratory application.

NETs were also generated *in vivo* using a *Mycobacterium tuberculosis* guinea pig model and quantified *in situ* using semi-automated methods (50). Using ImageJ, the authors quantify NETs based on pixel density per area. Tissue sections were stained using Hoechst. Thus, NET quantification was based on an increase in the observed DNA area. As mentioned, this is not specific to NET formation which requires additional staining for NET markers, such as MPO, elastase etc. Thus, the authors went further to prove that the increase in DNA area is colocalised with certain NET markers. A more accurate method involving the quantification of NETs based on specific markers, such as MPO would prove to be more accurate i.e., that of Santos et al. (49).

## CONCLUSION

Imaging of NETs can be a tedious task subject to sampling bias. Fortunately, a large number of groups are working towards high quality and easy to implement software packages that allow for high throughput and accurate quantification of NETs. This further will allow for reduction in sampling bias and allow for better reproducibility across laboratories.

## AUTHOR CONTRIBUTIONS

SvB conceptualized and wrote the manuscript. LV prepared the table. CN assisted with references. SR, SH, and PH read and revised the manuscript.

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# Self-DNA at the Epicenter of SLE: Immunogenic Forms, Regulation, and Effects

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Self-reactive B cells generated through V(D)J recombination in the bone marrow or through accrual of random mutations in secondary lymphoid tissues are mostly purged or edited to prevent autoimmunity. Yet, 10–20% of all mature naïve B cells in healthy individuals have self-reactive B cell receptors (BCRs). In patients with serologically active systemic lupus erythematosus (SLE) the percentage increases up to 50%, with significant self-DNA reactivity that correlates with disease severity. Endogenous or self-DNA has emerged as a potent antigen in several autoimmune disorders, particularly in SLE. However, the mechanism(s) regulating or preventing anti-DNA antibody production remain elusive. It is likely that in healthy subjects, DNA-reactive B cells avoid activation due to the unavailability of endogenous DNA, which is efficiently degraded through efferocytosis and various DNA-processing proteins. Genetic defects, physiological, and/or pathological conditions can override these protective checkpoints, leading to autoimmunity. Plausibly, increased availability of immunogenic self-DNA may be the key initiating event in the loss of tolerance of otherwise quiescent DNA-reactive B cells. Indeed, mutations impairing apoptotic cell clearance pathways and nucleic acid metabolism-associated genes like DNases, RNases, and their sensors are known to cause autoimmune disorders including SLE. Here we review the literature supporting the idea that increased availability of DNA as an immunogen or adjuvant, or both, may cause the production of pathogenic anti-DNA antibodies and subsequent manifestations of clinical disease such as SLE. We discuss the main cellular players involved in anti-DNA responses; the physical forms and sources of immunogenic DNA in autoimmunity; the DNA-protein complexes that render DNA immunogenic; the regulation of DNA availability by intracellular and extracellular DNases and the autoimmune pathologies associated with their dysfunction; the cytosolic and endosomal sensors of immunogenic DNA; and the cytokines such as interferons that drive auto-inflammatory and autoimmune pathways leading to clinical disease. We propose that prevention of DNA availability by aiding extracellular DNase activity could be a viable therapeutic modality in controlling SLE.

**Keywords: DNases, systemic lupus erythematosus, toll-like receptors, interferons, autoantibodies**

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# INTRODUCTION

## Anti-DNA Antibodies as a Biomarker for SLE

Anti-DNA antibodies (Abs) are not exclusive to systemic lupus erythematosus (SLE or lupus), yet, their persistence in serum is the most reliable serological marker for lupus diagnosis (1–4). High titers of anti-DNA Abs directly correlate with disease activity (3, 5), predictions of lupus flares (6, 7), hypocomplementemia (8), and proliferative lupus nephritis (9, 10). 70–80% of SLE patients have detectable levels of anti-DNA Abs, of which ∼45–50% have high titers (3, 8, 11). This is in contrast with anti-DNA Ab- positive non-SLE patients with rheumatoid arthritis (RA), scleroderma, vasculitis, tuberculosis, autoimmune hepatitis, viral hepatitis or cancer, where the titers are predominantly low-to-moderate (3, 8). Additionally, a fraction of aged healthy individuals also have anti-DNA Abs but rarely at high titers (12). The correlation of high titers of anti-DNA Abs with SLE disease severity is indicative of a requirement for the persistent availability of DNA as an immunogen. Additionally, many pathological conditions including infections, and cancer can induce anti-DNA Abs which invokes a status for DNA as a readily available adjuvant associated with various proteins under different conditions.

## B Cells in Anti-DNA Responses

Rheumatic diseases like SLE, RA, Sjogren's syndrome, vasculitis, antiphospholipid syndrome etc., which cause development of anti-DNA Abs in several patients, are driven by B cells (13, 14). Moreover, DNA-specific B cells can readily expand in all individuals upon exposure to microbial DNA (4). In healthy individuals, the microbial DNA-specific B cell expansion is transient. However, under autoimmune conditions, the bacterial DNA-reactive B cells also recognize self-DNA and are retained after the infection is cleared (15). It is therefore of clinical relevance to understand the conditions which cause the persistence of DNA-reactive B cells in autoimmune diseases like SLE. Toward this goal, significant advances have been made in the area of B cell biology to understand the regulation of autoreactive B cells. A recent comprehensive review on B cell genetic risk factors involved in SLE highlighted the importance of examining specific B cell subsets for better targeted therapeutic intervention (16). The major B cells subsets implicated in anti-DNA antibody production include germinal center (GC) B cells that produce long-lived plasma/ memory cells and the extrafollicularly generated short-lived plasmablasts (17, 18). Several studies in mice outline a significant role of the extrafollicular pathway in anti-DNA/ chromatin Ab production, showing that B cells can undergo both isotype switching and affinity maturation outside of the GCs (19–22). Notably, expansion of extrafollicular B cells in active human SLE patients has also been reported (23, 24). In a recent study, specific subsets of B cells involved in the extrafollicular pathway of autoantibody production in SLE were defined in patients with active disease (25). Unlike the GC pathway, the absence of extrafollicular tolerance checkpoints might explain the preferential emergence and amplification of anti-DNA responses via the extrafollicular route.

In accordance with the predominantly short-lived nature of DNA-reactive B cells, B cell targeting therapies like Rituximab (anti-CD20) and Belimumab (anti-BAFF) have been partially effective in SLE treatment (13, 14, 26). There was a modest yet significant reduction of SLE disease severity in patients with serologically and clinically active SLE upon treatment with Belimumab (Benlysta), alongside standard therapy (7, 27–29). Notable observations from phase III Belimumab trials BLISS−52 and BLISS−76 (30, 31) were that increased anti-DNA Ab titers predicted lupus flares (6, 7), while successful treatment resulted in reduced anti-DNA Abs (29), positively correlating anti-DNA Abs with disease manifestations. Although anti-B cell therapies are promising (14, 32), there remains great variability in the reduction of autoantibodies and disease severity upon treatment, in part due to the variable B cell subsets involved in antibody production. Additionally, most patients receive supplemental concurrent administration of corticosteroids that have several adverse side effects, including infections, hypertension, hyperglycemia, osteoporosis, cataracts, glaucoma, and cognitive impairment (33, 34). Therefore, effective treatment of SLE with minimal side effects requires newer approaches and interventions in addition to and beyond B cell-targeted therapy.

## T Cells in Anti-DNA Responses

Along with B cells, the generation, and amplification of anti-DNA antibodies requires a T-cell dependent antigenic stimulation process, which indicates that anti-DNA antibody production is not just a consequence of polyclonal stimulation of immune cells. Indeed, autoreactive T cell clones have been identified in mice (35) and humans (36–38) and are essential for the amplification of autoreactive B cells (**Figure 1**). A subset of $CD4^+$ T cells expressing high CXCR5, ICOS, and PD-1, named follicular helper T cells (Tfh) are particularly implicated in several autoimmune diseases. Tfh promote the generation of germinal center-driven anti-DNA Abs in several lupus mouse models by providing key cytokines like IL-21 and IL-4 to B cells in the germinal centers (39–41). Likewise, a subset of SLE patients have increased numbers of $CD4^+CXCR5^+ICOS^{hi}PD\text{-}1^{hi}$ circulating T cells, resembling mouse Tfh cells (39, 42, 43). Another distinct population of helper T cells has also been identified in the generation and amplification of anti-DNA/ chromatin responses through the extrafollicular pathway in mice (44–46), and more recently in SLE patients (47). Given the pleiotropic roles of T cells as B cell helpers (Tfh), cytokine producers (Th1, Th17) and suppressors of autoimmunity (Tregs) in SLE, it is no surprise that several T-cell targeted therapies are in use and/ or under investigation for lupus (48).

## pDCs in Anti-DNA Responses

In addition to the direct role of B and T cells in anti-DNA Ab production, high serum type-I interferon levels and activity directly correlate with high anti-DNA Ab titers in SLE patients (49–51). Plasmacytoid dendritic cells (pDCs) are considered as professional IFN-I producing cells and are implicated in

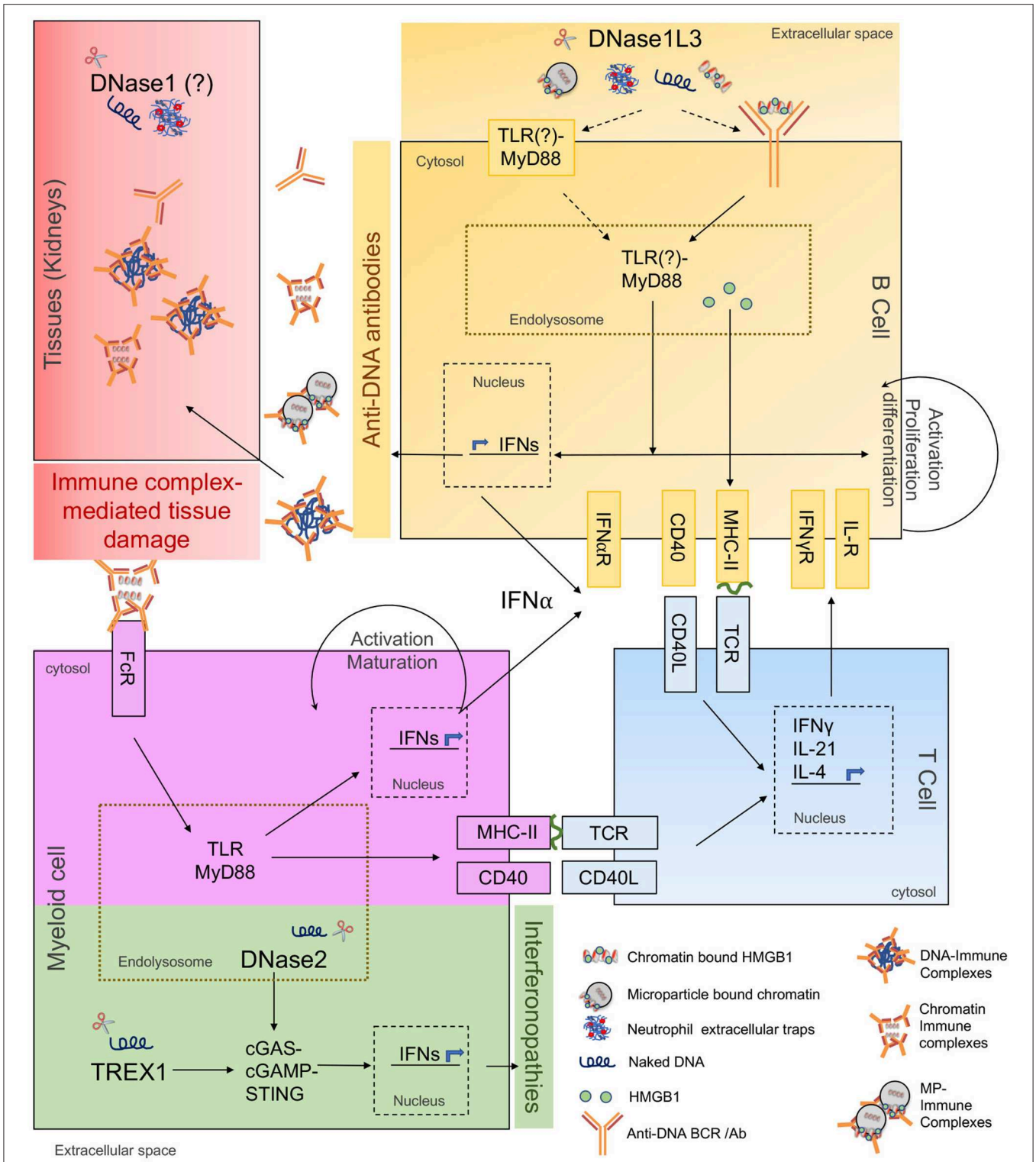


**FIGURE 1 |** Cellular and molecular responses to extracellular and intracellular DNA. The schematic shows involvement of extracellular DNases in anti-DNA responses/SLE pathogenesis and intracellular DNases in interferonopathies. The major molecular pathways of autoantibody and autoinflammatory responses are highlighted in different colors as described below. **Yellow**: Primary cellular and molecular pathways of anti-DNA Ab production. DNase1L3-deficiency increases availability and uptake of cfDNA (naked DNA, NET-DNA, cell-free chromatin, and microparticle-associated chromatin), along with associated proteins potentially through self-reactive BCRs or through cell-surface TLRs. Internalized self-DNA causes TLR-MyD88 dependent B cell activation, differentiation, IFN production, and presentation of

*(Continued)*

**FIGURE 1 |** cfDNA-associated peptides to T cells. **Blue**: T cells help in anti-DNA Ab production. Costimulatory and cognate MHC-TCR interactions between DNA-reactive B and T cells stimulate activation, proliferation, and differentiation of B cells into anti-DNA Ab secreting cells. **Purple**: Amplification of anti-DNA Abs through myeloid cell help. Anti-DNA antibodies accumulate and form immune complexes with cfDNA which are internalized through Fc-receptors on myeloid cells i.e., DCs, pDCs, macrophages, further inducing IFN production through TLR-MyD88 pathway. Myeloid cells also present self-antigen to T cells further amplifying the B-T cell interaction loop and anti-DNA Ab production. **Red**: Undigested DNA promotes IC formation and deposition in target organs. DNase1 expressed in kidneys digests locally produced apoptotic cell-derived DNA. IC-formation is enhanced in the presence of extracellular DNA. ICs deposit in kidneys causing immune complex-mediated tissue damage. **Green**: DNases and signaling pathways regulating interferonopathies. DNase2 cleaves endocytosed apoptotic cell-derived DNA while TREX1 cleaves cytosolic DNA. Absence of DNase2 and TREX1 trigger activation of cGAS-STING pathway causing IFN production leading to interferonopathies. DNase2 and TREX1 do not directly contribute to anti-DNA antibody production.

autoimmunity (52, 53). Stage III-IV lupus nephritis (LN) patients also show increased infiltration of pDCs in kidneys (54). Consistently, pDC depletion in BXSB and B6. *Nba2* models of SLE ameliorated disease (55, 56). Furthermore, functional impairment of pDCs by monoallelic deletion of *Tcf4* was sufficient to reduce autoantibody production and disease manifestations in two genetic mouse models (57). Clearly, the role of pDCs in autoimmunity is evident but their precise role in anti-DNA antibody production needs further investigation. In humans, pDCs were shown to promote B cell differentiation into plasmablasts/ plasma cells by producing IFNα and IL-6 *in vitro* (58), while activated human B cells were able to induce IFNα production by pDCs (59). In another study, pDCs from healthy subjects promoted the expansion of IL-10 producing regulatory B cells through IFNα, while pDCs from SLE patients did not (60). This evidence for a reciprocal interaction between B cells and pDCs with the involvement of IFNα, warrants further investigation of the role of pDCs in anti-DNA antibody production.

Taken together, the generation of anti-DNA Abs in SLE requires the activation and interaction of several key immune cell types, depicted in **Figure 1**. In the following sections we will review what we know so far about the forms of antigenic DNA, its regulation and sensing, and the effector responses that drive anti-DNA Ab production.

## Immunogenic DNA: Sources and Protein Partners

DNA by itself is a weak antigen compared to macromolecules like proteins, lipids, and glycans. However, certain nucleotide sequences and structural determinants can be immunogenic. Anti-DNA Abs to specific bacterial DNA are present in healthy individuals and do not react with other bacterial or endogenous DNA (61). On the other hand, antibodies to bacterial DNA in SLE patients cross react with all DNA irrespective of its source (61–63). Such promiscuity of anti-DNA Abs in SLE patients could be explained through: (1) positive selection of BCR clones recognizing common determinants of DNA, e.g., phosphodiester backbone due to B cell tolerance checkpoint defects; (2) epigenetic/ structural modification of endogenous DNA through chemical modifications or interactions with DNA-binding proteins; or (3) the excessive availability of immunogenic cell-free DNA (cfDNA) due to clearance or DNA digestion defects. Overall, it is likely that the availability of modified immunogenic DNA to DNA-reactive B cells precipitates SLE-associated pathogenic anti-DNA responses (**Figure 1**). cfDNA is detectable in the serum and plasma of healthy subjects (64), while its levels increase in conditions associated with excessive cell death, e.g., pulmonary embolism, mechanical, or drug induced injury/ trauma, cancer, pregnancy, sepsis, organ transplantation, RA and SLE (65, 66), summarized in **Table 1**. The common forms of cell death that cause cfDNA release include apoptosis, necrosis, and NETosis.

## Neutrophil Extracellular Traps

NETosis is a form of neutrophil cell death involving release of neutrophil extracellular traps—NETs (92). NETs are released through a process of nuclear decondensation followed by either slow (lytic) or rapid (non-lytic) release of chromatin studded with neutrophil granular proteins. The complex biology of NETs/ NETosis and its roles in antimicrobial immunity, pathological conditions like allergic asthma, vasculitis, RA, psoriasis, and SLE were recently comprehensively reviewed (74). Increased NETosis was identified in kidney and skin biopsies from SLE patients with lupus nephritis and cutaneous SLE, respectively (93). Moreover, a positive correlation was observed in SLE patients with reduced NET-associated DNA (NET-DNA) degradation and lupus nephritis (94). The pathogenic effects of NETs in psoriasis (75) and SLE (76, 77) have been attributed to their stimulatory activity on pDCs, wherein nucleic acid-mediated TLR9/7 stimulation causes type I IFN secretion, which in turn potentiates the autoinflammatory loop (76–78).

The stimulatory NET components are a composite of neutrophil genomic DNA (gDNA), mitochondrial DNA (mtDNA) and neutrophil granular proteins, which are interferonogenic (78, 83, 84). Like gDNA MtDNA is associated with DNA-binding proteins to form complexes called nucleoids, akin to chromatin. Transcription factor A-mitochondria (TFAM), is a high-mobility group (HMG) protein involved in the compaction of mitochondrial DNA into nucleoids. Unlike other cells, damaged mtDNA in neutrophils is not degraded through "mitophagy"; instead, damaged-unoxidized mtDNA is decondensed and expelled, while oxidized mtDNA (ox-mtDNA) is degraded through lysosomes within neutrophils or after uptake by macrophages. Both these pathways are non-inflammatory in healthy individuals (95). However, in several SLE patients, due to the blocking effect of anti-RNP Abs or IFNs on TFAM, neutrophil-mtDNA is unable to dissociate from nucleoids, hence ox-mtDNA is retained within the neutrophils and expelled with NETs, which induces the production of type-I IFNs through pDCs (78). Indeed, in about 50% of SLE patients ($n = 14$) with anti-RNP Abs, ox-mtDNA is present, and so are antibodies to

**TABLE 1 |** Autoimmune responses to extracellular DNA—Antigens, regulators, and sensors.

| cfDNA-Association or source | Generated through | Associated proteins | Sensitive to | Sensors | Associated pathologies | Key References |
|---|---|---|---|---|---|---|
| Chromatin | Apoptosis<br>Necrosis<br>NETosis<br>Pyroptosis | Histones<br>HMGB1 | DNase1L3<br>> Dnase1 | TLR9<br>TLR2<br>TLR4<br>RAGE | SLE<br>RA<br>Sjogren's Syndrome | (67–69) |
| Microparticles | Apoptosis<br>Cellular-activation<br>Necrosis | Histones<br>HMGB1<br>G3BP | Dnase1L3 | MyD88-signaling pathway | SLE<br>HUVS | (67, 70–73) |
| Neutrophil Extracellular Traps (NETs) | NETosis | Histones<br>HMGB1<br>LL-37<br>MPO<br>HNP<br>Other granular proteins | Dnase1L3 and Dnase1 | TLR4<br>TLR9 | SLE<br>RA<br>Psoriasis | (74–82) |
| Mitochondrial | NETosis | TFRAM | Dnase1L3 (?)<br>Dnase1 (?) | TLR9<br>RAGE | SLE | (78, 83, 84) |
| Bacterial | Infection | Curli Amyloid<br>ERV gp70<br>$\beta_2$GPI | DNase1<br>Dnase1L3 (?) | TLR2/<br>TLR9<br>? | SLE<br>AIH | (85–87) |
| Cancer | Tumor cell apoptosis,<br>Necrosis | ? | Dnase1<br>Dnase1L3 (?) | ? | Anti-DNA Abs<br>? | (3, 65, 66, 88, 89) |
| Fetal | Apoptosis of fetal cells | ? | DNase1L3 | ? | ? | (66, 90, 91) |

*ERV gp70, Endogenous retrovirus glycoprotein 70; HMGB1, High mobility group box 1; G2RB, galectin 3 binding protein; LL-37, cathelicidin-derived antimicrobial peptide; MPO, Myeloperoxidase; TFRAM, Transcription factor A-mitochondria; $\beta_2$GPI, $\beta_2$ Glycoprotein I; SLE, Systemic lupus erythematosus; AIH, Autoimmune hepatitis; HNP, Human Neutrophil protein; HUVS, Hypocomplementemic urticarial vasculitis syndrome; ?, unknown.*

it (78). Increased NETosis (77, 84) and increased anti-mtDNA Abs are associated with increased anti-dsDNA, IFN-signature and disease activity index in SLE patients (83), indicating an important role of neutrophil mtDNA in SLE pathogenesis.

Apart from self-DNA and ox-mtDNA, the DNA-associated neutrophil microbial peptides LL37 and human neutrophil proteins (HNPs), human beta-defensin 2 and 3 are strong potentiators of IFN responses. LL-37 cause aggregation of DNA fragments, making them resistant to nucleases and facilitating their endocytosis in pDCs via autoantibody-Fc receptor-mediated uptake and IFN production (75, 77, 96). In monocytes, LL37 promoted the uptake of self-DNA to activate type I IFN responses through cytosolic DNA sensor cGAS-STING (79). Overall, in different cell types LL37-DNA complexes are potent inducers of type-I IFN through cytosolic or endosomal sensing. Not surprisingly, 40–55% of SLE patients were also found to develop anti-LL37 and anti-HNP antibodies, which significantly correlated with serum IFNα and disease activity score (77). These data suggest that increased NETosis drives chronic IFN production from pDCs in SLE patients, via production of high molecular weight immune complexes containing gDNA, ox-mtDNA and LL37. It was recently shown in human SLE patients that LL37-DNA complexes from netting neutrophils promoted internalization of self-DNA resulting in activation of LL37-specific human memory B Cells via TLR9 stimulation and production of anti-LL37 Abs (80).

In summary, autoimmune responses to NETs studied so far provide evidence for NET-DNA (gDNA/ mtDNA) as a TLR9 ligand and as an adjuvant promoting IFN production and polyclonal proliferation of B cells, including DNA reactive B cells in SLE, RA (81), psoriasis etc. However, there is little evidence to suggest that NET-DNA serves as a direct autoantigen for DNA-reactive B cells. Further experiments need to be undertaken to answer these questions.

## Intracellular and Apoptotic DNA

Oxidized mtDNA generated within the cells due to oxidative stress can be immunogenic if not processed and purged efficiently. Autophagic clearance of cytoplasmic substrates in the lysosomes has been suggested to prevent the availability of altered self-antigens including modified nuclear-DNA and ox-mtDNA in the cytosol (97, 98). A recent study using monocytes from SLE patients found that autophagic degradation of mtDNA in lysosomes is essential to prevent its accumulation in the cytosol. When accumulated, mtDNA activated the cGAS-STING pathway causing differentiation of monocytes into autoinflammatory DCs (99). Interestingly, IFNα signaling triggered increased mitochondrial respiration, oxidative stress and impaired lysosomal degradation in monocytes, suggesting

a direct role of IFNα in autoinflammation (99). This study highlights the importance of efficient mitochondrial recycling through autophagy in the maintenance of peripheral tolerance. In addition to mtDNA, apoptotic DNA internalized by phagocytes is also digested within acidified lysosomes. Inefficient lysosomal maturation in macrophages derived from lupus-prone MRL/*lpr* mice caused increased oxidative stress and impaired acidification of lysosomes. This promoted prolonged accumulation of internalized nucleic acids in endolysosomes and leakage into the cytosol, activating TLRs, and cytosolic sensors (100). Overall, autophagic and lysosomal degradation of self/internalized nucleic acids and associated proteins prevents autoinflammation.

## Microparticles

Apoptotic cells are quickly efferocytosed by macrophages under an anti-inflammatory program, the impairment of which can contribute to SLE (101). Upon cell death, DNA could be exposed extracellularly on apoptotic bodies (102), microparticles (MPs) (70), or as nucleosomes (103). MPs are small lipid membrane bound vesicles of 0.2–1 μm in diameter, generated during late apoptosis/ early necrosis of platelets, leukocytes, endothelial cells, or upon cellular activation through TLRs (70). MPs are decorated with different proteins like transporters, adhesion molecules, surface receptors etc., depending on their cellular parent, along with several constitutive proteins like galectin 3 binding protein (G3BP) (71), HMGB1 (104) and histones. MPs also contain nucleic acids like DNA, RNA, and microRNAs which could be surface exposed or encapsulated (105). MP-associated DNA appears concealed from the most abundant extracellular nuclease—DNase1, and specifically requires the activity of DNase1L3 for efficient degradation (67). Due to their ubiquitous production by all cells and unique structural/ antigenic properties, MPs present the most abundant and enduring source of autoantigens including cfDNA.

Although MPs are produced in all individuals and were proposed to have homeostatic functions (106, 107), several pathologies are also associated with them. Considerable increase in numbers, alterations in cellular origin and composition of circulating MPs have been implicated in atherosclerosis, thrombosis, vasculitis, systemic sclerosis, diabetes, thrombocytopenia, and rheumatoid arthritis (72, 107–109). MP-associated DNA and proteins have also emerged as important contributors to SLE pathogenesis. Antibodies from SLE patient sera and mouse models, as well as monoclonal anti-dsDNA Abs, have been shown to bind DNA in microparticles (67, 73, 110). There is also a significant increase in proportions of MPs in SLE patients with surface bound IgG2, IgM, and C1q, which positively correlates with disease activity, anti-DNA Abs titers and complement activation in patients (110, 111). There is also an increase in the concentration/ proportion of circulating MPs in SLE sera with altered protein composition—expressing VCAM-1, CD40L, HMGB1, or G3BP (71, 110, 112), which could serve to further engage ICs. In agreement, MPs-expressing G3BP were found to predominate in SLE patient sera ($n = 44$) (71). Moreover, colocalization of G3BP with IgG was imaged by immune electron microscopy in the glomeruli of nephritic kidneys, suggesting local cell-derived MPs as additional source of autoantigen for tissue IC-deposition (71). Overall, it is likely that circulating ICs form early in lupus development and initially may not reach the threshold of pathogenicity. Their eventual deposition in tissues and the ensuing organ damage could be enhanced by additional local factors such as impaired degradation of DNA. This multistep process may also explain why not all lupus patients develop lupus nephritis.

It was reported that MP-associated ICs from SLE patients promote ROS production in neutrophils and prime them for LPS-mediated NETosis (113, 114). MPs derived from SLE patients activated blood-derived pDCs and monocyte-derived DCs to express increased CD80, CD83, IL-6, and TNFα (113). Notably, unlike SLE-MPs, MPs from controls, RA and systemic sclerosis patients lacked MP-associated chromatin and did not activate DCs, nor induced NETosis (113). This agrees with our observations that about 1/3$^{rd}$ of the SLE patients with sporadic SLE, have DNase-sensitive chromatin on the surface of their MPs (67). The loss of DNase1L3 activity causes preferential accumulation of DNA in MPs (67) as well as the presence of higher molecular weight DNA in the plasma (90). These higher order structures are much more capable of engaging multiple BCRs in a stable interaction (4), and therefore could be potent stimulators of B cells with DNA-reactive BCRs. Together these studies are suggestive of a significant role of MP-associated chromatin as an abundant source of self-DNA in SLE, for activation of pDCs and DCs via the Fc receptors and potentially direct activation of DNA-reactive BCRs.

## Microbial (Bacterial/ Viral) DNA

SLE is a multifactorial disease requiring genetic susceptibility and environmental triggers for complete loss of tolerance and pathogenic manifestations. A major cause of lupus flares and increased disease activity in SLE patients is due to infections (7). Bacterial infections are most common in SLE patients and thought to contribute to SLE pathogenesis by enhancing inflammation and generating cross-reactive B cells which recognize bacterial as well as self-DNA (61). Bacterial amyloid protein-DNA composites were shown to stimulate a potent IFN response and trigger autoantibody production including anti-dsDNA Abs in lupus-prone as well as wild type mice (115, 116). Infections by all bacterial strains expressing amyloid-DNA complexes could potentially trigger autoimmunity in predisposed individuals, which could contribute to sporadic SLE and also lupus flares.

The role of microbiota in autoimmunity is well-appreciated, although poorly understood (117). A recent study showed that the pathobiont *Enterococcus gallinarum* was able to translocate to the liver and activate autoantigenic T cells, induce IFN-responses through TLR7 stimulation and anti-dsDNA Ab production in lupus prone mice. Accordingly, the pathological responses could be alleviated by antibiotic treatment (85). In several SLE patients, reactivation of human polyomavirus (BK virus) generates antibodies to T-antigen, DNA and DNA-binding proteins—TBP (TATA-box binding protein) and CREB (cAMP response element binding protein). Specifically, anti-dsDNA Ab were confined to patients with frequent polyomavirus reactivations and expression of T antigens (86), indicating a role for T-Ag-DNA complexes

in the stimulation of DNA-reactive B cells. Other potential sources of cfDNA in autoimmunity include tumor-derived DNA in cancer patients and fetal-DNA in pregnant females (66, 91). Altogether, these studies suggest that microbial DNA may promote autoimmune responses including the production of anti-DNA Abs; however, its primary antigenic role in the loss of tolerance to self-DNA has not been firmly established.

## DNases as Key Regulators of Immunogenic DNA

Innate nucleic acid (NA) sensors do not discriminate between foreign and self-NAs, hence the processing or metabolism of endogenous NAs is of paramount importance to prevent immune stimulation. Therefore, it is not surprising that ∼40% of the genes involved in monogenic or Mendelian-inherited forms of autoimmunity are nucleases. Nucleases can be broadly classified into two main categories depending on their spatial expression: (1) Intracellular nucleases—cleave NAs inside the cells, during apoptosis or after uptake of apoptotic bodies. (2) Extracellular nucleases—cleave NAs exposed extracellularly during apoptosis or generated outside of the cells. The tissue expression profile, structure, enzymatic activity, and functions of the two main classes of DNases in various pathological conditions were recently reviewed (118, 119).

## Intracellular Nucleases: Major Negative Regulators of Autoinflammation

### Cytosolic Nucleases

Genetic autosomal recessive mutations in RNA processing enzymes of the RNASEH2 complex, *ADAR1*, and *SAMHD1* cause abnormal induction of type-I IFNs and lead to Aicardi-Goutières syndrome (AGS) and related interferonopathies. In addition to these RNases, an autosomal recessive mutation in the cytoplasmic–ER membrane-resident 3′-DNA repair exonuclease1 (TREX1 or DNASEIII) also causes AGS and SLE (120, 121). Classical AGS is identified very early in age, mainly as a neuroinflammatory disorder of the central nervous system with very high levels of IFNα in the cerebrospinal fluid. Glaucoma, thrombocytopenia, hepatomegaly, chilblain-like skin lesions, and late onset of SLE like symptoms are also typical of AGS (122). Analysis of serum autoantibodies from 56 AGS patients (23.4%-*TREX1*; 57.1%-*RNASEH2B*; 2.1% *RNASEH2A;* 4.3%; 8.5% *RNASEH2C;* 4.3% *SAMHD1*; and 4.3% *ADAR1* mutants) was performed, using an autoantibody array to assess their antigen-specificity. The study revealed their specificity to nuclear antigens like gp210, PCNA, Ro/SSA, Sm/RNP, SS-A/SS-B etc. Even though AGS and SLE share several overlapping disease manifestations, ss/dsDNA specific antibodies were not detected in any of the AGS patient sera in this study (123). Moreover, in a previous AGS clinical study, only 3 patients (all <3 years age) from a cohort of 24 had anti-dsDNA Abs. Among the three, one patient had a mutation in *TREX1*, one in *RNASEH2C* and one had an unknown mutation (124). *Trex1*$^{-/-}$ mice do not develop classical AGS, but rather develop lethal inflammatory myocarditis, without anti-chromatin/ DNA Abs (125, 126). These studies indicate a limited role for the intracellular exonuclease *TREX1* in anti-DNA B cell responses.

## Lysosomal DNases

DNASE2 is an endonuclease that functions in the lysosomes and is known to process DNA internalized with apoptotic cells. DNASE2 is expressed by macrophages in almost all tissues. Mice deficient in *DNASE2* die *in-utero*, due to an overwhelming IFNα response and lethal anemia (127, 128). Sequencing analysis on 24 SLE patients from a Korean cohort revealed 6 sequence variants of *DNASE2*, all of which were at a higher risk for renal disorders but showed no significant association with SLE (129). Recently, three individuals from two families of Algerian or Italian ancestry were identified with biallelic mutations in *DNASE2*, causing complete loss of DNASE2 endonuclease activity. They were able to survive with medical intervention but had severe neonatal anemia, glomerulonephritis, liver fibrosis, and arthropathy. The hallmark yet again was the excessive production of IFNα and associated interferonopathies (130). Remarkably, all the patients with *DNASE2* mutations had high titers of anti-DNA Abs and renal disorders. Further analysis of DNASE2 in SLE patients will shed more light on its role in SLE pathogenesis and anti-DNA responses. Notably, both the intracellular DNases—TREX1 and DNASE2, signal through the cGAS-STING pathway for IFN production (131).

Most recently, two endolysosomal proteins phospholipases D3 and D4 (PLD3/PLD4) with putative phospholipase activity were shown to have a functional 5′ exonuclease activity preferentially on unstructured ssDNA. PLD3 or PLD4-deficient mice displayed a TLR9-stimulated inflammatory syndrome while PLD3/4 double-deficient mice were unable to survive beyond the age of 21 days due to severe liver inflammation. Interestingly, the observed autoinflammatory syndrome was mediated by IFNγ instead of IFNα. Although there was excessive TLR9 activity causing IFNγ production, no autoantibody responses were reported (132). Polymorphisms in PDL4 linked to RA and systemic sclerosis (133, 134) have also been reported. Altogether, these studies identify the predominant function of intracellular nucleases in preventing autoinflammatory conditions, whereas their contribution toward anti-DNA antibody responses may be limited, as shown in **Figure 1**.

## Extracellular DNases: Negative Regulators of Extracellular Immunogenic DNA

### DNase1: A Potential Negative Regulator of Lupus Nephritis?

DNase1 is the most abundant secreted endonuclease, that is primarily expressed in the salivary glands, kidneys and gut (135). The association of DNase1 with SLE was initially identified through the *DNase1*$^{-/-}$ mouse model generated on a mixed B6/129 background, in which some mice developed anti-DNA and anti-nucleosome-Abs (predominant), as well as glomerulonephritis in a gender-independent manner. However, in subsequent studies it was shown that the B6/129 mixed background itself caused most of the observed SLE phenotype (136), as *DNase1*$^{-/-}$ mice on a pure B6 background did not develop SLE features (68).

Similarly, limited association of DNASE1 with anti-DNA antibody production in human SLE has been identified. Two Japanese patients that developed serological features of SLE with high titers of anti-DNA and anti-Nuc Abs, were identified with an A→ G mutation in exon 2 of human *DNASE1* causing a 3–4 fold reduction in enzymatic activity (137). Till date there are no further reports on SLE patients identified with similar or other mutations in *DNASE1* (138). However, in another study with 113 SLE patients, Dnase1 activity was found to be significantly lower in SLE patients compared to healthy controls, which negatively correlated with anti-Nucleosome antibody titers. No correlation was found between reduced DNase1 activity and SLE disease flare-ups or kidney complications in this cohort (139). Notably, kidney biopsies from 10 patients were screened for DNASE1 activity of which 4 patients had SLE-associated nephropathy. These 4 patients showed a concurrent low enzymatic activity of DNASE1 compared to healthy controls (140). In agreement with these observations, a reduction in DNASE1 expression in kidney, and urine directly correlated with progression of lupus nephritis in mouse and in humans with self or transplanted kidneys (141, 142). These studies suggest a potential role of locally produced DNase1 in the prevention of immune complex deposition and subsequent kidney nephritis (143).

Unlike its role in anti-DNA responses, the function of Dnase1 in the degradation of NETs is better established. Healthy human serum was able to degrade NETs *in vitro* and the functional component was identified to be DNase1. Conversely, sera from 36.1% of 61 SLE patients had poor or no ability to degrade NETs *in vitro*. These patients were found to have high anti-NET Abs which hampered the accessibility of DNASE1 to NETs, Notably, in this cohort of SLE patients the poor NET degraders had severe kidney nephritis (94)—further supporting a potential role of DNase1 in preventing kidney nephritis. Recently, NET degradation activity of Dnase1 was further corroborated when Dnase1 was also shown to have a redundant function along with DNase1L3 in the degradation of NETs formed during sterile neutrophilia and septicemia. The absence of DNase1 and DNase1L3 both caused vascular obstruction and organ damage. The results were consistent with human sera samples and in two different mouse models of DNase1 deficiency (82). This chromatin-degrading effect of Dnase1 seems to be specific to NET-associated DNA as the quality and quantity of cfDNA fragmentation was indistinguishable between plasma from WT and *DNase1*$^{-/-}$ mice (144). Overall, the available data from SLE patients and *DNase1*$^{-/-}$ mice do not indicate its involvement in anti-DNA antibody responses, whereas its role in SLE-related renal pathogenesis is prominent and deserves further exploration.

## DNase1 Like 3: Major Negative Regulator of Anti-DNA Responses

As the name indicates, DNase1L3 bears close structural and functional resemblance with DNase1 and together they comprise the secreted endonucleases in the serum (145). DNase1L3 is one of the family members of three homologous DNase1 like proteins. The DNase domain of all the Dnase1 family of enzymes is highly conserved, while the C terminal domains are most variable and may impart unique attributes to the enzymes. DNase1 and DNase1L2 lack a C-terminal domain, while DNase1L1 has a GPI-anchored hydrophobic region. DNase1L3 contains a positively charged C terminal domain (146). Homology modeling suggested that the C-terminal peptide of DNase1L3 may stretch out at a fixed angle from the main DNase domain with a stable α-helical secondary structure bearing a positive charge (67). Upon deletion of the C-terminal domain or modulation of its conformation, DNase1L3 lost its unique abilities of (1) efficiently degrading DNA within polynucleosomes and (2) digesting liposome-coated DNA (67, 147). Although the exact mechanism by which the C-terminal domain of DNase1L3 imparts the protein its unique ability to access lipid-encapsulated and histone-protected DNA is not clear, the positive charge on the α-helix may facilitate lipid membrane binding/ penetration and dislocation of histones from DNA. This unique structural property of DNase1L3 poises it to digest MP-associated DNA and prevent accumulation of extracellular DNA, thereby suppressing SLE. Indeed, we found that IgG from sera of at least 2/3rd of the 53 patients with sporadic SLE, bound to the surface of MPs. Pre-treatment of MPs with DNase1L3 abolished this binding in half of the patients indicating that the IgG binding on the surface of MPs was DNase1L3-sensitive (67). This finding could have implications in using DNase1L3 as a therapeutic to reduce MP-DNA-dependent immune complex formation. DNase1L3 was also recently shown to degrade intravascular NET-DNA and prevent vascular occlusion by disrupting NET clots similar to DNase1 (82).

The role of DNase1L3 in autoimmunity was discovered during clinical human patient analysis, summarized in **Table 2**. A homozygous 1-bp deletion in *DNASE1L3* (c.643delT) caused pediatric-onset familial SLE (148). Homozygous frameshift mutations—c.289_290delAC and c.320+4delAGTA in *DNASE1L3* led to exon skipping and pediatric SLE in two respective families (149). Recently, four Italian affected individuals were identified with similar mutations in *DNASE1L3* (150). In addition to null mutations, SNPs have also been reported in DNASE1L3 in humans. A heterozygous SNP C686/T686 resulting in R206C substitution in DNASE1L3 was found to reduce the DNase1L3 enzymatic activity (151). Furthermore, SNPs in DNase1L3 gene have also been associated with a related autoimmune disease Scleroderma (152, 153). Similar to humans, DNase1L3-deficient (DNase1L3KO) mice (on a pure B6 or pure129 background) develop SLE-like symptoms, including the gender-neutral formation of high titers of anti-DNA abs at an early age (67). Recently, another strain of DNase1L3KO mice was shown to develop anti-DNA responses that were further enhanced in SLE-prone mice (154). In striking contrast with all other nucleases, the anti-DNA responses in DNase1L3KO mice were STING-independent but MyD88-dependent (67). These data identify DNase1L3 as a DNase that is uniquely structured to access and degrade DNA associated with lipids and DNA-binding proteins. It forms an essential component of the DNase arsenal, in the absence of which extracellular DNA escapes degradation and can be a direct autoantigen for the activation and proliferation of DNA-reactive B cells. In

**TABLE 2 |** Known cases of *DNASE1L3* mutations in human subjects.

| | Cohort details | Identified mutations in *DNASE1L3* | Disease characteristics | Reference |
|---|---|---|---|---|
| 1. | 6 families<br>17 affected subjects (6 Females. 11 males) | Homozygous 1-bp deletion c.643deiT | $ANA^{+ve}$, Anti-$dsDNA^{+ve}$, $ANCA^{+ve}$<br>Hypocomplementemia<br>Nephritis in 10 subjects<br>SLE in all subjects with SLEDAI: 8-22 | (148) |
| 2. | 2 families<br>5 affected subjects<br>(All females) | Homozygousframeshift mutation.<br>c.289_290deiAC and c.320+4deiAGTA | HUVS in all subjects<br>SLE in 4 subjects<br>$ANA^{+ve}$, Anti-$dsDNA^{+ve}$, $ANCA^{+ve}$<br>Hypocomplementemia<br>Nephntis(classll-111) in 3 subjects | (149) |
| 3. | 1 family<br>4 affected subjects<br>(2 females. 2<br>males) | Homozygous 2b frameshift deletion<br>c.289_290deiAC | $ANA^{+ve}$, Anti-$dsDNA^{+ve}$, $ANCA^{+ve}$<br>Polyarthritis Glomerulonephritis<br>Vasculitis Hypocomplementemia | (150) |
| 4. | 9 populalons<br>>90 subjects per group | Heterozygous SNP C686fT686 resulting in<br>R206C substitution<br>Found mainly in European<br>Populations | Reduced Dnase1L3 enzymatic activity<br>Disease association not studied | (151) |

agreement, DNase1L3-deficient patients and DNase1L3KO mice show the development of anti-DNA Abs at a very early age.

Altogether, as depicted in **Figure 1**, these studies on nucleases provide evidence that (1) Anti-DNA responses are not induced by excessive IFN production *per se*, arguing that IFNs play a major role in the amplification of anti-DNA responses but not in the breakdown of tolerance to DNA. (2) Extracellular availability of immunogenic DNA as a direct antigen drives anti-DNA reactive B cell responses in SLE, making a strong case for the regulatory role of extracellular DNases in anti-DNA antibody production.

## Nucleic Acid Sensors: The Double-Edged Sword

It is now well-established that microbial NA sensors also recognize self-NAs under autoimmune conditions (69, 155, 156). For that reason, self-NA availability to NA-sensors is limited by nucleases and the availability of several NA-sensors is stringently controlled by their localization inside endosomes and by post translational processing for function (157). Together, they prevent self-NA availability and sensing. The contribution of DNA and RNA-sensors in autoimmunity has been the topic of several comprehensive reviews (158, 159). Here we highlight some key points related to the DNA-sensors involved specifically in the antibody response to DNA. We discuss literature which emphasizes the prominent role of endosomal TLRs in DNA sensing to generate an "autoantibody response," unlike the cytosolic DNA sensors which mainly engage an "autoinflammatory response." All the DNA and RNA-sensors are intracellular and further divided into two groups—cytosolic or endosomal. In keeping with the main theme of this review, we discuss the known NA-sensing pathways regulated through intracellular and extracellular nucleases.

## NA-Sensing Regulated Through Intracellular Nucleases

Mice deficient in RNaseH2 complex proteins, SAMHD1 and ADAR1 are either embryonically lethal or do not recapitulate human AGS. Yet, they revealed that RNaseH2 or SAMHD1-dependent NA-accumulation led to the stimulation of the cGAS-STING pathway, while loss of ADAR1 (deaminase) stimulated the RNA sensing MDA5-MAVS pathway (118). Cytosolic DNA sensing due to DNaseIII or Trex1 deficiency stimulated the cGAS→ cGAMP→ STING→ IRF3→ IFNα signaling axis (121, 126, 131). Overall, the predominant response to DNA sensing in the cytosol was autoinflammatory in both humans and mice. Additionally, although antibodies with other specificities were observed, DNA specific antibodies were detected only in a minority of patients (118, 122, 124). As highlighted in **Figure 1** in green, these studies point toward the role of cytosolic cGAS-cGAMP-STING signaling pathway in autoinflammation but not in the initiation of anti-DNA antibody responses.

The contribution of lysosomal DNase2 in anti-DNA antibody production is complicated partly because of the absence of a viable mouse model and partly due to the paucity of human patients identified with *DNASE2* mutations. The absence of DNase2 in mice causes accumulation of apoptotic cell derived DNA in the lysosomes of liver and bone marrow macrophages causing lethal anemia and cell death. DNase2KO mice can be rescued by the deletion of IFNAR. However, "IFNAR-deficient DNase2KO mice develop chronic polyarthritis, splenomegaly, and ANA. The accumulated DNA stimulates the cGAS-STING-IRF3/7 pathway leading to massive type-I interferon production, because the deficiency of either cGAS (131)/ STING (160, 161)/ IRF3 or IRF7 (162)/AIM2 (161) can rescue the mice form prenatal anemia and severe arthritis. While the generation of IFNα and TNFα in DNase2-deficient mice as well as humans is documented, anti-DNA antibody production in DNase2KO mice is not predominant (131, 160, 163). Instead, DNase2-IFNAR-double-deficient mice developed

antibodies against RNA-associated antigens and splenomegaly in an RNA-driven TLR-dependent manner (161, 163, 164). Further analysis of DNase2 in anti-DNA responses is required as the three DNASE2-deficient patients (age 11–17) reported thus far had fluctuating significant elevations in circulating anti-DNA Abs (130). It is possible that the formation of anti-DNA Abs in these patients and mice is an "after-effect," as a result of polyclonal activation of B cells due to chronic inflammation and IC formation.

Altogether, the current literature suggests that the cytosolic DNA is detected primarily through the cGAS-STING pathway which induces a potent autoinflammatory response, while the minimal anti-DNA antibody production seems to be a secondary effect. This agrees with the seemingly confounding role of STING in autoimmunity. STING appears to be a potentiator of autoimmune responses by inducing IFNα and downstream ISGs (165, 166). Remarkably, patients with overactive STING do not develop detectable anti-DNA Abs (165, 166)—strengthening the idea that cytosolic STING signaling pathway is not directly involved in anti-DNA responses. Contrary to its autoimmune-stimulatory activity, STING-deficiency was shown to exacerbate autoimmune manifestations including anti-DNA antibody production in MRL. $Fas^{lpr}$ lupus mouse model (167). It is likely that when cytosolic DNA is absent STING functions as a negative regulator of endosomal TLR signaling through yet undiscovered mechanisms. Alternately, when cytosolic DNA is present STING induces IFNα signaling and autoinflammation. Overall, what we can conclude with confidence is that the cGAS-STING pathway does not seem to induce anti-DNA Ab production.

## NA-Sensing Regulated Through Extracellular Nucleases

As discussed earlier, DNase1 deficiency does not seem to induce anti-DNA responses by itself. However, its local functions reported in the kidney may promote formation of immune complexes laden with DNA, which are known to engage the endosomal TLR9-MyD88 signaling pathway (168). Further studies on DNase1 need to be performed to establish its NA-sensing partners.

DNASE1L3-deficient patients and DNase1L3KO mice both develop high titers of anti-DNA Abs at an early age, without a female gender bias. Using DNase1L3KO mice deficient in either STING or MyD88 we found that anti-DNA and anti-nucleosome responses in DNase1L3KO mice are dependent on the TLR-MyD88 pathway. Further studies are underway to delineate which MyD88-dependent TLRs are involved in the autoimmune responses to MP-associated DNA in D1L3KO mice. Some studies suggest that DNase1L3 may have intracellular localization with functions in apoptosis and inflammation, reviewed in Keyel PA (119). However, as autoimmunity in DNase1L3KO mice is independent of the cytosolic DNA sensor STING (67), it indicates that the anti-DNA responses are specifically due to the extracellular DNase function of DNase1L3. Indeed, we were able to temporarily reduce the anti-DNA Ab titers in DNase1L3KO mice by transient replenishment of circulating DNase1L3 enzyme (67). Overall, extracellular DNases predominantly regulate the stimulation of TLR-MyD88 pathway of DNA sensing, highlighted in yellow in **Figure 1**.

## Role of Toll-Like Receptors in Anti-DNA Antibody Production

Deficiency of the adaptor protein MyD88 ameliorates SLE specific autoantibodies and associated pathology in several lupus mouse strains (169). Expression levels of TLR2, TLR7, TLR9, IFN-alpha, and LY6E (Sca-1) mRNAs in SLE patients are significantly higher than healthy controls, indicating contribution of TLR-MyD88 signaling pathways in the pathogenesis of human lupus (170). Several studies show that circulating DNA-containing ICs correlate positively with anti-dsDNA Ab production in SLE patients (171, 172). Indeed, the importance of BCR/TLR–dual signaling in autoimmune B cell responses was originally identified by using IC-mediated activation of IgG2a-reactive murine AM14 B cells (69). However, it is now clear that TLR engagement also promotes activation, proliferation/ differentiation of B cells that directly bind DNA (or other autoantigens) through the BCR and may therefore play an important role in the early stages of autoantibody production. Among the MyD88-dependent TLRs—TLR7 and TLR9 are the prominent ones involved in the development of anti-DNA Abs in mouse models of lupus and altered TLR7 and 9 expression has been reported in human SLE patients as well (173). Perhaps the most convincing evidence for the involvement of TLR7/9 signaling in B cells for anti-DNA antibody production comes from the case studies of SLE patients that develop an antibody deficiency syndrome similar to common variable immunodeficiency (CVID). These patients have a complete remission from SLE, with absence of anti-DNA Abs, and B cells are unresponsive to TLR7/9 stimulation—indicating the crucial role of TLR7/9 mediated B cell responses in SLE (174, 175).

## TLR7- the Master of RNA-Driven SLE Pathogenesis

The seminal role of TLR7 in SLE pathogenesis is firmly established. TLR7 promotes the formation of autoantibodies against RNA and RNA-associated proteins. Deletion of TLR7 reduces anti-SmRNP and other RNA-associated antibody responses, however, in most cases there is no reduction in anti-DNA responses (158, 176, 177). It was shown that B cell-intrinsic TLR7 signaling is essential for the formation of spontaneous germinal centers (178). Therefore, in the B6. *Sle1b* lupus mouse model where autoantibody formation is driven predominantly through the GC-pathway (179), TLR7 deficiency also reduces anti-DNA Abs (178, 180). Most-importantly, deficiency of TLR7 ameliorates SLE-associated splenomegaly and nephritis (176, 181), while expression of an extra copy of TLR7 exacerbates it (182). Altogether, TLR7 is the master regulator of RNA-driven TLR-dependent systemic autoimmune manifestations. However, it does not seem to play a direct role in anti-DNA antibody production and yet appears indirectly involved in SLE though

its functions in molecular pathways necessary for antibody production and inflammation.

## The Dichotomous Pathogenic and Tolerogenic Role of TLR9 in Autoimmunity

By far the most confounding endosomal TLR is TLR9, which is endowed with both pathogenic and regulatory functions. It is required for the generation of high-titer antibodies to DNA and DNA-associated proteins in several murine lupus models including MRL/$Fas^{lpr}$, B6. *Sle1b* and FcγRIIB$^{-/-}$ (158, 169, 176, 178). However, even though anti-DNA specific B cells and antibody titers are reduced in the absence of TLR9, there is an exacerbation of lupus pathogenesis (splenomegaly, nephritis, etc.) and an increase in autoantibodies against RNA-associated antigens (176, 177, 181, 183, 184)—suggesting a negative regulatory or tolerogenic role for TLR9 in the pathogenesis of lupus, by suppressing TLR7-mediated autoimmunity. Similar regulatory role of TLR9 was demonstrated in pristane-induced murine lupus. Pristane exposed TLR9-deficient BALB/c mice had an exacerbated production of autoantibodies against RNA, neutrophil cytoplasmic antigens, and myeloperoxidase and worse renal disease than TLR9-sufficient mice (185). TLR9 has also been shown to promote production of protective IgM antibodies by B-1b cells and prevent expansion of proinflammatory Th17 T cells, thereby regulating systemic autoimmunity (186). Recently, another potential mechanism contributing to the regulatory role of TLR9 was described. Exposure of phagocytes to apoptotic cell-associated DNA (a common antigenic source in experimental lupus), upregulated the expression of the transcription factor AhR (aryl hydrocarbon receptor) in a TLR9-dependent manner. AhR in turn drove the production of the immunoregulatory cytokine IL-10. Therefore, loss of TLR9 or AhR in lupus prone mice exacerbated disease (187). These studies indicate that TLR9 stimulation by DNA in macrophages could be immunosuppressive. However, B cells may not be subject to the same suppressive effects as they are poor phagocytes and this aspect needs further investigation.

## A Potential Role of Surface TLRs in Anti-DNA Responses

Several studies indicate an indirect contribution of cell surface TLRs in anti-DNA antibody production through HMGB1 which is a DNA binding protein known to stimulate TLR-signaling and induce a proinflammatory program (188). In C57BL/6$^{lpr/lpr}$ mice deficient in TLR2 or TLR4, glomerular IgG deposition and mesangial cell proliferation were remarkably decreased, and ANA, anti-dsDNA, and anti-cardiolipin autoantibody titers were significantly reduced (189). Moreover, TLR2-deficiency significantly reduced anti-DNA/nucleosome antibodies, renal disease, and IL-6 production in a pristane-induced lupus mouse model (190). Similarly, in SLE patients, HMGB1 in circulating DNA-containing ICs from SLE patients induced production of anti-dsDNA Abs through the TLR2-MyD88 pathway *in-vitro* (172). Recently, amyloid curli-DNA complexes were also shown to stimulate TLR9 via TLR2 (87).

In summary, the studies so far suggest a central role for TLR9 in the induction of anti-DNA antibody responses, supported by TLR7, 2, and 4. TLR9 is the only direct endosomal DNA sensor and induces a robust IFN response upon stimulation. However, its endosomal localization limits its accessibility. Therefore, IC-mediated internalization through Fc-receptors or direct BCR mediated uptake of DNA are the most potent inducers of TLR9 and anti-DNA Abs. Surface TLRs might play a role in aiding the delivery of DNA to TLR9. However, for long-lived plasma cell formation the germinal center pathway of differentiation is necessary. TLR7 plays a key role in the formation of spontaneous GCs, probably via stimulation through endogenous retroelements (191). Therefore, TLR7 signaling could further promote anti-DNA responses via the GC-pathway. It is more likely that for an effective anti-DNA B cell response all these TLRs—TLR9, TLR7, and TLR2/4 are required in tandem and the inflammatory program induced by them which includes cytokines like IFNα, IFNγ, IL-6, IL-10, and TNFα is necessary for the amplification of DNA-reactive B cells.

## Interferons: Key Effectors in the Development and Progression of Anti-DNA Responses and SLE Pathogenesis

### Type-I Interferons: Prominent Role of IFNα in Anti-DNA Responses

The three main pathways of type-I interferon induction include sensing by (1) cGAS-STING, (2) RIG-I/MDA5-MAVS, and (3) TLR-MyD88/TRIF. Their involvement in the pathogenesis of several rheumatic diseases and the current therapeutic interventions targeting the type-I interferon pathway have been extensively discussed in excellent reviews elsewhere (192, 193). Type1 interferons are at the core of several disease manifestations in monogenic disorders discussed above, so much so that most of the symptoms have been classified separately as "type-I interferonopathies," with AGS as the prototype. Loss of function mutations in cytosolic DNA/RNA processing enzymes and gain of function mutations in the ds-RNA receptor gene IFIH1 (MDA5) (194, 195) and adapter protein *TMEM173* (STING) cause excessive type-I IFN production leading to severe disease pathologies (122, 196). However, as discussed in previous sections, the induction of type-I interferons by the stimulation of cytosolic NA sensors does not seem to engage the pathways directly responsible for anti-DNA antibody production, although it is crucial for disease manifestations with features overlapping that of rheumatic diseases.

On the other hand, accumulating evidence implicates the TLR pathway of type-I IFN production as a major pathway of anti-DNA Ab production. The most convincing data is from SLE patients with genetic variations in the major proteins involved in the TLR-IFN signaling axis. Polymorphisms in TLR signaling pathway proteins such as IRF5, IRF7, IRF8, and IRAK1 are associated with SLE (197, 198). Most of the polymorphisms in IRFs directly correlate with high anti-DNA and anti-Ro/La/Sm antibodies which result in increased IFNα activity (199–201). These studies suggest a crucial role of autoantibody-DNA/ RNA complexes in the stimulation of the TLR pathway resulting in

increased type I IFN production (**Figure 1**), which feeds into an amplification loop. Although the role of ICs in the production of type-I IFNs is clear, whether they are directly involved in the generation of autoantibodies found in those ICs is less understood. In this regard, understanding the direct vs. indirect effects of type-I IFNs on B cells may be critical. Indeed, a number of studies have looked at the effect of IFNα on B cells in various lupus-prone mouse models by overexpressing IFNα through adenovirus in NZB/W-F1, NZW/BXSB and B6. *Sle1,2,3*, reviewed in Liu et al. (202); or by deleting the IFNα receptor in B cells of B6. *Sle1b* (203) and WASp-chimeric (204) experimental lupus models. In all these models IFNα signaling positively correlated with B cell activation and differentiation into GC B cells and antibody-forming cells and high anti-DNA Ab titers. In the B6. *Sle1b* model, IFNα specifically promoted autoreactive B cell expansion and positive selection through the germinal center pathway but was dispensable for B cell responses against foreign antigens (203). Interestingly, in most experimental models, IFNα production through the TLR7 signaling pathway seems to play a major role in SLE by either regulating germinal center formation or by promoting the generation of IC-forming pathogenic autoantibodies that ultimately cause kidney pathology. TLR9-deficieny in MRL.Fas(lpr) mice caused exacerbated renal disease which was abrogated in the absence of IFNAR-signaling through specific reduction of anti-RNA specific antibodies (205), suggesting a crucial role of TLR7-IFNα signaling axis in SLE pathogenesis. In DNase1L3-deficient mice where increased accumulation of undigested cfDNA leads to specific production of anti-chromatin and anti-DNA antibodies through the TLR-MyD88 pathway, IFNα overexpression exacerbated anti-DNA responses and mortality (67). Collectively, these studies establish an important role of type I IFN in autoantibody-driven inflammation, although it has been difficult to distinguish its effects on autoantibody production *per se* vs. the downstream inflammatory process.

### IFN Gamma: Initiator of Anti-DNA Antibodies in SLE?

Type-II IFN (IFNγ) has been implicated in both human and mouse lupus (206). Accumulation of autoantibodies has been shown to precede clinical presentation of SLE disease by several years (2). A comprehensive longitudinal analysis of lupus autoantibodies, IFNα, and IFNγ from serum samples of 55 patients before and after clinical onset of SLE, with matched controls was performed. The study revealed that in SLE patients, autoantibodies appear years before clinical SLE is detectable but notably they either coincided with or followed an increase in IFNγ. In contrast, increase in IFNα was observed mostly at the time of detectable clinical disease (207). These findings suggest IFNγ as the initiator and IFNα as the propagator of autoantibody production. In line with this model, recently, in two independent studies with B6. *Sle1b* or WASp murine models of lupus, deletion of IFNγ receptor in B cells led to a complete loss of germinal centers, abolishment of anti-dsDNA Abs and systemic autoimmune manifestations (204, 208). In WASp chimeric mice and in human B cells, IFNγ signaling along with BCR/CD40 and TLR signaling was shown to be necessary for the induction of Bcl-6, the master regulator of GC responses (204). Alternately, in the B6. *Sle1b* model, IFNγ signaling through STAT1 was required for the expression of transcription factor T-bet, and IFNγ production in B cells, which then differentiated into pathogenic anti-DNA IgG2b/2c producing cells (208). Interestingly, a subset of B cells expressing T-bet, CD11c, and IFNγ named age-associated B cells (ABCs), have been implicated in SLE pathogenesis as well (209). Moreover, upon deletion of IFNγ a dramatic decrease in anti-dsDNA antibodies of IgG2a subclass and reduced proliferation of B cells was also observed in the MRL-FAS (lpr) mice (210), where anti-DNA antibody production occurs mainly through the extrafollicular pathway (20). Overall, these studies highlight the important role of IFNγ signaling in the GC, extrafollicular, and ABC-associated pathways of anti-DNA antibody production. Several studies describing IFNγ in SLE, highlight the role of T cells in the production of pathogenic IFNγ (204). Indeed, a higher percentage of $CD4^+$ and $CD8^+$ T cells from SLE patients produce excessive IFNγ (211). Additionally, Tfh mediated autoimmunity in $Roquin^{san/san}$ mice was induced by the accumulation of excessive IFNγ producing T cells due to delayed degradation of IFNγ mRNA (212). Considering the evident importance of IFNγ in autoimmunity, neutralization, or reduction of IFNγ has been tried as a therapeutic modality in mice and human SLE. Significant reduction in autoimmunity was observed in NZB/NZW-F1, MRL. $Fas^{lpr}$ and pristane induced mouse model of lupus, however, due to the crucial role of IFNγ in antiviral immunity in humans, the usefulness of IFNγ blockade in SLE may be limited (206).

Overall, the role of interferons in anti-DNA Ab production is well-established. However, due to the significant overlap between the genes induced by IFNα and IFNγ (213, 214), it is harder to delineate their individual contribution to autoantibody production and SLE pathogenesis. Based on the evidence discussed above, it is plausible that under autoimmune conditions (in the presence of cfDNA/RNA or cell-intrinsic defects), autoantibody production is initiated by the stimulation of B cells by antigen-stimulated IFNγ-producing T cells, leading to autoreactive B cell proliferation and differentiation. The autoantibodies produced thereby form complexes with the cfDNA/RNA may then promote pDC/DC activation and IFNα production, which would further amplify the response, as shown in the schematic in **Figure 1**.

## CONCLUSIONS

Emerging genetic and functional evidence suggests that the efficient degradation of extracellular DNA is an important checkpoint in preventing the stimulation of DNA-reactive B cells. As depicted in **Figure 1**, a B cell-intrinsic TLR-MyD88 pathway of DNA recognition seems to be necessary for the break in tolerance to DNA, supported by helper T cells recognizing DNA-associated antigens. These autoreactive B cells could further proliferate and differentiate in response to type-I IFNs produced by themselves and by myeloid/ stromal cells, thereby amplifying the autoantibody response. Therefore, using interventions that could enhance or amplify the degradation of extracellular DNA may work to impede the production of anti-DNA antibodies and could be tested as a therapeutic for SLE.

## AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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# Johnny on the Spot-Chronic Inflammation Is Driven by HMGB1

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Although much has been made of the role of HMGB1 acting as an acute damage associated molecular pattern (DAMP) molecule, prompting the response to tissue damage or injury, it is also released at sites of chronic inflammation including sites of infection, autoimmunity, and cancer. As such, the biology is distinguished from homeostasis and acute inflammation by the recruitment and persistence of myeloid derived suppressor cells, T regulatory cells, fibrosis and/or exuberant angiogenesis depending on the antecedents and the other individual inflammatory partners that HMGB1 binds and focuses, including IL-1β, CXCL12/SDF1, LPS, DNA, RNA, and sRAGE. High levels of HMGB1 released into the extracellular milieu and its persistence in the microenvironment can contribute to the pathogenesis of many if not all autoimmune disorders and is a key factor that drives inflammation further and worsens symptoms. HMGB1 is also pivotal in the maintenance of chronic inflammation and a "wound healing" type of immune response that ultimately contributes to the onset of carcinogenesis and tumor progression. Exosomes carrying HMGB1 and other instructive molecules are released and shape the response of various cells in the chronic inflammatory environment. Understanding the defining roles of REDOX, DAMPs and PAMPs, and the host response in chronic inflammation requires an alternative means for positing HMGB1's central role in limiting and focusing inflammation, distinguishing chronic from acute inflammation.

**Keywords: HMGB1, chronic inflammation, autoimmunity, cancer, autophagy**

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> *"A 'Johnny on the spot' is a man or youth who may be relied upon to be at a certain stated place when wanted and on whose assured appearance confident expectation may be based. It is not sufficient that an alert and trustworthy individual, to be thought deserving of the name 'Johnny on the spot,' should restrict his beneficent activity to the matter of being at a certain place when needed. He must, in addition, render such service and attend to such business when there as the occasion may require, and such a 'Johnny' must be on the spot not merely to attend to the business of others, but also to look after his own. Hence an individual who is prompt and farseeing, alive to his own interests and keenly sensible of means for promoting his own advantage is a 'Johnny on the spot.'" Anonymous, April 1896.*

## INTRODUCTION

### DAMPs and Inflammation

In the context of sterile inflammation, damage-associated molecular pattern molecules (DAMPs) are considered the "signal 0" that mediates engagement of pattern recognition receptors (PRRs) in immune cells, leading to the activation (signals 1–4) and tissue integration and persistence (signal 5) of their effector functions. Polarization of the immune infiltrate relies on cues present at the site

of injury, including DAMPs, as well as on its REDOX state (1). DAMPs include proteins such as heat shock proteins (HSP), histones and cytokines, but some non-protein molecules can also act as "danger signals" such as ATP, extracellular DNA or RNA, and mitochondrial DNA. The prototypic DAMP and focus of this review is the high mobility group (defined by mobility on electrophoretic gels) box 1 protein, HMGB1, a member of the extended HMG-box containing proteins. These molecules only bind with high affinity non-B-type DNA conformations that are either bent or unwound. HMG-box domains regulate transcription, replication, and DNA repair, all of which change chromatin conformation. The HMG family of proteins includes such molecules as the three superfamilies each containing a specified functional domain: 1) HMGA (AT-hook domain)-HMGA1 and 2; HMGB (HMG-box domain) including homologous functional proteins HMGB1, 2, 3, and 4; HMGN (nucleosomal binding domain) including HMGN1, 2, 3 and 4; the SRY, Sex-Determining Region Y Protein located on the Y chromosome; and the TCF/LEF Transcription Factors including Lymphoid enhancer-binding factor 1 and T Cell Transcription Factor 1 that are involved in the Wnt signaling pathway, recruiting beta-catenin as a coactivator to enhancer elements of genes (2).

HMGB1 was first described as a chromatin-associated molecule, but other properties related to its cytosolic roles, promoting autophagy and its extracellular roles, promoting inflammation, have since been described. HMGB1 is a non-histone nuclear protein that contains two lysine-rich DNA binding regions, A and B boxes, and an unusual C terminal acidic tail composed largely of aspartic and glutamic acid residues (3). As a component of nucleosomes, HMGB1 has attracted attention in the context of autoimmunity, a topic which will be discussed below.

Although other receptors for HMGB1 may be identified given its "sticky" properties and unusual acidic and basic domains, known receptors include Toll-like receptors (TLRs) 2, 4, and 9, as well as receptor for advanced glycation endproducts (RAGE), CXCR4/CXCR7 and T-cell immunoglobulin and mucin-domain containing-3 (TIM-3) (3, 4). Endothelial cells are early responders to tissue damage, and readily upregulate adhesion molecules and initiate neutrophil recruitment following HMGB1 binding to its receptors (1). Cells of the immune system also respond to HMGB1, which promotes pro-inflammatory cytokine release by macrophages including tumor necrosis factor alpha (TNF-α) and interleukin (IL)-6 and activation of dendritic cells (DCs) (1).

## Autophagy and Cell Death

Autophagy is a highly conserved multistep mechanism that allows maintenance of metabolic homeostasis in cells undergoing stress, i.e., nutrient and oxygen deprivation, infection or oxidative stress, among others. During autophagy, bulk portions of the cytoplasm or ubiquitinated targets, such as protein aggregates or damaged organelles, are enclosed in the autophagosome, a double-membraned structure which then fuses with a lysosome for cargo degradation and ATP generation, under tight regulation of a set of proteins collectively named the ATG (autophagy-related gene) proteins. Autophagy is therefore a survival mechanism which has, however, also been associated at extremes with cell death, specifically "autosis," where dying cells display numerous autophagosomes in their cytoplasm, often as a prelude to apoptosis (5). Thus, in most instances of cell death, autophagy accompanies cell demise, but it is not necessarily its cause, with several components of the autophagic machinery being involved in processes related to cell death (6) including ferroptosis.

## Microvesicles and HMGB1

The efficiency in inducing an inflammatory response depends on the success of cellular communication, which in turn, depends on ligand-receptor interaction, wherein the ligand can be on the membrane of the stimulatory cell, free in the extracellular medium and in extracellular vesicles (EVs) (7, 8). In general, there are 3 different types of EVs, which are classified based on their size, biogenesis and composition (9, 10). The small vesicles originate in endosomal compartments called multivesicular bodies (MVBs), which fuse with the cell membrane to release their intraluminal vesicles to the extracellular medium, where they are identified as Exosomes (Exo), ranging from 30 to 150 nm (7, 11). Microvesicles/Microparticles (MPs) are vesicles ranging in size from 100 to 1,000 nm, budding directly from the plasma membrane (12). The last and largest type are apoptotic bodies, from 1,000 to 3,000 nm, originating from the cytoplasmic membrane of apoptotic cells (12). Interestingly, MPs and apoptotic bodies can be released from activated or apoptotic cells (13–15).

EVs are found in bodily fluids, such as amniotic fluid, breast milk, semen, plasma, saliva, nasal secretion, cerebrospinal fluid (CSF), ascites, synovial fluid, etc. (16). Several studies have stressed the importance of EVs in cell to cell communication because they are carriers of bioactive molecules, such as DNA, mRNA, miRNAs, lipids, adhesion molecules, cytokines, molecules involved with antigen presentation, autoantigens, DAMPs and PAMPs (7, 8, 17–21). Furthermore, during pathological processes, EV levels are increased, contributing to the development, progression and persistence of inflammatory and autoimmune diseases and cancer (19, 22–25).

# COMPARTMENTAL ROLES OF HMGB1

## Nuclear

The various functions of HMGB1 are highly context dependent. In the nucleus of homeostatic cells (**Figure 1**), HMGB1 is loosely associated with DNA, mediating its replication, transcription, recombination, repair and overall stability (1, 3). For example, HMGB1 preferentially binds damaged DNA following ionizing radiation, treatment with platinum or other DNA damaging chemotherapy and undergoes post-translational modifications to recruit and directly interact with other proteins involved in DNA repair, such as p53 (26, 27). Additionally, HMGB1 and p53/p73 interact in the nucleus, promoting access to their transcriptional complexes on DNA. p53 regulates the subcellular localization of HMGB1, inhibiting its translocation to the cytoplasm and *vice-versa* (28). This has proven to be a pivotal mechanism

for balancing subsequent apoptotic and autophagic processes. HMGB1 contributes to chemoresistance by exporting p53 out of the nucleus and into the cytoplasm, where it undergoes autophagic degradation (29). HMGB1 is important for all of the DNA repair enzymes in the nucleus (30). There it serves as a "Jack-of-all-trades" in addition to its "Johnny-on-the-spot role," facilitating the nucleotide excision repair (NER) pathway, the base excision repair (BER) pathway, the mismatch repair (MMR) pathway, the non-homologous end-joining (NHEJ) pathway, and V(D)J recombination in T and B cells.

Unlike histones, which are associated with maintenance of chromatin structure, HMGB1 can both compact and destabilize chromatin, facilitating access to numerous transcription factors (26). HMGB1 has previously been associated with regulation of transcription factors related to cell death (31), hormonal (32), and immune responses (33). HMGB1 contributes to liver tumorigenesis by positively regulating the expression of yes-associated protein, which forms a complex with hypoxia-inducible factor 1α to drive hepatic cells to acquire a glycolytic metabolic profile (34). Additionally, HMGB1 controls transcription of HSP beta-1/HSP27, essential for mitochondrial quality control, resultant mitophagy, and thus maintenance of metabolic homeostasis (35).

### Cytoplasmic

In the face of stress, HMGB1 is actively translocated from the nucleus to the cytoplasm through oxidation of the cysteine encoded at position 106 as well as post-translational modifications posited including ADP ribosylation (36) and acetylation (37). There, it frees Beclin-1 from Bcl-2/BCLxL interaction sites, promoting formation of autophagosomes. Importantly, binding of HMGB1 with Beclin-1 is dependent on disulfide bond formation between cysteines 23 and 45. Treatment of MEFs and tumor cells with autophagy inhibitors prevents LC3 puncta formation and HMGB1 translocation. Knockdown of Atg 5 has a similar effect (38). Thus, HMGB1 translocation to the cytoplasm induces autophagy but the occurrence of autophagy also regulates HMGB1 translocation.

Autophagy is essentially a pro-survival cellular mechanism. Given the close relationship between HMGB1 and autophagy, there has been extensive research into the role of HMGB1 and chemoresistance in various types of tumors and classes of drugs, which will be discussed below.

### Extracellular

HMGB1 is actively secreted to the extracellular milieu by components of the immune system, platelets and endothelium following infection and exposure to inflammatory mediators (39). Oxidative stress-mediated autophagy is also a trigger for HMGB1 active release (38). As a leaderless molecule, HMGB1 undergoes lysosomal exocytosis and passive diffusion, given its polybasic tracts and protein transduction domain like properties, to gain access to the extracellular space (3, 40). It is also released from dying cells, both necrotic (with loss of membrane integrity) and late apoptotic, partly as a result of a cell's failure to undergo efferocytosis (41–45). Apoptotic cell-derived HMGB1 usually does not possess immune activating properties due to its oxidized state (39). Once out of the cell, HMGB1 can act as a DAMP through direct binding with its receptors or it can interact with other molecules including IL-1, DNA, RNA, miRNA, LPS and nucleosomes, dictating the range of different receptors that can be bound and the consequent biologic effect. The oxidative state of HMGB1 determines its roles as a chemokine, when reduced, partnering with CXCL12 to attract leukocytes, or as a cytokine, when partially oxidized, forming disulfide bonds and binding TLR4. Cell fate also seems to be regulated by HMGB1with reduced forms of HMGB1 decreasing the cytotoxicity of various chemotherapeutic agents by inducing autophagy whereas its oxidized form has the opposite effect, contributing to cell death (46).

## HMBG1 AND AUTOIMMUNITY

### HMGB1 and Human Disease

HMGB1 is upregulated in the serum/plasma of patients with various autoimmune disorders including vessel vasculitis, systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) (47). Furthermore, patients with active disease present with higher levels of serum/plasma HMGB1 than those with inactive disease (47), highlighting its importance as a mediator of autoimmunity.

#### Systemic Lupus Erythematosus

SLE is an autoimmune disease characterized by the production of antinuclear antibodies, with nucleosomal antigens being the main targets, which induces systemic, potentially life-threatening symptoms (48, 49). In this disease, a high rate of cell death or persistence of non-cleared apoptotic cells lead to a large load of nuclear autoantigens being released in tissues or the blood, triggering autoimmunity (50). It has been speculated that non-cleared apoptotic cells can assume characteristics of "secondary necrotic cell death," leading to HMGB1 release to the extracellular milieu and driving autoimmunity further, but whether this effect relies on HMGB1 alone remains to be clarified. Accordingly, genetic defects in the complement component C1q, an opsonin involved in apoptotic cell clearance, are associated with SLE in humans (51, 52). Neutrophils from patients with SLE are more prone to undergo NETosis, a form of cell death where a mesh of chromatin along with other nuclear components and antimicrobial effectors (termed NET, neutrophil extracellular trap) is externalized (53, 54). It has been proposed that the presence of HMGB1 in NETs can prevent their clearance through inhibition of DNAse I activity, leading to lupus nephritis, a complication of SLE (55, 56). Moreover, there can be an increase in HMGB1 serum levels from patients with pedriatic lupus nephritis in comparison to SLE patients without renal involvement (57). Defects in NET degradation have also been associated with other autoimmune disorders characterized by the presence of autoantibodies (48, 58, 59) but the role of HMGB1 in these settings has not been thoroughly explored. Dendritic cells (DCs) preferentially present antigens from NETotic neutrophils (60) and HMGB1 present in NETs released by neutrophils from pedriatic SLE patients up-regulate type I interferon production by tissue plasmacytoid DCs, which

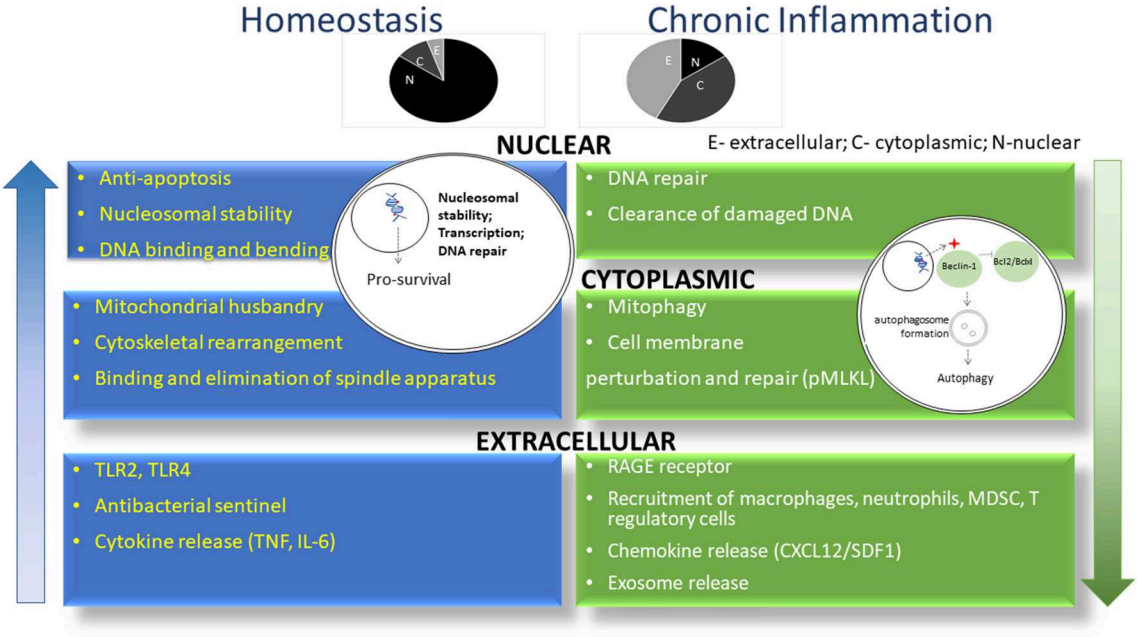


**FIGURE 1 |** HMGB1 exerts differential roles in homeostasis and chronic inflammation. As shown in blue, HMGB1 is predominantly located in the nucleus of cells in the setting of homeostasis, where it promotes nucleosomal stability and facilitates access of transcriptional factors to DNA. In chronic inflammation (green), HMGB1 leaves the nucleus to drive autophagy in the cytoplasm and act as a DAMP in the extracellular *milieu*. Shown above are the relative proportion of HMGB1 in individual compartments during homeostasis and chronic inflammation. N, nucleus; C, cytoplasm; E, extracellular.

further stimulates the release of NETs and aggravates the disease (61). Macrophages can also respond to HMGB1, upregulating the production of inflammatory cytokines TNF-α and IL-6 both *in vitro* and *in vivo* (62, 63).

## Rheumatoid Arthritis

In rheumatoid arthritis (RA), inflammation and hyperplasia of the synovial membranes can be worsened by angiogenesis and consequent increase in the influx of inflammatory infiltrate. Fibroblasts from RA patients readily enhance HIF-1α expression following HMGB1 treatment via TLR4 engagement and signaling through NF-κB (64). Furthermore, conditioned medium from HMGB1-treated fibroblasts from RA patients induce endothelial cell tube formation via VEGF release. HMGB1 neutralization attenuates symptoms of experimental arthritis, with significant lower expression of HIF-1α and VEGF *in vivo*. The anti-inflammatory drug cilostazol reduces these angiogenic effects of HMGB1 (65). HMGB1 is upregulated in the spinal cord of arthritic mice and intra-thecal administration of anti-HMGB1 Ab reverses mechanical hypersensitivity in these animals, a symptom that is attributed to the action of disulfide HMGB1 alone (66). Monocytes from patients with active RA require lower amounts than healthy controls of HMGB1 to acquire a migratory phenotype dependent on CXCL12, a chemokine known to form a heterocomplex with HMGB1 in the extracellular milieu to drive inflammation (67). Interestingly, high levels of the enzymes thioredoxin and thioredoxin reductase (which have previously been associated with disease severity) are found in the plasma of RA patients and maintain the reduced status of HMGB1, its ability to bind CXCL12 and therefore exert inflammatory activity. Another important partner of HMGB1 in the pathogenesis of RA is LPS, which activates synovial fibroblasts to produce inflammatory cytokines, matrix-metalloproteinases, increase autophagic flux and decrease apoptosis (68, 69). Methotrexate, a drug commonly used in the treatment of patients with RA, decreases HMGB1 levels and hyperplasia in the synovial tissue. HMGB1 knockout in fibroblasts from these patients renders them less proliferative and invasive (70). However, methotrexate treatment can also stimulate autophagic flux in RA fibroblasts via an HMGB1-Beclin1-dependent fashion, culminating in chemoresistance (71).

## Multiple Sclerosis

Multiple sclerosis is an autoimmune demyelinating disease characterized by high concentrations of extracellular HMGB1 and its receptors, RAGE and TLR's, in patients' plasma (72) or cerebrospinal fluid (73). Additionally, there is enhanced cytosolic expression of HMGB1 in cells within MS lesions. In experimental autoimmune encephalomyelitis (EAE), HMGB1 neutralizing antibody has both prophylactic and therapeutic effects, preventing oligodendrocyte loss and $CD4^+$ T cell recruitment to the central nervous system of treated mice (74). Some of the anti-inflammatory drugs that are currently being studied for the treatment of EAE act through inhibition of HMGB1, decreasing inflammatory infiltrate, production of cytokines, neuronal damage, activation of cells in the central nervous system and overall diminishing the severity of the disease (75, 76). In experimental autoimmune myocarditis, silencing of

HMGB1 in macrophages prevents their polarization to the M1 phenotype following activation with LPS and prevents activation of the NF-κB pathway (77). Furthermore, i*n vivo* silencing of HMGB1 prevented M1 macrophage infiltration and protected the cardiac tissue of treated mice. Fingolimod (Gilenya), the first FDA-approved oral disease-modifying drug for the treatment of MS, reduces serum levels of HMGB1 in patients which may be a suggested marker for clinical relapse (78). HMGB1 can be found in the nucleus of astrocytes and macrophages during the progression of EAE but in neurons, HMGB1 is found mainly in the cytoplasm during the onset phase, indicating that different cell types and subcellular localization of HMGB1 can contribute to pathology in this setting (79).

### Psoriasis Vulgaris

Psoriasis vulgaris (PV), a dermatological disease initially categorized as a hyperkeratotic disorder, has more recently been redefined as to include an immune-mediated chronic inflammatory aspect to its pathophysiology, involving systemic activation of T cells and production of inflammatory cytokines, including HMGB1 (80–82). A role for the reprogramming of Tregs into IL-17 producing cells in psoriatic lesions has also been reported (83). Serum concentrations of HMGB1 in PV patients are higher than healthy controls and have been found to correlate with disease severity according to the Psoriasis Area Severity Index (80, 81). Furthermore, patients undergoing treatment with TNF-α blockade, fumaric acid and methotrexate, but not IL-12/IL-23 inhibitors, presented with a reduction in serum levels of HMGB1 (80). Skin lesions from PV patients show increased positivity for extranuclear HMGB1 (81, 84) and in patients with severe PV, healthy skin biopsies also show such an increase (84). It has also been reported that circulating $CD8^+$ T cells as well as Tregs from PV patients have increased expression of HMGB1's receptor RAGE, further indicating the involvement of T cells in the onset and progression of disease and suggesting possible therapeutic targets. However, the defined role for HMGB1 in PV has not been fully elucidated. There is evidence that autophagy limits keratinocyte inflammatory responses and since HMGB1 is a known inducer of autophagy, one can speculate that the higher levels of HMGB1 in the serum of patients with severe disease could be a regulatory mechanism rather than a driver of inflammation (80, 85).

### Atopic Dermatitis

Atopic dermatitis (AD) is a chronic inflammatory skin disease characterized by high levels of serum IgE and pruritic skin lesions that are infiltrated by mast cells, eosinophils, macrophages, DCs and T cells, particularly those of the Th2 profile, with cytokines like IL-4, IL-13, and IL-31 playing important roles in its pathophysiology (86). In a murine model of 2,4-dinitrochlorobenzene (DNCB)-induced AD, HMGB1 and RAGE were found in high concentrations within the lesions of DNCB treated mice, along with higher levels of TNF-α, IL-6, and phosphorylated NF-κB, all of which were reduced after treatment with glycyrrhizin, a compound that targets HMGB1 (87). Interestingly, while in healthy controls HMGB1 was confined to the nucleus, in AD lesions it was found in the cytoplasm. Moreover, the authors show that HMGB1 can activate and recruit mast cells *in vivo,* thus contributing to the pathogenesis of AD, an effect which was also abrogated by glycyrrhizin. In human samples, the highest extracellular HMGB1 concentrations were found in skin lesions from AD patients, followed by PV patients and then healthy controls (88). The same pattern is observed when comparing HMGB1 expression on immune cells that infiltrate the lesions or reside in normal skin. Additionally, epithelial cells from AD samples present with more nuclear p65 than PV samples and healthy controls, suggesting an HMGB1-NF-κB axis that may be at play in AD. This axis was also explored *in vivo*, in a study where treatment with the flavonoid quercetin inhibited HMGB1 translocation from the nucleus to the cytoplasm in lesions and decreased levels of RAGE, TLR-4, and nuclear NF-κB proteins in tissue homogenates (89). In an organotypic human epidermis model, treatment with HMGB1 or IL-4 downregulates the expression of several proteins related to skin barrier function and increases production of IL-33, an inflammatory cytokine known to be upregulated in skin lesions of AD patients (90). Furthermore, HMGB1 stimulation of keratinocytes also impairs epidermal growth and maturation *in vitro*. These results suggest that HMGB1 could also act on AD through disrupting homeostasis of keratinocytes and impairing the skin's barrier function. In addition to loss of skin barrier function, AD can be accompanied, amongst other clinical features, by high levels of circulating IL-17 and IL-23. Recently, it has been reported that HMGB1 serum levels positively correlate to disease severity, serum IgE, IL-17, and IL-23 concentrations and inversely correlate to circulating IL-10 levels (91).

### Allergic Rhinitis

Allergic rhinitis is a very common disorder caused by IgE-mediated nasal inflammation which is further propagated by the cytokines produced by the immune infiltrate (92). HMGB1 levels in the nasal lavage of children with untreated rhinitis is significantly higher that healthy controls and correlates with severity of disease (92, 93). Similar results were reported with patients with chronic rhinosinusitis (94). As with AD, glycyrrhizin also seems to attenuate the symptoms of allergic rhinitis by targeting HMGB1 and reducing its concentration in the nasal fluid of treated patients (93). The authors also show that glycyrrhizin selectively kills eosinophils isolated from peripheral blood of healthy donors *in vitro*, which could also account for its effects *in vivo*. In an OVA-induced model of AR, treatment with SIRT1 had both systemic and local anti-inflammatory effects (95). Interestingly, the drug significantly decreased the levels of HMGB1 found in the nasal mucosa *in vivo* and reduced signaling through the HMGB1-NF-κB pathway *in vitro*.

## Microvesicles and Autoimmunity

In autoimmunity, platelet EVs seem to influence the course of disease, being present in high levels in patients with RA (96–98), SLE (98, 99), Grave's disease (100) and systemic sclerosis (101). In RA, EVs can act through several different pathways, presenting antigens to the immune system, degrading extracellular matrix, carrying miRNA and autoantigens, such as citrullinated proteins,

resulting in induction of inflammation, as well as perpetuating it by formation of bioactive immunocomplexes (ICs) (23, 98).

RA patients who are seropositive for $CCP^{+}/RF^{+/-}$ (anti-cyclic citrullinated peptides and rheumatoid factor, respectively) present systemic high levels of inflammatory cytokines and $CD14^{+/-}/CD16^{+}$ monocytes (called intermediate profile) in comparison to their seronegative counterparts (96). Interestingly, these patients have high levels of $HMGB1^{+}$ EVs in both the blood and synovial fluid. Systemic EVs associate with IgG and IgM to form ICs (EV-ICs), which are internalized by patient-derived mononuclear phagocytes *in vitro* and induce IL-1β, IL-6, and TNF-α production. $HMGB1^{+}$ EVs may thus have a role in the maintenance of systemic inflammation seen in seropositive patients, through their action on monocytes and/or DCs. Moreover, HMGB1 alone or in EVs can also associate with autoantigens (102), which in the extracellular microenvironment can act as a potent inflammatory cytokine, inducing production of other pro-inflammatory factors by monocytes (103). Not only monocytes, but also neutrophils can be influenced by EV-ICs in RA patients, since they induce leukotriene production by these cells *in vitro* (97).

Similar to what happens in RA, microparticles from SLE patients form ICs (IgG and IgM), and MPs associated with IgG are correlated with active disease (104). ICs directly influence the course of SLE, as they lead to deposition in tissues such as kidneys or skin and activate cells of the immune system to induce lesions (105). Contributing to the progression of the disease, phosphatidylserine negative MPs have significant expression of HMGB1, tissue factor (TF) and vascular cell adhesion protein 1 (VCAM-1) (99), proteins that are involved with inflammation, thrombotic events and cardiac disorder, phenomena that are characteristic of SLE. Moreover, these MPs show a relationship with the decrease of cystatin C, reducing renal function, as well as increasing expression of TNF receptor, which is associated with active nephritis (106).

Systemic sclerosis associated with pulmonary arterial hypertension (101) is another multisystemic disorder with high levels of HMGB1 in MPs, reinforcing the notion that vascular damage and inflammation are prominent in these patients.

The high levels of anti-TSH receptor in Grave's disease indicate intense immune system activation (107) with inflammatory response. This phenomenon seems to reflect in serum MPs of the patients, presenting high expression of pro-inflammatory molecules such as HMGB1, P-selectin, and CD40L (100). Furthermore, the authors demonstrate the presence of monocyte-derived MPs with double positivity to HMGB1 and SYTO 13 (which stains RNA/DNA), indicating they originate from apoptotic cells. After antithyroid treatment, patients present less $HMGB1^{+}/SYTO\ 13^{+}$ MPs, but not equal to the levels of healthy donors.

Platelet and endothelial MPs from relapsing-remitting MS patients increase endothelial barrier permeability (108), besides attracting leucocytes, extending the inflammation of the central nervous system and exciting endothelial cells to produce TNF-α (109). Since EVs reflect their cell of origin, which are directly influenced by the microenvironment, it is possible to speculate that HMGB1 is present in these vesicles, even though, this has not been thoroughly investigated. $HMGB1^{+}$ MPs are eligible candidates for participation in the pathogenesis of inflammatory bowel diseases, since EVs isolated from colonic luminal fluid induce a pro-inflammatory profile on macrophages and epithelial cells *in vitro* (110).

# HMGB1 AND CANCER

## Cancer Is the End Stage of Chronic Inflammation

Pathologists distinguish between chronic (>30 days) and acute (<30 days) inflammation based on the presence and prevalence of lymphocytes (chronic) or neutrophils (acute). DAMPs and PAMPs promote the initiation of an immune response driven by tissue injury or pathogens respectively, which recruits innate inflammatory cells. Adaptive immune cells start to replace innate cells within 3–10 days. HMGB1 plays an important and decisive role in regulating the phenotype, maturity, and behavior of DCs. Firstly, HMGB1 is a chemoattractant for immature DCs (111). HMGB1, specifically the B box motif, stimulates DCs to mature via TLR4 signaling (111–113). This has been shown by the presence of co-stimulatory surface molecules CD80, CD86, and HLA-A, -B, -C, as well as various other marker molecules that demonstrate maturation of DCs (111). As DCs mature, they also secrete inflammatory cytokines, such as IL-6,−8,−12,−1α, TNF, and RANTES (112). Furthermore, signaling via the p38-MAPK pathway, HMGB1 drives DC to express CD83 and secrete IL-6 (112). HMGB1 can also promote secretion of IL-23, another IL-6/IL-12 family member, in bone marrow-derived DCs, which then in turn promotes Th17 cell differentiation (114). In effect, HMGB1 promotes an inflammatory microenvironment through its signaling and stimulation of DCs.

In the context of cancer, HMGB1-mediated inflammation is certainly significant. In the presence of HMGB1, DC receptors CXCR3 and CCR5 are upregulated in the tumor microenvironment of lung cancer tissue, enhancing migration of DCs (5). HMGB1 originating from tumor cells promote DC recruitment into the tumor microenvironment. HMGB1 and IFN-γ from $CD8^{+}$ T cells are positively correlated, forming a positive network: IFN-γ promotes HMGB1 secretion in tumors, which in turn promotes $CD8^{+}$ T cells to secrete more IFN- γ (115). Additionally, IFN- γ stimulated tumor cells to produce CCL5, CXCL10, and CXCL11, which further supports the notion that DCs will be attracted toward tumor (115). HMGB1 attraction of DCs to an inflamed site, may also promote tolerization without additional PAMPs or other TLR signals.

With chronic release of DAMPs or PAMPs, professional antigen presenting cells (both recruited inflammatory DCs as well as tissue resident CD103+/CD141+ DCs) promote integration and maturation of the inflammatory response, promoting cells of the so-called "adaptome." The adaptive immune system is the "best doctor" in wartime for both diagnosing and treating diseases, integrating five elemental, highly networked lymphoid cells that both support and counter-regulate each other: NK cells, NKT cells, αβ T-cells, γδ T-cells, and B cells expressing an IgH and κ or λ light chains. It carries out

these tasks with unmatched precision with the help of rearranged T and B cell receptors, our most diverse set of expressed and rearranged genes, fundamentally distinguishing one individual from another. This autologous potential receptor diversity, ranging from $10^{15}$ to $10^{25}$ for each chain of the rearranged receptors, contains only two chains expressed in each cell. The immune repertoire is the sum of the individual clonotypes within one chain, including individual CDR3 sequences.

The primary role of HMGB1 within the cell is to regulate transcription in the nucleus. However, as mentioned previously, when a cell undergoes necrosis or necroptosis, HMGB1 is released, acting as a DAMP and promoting inflammatory pathways. While HMGB1 plays a role in sustaining chronic inflammation and promoting wound healing, it can also trigger pathways which promote tumor growth including angiogenesis, reparative epithelial proliferation, efferocytosis by recruited macrophages and inhibition of immune effectors mediated by myeloid derived suppressor cells (MDSCs) and T regulatory cells (Tregs) (116). This has led to the notion that chronic inflammation, a wound healing phenotype, and cancer progression are closely related.

Indeed, HMGB1 contributes to immune escape and tumor progression *in vivo* by stimulating the proliferation of MDSCs as well as increasing their T cell-inhibitory properties (117, 118). Lewis lung carcinoma cells treated with resveratrol express less HMGB1 and induce less MDSC mobility when co-cultured, an effect that was partially reversed by treatment with exogenous recombinant HMGB1 (119). The supernatant from MCF-7 breast tumor cells is rich in HMGB1 and skews bone marrow progenitors into MDSCs, a phenomenon which is abrogated by treatment with ethyl pyruvate or anti-HMGB1 antibody (120). Serum levels of HMGB1 and the frequency of MDSCs are correlated and increased in breast cancer patients, MDSCs cultured under starvation conditions and in the presence of ethyl pyruvate do not upregulate autophagic markers and have their viability drastically reduced (121). HMGB1-induced autophagy not only promotes tumor growth by directly enhancing tumor cell survival, but also by boosting the immunosuppressive nature of the TME by perpetuating regulatory cells. HMGB1 can also act on MDSCs to facilitate metastatic dissemination. HMGB1-mediated recruitment of MDSCs to the peritoneal cavity of mice can increase their metastatic burden post-resection surgical extirpation (122). MDSCs are also associated with immunosuppression after trauma and sepsis. Accumulation of MDSCs in the spleen of mice following trauma is dependent on tissue-derived HMGB1 (123).

Tregs are another major population at play in chronic inflammatory environments as well as within the TME. In autoimmunity, there are many reports demonstrating that neutralizing antibodies or drugs that directly target HMGB1 can reduce inflammation and stimulate the development of Tregs, ameliorating symptoms of diseases like type 1 diabetes (124), autoimmune thyroiditis (125), and graft-vs.-host disease (126). In the chronic inflammatory setting, HMGB1 levels in the serum of atherosclerotic patients is increased, which negatively correlates with the Treg/Th17 ratio, promoting progression of the disease (127). In chronic HBV infection, HMGB1-mediated autophagy is important for Treg survival and functionality (128).

In cancer, HMGB1 can be associated with differentiation of Tregs, recruitment to the TME and enhancement of suppressive features. Tumor cell-derived HMGB1 increases the absolute numbers of Tregs in the spleen and draining lymph nodes of tumor-bearing mice, while also stimulating Tregs to produce IL-10 and suppress T cell activation (129). Co-culture of PBMCs with the neuroblastoma cell line SK-N-SH and its supernatant induces differentiation of $FoxP3^+$ $CD4^+$ T cells. The suppressive function of these cells was not tested. This effect is abrogated by HMGB1 neutralization (130). An HMGB1-TSLP (thymic stromal lymphopoietin) axis has been demonstrated, where tumor-derived HMGB1 and TSLP enhance DC-mediated activation of Tregs, a phenomenon that was dependent on the presence of the TSLP receptor on DCs (131). Furthermore, intratumoral inhibition of HMGB1 boosted T-cell dependent antitumor immune response *in vivo*. In head and neck cancer patients, HMGB1 acts as a chemokine for Tregs and enhances their suppressive capacity, with both tumor-infiltrating and circulating Tregs expressing the HMGB1 receptors TLR4 and RAGE (132). In breast cancer patients, the presence of intranuclear HMGB1 in tumor cells (that is, non-secreted HMGB1) is a favorable prognostic factor and negatively correlates with infiltration of Tregs or tumor-associated macrophages (133).

Chronic inflammation in the setting of cancer arises with cell death and release of DAMPs from necroptotic and necrotic cells. These cells break down and end up releasing HMGB1 into the extracellular space along with other DAMPs, including ATP, histone H1, S100 molecules, heat shock proteins, DNA, and RNA. HMGB1, often coupled with free-nucleotide-containing molecules, activates PRRs, which then activate inflammatory and wound healing pathways. These downstream effects provide the scenario that eventuates in the cycles of cell death, DAMP release, and reparative proliferation, conditions that ultimately lead to chronic inflammation (116).

Individual PRRs activated by HMGB1, such as TLRs, also contribute to tumor progression. TLRs play significant roles in metastasis promotion, immune evasion, and nascent and perpetual neoangiogenesis (116). This promotes tumor replication, emergent genomic instability, and Darwinian selection in promoting its ability to develop and grow.

Once a tumor has been established, it can continuously produce DAMPs and release them into the surrounding tumor microenvironment (116). Although somewhat higher levels of HMGB1 are expressed in some tumors, its localization to the cytosol and extracellular release associated with emergent autophagy more often dictates the outcome (in addition to receptor expression including RAGE and the TLRs) (134). The emergent tumor now contributes to releasing DAMPs into the local area, thereby promoting both cancer development and inflammation. These two pathways play off each other, causing more and more HMGB1 to be released and leading to a positive feedback of both pathways. Therefore, chronic inflammation directly initiates cancer. This apparent positive feedback stems

from DAMP release and emergent secondary genomic instability, showing that chronic inflammation and cancer are intertwined.

## Microvesicles and Cancer

Inflammation is a core hallmark of cancer (135), which may be related with initiation, promotion, invasion and metastasis (136, 137). In this context, EVs, being mediators of cellular communication, have pivotal roles in facilitating progression, metastatic niche formation and metastases, driving tumor-promoting inflammation (136–138). Tumor EVs, such as Exo, may contain pro-inflammatory molecules, such as HSP, PGE-2, and HMGB1 (25, 139). The paracrine or autocrine action of HMGB1 can participate on chemoresistance to drugs such as oxaliplatin and 5-fluorouracil (5-FU), by the induction of autophagy (140) and repair of DNA adducts. Interestingly, culture supernatant of chemoresistant cells are rich in HMGB1. Although not the focus of that study, it is possible to speculate that at least part of the measured HMGB1 could be found within EVs in the medium.

Tumor-derived exosomes containing HMGB1 can modulate the microenvironment through subversion of the immune response. Patients with gastric cancer have high plasma and tumor tissue HMGB1 expression and this correlates with poor prognosis. Furthermore, Exo from culture supernatant and tumor tissue induce a pro-tumor profile in neutrophils, which is dependent on expression of exosomal HMGB1 (25). This recognition occurs via signaling through TLR4, activating NF-kB and STAT3, which confers on neutrophils resistance to apoptosis, increased formation of autophagosomes and production of pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α) and molecules involved with migration and invasion, such as oncostatin M (OMS), MMP9, and VEGF. These phenotypic changes induce increased gastric tumor cell migration *in vitro*.

HMGB1 is highly expressed in the tumor tissue, often with enhanced cytosolic translocation, in almost all cancers including breast, colorectal, pancreatic, and hepatocellular carcinoma (134, 141, 142). Exosomal HMGB1 of human liver cancer cell lines induces B regulatory (Breg) differentiation, proliferation, and activation in healthy donors (143). This differentiation occurs through engagement of TLR2/TLR4 in B lymphocytes, which express TIM (T cell Ig and mucin domain)-1 membrane glycoprotein. Additionally, TIM-$1^+$ Breg cells in contact with $CD8^+$ T lymphocytes induce suppression, decrease proliferation and TNF-α and IFN-α expression. TIM-$1^+$ Breg are excellent producers of IL-10, presenting a correlation with increased recurrence, decrease of overall survival and disease-free survival of hepatocellular carcinoma patients (143), which together are associated with a poor prognosis.On the other hand, treatment of cervical cancer cell lines with 5-Aminolevulinic acid photodynamic therapy (ALA-PDT) can increase HMGB1 content in tumor-derived Exo, which was found to be beneficial to the activation of APCs, inducing secretion of IL-6, IL-12, IL-18, IFN-γ, and TNF-α *in vitro* (144).

Thus, extracellular vesicles are efficient messengers, systemically or locally, which reflectthe phenotype of the cell of origin, being able to also act as biomarkers. They are loaded with nucleic acids, lipids and protein, including HMGB1 and other DAMPs, inducing changes that contribute with progression of autoimmune diseases and cancer, by increasing the inflammatory response. However, there is also evidence pointing to the fact that Exo can carry immune-activating molecules and that HMGB1 could be one of them.

## HMGB1 Expression in Human Cancers and Its Prognostic Value

Both systemic and local HMGB1 evaluation demonstrate that its expression frequently accompanies advanced disease stages and poorer prognosis in most epithelial tumors including lung, colorectal (CRC) and pancreas (**Table 1**). Chemotherapy can induce higher levels of circulating HMGB1-containing nucleosomes in lung cancer patients, a factor predictive of low differentiation of tumor cells and a more invasive phenotype (176). In esophageal cancer patients, neoadjuvant radiation and chemotherapy correlate with increased serum levels of HMGB1 and pulmonary complications (169). Similarly, in patients undergoing heated intraperitoneal chemotherapy, higher rates of HMGB1 released into the extracellular space positively correlate with complication rates (177). Following radioembolization therapy in CRC patients, high serum levels of HMGB1 are a predictive factor for failure to respond to treatment while both pre and post therapeutic high HMGB1 correlate with poor overall survival (167). There is also evidence linking HMGB1 expression in tumors to recruitment of immune infiltrate [although the opposite phenomenon has also been observed (158, 159)] associated with better activation of anti-tumor immune responses (149, 150, 170). The absence of correlation between HMGB1 in the tumor microenvironment and immune infiltrate has also been reported in both breast and esophageal cancers (178). In a recent study that evaluated The Cancer Genome Atlas (TCGA) of the pancreatic adenocarcinoma dataset, HMGB2, but not HMGB1, was found to have predictive prognostic value, with high expression associated with worse outcome (179). In a pancreatic ductal adenocarcinoma (PDAC) cohort, high serum HMGB1 levels proved to have diagnostic potential, besides positively correlating with stage of disease, presence of metastasis, tumor size and worse prognosis (174). Subcellular localization of HMGB1 can be of importance regarding disease outcome. In stage III CRC patients, localization of HMGB1 to the nucleus correlates with better recruitment of $CD45^+$ cells and survival rate, whereas co-localization to both nucleus and cytoplasm has the opposite effect (158). In PDAC patients, the presence of nuclear $HMBG1^+/HMGB2^-$ tumor cells correlate with significantly shorter postoperative survival (172). However, low nuclear HMGB1 expression can also be associated with shorter median survival time in pancreatic (173) and with poor prognosis in breast cancer (133).

Although information available in the current literature can vary regarding the role, localization and clinical relevance of HMGB1, it is of extreme interest to explore its prognostic and biomarker potential, since it appears to be altered in so many human cancers (142). Also, some evidence present HMGB1 as a candidate predictive of therapy responsiveness in a plethora of treatment settings. However, it is yet to be

**TABLE 1 |** HMGB1 expression and subcellular localization in epithelial human cancers.

| Tumor type | Site | Prognosis | Stage | Subcellular localization | Observations | References |
|---|---|---|---|---|---|---|
| NSCLC | Serum | – | Advanced | n/a | Serum HMGB-1 negatively correlated with response to chemotherapy and survival (145) | (145, 146) |
| NSCLC | Tissue | – | Advanced | n/a (147); n/s (148) | Significant association observed between the gene expression levels of HMGB1 and MMP-9 (147); high expression of HMGB1 was closely related to the poor prognosis of patients with lung cancer (148) | (147, 148) |
| NSCLC | Tissue | + | Advanced (149); I-IV (150) | Mainly cytoplasm (149); | Low HMGB1 associated with poor immune activation | (149, 150) |
| BREAST | Tissue | + (151) | I-IV (151); I-III (152) (153) (133); early (154) | Nucleus (155) (133); mainly cytoplasm (151); both (153) | Less differentiated carcinoma presented more diffused localization of HMGB1 in the nucleus (155); Cytoplasmic HMGB1 associated with small tumor size and early stages (151); no prognostic significance (152); cytoplasmic HMGB1 associated with TIL, but no prognostic significance (153) (156); post-chemotherapy increase in circulating HMGB1 correlated with better survival (154) | (133, 151–156) |
| BREAST | Serum | – | Advanced | n/a | High pre-chemotherapy HMGB1 levels predicted a later therapy response | (157) |
| CRC | Tissue | – | Advanced | Nucleus (155, 158) | Less differentiated carcinoma presented more diffused localization of HMGB1 in the nucleus (155); HMGB1 in the cytoplasm reduces infiltration of $CD45^+$ cells (158); High HMGB1 in tumor tissue correlated with metastasis and lower DCs infiltration (159) | (155, 158–165) |
| CRC | Serum | – | Advanced and Early (166) | n/a | No correlation between serum HMGB1 and patient survival (166) | (166–168) |
| ESOPHAGEAL | Serum | – | I-IV | n/a | HMGB1 increment after neoadjuvant chemotherapy; Preoperative serum HMGB-1 may be associated to response to preoperative treatment | (169) |
| ESOPHAGEAL | Serum | + | I-IV | n/a | HMGB1 linked to immunogenic cell death | (170) |
| ESOPHAGEAL | Tissue | – | I-III | Both | HMGB1 expression positively correlated with expression of VEGF-C, lymph node metastasis, MLD and stage | (171) |
| PANCREATIC | Tissue | – | Advanced and Early | Mainly nucleus | The combination of $HMGB1^+/HMGB2^-$ expression linked to poor prognosis | (172) |
| PANCREATIC | Tissue | + | Advanced and Early | Mainly nucleus | Diminished nuclear and total cellular expression of HMGB1 in PDAC correlates with poor overall survival | (173) |
| PANCREATIC | Serum | – | Advanced and Early | n/a | HMGB1 as a potential diagnostic biomarker for PDAC (174); Circulating nucleosomes and HMGB1 as prognostic factors (175) | (174, 175) |

*NSCLC, non-small cell lung carcinoma; n/a, not applicable; n/s, not specified; TIL, tumor infiltrating lymphocytes; DCs, dendritic cells; MMP-9, matrix metalloproteinase; VEGF-C, vascular endothelial growth factor C; MLD, micro-lymphatic vessel density.*

**TABLE 2 |** HMGB1-mediated autophagy is controlled by microRNAs.

| miR | Predicted targets (top 5) | Disease association | Observations | References |
|---|---|---|---|---|
| miR-410-3p (http://www.targetscan.org/cgi-bin/targetscan/vert_71/targetscan.cgi?mirg=hsa-miR-410-3p) | NPPC<br>DCTN6<br>CBFB<br>TRAPPC3<br>ARFIP1 | PDAC | Chemosensitization via inhibition of HMGB1-mediated autophagy | (180) |
| miR-34-a (http://www.targetscan.org/cgi-bin/targetscan/vert_71/targetscan.cgi?mirg=hsa-miR-34a-5p) | MDM4<br>HCN3<br>FAM76A<br>SCN2B<br>SYT1 | AML, retinoblastoma | Chemosensitization via inhibition of HMGB1-mediated autophagy | (181, 182) |
| miR-142-3p (http://www.targetscan.org/cgi-bin/targetscan/vert_71/targetscan.cgi?mirg=hsa-miR-142-3p.1) | BOD1<br>WASL<br>RHOBTB3<br>FAM114A1<br>MANBAL | AML | Chemosensitization via inhibition of HMGB1-mediated autophagy | (183) |
| miR-142-3p (http://www.targetscan.org/cgi-bin/targetscan/vert_71/targetscan.cgi?mirg=hsa-miR-142-3p.1) | BOD1<br>WASL<br>RHOBTB3<br>FAM114A1<br>MANBAL | NSCLC | Chemosensitization via inhibition of HMGB1-mediated autophagy | (184) |
| miR-129-5p (http://www.targetscan.org/cgi-bin/targetscan/vert_71/targetscan.cgi?mirg=hsa-miR-129-5p) | CACNG2<br>IGIP<br>CAMK2N1<br>YIPF5<br>LRRC4C | Breast | Chemosensitization via inhibition of HMGB1-mediated autophagy | (185) |
| miR-218 (http://www.targetscan.org/cgi-bin/targetscan/vert_71/targetscan.cgi?mirg=hsa-miR-218-5p) | TUB<br>VOPP1<br>SGCZ<br>TPD52<br>C3orf70 | Endometrial | Chemosensitization via inhibition of HMGB1-mediated autophagy | (186) |
| miR-22 (http://www.targetscan.org/cgi-bin/targetscan/vert_71/targetscan.cgi?mirg=hsa-miR-22-5p) | PCDH15<br>DST<br>ATP6V1G3<br>MINPP1<br>MAS1 | Osteosarcoma | Chemosensitization via inhibition of HMGB1-mediated autophagy; Inhibition of proliferation, migration and invasion of tumor cells (187) | (187, 188) |
| miR-200c (http://www.targetscan.org/cgi-bin/targetscan/vert_71/targetscan.cgi?mirg=hsa-miR-200c-5p) | GNG13<br>SYT4<br>PGPEP1L<br>LSMEM1<br>CPXCR1 | NSCLC | Chemosensitization via inhibition of HMGB1-mediated autophagy | (189) |
| miR-129-2 (http://mirdb.org/cgi-bin/search.cgi?searchType=miRNA&full=mirbase&searchBox=MIMAT0004605) | BDKRB2<br>TMEM136<br>CCP110<br>SCN3B<br>SEC14L1 | Glioma | Chemosensitization via inhibition of HMGB1-mediated autophagy | (190) |

*(Continued)*

**TABLE 2** | Continued

| miR | Predicted targets (top 5) | Disease association | Observations | References |
|---|---|---|---|---|
| miR-505 (http://www.targetscan.org/cgi-bin/targetscan/vert_71/targetscan.cgi?mirg=hsa-miR-505-5p) | CLEC2A<br>SMIM6<br>PITPNM3<br>CFC1B<br>AKT1S1 | HCC | Inhibition of HMGB1-mediated DNA repair and inactivation of Akt pathway | (191) |
| miR-141 (http://www.targetscan.org/cgi-bin/targetscan/vert_71/targetscan.cgi?mirg=hsa-miR-141-5p) | DST<br>CDR1<br>C1orf54<br>HMGB1<br>RSPH4A | Acute pancreatitis | Inhibition of HMGB1-mediated autophagy may decrease tissue injury *in vivo* | (192) |

*PDAC, pancreatic ductal adenocarcinoma; AML, acute myeloid leukemia; NSCLC, non-small cell lung carcinoma; HCC, hepatocellular carcinoma; Human predicted targets retrieved from the TargetScan and miRDB databases.*

determined where best to look–within the circulation or in tumor tissues–and if subcellular localization can offer any clinically relevant information.

## HMGB1 and miRNAs–an Unusual Liaison

One of the most extensively studied features of HMBG1 involves its ability to induce autophagy in situations of stress, including therapy, leading to drug resistance. Interestingly, miRNAs, short sequences that act as non-coding, post-translational regulators of multiple target genes, also seem to be regulators of this biologic phenomenon through direct modulation of HMGB1 expression. The role of individual miRNAs in controlling autophagy mediated by HMGB1 and their predicted targets in humans, according to the TargetScan or miRDB databases, are shown in **Table 2**.

In PDAC pre-clinical models, expression of HMGB1 and miR-410-3p inversely correlate with responsiveness to gemcitabine and in patients, expression of this miRNA is associated with good prognosis. From an array of 30 genes, HMGB1 was the only gene under the control of miR-410-3P which was overexpressed in gemcitabine-resistant human PDAC cells. Importantly, it was demonstrated that overexpression of miR-410-3P inhibits formation of HMGB1-mediated LC3 puncta in gemcitabine-treated PDAC cells (180). Similar results were found in doxorubicin and cisplatin-treated osteosarcoma cells *in vitro*, where miR-22 regulates HMGB1-induced post-chemotherapy autophagy (188) and in paclitaxel-treated endometrial cancer cells where miR-218 plays this role (186). miR-34a has previously been identified as a tumor suppressor, downregulated in various cancers (193–195). In acute myeloid leukemia cells, miR-34a overexpression stimulates apoptosis through regulation of Bax and Bcl-2, in addition to inhibiting autophagy following treatment with all-trans retinoic acid (181). This autophagy limiting, apoptosis inducing property of miR-34a has also been demonstrated in retinoblastoma, where transfection with a miR-34a mimic enhanced *in vitro* sensitivity to vincristine, etoposide and carboplatin, in addition to increasing markers of DNA damage, ROS production and loss of mitochondrial membrane potential (182). In breast cancer, miR-129-5p direct regulation of HMGB1 and consequently of autophagy contributes to ameliorate radiosensitivity *in vitro* (185). Additionally, even though the effects on chemosensitivity were not evaluated, miR107 was found to be downregulated in human breast tumors and cell lines (196). This miR targets HMGB1 directly and regulates its autophagy inducing properties *in vitro*, while overexpression reduces tumorigenesis *in vivo*. miR-142-3p can both down-regulate expression of HMGB1 and increase signaling through the PI3K/Akt/mTOR pathway, decreasing post-chemotherapy autophagy in lung tumor cells *in vitro* (184). Additionally, miR-142-3p-overexpressing lung tumors have lower expression of HMGB1 and increased sensitivity to doxorubicin and cisplatin *in vivo*. In AML, miR-142-3p is also implicated in the direct regulation of HMGB1, inhibiting autophagy and enhancing drug sensitivity (183). Lower expression of miR-142-3p and higher expression of HMGB1 in PBMCs from pediatric AML patients is found.

In hepatocellular carcinoma (HCC) cell lines, miR-505 negatively regulates HMGB1, increasing doxorubicin cytotoxicity *in vitro* via enhanced caspase 3 activity, induction of DNA damage and decreased phosphorylation of Akt, a pathway known to be closely involved in drug resistance (191).

Some chemotherapeutic agents such as doxorubicin, mitoxantrone, oxaliplatin and bortezomib can elicit so-called "immunogenic cell death" (ICD). Markers of ICD include calreticulin CRT exposure on the cell membrane, secretion of ATP and HMGB1, which are often accompanied by increases in autophagic flux. In the context of chemoresistance, HMGB1 is detrimental because it prevents cells from dying (inhibiting apoptosis and promoting autophagy) whereas in ICD, HMGB1-induced autophagy is considered an immunogenic signal that will ultimately lead to tumor elimination. In this context, miR-27a controls CRT translocation and secretion of ATP and HMGB1 after treatment of colorectal cancer cells with ICD inducers (197). miR-27a low-expressing tumor cells released more HMGB1 into the extracellular space, displaying a more autophagic phenotype able to induce DCs phenotypical and functional maturation *in vitro*.

## A TEASER: HGF AND HMGB1 AS RECIPROCALLY REGULATED HORMONAL MEDIATORS IN CELL DEATH

Although we usually consider apoptotic cell death as a "quiet death," reparative proliferation of epithelia requires some form of communication to persisting cells that replication is in order. Interestingly, hepatocyte growth factor is released from apoptotic cells (198–203) and HMGB1, as detailed above, from necrotic and necroptotic cells (204). HGF release is associated with signaling through the Met receptor to upregulate CXCR4 expression (205–207). Interestingly, CXCR4 is an important receptor, as noted above, for SDF1/CXCL12/HMGB1 heterodimers that promote recruitment of inflammatory cells (67, 208–213). HMGB1 is released following tissue injury, forming a heteroduplex with CXCL12. Signaling through CXCR4 promotes response to injury, tissue regeneration and increase in cell cycling by promoting quiescent stem cell transition from G0 to GAlert. Most of the "reparative" program of tissues involved with wound healing and normal epithelial barrier function involves this pathway. In the setting of cancer, neoplastic epithelia exhibit an exaggerated program of tissue repair associated with premature and unscheduled cell death leading to a folie a' deux of DAMP release, reparative proliferation, and inhibition of immunity, something we refer to as the DAMP hypothesis to explain cancer and perhaps other chronic inflammatory states.

## CONCLUSIONS

Even though HMGB1 was first described as a nuclear protein, today we know that it is also one of the major cytokine-like mediators of inflammation and its pathological discontents, such as autoimmunity and cancer. HMGB1 is a highly context-dependent molecule, exerting various biologic effects depending on its partnering molecule, redox state, and subcellular location. Furthermore, HMGB1-mediated autophagy participates in important cellular processes such as cell fate decisions, chronicity of inflammatory responses and chemosensitivity to cancer-ablative drugs. While HMGB1, as a DAMP, can attract immune cells to the tumor site and engage receptors such as TLRs, culminating in immune activation, its presence in human tumor tissues and circulation is frequently associated with disease severity and progression. In autoimmunity, patient's HMGB1 levels increase in the active phase of disease, worsening inflammation and symptoms. Thus, it is of extreme importance to further elucidate underlying mechanisms involving HMGB1 signaling in pathology, in order to possibly one day use it as a therapeutic target, prognostic or even diagnostic biomarker in patients suffering from autoimmune disorders and cancer.

## AUTHOR CONTRIBUTIONS

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# Proinflammatory Differentiation of Macrophages Through Microparticles That Form Immune Complexes Leads to T- and B-Cell Activation in Systemic Autoimmune Diseases

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Patients with rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) demonstrate increased circulating microparticles (MP). These vesicles, primarily those that form immune complexes (MP-IC), may activate monocytes. We evaluated the effect of MP and MP-IC in the differentiation of monocytes to macrophages (monocyte-derived macrophages; MDM) and for consequences in autologous lymphocyte activation. Monocytes from healthy controls (HC) and patients with RA and SLE that differentiated into MDM in the presence of MP-IC showed a proinflammatory (M1-like) profile, which was more evident using MP-IC from patients with RA than those from patients with SLE. Notably, MDM from HC and patients with RA that differentiated with MP-IC were more prone to M1-like profile than those from patients with SLE. In HC and patients with RA, monocyte differentiation using MP-IC decreased the frequency of MDM that bound/internalized latex beads. The M1-like profile did not completely revert following IL-4 treatment. The effect of M1-like MDM on T lymphocytes stimulated with phytohemagglutinin was further evaluated. MDM differentiated with MP enhanced the proliferation of T cells obtained from patients with RA compared with those differentiated with MP-IC or without vesicles. Neither MP nor MP-IC induced interferon (IFN)-γ+ and tumor necrosis factor (TNF)-α+ T cells in patients with RA. Conversely, unlike MDM differentiated with or without MP, MP-IC enhanced the proliferation and increased the frequencies of IFN-γ+CD4+ T, TNF-α+CD4+ T, and IFN-γ+CD8+ T cells in patients with SLE. The co-culture of B cells with MDM obtained from patients with RA and SLE and differentiated with MP-IC increased the expression of B-cell activation markers and prevented B lymphocyte death. Strikingly, only for patients with SLE, these responses seemed to be associated with a significant increase in B-cell activating factor levels, high plasmablast frequency and immunoglobulin production. These results showed that MP-IC from patients with systemic autoimmune diseases favored the polarization of

MDM into a proinflammatory profile that promotes T-cell activation, and additionally induced B-cell activation and survival. Therefore, the effect of MP-IC in mononuclear phagocytes may be an important factor for modulating adaptive responses in systemic autoimmune diseases.

**Keywords: microparticles, macrophage, M1-like activation, M2-like activation, systemic autoimmune diseases, rheumatoid arthritis, systemic lupus erythematosus**

## INTRODUCTION

Rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) are chronic systemic autoimmune diseases affecting a large number of people globally (1). The etiology of SLE and RA is not completely known; however, both diseases are characterized by the presence of antibodies against self-antigens (2, 3). These autoantibodies have been associated with a loss of central and peripheral mechanisms of tolerance, as well as with tissue damage and the maintenance of chronic inflammatory responses, through immune complex (IC) formation, recognition and tissue deposits (2, 3). Recently, extracellular vesicles such as microparticles (MP) were reported as one of the main sources of circulating IC in RA and SLE (4–6). Such cell membrane-derived vesicles are primarily formed during cell death and activation and exhibit a broad spectrum of physiological functions, such as intercellular communication, different phases of innate and adaptive immunity, apoptosis, and cellular homeostasis, in healthy individuals (7).

In RA and SLE, alterations in the count, phenotype, recognition, and function of MP and MP forming IC (MP-IC) have been reported (4, 5). MP from patients with RA and SLE reportedly contain proinflammatory components and autoantigens such as citrullinated peptides, high-mobility group protein 1 (HMGB1), and nucleic acids (4, 8). Mononuclear phagocytes, mainly monocytes and macrophages, play critical roles in depurating apoptotic cells, MP and IC (9, 10); interestingly, MP-IC are more efficiently bound and internalized by these phagocytes than MP alone (4). Therefore, MP have been postulated to activate and define the functional profile of monocytes and macrophages obtained from patients with RA and SLE via the activation of Toll-like receptors (TLR) -4, -9, and -7, which recognize oxidized HMGB1, DNA, and RNA, respectively. In addition, MP-IC seem to additionally signal mononuclear phagocytes via Fcγ and complement receptors, thus perpetuating the inflammatory process in these patients (11, 12).

Monocytes and macrophages are key components of the innate immune system and have numerous functions such as phagocytosis, antigenic presentation, and cytokine production (10, 13). In the murine model, many tissue macrophages, such as microglia in the brain, peritoneal macrophages, and Kupffer cells in the liver, originate from the yolk sac, or fetal liver progenitors (14). In adult mice, macrophage populations in the lung, peritoneal cavity, and spleen are more heterogeneous owing to the presence of bone marrow-derived macrophages during steady state and inflammation (14, 15). Under inflammatory conditions, resulting from damaged tissues following an infection or injury, monocytes are recruited from the circulation and are differentiated into monocyte-derived macrophages (MDM) while migrating to the affected tissues (10). MDM often show a proinflammatory phenotype and function, which is primarily attributed to the local inflammatory environment. MDM can secrete various inflammatory mediators, including tumor necrosis factor (TNF)-α, interleukin (IL)-1β, -12, and -6. If the inflammatory response is not quickly controlled, these phagocytes can become pathogenic and contribute to disease progression, as demonstrated in numerous chronic inflammatory and autoimmune diseases including atherosclerosis, multiple sclerosis, inflammatory bowel diseases, RA, and SLE (16–19). The number of macrophages in the synovia and kidneys of patients with RA and SLE correlates with joint damage and glomerulonephritis development, respectively (20, 21), as well as with clinical responses to therapy for both diseases (22, 23).

Reportedly, activated macrophages exhibit significant functional heterogeneity and may polarized into one of two main phenotypes: classically activated M1 (proinflammatory) and alternatively activated M2 (anti-inflammatory) (24). Macrophages stimulated by lipopolysaccharide (LPS) and interferon (IFN)-γ exhibit an M1 profile. As a result, M1 macrophages release high levels of proinflammatory cytokines and chemokines, which in turn promote the recruitment and activation of Th1 and NK cells. M1 macrophages exhibit plasticity and switch their phenotype toward an alternative profile *in situ.* Alternatively, IL-4 induces the production of M2 macrophages that counter inflammation via the phagocytosis of apoptotic neutrophils, production of anti-inflammatory cytokines and increased synthesis of mediators involved in tissue remodeling, angiogenesis and wound repair (25).

Currently, the components of the inflammatory cascade that may favor the perpetuation of the M1 phenotype in macrophages of patients with RA and SLE are still unclear. In this study, we propose that circulating MP, and especially MP-IC, of patients with SLE and RA can directly alter the differentiation of monocytes into proinflammatory macrophages, contributing to the amplification, and perpetuation of autoimmune phenomena and chronic inflammation in affected tissues. The effect of circulating MP and MP-IC obtained from patients with RA and SLE in monocytes was evaluated for their differentiation into macrophages; the functional consequences of this process were also assessed in T- and B-cell activation.

# MATERIALS AND METHODS

## Reagents, Materials, and Antibodies

RPMI-1640 GlutaMAX medium, Dulbecco's phosphate-buffered saline (DPBS) and fetal bovine serum (FBS) were purchased from Gibco–BRL (Grand Island, NY, USA). Histopaque®-1077, trypan blue, dimethyl sulfoxide anhydrous ≥99.9% (DMSO), tween-20, phytohemagglutinin leukoagglutinin (PHA) and bovine serum albumin (BSA) were obtained from Sigma Aldrich (St. Louis, MO, USA). Penicillin and streptomycin were purchased from Cambrex-BioWhittaker (Walkersville, MD, USA). The BD™ Human Inflammatory Cytometric Bead Array (CBA) was purchased from BD Pharmingen (San Diego, CA, USA). The Rosette Sep Human B cell and T cell Enrichment Cocktails were obtained from STEMCELL Technologies (British Columbia, Vancouver, Canada). Brefeldin A was procured from eBioscience (San Diego, CA, USA). The probes carboxyfluorescein succinimidyl ester (CFSE), LIVE/DEAD Fixable Aqua Dead Cell Stain and FluoSpheres latex beads OR were procured from Invitrogen (San Diego, CA, USA). Recombinant human (rh) CD40 Ligand (CD40L), rhIFN-γ, and rhIL-4 were purchased from R&D Systems; rhIL-2 from Biolegend (San Diego, CA, USA) and the affinity-purified $F(ab')_2$ fragment anti-human IgM (anti-BCR) and $F(ab')_2$ anti-IgG fragment Alexa Fluor 488 conjugated from Jackson ImmunoResearch (New Baltimore, PA, USA). Monoclonal anti-human MY4 (CD14)-FITC (Clone 322A-1) antibody was obtained from Beckman Coulter; monoclonal antibodies against human CD16-V450 (Clone 3G8), CD32-PE (Clone 3D3), CD36-APC (Clone CB38, also known as NL07), CD3-PerCP (clone SK7), CD4-PE-Cy7 (Clone RPA-T4), CD19-V450 (Clone HIB19), CD69-PE (Clone FN50), CD80-FITC (Clone MOPC-21), CD86-PE-Cy5 (Clone IT2, 2), IFN-γ-APC (clone B27), TNF-α-PE (Clone 6401.1111), CD163-PE (Clone GHI/61), CCR2-Alexa Fluor 647 (Clone 48607), and CD20-PE (Clone L27) were acquired from BD Pharmingen (San Diego, CA, USA). Anti-human HLA-DR-APC-Cy7 (Clone L243), CD209-APC (Clone 9E9A8), CD19-Brilliant Violet 650 (Clone HIB19), CD38-Brilliant Violet 785 (Clone HIT2), CD138-APC-Cy7 (Clone MI15), and CD27-PE-Cy7 (Clone O321) antibodies were purchased from Biolegend. Anti-human CD8-eFluor 450 (clone OKT8) antibody was obtained from eBioscience.

## Patients and Controls

In total, 34 patients with SLE (diagnosed according to the American College of Rheumatology criteria) (26) and 34 patients with RA (diagnosed according to the American College of Rheumatology and European League Against Rheumatism criteria) (27) who were recruited at the Rheumatology Service of the Hospital Universitario San Vicente Fundación (HUSVF) in Medellín, Colombia were included in this study. Patients with SLE had a median age of 39 (24–54) years, and 85% of these were women. Twenty patients with SLE were classified to have inactive SLE according to the SLE Disease Activity Index (SLEDAI $< 4$), and 14 patients were classified with active SLE (SLEDAI $\geq 4$) (28). The median age of patients with RA was 45 (29–62) years, and 84% of these were women. Sixteen patients with RA were identified to be in remission based on the disease activity score (DAS)−28 (DAS−28 $\leq 2.6$); and 18 patients with RA were identified to have moderate activity (DAS−28 $> 3.2$ and $\leq 5.1$). None of the patients received biological therapy. Fourteen patients with SLE and RA each, who had 80% of the disease in the inactive form, were included in our *in vitro* assays with monocyte cells. On the other hand, 10 patients with seropositive RA and 10 with active SLE were included in the MP and MP-IC groups; Additionally, fourteen healthy controls (HC), matched for sex and age, were included. This study was conducted in accordance with the Declaration of Helsinki; the research protocol and informed consent forms were approved by the Universidad de Antioquia's Medical Research Institute and HUSVF Ethic Committees. All patients and HC provided consent for participation in the study.

## MP Isolation and MP-IC Formation

Circulating MP and MP-IC from patients with SLE (LMP and LMP-IC, respectively) and MP and MP-IC from patients with RA (RMP, and RMP-IC, respectively) from poor-platelet plasma were obtained as previously described (4) and were frozen at −70°C until use. Every batch of MP and MP-IC were generated by mixing respective vesicles from 3 to 4 patients. These patients belong to previously published cohorts, in which a detailed characterization of MP was performed. Because the formation of IC by MP was one of the main characteristic associated with the clinical involvement of both SLE (active disease by SLEDAI) (4) and RA (systemic inflammation by inflammatory cytokines) (29) patients in our previous studies, this was the variable specifically evaluated in the present work for MP. The phenotypic characteristic of the MP and MP-IC *ex vivo* before their storage and *in vitro* opsonization are shown in **Supplementary Table 1** and **Supplementary Figure 1A** MP-IC pools were those that formed ≥28.45% of IC for RA patients and ≥38.85% for SLE; MP pools were those that formed ≤6% of IC (**Supplementary Figure 1B**). The MP-IC thresholds were established according to the distribution of the circulating MP-IC frequency in a population of patients with SLE (4) and RA (29); the MP thresholds were established according to the distribution of the circulating MP-IC frequency in a population of HC (4), which was previously studied by us. To MP-IC formation the total IgG was previously obtained from pooled serum samples taken from 16 seropositive patients with SLE [with high levels of antinuclear antibodies (ANAs), anti-DNA and/or anti-Smith] and 16 seropositive patients with RA [with high levels of anti-cyclic citrullinated peptides antibodies (anti-CCP)] by using a NAb™ Protein G Spin Kit (Thermo scientific, Waltham, MA) according to the manufacturer's instructions. IgG enrichment was verified by protein electrophoresis with silver staining and western blot (data not shown). The final IgG preparation of SLE patients used for opsonization had 1:1.280 ANAs [speckled pattern, indirect immunofluorescence (IIF) using HEP-2 cells], 1:40 anti-DNA (IIF), 1220 Units anti-Smith (ELISA), 1270 Units anti-Ro/SSa (ELISA), 90 Units anti-La/SSb (ELISA), and 7630 Units anti-ribonucleoprotein (RNP, ELISA). The final IgG preparation of patients with RA used for opsonization had 286.3 Units anti-CCP (CCP3 IgG ELISA) (30). All these kits were

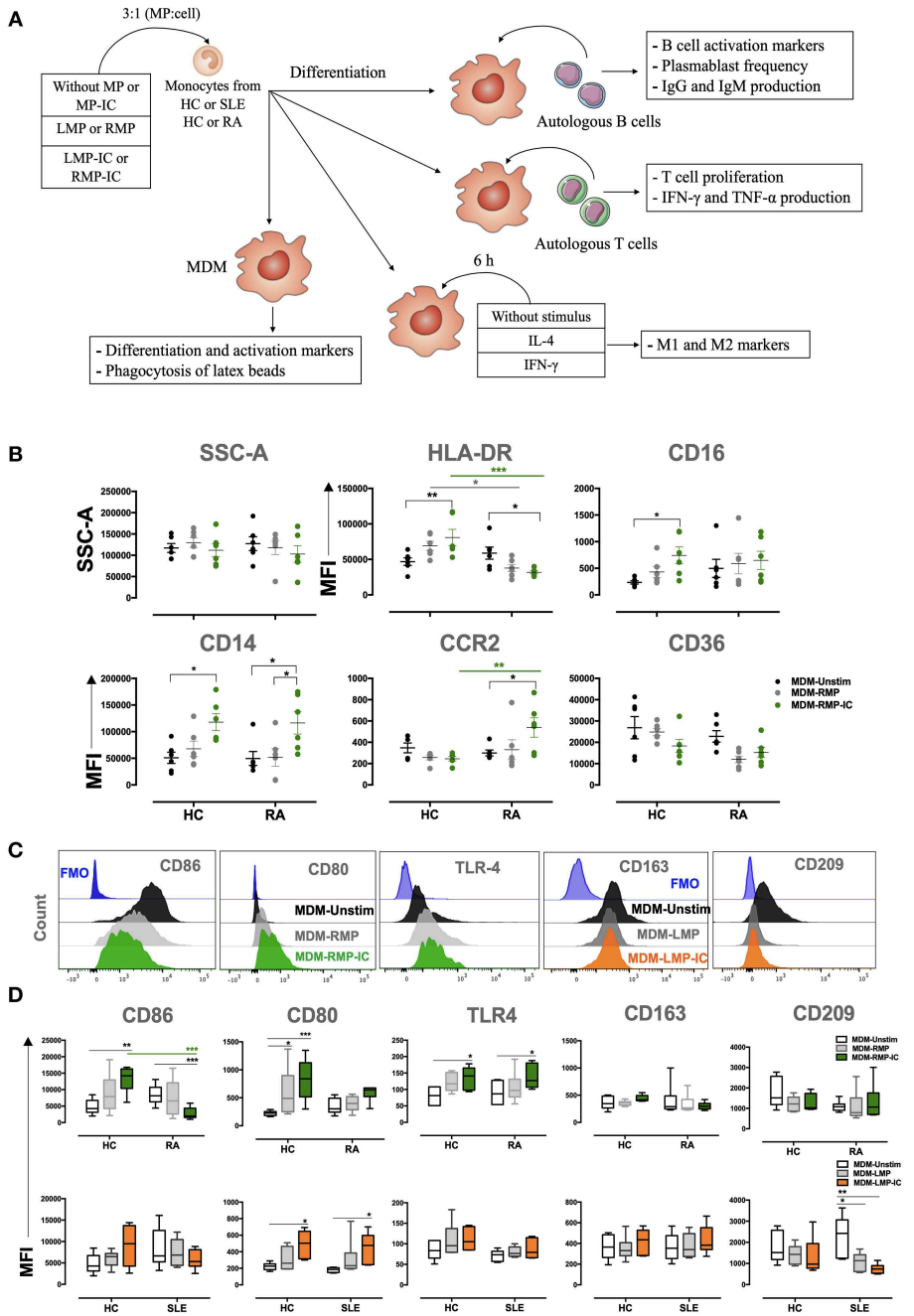


**FIGURE 1 |** MP-IC change the expression of differentiation and activation markers in MDM. **(A)** Summarized methodological strategy used in this article is presented. **(B)** The MFI of markers associated with the differentiation of MDM: CD36, HLA-DR, CD16, CD16, CCR2, and SSC-A; MDM differentiated without [Unstimulated (Unstim), black dots] or with RMP (light gray dots) or RMP-IC (green dots) from patients with RA ($n = 6$) and HC ($n = 6$). **(C)** Representative histograms of markers associated with M1 polarization (from left to right: CD86, CD80, and TLR4) in MDM from patients with RA differentiated without (Unstim, black histograms) or with RMP (light gray histograms) or RMP-IC (green histograms); representative histograms of markers associated with M2 polarization (CD163 and CD209) in MDM from patients with SLE differentiated without (Unstim, black histograms) or with LMP (dark gray histograms) or LMP-IC (orange histograms). Blue histograms represent the FMO control for each marker. **(D)** Top, the MFI of markers associated with M1 and M2 polarizations in MDM from patients with RA ($n = 6$) and HC ($n = 6$) differentiated without (Unstim, black whisker box) or with RMP (light gray whisker box) or RMP-IC (green whisker box). Below, the MFI of markers associated with M1 and M2 polarizations in MDM from patients with SLE ($n = 6$) and HC ($n = 6$) differentiated without (Unstim, black whisker box) or with LMP (dark gray whisker box) or LMP-IC (orange whisker box). Comparisons among the groups were performed using ANOVA II and the Bonferroni *post-hoc* test.

purchased from Inova (San Diego, CA). For opsonization, ∼1 × $10^6$ LMP and RMP were mixed and incubated with 15-μg/mL purified IgG (from SLE and RA, respectively) for 60 min at 37°C. Unbound antibodies were washed with 1 mL of DPBS at 17,000 × g for 60 min. The MP from patients with SLE and those from patients with RA forming IC (LMP- and RMP-IC, respectively) was assessed after staining with an *F*(*ab*')2 anti-IgG fragment conjugated with Alexa Fluor-488 for 30 min at 4°C (MP-IgG+ > 28%) using flow cytometry as previously described (**Supplementary Figure 1B**) (4). For use, each batch was thawed and quantified using flow cytometry as previously reported (4).

## Monolayer Culture of CD14+ Phagocytes and Their Differentiation Into MDM

CD14+ cells were enriched and subsequently differentiated into MDM as previously reported (31). Briefly, peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll density gradient centrifugation from defibrinated venous blood samples from HC and patients with SLE and RA. For monocyte adherence, PBMCs containing 1.2 × $10^5$ CD14+ cells were plated in 96-well plates (Corning Incorporated Life Science, Lowell, MA, USA) using 250-μL RPMI-1640 GlutaMAX with 0.5% inactivated autologous serum-depleted MP (iSA-d) (4) for 4 h at 37°C in 5% $CO_2$. Subsequently, wells were washed with pre-warmed DPBS plus 0.5% iSA-d to remove non-adherent cells. The adherent cells obtained were cultured in 250-μL RPMI-1640 supplemented with 10% iSA-d plus 100-μg/ml streptomycin and 100-IU/ml penicillin in the presence or absence of MP (RMP or LMP) and MP-IC (RMP- or LMP-IC) in a ratio of 1:3 (cells:MP) for 6 days at 37°C in 5% $CO_2$ to facilitate their differentiation into MDM. After the culturing, supernatants were collected and frozen at −20°C until the level of various cytokines [IL-8, IL-6, IL-10, TNF-α, IL-1β, IL12p70, BAFF (B-cell activating factor) and APRIL (A proliferation-inducing ligand)] was assessed. Morphological changes and the expression of differentiation markers were evaluated in MDM using flow cytometry through specific anti-human antibodies against CD36, HLA-DR, CD16, CD14, and CCR2. Cells were blocked (DPBS plus 1% BSA, 0.01% $NaN_3$ and 10% inactivated FBS) and stained for 30 min at 4°C followed by washing twice with washing buffer (DPBS plus 1% BSA and 0.01% $NaN_3$). Fluorescence minus one (FMO) controls were established for each antibody using HLA-DR staining. Using the LSR Fortessa flow cytometer with the FACS DIVA software (BD), 50,000 cells were immediately acquired.

In other experiments, the frequency of phagocytic cells, repolarization to M1 and M2 profile, and autologous B- and T-cell co-cultures were performed as detailed below.

## Phagocytosis Assay

To evaluate the phagocytosis of MDM differentiated with or without of MP (RMP or LMP) and MP-IC (RMP- or LMP-IC), MDM were incubated with fluorescent latex beads in a ratio of 1:5 (cell:beads). Subsequently, macrophages were centrifuged for 5 min at 900 × g and incubated for 2 h at 37°C in 5% $CO_2$. Then, MDM were repeatedly washed with DPBS and were stained with anti-HLA-DR antibody; ∼30,000 cells were immediately acquired using a flow cytometer.

## MDM Repolarization to M1 and M2 Profiles

To evaluate repolarization of MDM differentiated with or without of MP (RMP or LMP) and MP-IC (RMP- or LMP-IC), phagocytes differentiated with and without these extracellular vesicles were treated for 6 h with 20-ng/mL hrIL-4 or 20-ng/mL hrIFN-γ at 37°C in 5% $CO_2$ to favor M1 and M2 activation, respectively. Supernatants were collected and frozen at −20°C until the assessment of proinflammatory (M1 markers: IL-8, IL-6, TNF-α, IL-1β, and IL12p70) and anti-inflammatory (M2 marker: IL-10) cytokines. In MDM, M1, and M2 polarization was additionally determined by measuring the expression of classical M1 (CD80, CD86, TLR4, and CD32) and M2 (CD163 and CD209) membrane receptors. MDM staining using specific antibodies was performed as previously detailed. Assays with MDM from HC using different IL-4 and IFN-γ concentrations at differing stimulation times were previously performed to determine culture conditions for M1 and M2 activation. Treatment with 20-ng/mL IFN-γ (M1) or IL-4 (M2) for 6 h at 37°C in 5% $CO_2$ was selected (**Supplementary Figure 2**; Data not shown).

## Co-culture of MDM With Autologous T and B Cells

To evaluate the effect of MDM differentiated with MP (RMP or LMP) and MP-IC (RMP-or LMP-IC) on B- and T-cell activation, fresh autologous CD3+ and CD19+ cells were enriched with the Rosette Sep according to the manufacturer's instructions (purity > 95% and > 90%, respectively). T cells were independently labeled with 1-μM CFSE and repeatedly washed; T and B lymphocytes were co-cultured with previously differentiated MDM with and without extracellular vesicles in RPMI-1640 GlutaMAX that was supplemented with 10% FBS, 100-μg/mL streptomycin and 100-IU/mL penicillin, 10-ng/mL rhIL-2, and 2-mM L-glutamine (complete medium).

For co-culturing with CD3+ lymphocytes, the resultant T cells were left unstimulated (complete medium) or were stimulated with 10-μg/mL PHA and immediately added to pre-washed MDM (2:1, T cells:MDM) and incubated for 96 h at 37°C in 5% $CO_2$. Four hours before terminating the incubation of cell cultures, cells were treated with 1-μg/mL Brefeldin A. Lymphocytes were harvested by subjecting them to multiple washes with DPBS and subsequently blocking and staining with anti-CD4 and -CD8 antibodies for 30 min at 4°C. These cells were fixed with 2% paraformaldehyde and permeabilized with 0.5% Tween-20 and 0.2% BSA for 30 min. Subsequently, cells were stained with specific anti-human IFN-γ and TNF-α antibodies for 1 h.

For co-culturing with CD19+ lymphocytes, the resultant B cells were left unstimulated (complete medium) and immediately added to pre-washed MDM cultures (2:1, B cells:MDM) and incubated for 96 h. In parallel, as controls for B-cell activation (**Supplementary Figure 3**), B cells were unstimulated (complete medium) or stimulated with 2.5-μg/mL affinity-purified $F(ab')_2$ fragment anti-human IgM plus 1-μg/mL rhCD40L. Supernatants were collected and frozen at −20°C until the assessment of IgG and IgM levels. Lymphocytes were harvested by multiple washes

with DPBS and blocked and stained with anti-human CD19, CD20, CD38, CD27, CD138, CD80, CD86, CD69, and CD95 antibodies for 30 min at 4°C.

Finally, in both cases, 50,000 cells were acquired using a flow cytometer. FMO controls were also included. Cell viability was evaluated using the LIVE-DEAD probe and by changes in FSC-A and SSC-A parameters.

## Cytokine and Immunoglobulin Levels

The Human Inflammatory CBA kit was used to determine the levels of IL-8, IL-6, IL-10, TNF-α, IL-1β, and IL12p70 based on the manufacturer's instructions.

The levels of the cytokines BAFF and APRIL in supernatants were determined using commercial ELISA kits (Quantikine ELISA Human BAFF/BLyS/TNFS13B Kit and DuoSet ELISA Human APRIL/TNFS13 Kit, respectively; R&D Systems) in accordance with the manufacturer's instructions.

IgM and IgG antibody levels in supernatants were determined using commercial ELISA kits (Human IgM Uncoated ELISA kit and Human IgG Uncoated ELISA kit; Invitrogen by Thermo Fisher Scientific, Vienna, Austria) according to the manufacturer's instructions.

## Data Analysis

Two different categorically independent variables were compared using two-way ANOVA (ANOVA II) and the Bonferroni *post-hoc* test (data are presented as mean ± SD). Comparisons among groups of HC and patients with RA and SLE were performed via the Kruskal–Wallis test and Dunn's *post-hoc* test (data are presented as median ± interquartile range). Gating analyses, cell frequencies, mean fluorescence intensity (MFI) and the percentage of divided T cells after proliferation (proliferation modeling algorithm) were estimated using the FlowJo 10.2 software. Statistical analysis was performed using the GraphPad Prism version 7.2 (GraphPad Soft-ware Inc., San Diego, CA, USA). Statistical significance was set at $p \leq 0.05$; $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, and $^{****}p \leq 0.0001$.

# RESULTS

## MP-IC Induce the Differentiation of MDM to a Proinflammatory (M1-Like) Profile

The methodological strategy of this study is summarized in **Figure 1A.** The effect of MP and MP-IC in MDM, regarding changes in morphology (side and forward scatter), the expression of differentiation (HLA-DR, CD16, CD14, CCR2, and CD36) and activation (CD86, CD80, TLR-4, CD163, and CD209) markers and the expression of cytokine levels (IL-1β, IL-6, TNF-α, IL-10, IL-8, and IL12p70) were evaluated in mononuclear phagocytes obtained from HC and patients with RA and SLE, differentiated without (MDM-Unstimulated, -Unstim) or with extracellular vesicles (MP from SLE: MDM-LMP and MDM-LMP-IC; MP from RA: MDM-RMP and MDM-RMP-IC). No changes were observed in the granularity (side scatter) and size (forward scatter) of MDM among all the study groups (**Figures 1B**, **Supplementary Figure 4**, and data not shown).

MDM from HC showed increased HLA-DR, CD14, CD16, CD86, CD80, and TLR-4 expression in addition to increased IL-1β, IL-6, and TNF-α levels when these cells were differentiated with RMP-IC compared with those differenced with MDM-Unstim cells (**Figures 1B–D**, **2B**). Only CD16 and CD80 expressions and IL-1β and IL-6 levels increased in MDM from HC that were exposed to LMP-IC (**Figures 1D**, **2A,B** and **Supplementary Figure 4**). These results showed that MP-IC promote the differentiation of MDM from HC to a proinflammatory (M1-like) profile; this phenomenon was more evident with the vesicles of patients with RA than SLE.

With respect to mononuclear phagocytes from patients with RA, MDM differentiated with RMP-IC increased CD14, CCR2, and TLR4 expressions as well as IL-1β, IL-6, and TNF-α levels in supernatants but decreased HLA-DR and CD86 expressions compared with those differentiated with MDM-Unstim cells (**Figures 1B–D**, **2A,B**). Conversely, increased CD80 expression and decreased CD209 expression and high IL-1β and -6 levels in supernatants were noted in the MDM of patients with SLE that were differentiated with LMP-IC compared with those that were differentiated with MDM-Unstim cells (**Figures 1C,D**, **2A,B** and **Supplementary Figure 4**). These results suggested that MP-IC also promote the proinflammatory differentiation of mononuclear phagocytes in RA and SLE patients.

As an indirect measure of macrophage M1 or M2 activation (17), the frequency of MDM that bind/internalize latex beads was evaluated after their differentiation with or without MP and MP-IC. Few positive cells were found to be bound to latex beads when MDM from HC were exposed to RMP-IC and LMP-IC (**Figures 2C,D**). Similar results were observed with MDM from patients with RA, but no changes were noted in the cells of patients with SLE (**Figures 2C,D**). These results corroborated that the presence of MP-IC allow the differentiation of MDM to a M1-like profile. These changes were more evident in MDM from HC than in those from patients with RA and SLE.

## MDM Differentiated With RMP-IC Were Resistant to Repolarization to M2-Like Profiles Following IL-4 Treatment

To determine whether MDM differentiated with MP and MP-IC could reverse or potentiate their proinflammatory profiles, these MDM were further treated with IFN-γ or IL-4. As expected, IFN-γ treatment enhanced the MFI of M1-related molecules, such as CD86 and CD80, as well as TNF-α levels compared with those without treatment (Data not shown). However, IFN-γ treatment had no additive effect on MDM differentiated with MP and MP-IC regarding CD86, CD80, and TLR-4 expressions and IL-1β, IL-6, TNF-α, and IL-10 production (**Figures 3A,B**). IL-4 treatment did not result in an increase in CD86 and CD80 expressions in MDM of HC differentiated with RMP-IC, as noted in previous results (**Figure 1C**). However, after IL-4 treatment the TLR-4 expression and IL-1β and IL-6 levels remain increased in MDM of HC and patients with RA differentiated with RMP-IC. No differences were noted regarding TNF-α and IL-10 levels (**Figures 4A,B**). In patients with SLE, when MDM were differentiated with LMP-IC, IL-1β, IL-6, and

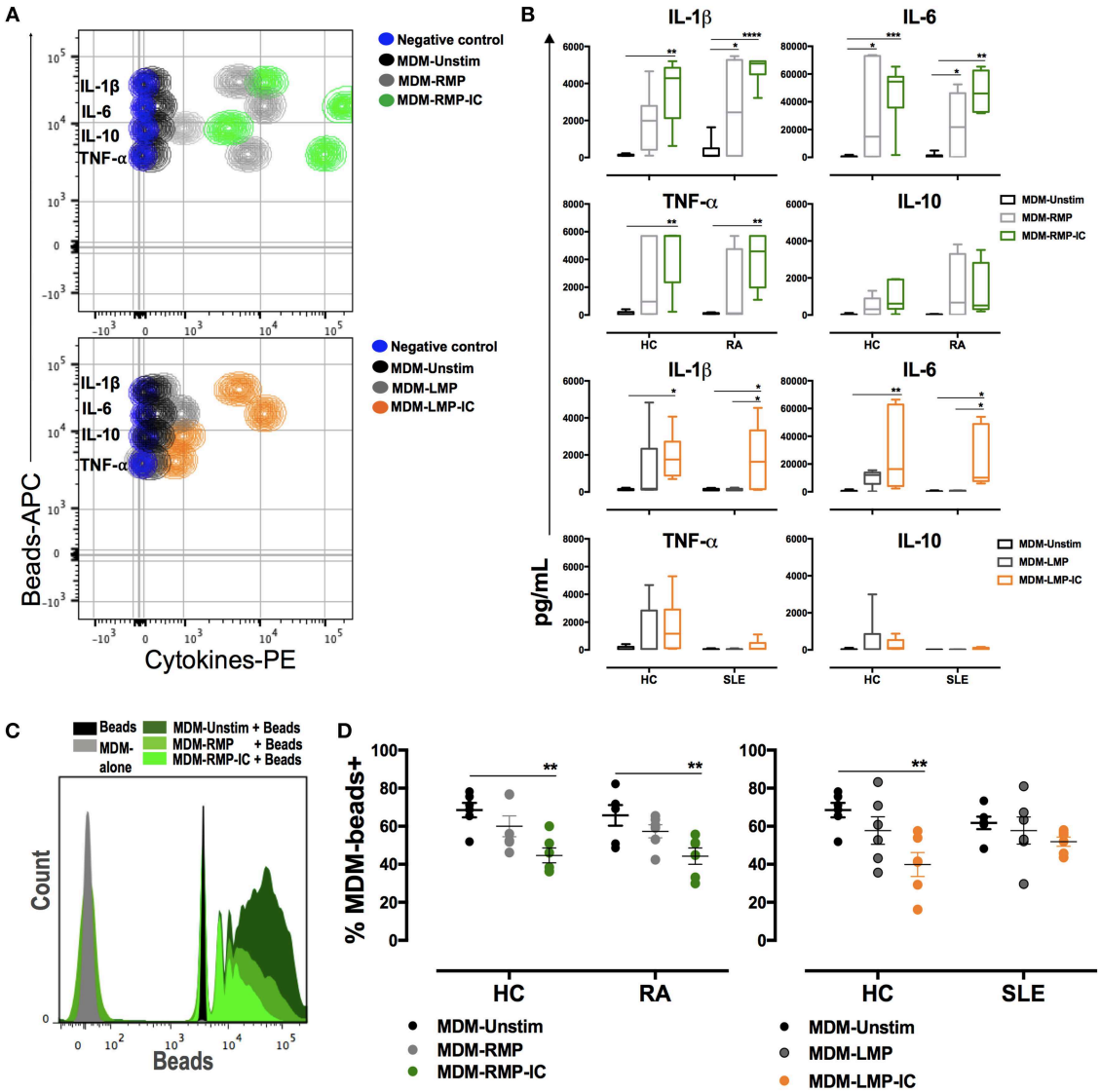


**FIGURE 2 |** MP and MP-IC induce the polarization of MDM toward a proinflammatory profile compatible with M1 activation and decrease the frequency of macrophages that phagocytose. **(A)** Top, CBA representative contour plot of cytokine levels in the supernatants of MDM from patients with RA differentiated without (Unstim, black contour plot) or with RMP (light gray contour plot) and RMP-IC (green contour plot). Below, a CBA representative contour plot of cytokine levels in the supernatants of MDM from patient with SLE differentiated without (Unstim, black contour plot) or with LMP (dark gray contour plot) and LMP-IC (orange contour plot). In both plots, the negative control (CBA beads alone) is shown as a blue contour plot. **(B)** The first two panels, cytokine levels in the supernatants of MDM from patients with RA ($n = 6$) and HC ($n = 6$) differentiated without (Unstim, black bar graph) or with RMP (light gray bar graph) and RMP-IC (green bar graph). The last two panels, cytokine levels in supernatants of MDM from patients with SLE ($n = 6$) and HC ($n = 6$) that were differentiated without (Unstim, black bar graph) or with LMP (dark gray bar graph) and LMP-IC (orange bar graph). **(C)** Representative histograms of latex beads alone (black); MDM from RA patients differentiated in absence of extracellular vesicles and incubated with fluorescent latex beads (Unstim, dark gray histogram) and MDM differentiated with RMP (medium green histogram) or RMP-IC (light green histogram) and incubated with fluorescent latex beads. **(D)** Left, the frequency of MDM from HC ($n = 6$) and patients with RA ($n = 6$) that bound/internalized fluorescent latex beads after these cells were differentiated without (Unstim, black dots) or with RMP (light gray dots) and RMP-IC (green dots). Right, the frequency of MDM from HC ($n = 6$) and patients with SLE ($n = 6$) that bound/internalized fluorescent latex beads after these cells were differentiated without (Unstim, black dots) or with LMP (dark gray dots) or LMP-IC (orange dots). Comparisons among the groups were performed using ANOVA II and the Bonferroni *post-hoc* test.

TNF-α levels did not increase after additional IL-4 treatment (**Figures 4A,B**), suggesting that these cells, but not MDM from HC and patients with RA, reversed the M1 profile induced by MP-IC (**Supplementary Figure 5**). These negligible changes cannot be explained due to a low effect of IL-4, since we observed that MDM treated only with IL-4 up regulated CD163, CD209, and IL-10, a phenotype compatible to M2 activation (**Supplementary Figure 2**).

The aforementioned results showed that RMP-IC induce a more prominent differentiation to an M1-like profile

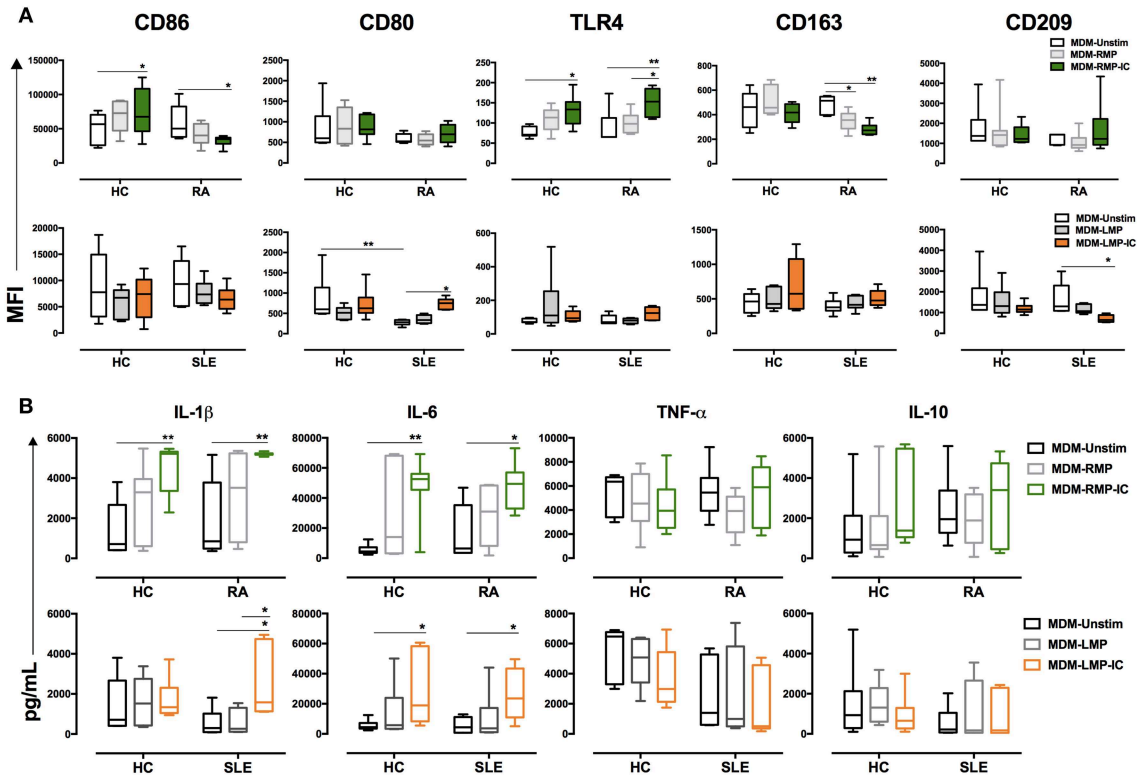


**FIGURE 3 |** IFN-γ enhances the proinflammatory profile of MDM differentiated with MP. **(A)** Top, the MFI of markers associated with M1 and M2 polarizations in MDM from patients with RA ($n = 6$) and HC ($n = 6$) that were differentiated without (Unstim, black whisker box) or with RMP (light gray whisker box) and RMP-IC (green whisker box) along with 6 h of IFN-γ treatment. Below, the MFI of markers associated with M1 and M2 polarizations in MDM from patients with SLE ($n = 6$) and HC ($n = 6$) differentiated without (Unstim, black whisker box) or with LMP (dark gray whisker box) and LMP-IC (orange whisker box) along with 6 h of IFN-γ treatment. **(B)** Top, cytokine levels in the supernatants of MDM from patients with RA ($n = 6$) and HC ($n = 6$) differentiated without (Unstim, black bar graph) or with RMP (light gray bar graph) and RMP-IC (green bar graph) along with 6 h of IFN-γ treatment. Below, cytokine levels in supernatants of MDM from patients with SLE ($n = 6$) and HC ($n = 6$) differentiated without (Unstim, black bar graph) or with LMP (dark gray bar graph) and LMP-IC (orange bar graph) along with 6 h of IFN-γ treatment. Comparisons among the study groups were performed using ANOVA II and the Bonferroni *post-hoc* test.

than LMP-IC in the MDM of HC and patients with RA (**Supplementary Figure 4**).

## MDM Differentiated With LMP-IC and RMP Induce Further Proliferation of Activated CD4+ T Cells From Patients With SLE and RA, Respectively

To evaluate the effects of MDM differentiated in the presence of MP and MP-IC on T-cell activation, MDM differentiated with or without these vesicles were co-cultured with T cells and stimulated with PHA. In patients with RA, MDM differentiated with RMP induced a higher proportion of dividing CD4+ T cells compared with those differentiated without extracellular vesicles or RMP-IC (**Figures 5A,B**). The induction of IFN-γ+ and TNF-α+ CD4+ T cells was not noted, and cytokine responses of CD8+ T cells to MDM from patients with RA differentiated with RMP and RMP-IC were not observed (**Figures 5B–E**). Additionally, no differences were detected in the frequency of live T cells in co-cultures (≥80%) compared with cells without MDM (≥83%). Notably, in MDM from HC differentiated with RMP-IC, an increased frequency of CD4+ T cells producing IFN-γ and TNF-α were observed.

MDM from patients with SLE differentiated with LMP-IC demonstrated a higher percentage of proliferating CD4+ T cells and an increased frequency of IFN-γ+ and TNF-α+ in CD4+ proliferating T cells than those in MDM differentiated without extracellular vesicles (**Figure 5**). Interestingly, although CD8+ T cells from patients with SLE proliferated in response to MDM regardless of the presence of LMP and LPM-IC, an increased percentage of IFN-γ+ cells were observed in these lymphocytes when MDM were differentiated with LMP and LPM-IC compared with those differentiated without these vesicles (**Figure 5**). Interestingly, T cells from HC did not respond to either MDM differentiated with or without these extracellular vesicles from SLE patients.

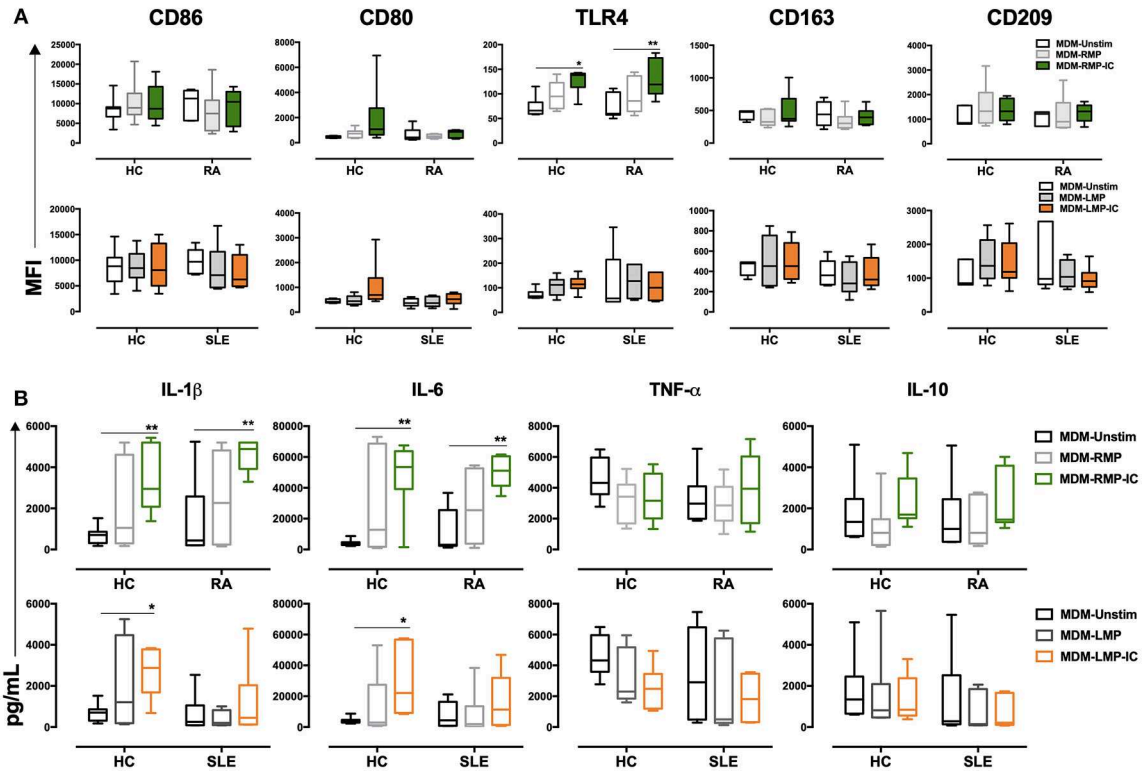


**FIGURE 4 |** IL-4 does not re-polarize the proinflammatory profile of MDM from HC and patients with RA differentiated with MP and MP-IC. **(A)** Top, the MFI of markers associated with M1 and M2 polarizations in MDM from patients with RA ($n = 6$) and HC ($n = 6$) differentiated without (Unstim, black whisker box) or with RMP (light gray whisker box) and RMP-IC (green whisker box) along with 6 h of IL-4 treatment. Below, the MFI of markers associated with M1 and M2 polarizations in MDM from patients with SLE ($n = 6$) and HC ($n = 6$) differentiated without (Unstim, black whisker box) or with LMP (dark gray whisker box) and LMP-IC (orange whisker box) along with 6 h of IL-4 treatment. **(B)** Top, cytokine levels in supernatants of MDM from patients with RA ($n = 6$) and HC ($n = 6$) differentiated without (Unstim, black bar graph) or with RMP (light gray bar graph) and RMP-IC (green bar graph) along with 6 h of IL-4 treatment. Below, cytokine levels in supernatants of MDM from patients with SLE ($n = 6$) and HC ($n = 6$) differentiated without (Unstim, black bar graph) or with LMP (dark gray bar graph) and LMP-IC (orange bar graph) along with 6 h of IL-4 treatment. Comparisons among the groups were performed using ANOVA II and the Bonferroni *post-hoc* test.

These results showed that MDM differentiated with extracellular vesicles favor CD4+ and CD8+ T-cell activation of patients with autoimmune diseases.

## MDM Differentiated With MP-IC Induce the Activation and Survival of B Cells From Autoimmune Patients

The co-culture of MDM differentiated with RMP-IC and B cells from patients with RA increased the expression of activation markers CD80, CD86, CD69, and CD95 on these lymphocytes compared with the co-culture of MDM differentiated without vesicles and B cells from patients with RA (**Figures 6A–C**). This activation phenotype was associated with a significant decrease in the frequency of dead B cells in co-cultures but not with an increase in BAFF levels in the supernatants of MDM from patients with RA differentiated with RMP-IC (**Figures 6D,E**). In addition, no differences were noted in the frequency of plasmablasts and plasmatic cells (**Supplementary Figure 3B** and data not shown) or the induction of IgM and IgG production by B cells from patients with RA in the co-cultures (**Figure 6E**). Interestingly, MDM differentiated with RMP, and not with RMP-IC, increased APRIL levels (**Figure 6E**). Moreover, MDM from HC differentiated with RMP-IC only increased CD95 level in B cells and induced IgM secretion. These cells were also more prone to cell death and showed no change in BAFF, APRIL, and IgG level compared with those from patients with RA (**Figure 6**).

The co-culture of B cells from patients with SLE and MDM differentiated with LMP-IC increased the expression of activation markers CD80, CD86, CD69, and CD95 on these lymphocytes compared with the co-culture of MDM differentiated without vesicles and B cells from patients with SLE (**Figure 7A**). This activation phenotype was associated with a significant decrease in the frequency of dead B cells and with an increase in BAFF levels in the supernatants of MDM from patients with SLE differentiated with LMP-IC (**Figures 7B,C**). In addition, all this was related to an increased plasmablast frequency, but not to plasmatic cells, and high IgM and IgG levels in co-cultures of B

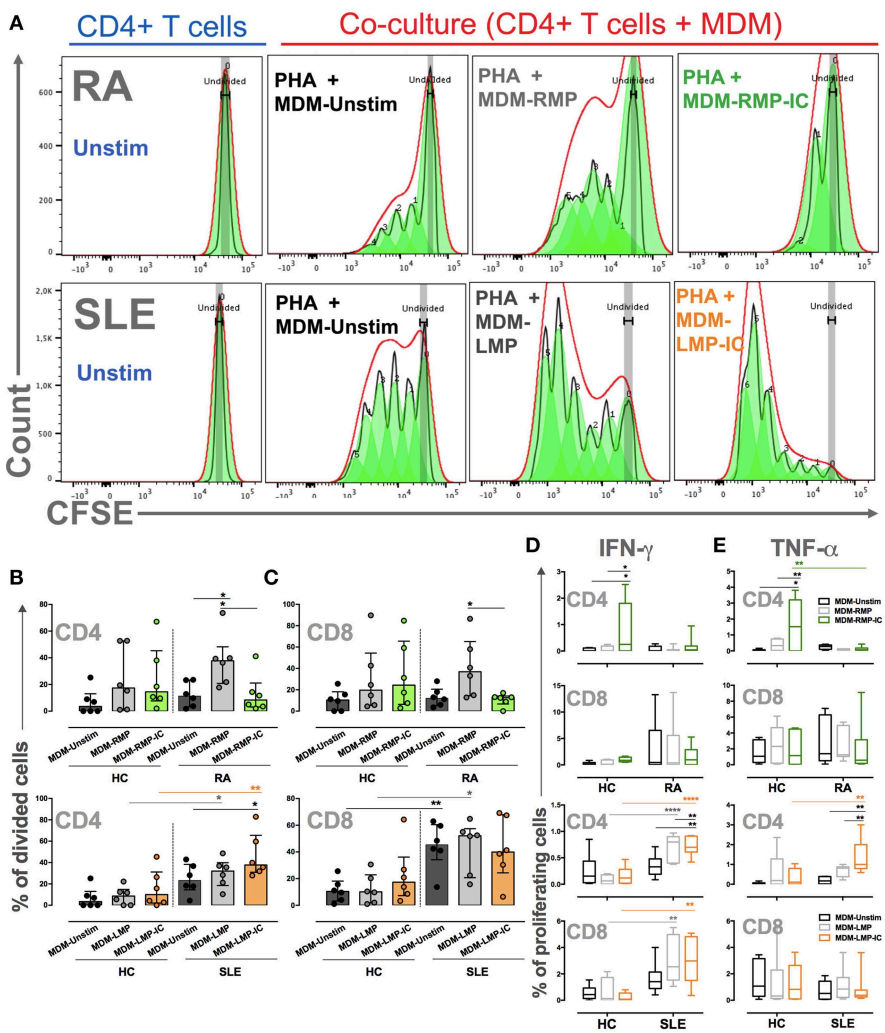


**FIGURE 5 |** MDM differentiated with MP and MP-IC induce the proliferation and activation of autologous T cells. **(A)** Representative proliferation modeling of CD4+ T cells alone (in complete medium) without (Unstim) or with stimulus with PHA and co-cultured with MDM from patients with RA (Top panel) and SLE (Below panel) differentiated without (MDM-Unstim) or with MP and MP-IC. **(B,C)** The frequency of CD4+ T cells **(B)** and CD8+ T cells **(C)** from patients with RA and SLE and HC that divided after PHA treatment and co-culture with MDM differentiated without (Unstim) or with MP or MP-IC. **(D)** The frequency of proliferating IFN-γ+ CD4+ T cells and IFN-γ+ CD8+ T cells and **(E)** the frequency of proliferating TNF-α+ CD4+ T cells and TNF-α+ CD8+ T cells from patients with RA and SLE and HC after PHA treatment and co-culture with MDM differentiated without (Unstim) or with MP and MP-IC. In all cases, patients with SLE: $n = 6$, patients with RA: $n = 6$, and HC: $n = 6$; comparisons among the groups were performed using ANOVA II and the Bonferroni *post-hoc* test.

cells of patients with SLE and MDM differentiated with LMP-IC (**Figures 7D–F**; data not shown). MDM from HC differentiated with LMP or LMP-IC tended to exhibit increased BAFF levels; moreover, these cells induced increased IgM secretion without a significant trend in the increase of CD95 level in B cells.

These results demonstrated that MDM differentiated in the presence of MP-IC can induce activation and survival of B cells of patients with autoimmune diseases.

## DISCUSSION

Macrophages are crucial in the pathogenesis of RA and SLE (16, 32). In the context of an inflammatory response, tissues recruiting blood monocytes are considered as the sources of inflammatory macrophages (10, 33), and M1- and M2-like disequilibrium of macrophages results in chronic inflammation (16, 34). In SLE (4, 7) and RA (29), MP and MP-IC exert proinflammatory effects in monocytes and MDM. However, to the best of our knowledge, this is the first report about the effect of MP and MP-IC on mononuclear phagocytes differentiation. Therefore, the findings of this study support the idea that the uptake of MP-IC in patients with RA and SLE by monocytes biases the differentiation toward M1-like MDM along with the expansion of proinflammatory responses and the induction of lymphocyte activation. Then, the effect of MP-IC in M1-like differentiation of MDM may contribute to the chronic inflammatory process and promote adaptive responses in systemic autoimmune diseases.

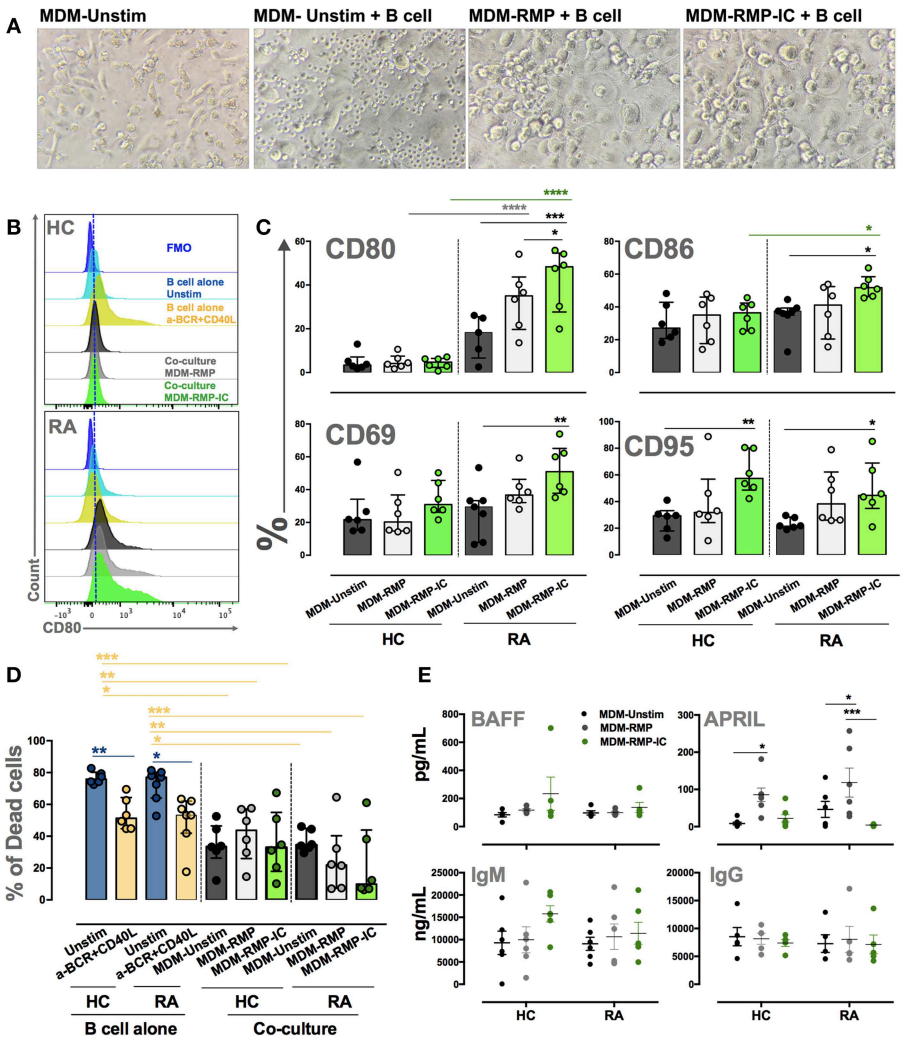


**FIGURE 6 |** MDM differentiated with MP-IC, and mainly with MP, induce the activation of autologous B cells from patients with RA. **(A)** From left to right: representative light microscopy pictures of MDM unstim alone; MDM unstim co-cultured with B cells; MDM differentiated in the presence of RMP or RMP-IC from patients with RA and co-cultured with B cells. **(B)** Representative histograms of CD80 expression on B cells from HC (top) and patients with RA (below) cultured alone (light blue) and with anti-BCR plus CD40L (yellow) or co-cultured with MDM differentiated without (Unstim, black) or with RMP (gray) and RMP-IC (green). Blue histograms represent the FMO control. **(C)** The frequency of CD80, CD86, CD69, and CD95 in B cells from patients with RA ($n = 7$) and HC ($n = 6$) co-cultured with MDM differentiated without (Unstim) or with RMP and RMP-IC. **(D)** The frequency of dead B cells (positive for LIVE-DEAD probe) from patients with RA ($n = 7$) and HC ($n = 6$) cultured alone (Unstim, in complete medium) and with anti-BCR plus CD40L (positive control) or co-cultured with MDM differentiated without (Unstim) or with RMP and RMP-IC. **(E)** BAFF and APRIL (Top panel) levels in supernatants of MDM from patients with RA ($n = 5$) and HC ($n = 5$) differentiated without (Unstim) or with RMP and RMP-IC. IgG and IgM (below panel) levels in supernatants from co-cultures of MDM differentiated with or without RMP and RMP-IC with autologous B cells from HC ($n = 5$) and RA ($n = 5$) patients. Comparisons among the groups were performed using ANOVA II and the Bonferroni *post-hoc* test.

## MP-IC Induce the Differentiation of Monocytes Into M1-Like MDM

Our results agreed with previous reports indicating proinflammatory effects of MP in different immune system cells (5, 35, 36). Despite a number of studies have shown that macrophages from patients with SLE have intrinsic defects in the phagocytosis of latex beads, apoptotic cells, bacteria, and yeast (31, 37, 38); the phagocytosis of IC is intact or even increased in these patients compared with that in patients with non-opsonized antigens (4, 39). In our previous studies, we demonstrated a more efficient uptake of MP-IC than that of MP by monocytes from patients with SLE (4) and RA (29); this may explain the higher proinflammatory response observed in MDM differentiated with MP-IC than those differentiated with MP. In addition, MP-IC induce the production of proinflammatory cytokines IL-6, IL-1β, TNF-α (RA and SLE), and IFN-α (SLE) in mononuclear phagocytes from patients with RA (29) and SLE (4). Considering these findings and that MP-IC induced higher proinflammatory differentiation than MP, it can be expected that signaling mechanisms and processing of these vesicles are similar to those reported for IC, for example the cross-linking of FcγR (CD16, CD32, and CD64) (12, 40).

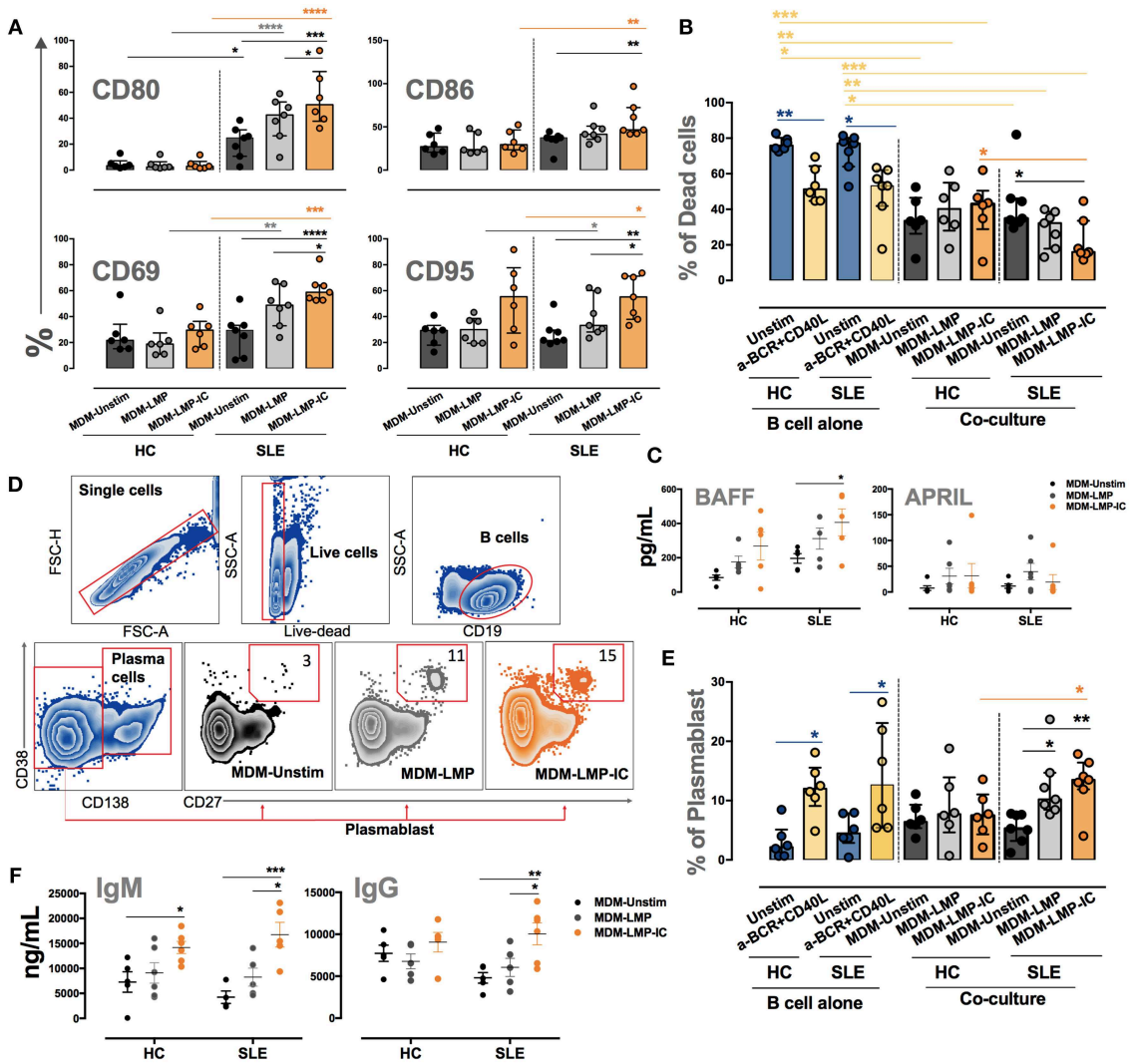


**FIGURE 7 |** MDM differentiated with MP and MP-IC induce the activation and plasmablast differentiation of autologous LB from patients with SLE. **(A)** The frequency of CD80, CD86, CD69, and CD95 in B cells from patients with SLE ($n = 7$) and HC ($n = 6$) co-cultured with MDM differentiated without (Unstim) or with LMP and LMP-IC. **(B)** The frequency of dead B cells (positive for LIVE-DEAD probe) from patients with SLE ($n = 7$) and HC ($n = 6$) cultured alone (Unstim, in complete medium) and with anti-BCR plus CD40L (positive control) or co-cultured with MDM differentiated without (Unstim) or with LMP and LMP-IC. **(C)** BAFF and APRIL levels in the supernatants of MDM from patients with SLE ($n = 5$) and HC ($n = 5$) differentiated without (Unstim) or with LMP and LMP-IC. **(D)** Representative gating strategy to determine the frequency of plasmablasts after the co-culture of B cells with MDM differentiated without (Unstim) or with LMP and LMP-IC. **(E)** The frequency of plasmablasts from B cells cultured alone (Unstim, in complete medium), with anti-BCR plus CD40L (positive control), or co-cultured with autologous MDM from patients with SLE ($n = 7$) and HC ($n = 6$) differentiated without (Unstim) or with LMP and LMP-IC. **(F)** IgG and IgM levels in supernatants from the co-cultures of MDM differentiated with or without LMP and LMP-IC with autologous B cells from HC ($n = 5$) and patients with SLE ($n = 5$). Comparisons among the groups were performed using ANOVA II and the Bonferroni *post-hoc* test.

Interestingly, IC can also induce monocyte differentiation; Tanaka et al. described that monocytes from healthy individuals differentiated into an immature dendritic cells (iDCs)-like phenotype in the presence of plate-immobilized human IgG (as a model for IC), and produce several proinflammatory cytokines, such as TNF-α, IL6, and GM-CSF, through the activation of FcγRI (CD64). These cells trigger autologous T-cell proliferation and cytokine production, including IFN-γ, TNF-α, and IL-4 (41). Several studies have shown that IC containing IgG against citrullinated peptides (ACPA-IC) induce FcγRIIa (CD32)-mediated TNF-α secretion in macrophages from patients with RA (42–45). Furthermore, in *in vitro* and *in vivo* models of SLE pathogenesis, IC can bind to FcγRs expressed on the surface of monocytes and plasmacitoid (p)-DCs. Their subsequent internalization allowed the DNA present in these IC to activate TLR9, inducing the production of proinflammatory cytokines, mainly TNF-α, IL-10, and IFN-α (46, 47). These studies support the hypothesis that MP-IC can activate

mononuclear phagocytes. In addition, our results not only agree with these reports but also showed the effect of MP-IC in the proinflammatory differentiation of MDM. Therefore, elevated amounts of circulating MP-IC in patients with active SLE and seropositive RA might have an impact in the proinflammatory differentiation of monocytes in patients once these cells migrate from blood to different tissues (**Figure 8**).

Although the proinflammatory differentiation of MDM was higher with MP-IC than that with MP, differences were also noted according to the source of monocytes. There were more prominent responses with monocytes from HC than that with monocytes from patients with RA and SLE. FcγR expression on circulating monocytes in patients with RA and SLE differed from those in HC. Several reports have shown an increased expression of CD64 in monocytes from patients with SLE (4, 48, 49), whereas CD32 expression was more predominant in monocytes from patients with RA (50). In addition, an imbalance was noted in the circulating monocyte subsets of these two diseases, both in murine models and in patient samples. Reportedly, intermediate monocyte subset in RA and non-classical subset in SLE seem to primarily migrate toward inflammation sites (9, 51). These monocytes are quite heterogeneous with regard to their phenotype and function and can respond in different ways to the same stimulus (10). These differences in both the monocyte phenotypes and sources may partly explain the variations observed in the differentiation of MDM from HC and patients with RA and SLE with MP-IC in our system. However, these differences could be also explained by other causes such as trained innate immunity phenomena (52) or immunosuppressive treatment. Thus, more studies are needed to further clarify these differences.

Moreover, although higher proinflammatory cytokine levels were noted during the differentiation of MDM with MP-IC than those with MP, differences were also noted according to the source of these vesicles as mainly appreciated in our HC data. Previously, MP from patients with autoimmune diseases were reported to contain alarmins and other TLRs ligands, such as HMGB1, citrullinated peptides (CPs), nucleic acids, and chromatin (4, 5, 35). In our case, the proinflammatory responses were observed primarily with MP from patients with RA. The content of post-translational modifications, e.g., CPs in RMP (5), can directly activate these phagocytes. CPs stimulate TNF-α, IL-6, IL-1β and IL-8 production by monocytes isolated from patients with RA, and these proinflammatory cytokine levels were abrogated by a TLR4 blockade (53). Therefore, we propose that MP from patients with RA may contain more alarmins, which activate mononuclear phagocytes and promote proinflammatory responses, than those from patients with SLE.

*In vitro* studies have demonstrated that human macrophages polarized to the M1-like phenotype can switch to M2-like phenotype following changes in micro-environmental conditions; this response has been associated with the regulation of inflammation by the production of anti-inflammatory cytokines such as IL-10 and transforming growth factor-(TGF)-β (27). The reversibility of polarization plays a critical physiological and therapeutic role, especially in diseases in which an M1/M2 imbalance plays a pathogenic role, such as RA and SLE. Strikingly, in our *in vitro* model, we found that the MDM of patients with RA and HC differentiated with RMP-IC were refractory to an M2 stimulus (IL-4). Although, IC have been reported to favor differentiation toward an alternative activation of macrophages (M2b) (54), Vogelpoel et al. reported a synergistic upregulation of proinflammatory cytokines TNF-α, IL-1β and IL-6 in macrophages derived from patients with RA and HC by IC exhibiting TLR ligands (55). It is tempting to propose that in chronic autoimmune diseases, such as RA and SLE, in which high levels of MP-IC are present in the circulation, these vesicles may favor a constant differentiation of monocytes into M1-like MDM in inflamed tissues (**Figure 8**).

## MDM Differentiated With MP and MP-IC Promote T- and B-Cell Activation

One of the most important implications of macrophages in the progression of autoimmune diseases is their function as antigen presenting cells (APCs). Activated monocytes and macrophages from the synovial fluid of patients with RA function as APCs to promote pathogenic CD4 T-cell responses at this inflammation site (56). Furthermore, the correlation of macrophage infiltration and kidney dysfunction in humans supports the contribution of macrophages in SLE (57, 58). Macrophages can perform canonical and non-canonical presentations to T (54) and B cells, respectively (59). Macrophages are considered as major APCs in tissues in second antigenic challenges, without the requirement of recirculation to lymph nodes (60). Considering our results, there may be migrant monocytes in patients with RA and SLE that differentiate into MDM with a M1-like profile in the presence of MP or MP-IC, presenting self-antigens contained in these vesicles to T and B cells localized in target organs (**Figure 8**).

MP-IC seems to have important implications regarding how monocytes/macrophages modulate the response of adaptive immune cells. Importantly, although extracellular vesicles from patients with RA induced a higher proinflammatory response compared with those from patients with SLE, M1-like macrophages differentiated with RMP and RMP-IC induced a more discrete activation of T and B cells than MDM differentiated with LMP and LMP-IC. This phenomenon can have different explanations such as differences in the treatment regimen and in the amount of autoreactive and memory cells in the evaluated patients (2, 3); it may be associated also with differences in the activation profile, differentiation, epigenetically modifications or other intrinsic defects of these cells in each disease. Therefore, we considered this aspect a limitation of our study. However, these results showed some contrasting manifestations, as previously demonstrated between these patients, such as a more significant involvement of proinflammatory cytokines including TNFα in patients with RA (2, 61), and B cells and antibody producing cells in patients with SLE (3). We found that RMP-IC induced a decreased expression of HLA-DR and CD86 in MDM from patients with RA compared with MDM differentiated without vesicles. This may explain the low CD4+ and CD8+ T-cell activation and proliferation in co-cultures with MDM from patients with RA differentiated with MP-IC compared with those from patients with SLE. Furthermore, it is possible that

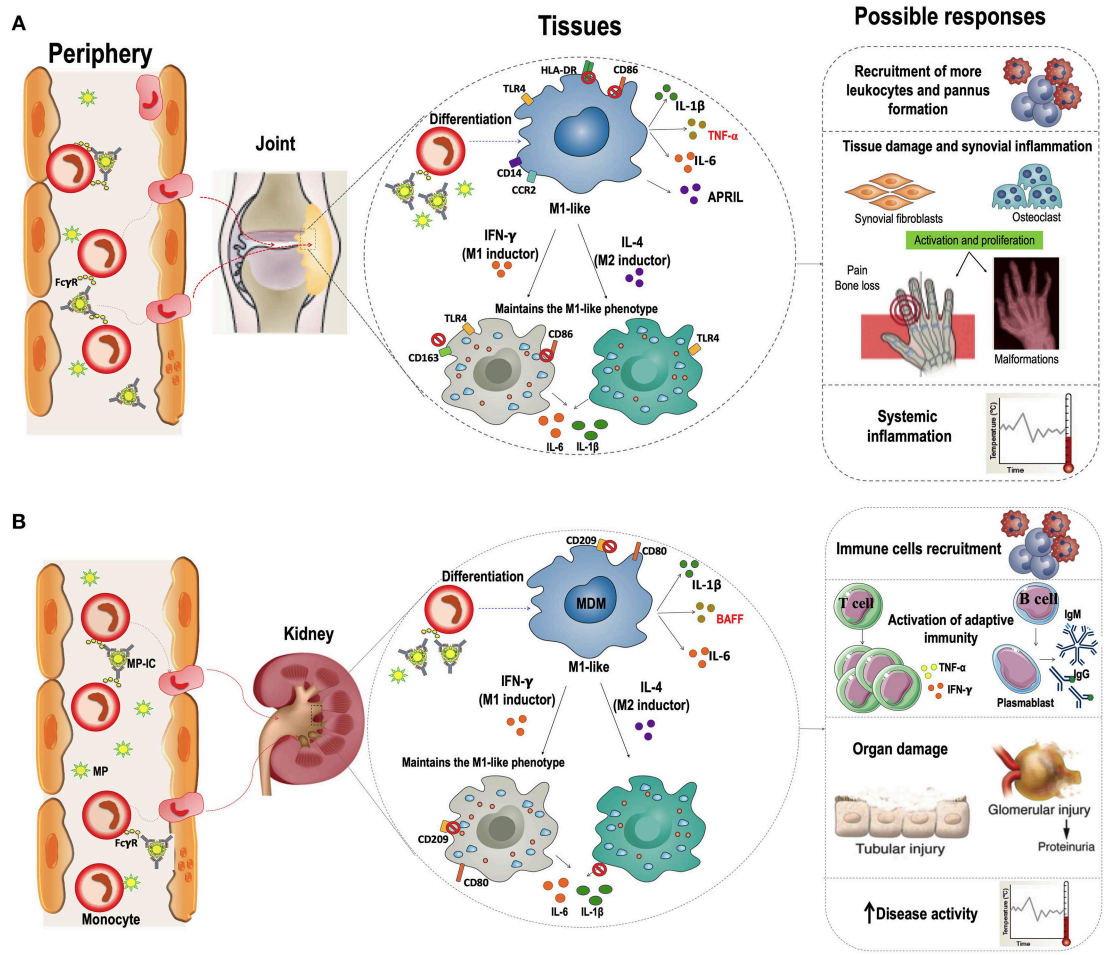


**FIGURE 8 |** Hypothetical models of the effect of macrophages differentiated in the presence of MP-IC in the RA and SLE pathogenesis. Proposed hypothetical model of the dynamic of interaction between MP-IC and monocytes in circulation and their potential contribution to promote macrophages differentiation to a proinflammatory profile in the tissues of patients with seropositive RA **(A)** and patients with active SLE **(B)**; these activated macrophages contribute to T cell activation and expansion, and B cell survival and production of antibodies; therefore, MP and specially MP-IC can participate in the pathophysiology of these autoimmune diseases, favoring the perpetuation of the chronic inflammation and autoimmune responses that affect the tissues of RA (joint, **A**) and SLE (kidney, **B**) patients.

MDM from patients with SLE make a more efficient semi-direct presentation of these vesicles than those from patients with RA; this antigenic presentation route has been primarily described as one of the mechanisms that govern the rejection of transplants (62) but until now has not been characterized in autoimmune diseases. Thus, the study of new underlying mechanisms and routes of crosstalk between these cells of innate and adaptive immunity is essential to further understand autoimmune diseases and in the design of alternative therapeutic strategies.

Interestingly in RA, only MP but not MP-IC, enhanced T-cell proliferation and activation. Indeed, APRIL was detected only in the supernatants of MDM differentiated with RMP. The APRIL receptor, a transmembrane activator and calcium-modulating cyclophilin ligand interactor (TACI), is expressed on resting and activated mature B cells (63) and activated T cells (64). Wang et al. showed that APRIL neutralization with soluble TACI immunoadhesin (TACI-Fc) *in vitro* inhibits antigen-specific T-cell activation (65). Another possible explanation of our results is related to TLR ligands present in MP (4, 35, 36). Hardenberg et al. showed that human monocyte-derived DCs secrete APRIL but not BAFF using TLR9 and TLR3 ligands (66). A different study demonstrated that synovial fibroblasts from patients with RA produce APRIL in response to TLR3 and TLR4 ligands present in the synovial fluid of these patients (67). Thus, MP-IC, at least in RA, may have contradictory roles in MDM differentiation, including the induction of an M1-like profile rather than T cells responses. RMP alone could expose more CP and induce the production of APRIL via TLR4, whereas in vesicles containing anti-CCP antibodies, there may exist some allosteric impediment for recognition by surface TLR4. However, additional studies are required to support this theory.

We found that the frequency of dead B cells decreases when they are co-cultured with autologous MDM from patients with SLE and RA and HC differentiated with or without MP and MP-IC. Strikingly, higher BAFF levels were detected in the

supernatants of MDM from patients with SLE. Reportedly, BAFF is essential for B-cell maturation and survival (68). Inflammatory stimuli, such as IC from patients with SLE, LPS, and IFN-α induce BAFF production by human monocytes and macrophages (69, 70); in addition, monocytes from patients with systemic autoimmune diseases produce more BAFF than monocytes from HC (71). This evidence suggested a potential survival mechanism for autoreactive B cells in patients with SLE, induced by MP and primarily by MP-IC indirectly. However, we should point out that autologous MDM-Unstim cells also promoted the expression of some activation markers and decreased the frequency of dead B cells of patients with RA and SLE, although the effect of MDM differentiated with MP-IC seem to be more significant. Possibly, MDM can provide soluble survival factors besides BAFF, such as IL-15 and TNF-α (72). In addition, the release of other factors such as IL-6 and IL-10 observed in these cultures may also play an important role in B-cell activation and survival.

Plasmablast differentiation was observed in the co-cultures of B cells and MDM from patients with SLE differentiated with LMP and LMP-IC. Previously, Kwissa et al. showed that CD14+CD16+ monocytes infected with dengue virus and their co-cultures with autologous B cells stimulated the differentiation of B cells into $CD27^{++}CD38^{++}$ plasmablasts, in a BAFF, TACI, and IL-10 depend manner (73). Other studies have shown that DC and monocytes stimulate B-cell differentiation into plasmablasts with BAFF- and APRIL-dependent mechanisms (74). Although we did not perform blocking assays for these cytokines, we observed that MDM-LMP-IC produce higher concentrations of BAFF in patients with SLE. Thus, this cytokine may be the potential mechanism by which MP and MP-IC indirectly stimulate plasmablast formation in patients with SLE.

Other possible routes via which MDM activate B cells include the non-canonical presentation of antigens and IC to B cells by macrophages (75). In addition, the ability to degrade the internalized cargo is impaired in the monocytes of patients with SLE (76) and in the macrophages of a lupus murine model (77). Monteith et al. showed that macrophages from lupus-prone MRL/*lpr* mice exhibit defective degradation of FcγR-bound cargo, induced by impaired lysosomal maturation and attenuated lysosomal acidification (77). Strikingly, the undegraded material of IC was recycled back to the cell membrane, and macrophages accumulated high levels of these IC on their surface. Considering this, we propose that macrophages from SLE in our model can activate B cells through a non-canonical presentation of extracellular vesicles. These defects have not been reported in phagocytic cells from patients with RA, and future studies are required to support this hypothesis.

## CONCLUSION

Despite the variability of the cell and MP sources in this study is quite large and increase the variability of results and limiting interpretation of those, we can conclude that MDM differentiated with MP-IC are more prone to an M1-like profile. As a consequence, mononuclear phagocytes differentiated with these vesicles are able to induce T-cell activation and support B-cell survival (hypothetical models presented in **Figure 8**). Excessive extracellular vesicles in the blood of patients with systemic autoimmune diseases may maintain the expansion of autoreactive T-cell clones and favor the survival of autoreactive B cells. The effect of MP and MP-IC on monocytes and macrophages seems to have clear consequences on the function and response of the adaptive immune system and therefore in the pathophysiology of autoimmune diseases. Our results also reaffirmed the notion that MP are promising therapeutic targets for patients with systemic autoimmune diseases.

## ETHICS STATEMENT

This study was conducted in accordance with the Declaration of Helsinki; the research protocol and informed consent forms were approved by the Universidad de Antioquia's Medical Research Institute and HUSVF Ethics Committees. All patients and HCs provided consent for participation in the study.

## AUTHOR CONTRIBUTIONS

CB and DC contributed to the study design, data acquisition, analysis and interpretation, and manuscript drafting. JV-V performed data acquisition and critical manuscript revision. CM-V performed clinical data acquisition and interpretation. MR and GV performed data analysis and interpretation and critical manuscript revision. All authors approved the final version of the manuscript and agreed to be accountable for all aspects of the work.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.02058/full#supplementary-material

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# Aggregated NETs Sequester and Detoxify Extracellular Histones

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In response to various infectious and sterile stimuli neutrophils release chromatin decorated with bactericidal proteins, referred to as NETs. Their scaffolds are formed from chromatin fibers which display an apparent diameter of 15–17 nm and mainly consist from DNA (2 nm) and DNA-associated histones (11 nm). The NET-forming strands are thus not naked DNA but higher ordered chromatin structures. The histones may be released from the NET, especially if their tail arginines have been citrullinated. Several studies indicate that extracellular histones are toxic for mammalian epithelia and endothelia and contribute to the microvascular dysfunction observed e.g., in patients suffering from autoimmune diseases or sepsis. NETs formed at sites of very high neutrophil densities tend to clump and form fairly stable enzymatically active aggregates, referred to as aggNETs. The latter are endowed with a bunch of enzymes that cleave, bind, and/or modify autologous as well as foreign macromolecules. The tight binding of the serine proteases to the matrix precludes the spread of these toxic enzymes into the tissue but still allows the access of soluble inflammatory mediators to the enzymatic active internal surfaces of the NETs where they are degraded. Here, we describe that externally added histones are removed from culture supernatants of aggNETs. We will address the fate of the histones and discuss the feature on the background of neutrophil-driven diseases and the resolution of inflammation.

**Keywords: histones, NET formation, aggNETs, proteolysis, autoimmunity, sepsis**

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## INTRODUCTION

Histones are a major part of nucleosomes, the basic structural unit of chromatin in the nuclei of eukaryotic cells (1). These nucleosomes each consist of two copies of the histones H2A, H2B, H3, and H4 forming an octamer with 140–150 base pairs of superhelical DNA wrapped around the histone core (2). The linker histone H1 assembles the repeating nucleosome cores into higher-order structures (3). Biosynthesis of histones takes places in the cytoplasm and many histones transiently remain there (4, 5); some reportedly accumulate on the plasma membranes (6). Extra-nuclear localization of histones is also found associated with the DNA structures in neutrophil extracellular traps (NETs), first described in 2004 as bactericidal mechanism (7). Furthermore, histones display anti-microbial activity reviewed in Hoeksema et al. (8), and have been implicated in tissue destruction, sepsis (9), and thrombosis (10). Mechanistically, histones reportedly display direct cytotoxic effect on eukaryotic cells (11), may directly activate phagocytes (12) and platelets (13).

Since their first description in 2004, NETs are now known to play a role in physiology and pathology (14). In high densities these NETs tend to aggregate; these aggNETs, first described to be induced by monosodium urate crystals (MSU) orchestrate the resolution of inflammation in gout by the degradation of inflammatory cytokines (15). With increasing cell densities the proteolytic degradation of cytokines/chemokines outweighs their release (16). The granular neutrophil elastase, a major protein of NETs and aggNETs, degrades various proteins of the extracellular matrix or immunoglobulins (17). Here, we describe that histones are sequestered and detoxified by aggNETs and that this increased viability of epithelial cells in contact to extracellular histones.

# MATERIALS AND METHODS

## Preparation of AggNETs

We isolated polymorphonuclear cells (PMN) from healthy donors (permit #193/13B from the local ethical committee; written informed consent of participants) by Ficoll density gradient (Lymphoflot, Bio-Rad Laboratories, Inc.) as described previously (18). The granulocytes were then incubated with 50 pg/cell monosodium urate crystals (MSU) for 18 h at 37°C. Successful formation of aggNETs is visible without magnification as depicted in **Figure S1E** in bright-field as well as under UV (∼312 nm excitation) after staining with 1 mg/ml propidium iodide (Sigma-Aldrich) for 2 h. Macrophotographs were taken using a Nikon D700.

## Biotinylation of Histones

We biotinylated calf thymus histones (Sigma-Aldrich) using the EZ-Link™ Sulfo-NHS-LC-Biotinylation Kit (Thermo Fisher Scientific) according to manufacturer's instructions.

## Treatment of Histones With AggNETs/Neutrophil Elastase/Proteinase3

We incubated 1 mg/ml of biotinylated histones with or without (1) aggNETs, (2) 5 mU Neutrophil Elastase (Sigma-Aldrich) or, (3) 5 mU Proteinase3 (Elastin Products Company) in RPMI 1640 medium (Thermo Fisher Scientific) for 24 h at 37°C. If indicated we added the neutrophil elastase inhibitors Sivelestat (6.6 μM) or Elafin (166 μM) (both Sigma-Aldrich).

## SDS-PAGE and Western Blot Analysis

We added 5x PAGE-buffer (2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue in 60 mM Tris-Cl pH 6.8) to the samples and denatured them at 95°C for 10 min. SDS-PAGE was performed using SERVAGel™ TG PRiME™ 4–20% gels (SERVA Electrophoresis GmbH) for 2.5 h at 100 V. Gels were either transferred onto an Immobilon-P$^{SQ}$ PVDF membrane (Merck Millipore Ltd.) using a Trans-Blot® SD Semi-Dry Transfer Cell (Bio-Rad Laboratories, Inc.) for 1 h at 350 mA or stained with 0.1% Coomassie Brilliant-Blue-G250 (Sigma-Aldrich). Macrophotographs of the Coomassie gels were taken using a Nikon D700. Membranes were blocked with 5% powdered milk (Carl Roth) in Tris-buffered saline (TBS) for 2 h at RT. We detected histone H1 employing rabbit anti-human histone H1.0 antibody [EPR6536] (ab134914, Abcam) overnight at 4°C followed by goat anti-rabbit IgG HRP Antibody (4030-05, Southern Biotech) for 1 h at RT. Biotinylation was detected with Pierce™ High Sensitivity Streptavidin-HRP (21130, Thermo Fisher Scientific). We developed Blots using Celvin® S-320+ (Biostep).

## Prediction and Visualization of Neutrophil Elastase Cleaving Sites

We used the sequence of bovine histone H1.3 (A7MAZ5, UniProtKB) to model its structure with SWISS-MODEL (19). Neutrophil Elastase cleavage sites on histone H1.3 were predicted using the ExPASy PeptideCutter tool (20) and were visualized using the RasMol Molecular Graphics Visualization Tool V2.7.5 (21).

## *In vitro* Histone Cytotoxicity Assay

Analyses by flow cytometry of HeLa cells treated with soluble histones or aggNET pre-treated histones was performed using the four color staining method adapted from Janko et al. (22) and Munoz et al. (23). Briefly, 24 h after seeding of HeLa cells into CELLSTAR® 24-well plates (Greiner Bio-One GmbH), the cells were treated for 1 h with 500 μg of histones, histones pre-incubated with aggNETs or aggNET supernatant in serum-free medium. Mock-treated cells served as controls. After removal of the media to fresh tubes, we washed the cells with DPBS (Thermo Fisher Scientific), detached them using Trypsin/EDTA (Merck) and combined them with the original media. After centrifugation, cells were resuspended in Ringer's solution (Fresenius Kabi) containing 1 μg/ml AnnexinA5, 1 μg/ml propidium iodide, 1 μg/ml Hoechst33342, and 10 nM 1,1′-dimethyl-3,3,3′,3′-tetramethylindodicarbocyanine iodide. Flow cytometry was performed using a Gallios Flow Cytometer (Beckman-Coulter) and Kaluza Analysis Software V2.1 (Beckman-Coulter). Graphs were created using Prism® V5.03 (GraphPad Software). Pictures of cells were taken using a Canon Eos 6D, the Eos Utility3 software (both Canon) in combination with an Axiovert 25 inverted microscope (Carl Zeiss) and the Adobe Photoshop CS5 V12.0.1 (Adobe Systems).

# RESULTS

## AggNETs Proteolytically Degrade Histones

Incubation of calf thymus histones with aggNETs for 24 h results in a complete degradation of histone H1 (**Figure 1A**) as shown by Coomassie staining of protein. Histone H1 was only detected by Western Blot analysis in the untreated sample, but neither in the aggNET-treated sample nor in the aggNET itself. We biotinylated the histone samples to exclude that the epitope recognized by the antibody was cleaved and therefore not recognized by Western Blotting. The biotinylation was again only detected in the untreated sample but neither in the aggNET-treated ones nor in the aggNETs. Proteinase3 (PR3) and Neutrophil Elastase (NE) are hallmark proteases located in the azurophilic granula of viable neutrophils and on the surfaces of aggNETs. As shown in **Figures 1B,C**, PR3 and NE degrade histone H1; the reaction is prevented by the specific inhibitors Elafin and Sivelestat, respectively. Prediction

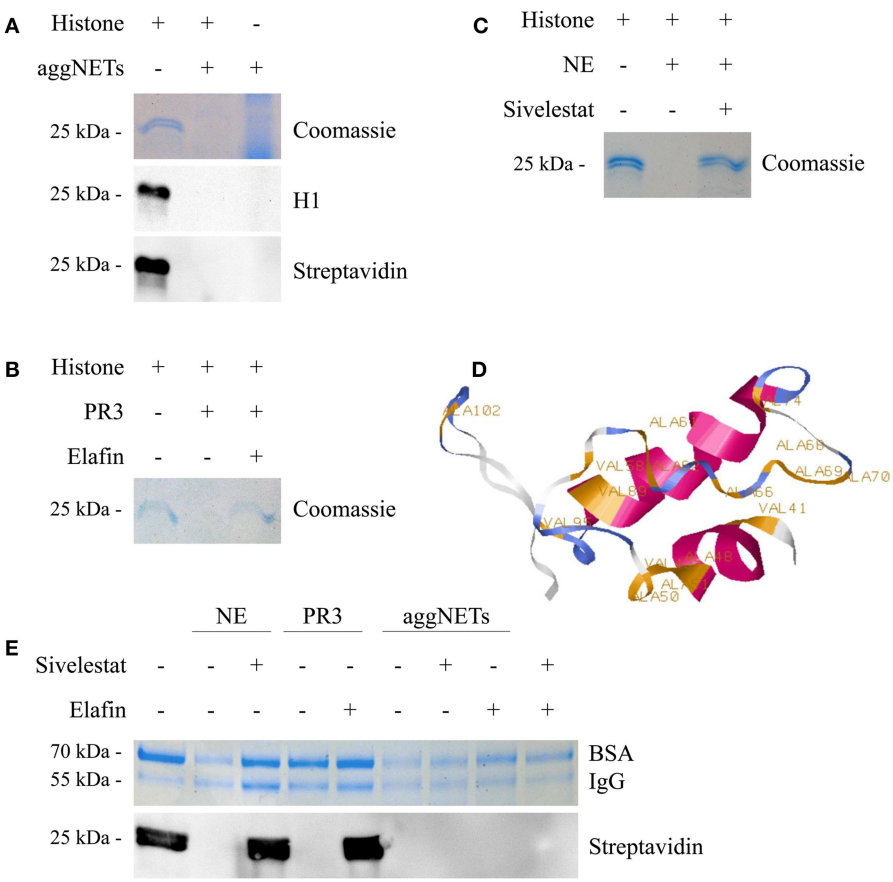


**FIGURE 1 |** aggNETs degrade histones. **(A)** Histones incubated with aggNETs are degraded as seen in Coomassie and staining and anti-histone H1 Blot. The biotinylationed histones are not bound by the aggNETs. **(B)** Proteinase3 (PR3) degrades histones. This degradation is inhibited by Elafin as seen in the Coomassie staining. **(C)** Neutrophil Elastase (NE) degrades histones, specifically inhibited by Sivelestat as shown in the Coomassie staining. **(D)** SWISS-MODEL of histone H1 (amino acids 39–119) with the cleavage sites for NE as predicted by ExPASy PeptideCutter. **(E)** NE and aggNETs favor histone over bovine serum albumin (BSA) and human Immunoglobulin G (IgG) for degradation; whereas PR3 can only degrade histones. Degradation of biotinylated histones by aggNETs is not inhibited by Sivelestat or Elafin or a combination of both as seen by the detection of Streptavidin HRP in Western Blot analysis. SDS-PAGE, Western Blot Analysis and Coomassie staining in **(A–C)** were performed after incubation of the samples for 24 h at 37°C. For **(E)** the incubation time was 8 h at 37°C. All images shown are representative images of at least three independent experiments. The full-sized images are shown in **Figures S1A–D**. The successful formation of an aggNET is shown in the macrophotographs in **Figure S1E** in bright-field and under UV after staining with propidium iodide.

by ExPASy PeptideCutter shows that bovine histone H1.3 (amino acids 39–119) exhibits various cleavage sites for NE (**Figure 1D**). Importantly, this degradation favors histone over bovine serum albumin (BSA) or human immunoglobulin G (IgG) (**Figure 1E**). Only NE and aggNETs but not PR3 slightly decrease the amount of full-size BSA and IgG. For NE, this was prevented by its specific inhibitor Sivelestat. Surprisingly, neither the addition of Sivelestat nor of Elafin nor a combination of both blocked the degradation of histones by aggNETs at any given time point and concentration.

## AggNET-Treatment of Histones Attenuates Cellular Cytotoxicity

As soon as 1 h after treatment with 500 μg/ml histone mix HeLa cells are in a bad shape, increase clustering and apparently die as displayed in the bright-field microscopic images (**Figure 2A**). Pre-treatment of histones with aggNETs prevented this fate. The supernatants of aggNET (aggNET-SN) did not affect the viability of the cells. Flow cytometry revealed that culture in the presence of histones markedly reduced viability and increased apoptosis and necrosis in HeLa cells (**Figure 2B**). This histone-mediated cytotoxicity is attenuated by pre-treatment with aggNETs. Detailed analyses of the different forms of cell death is depicted in **Figure 2C** and showed that the pre-treatment with aggNETs significantly decreased early apoptotic, apoptotic and primary necrotic cells; the population of secondary necrotic HeLa cells was only slightly increased. HeLa cells co-cultured with aggNET-SN show comparable viability as medium controls. Therefore, we can exclude that the incomplete rescue in aggNET pre-treated histones is caused by toxic aggNET- derived mediators.

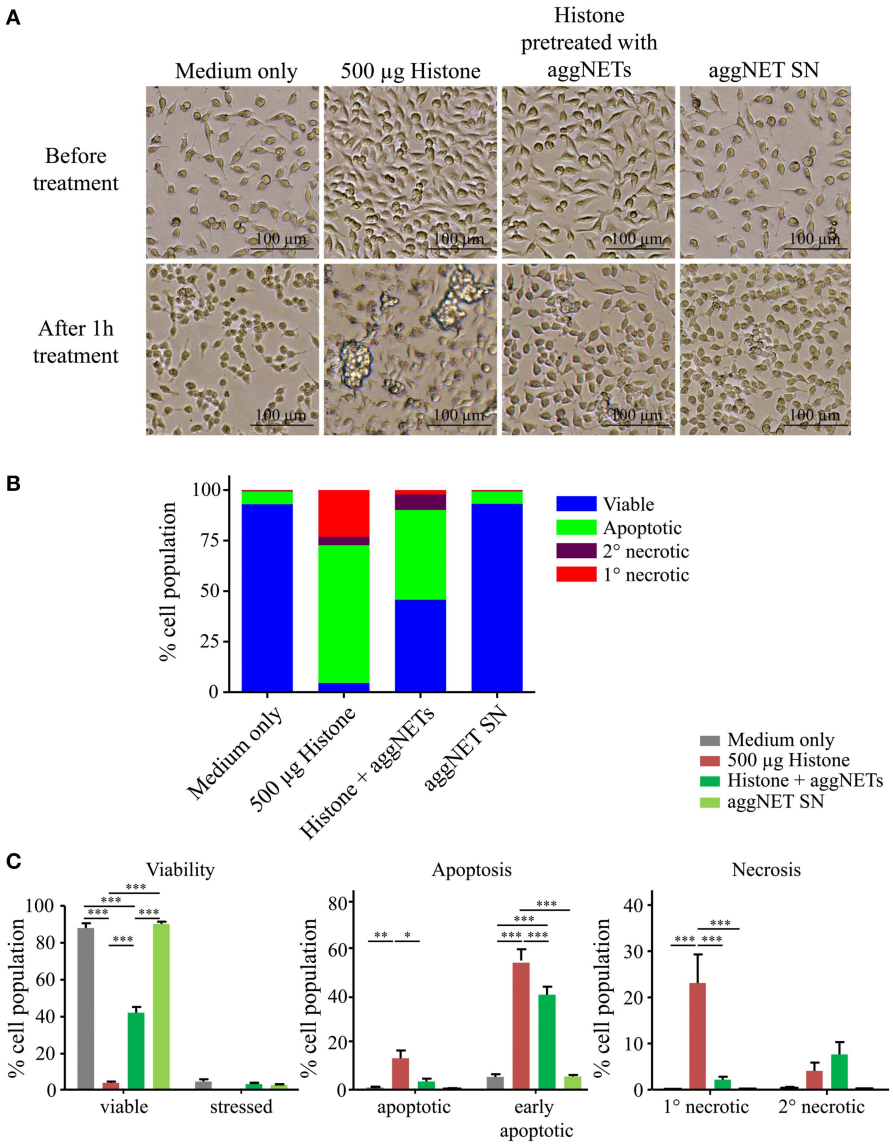


**FIGURE 2 |** Pre-treatment of histones with aggNETs attenuates histone-mediated cellular cytotoxicity. **(A)** Light microscope images in 10x magnification of HeLa cells before and after treatment with (1) 500 µg histones, (2) histones pre-treated with aggNETs for 24 h or (3) aggNET supernatant (SN). **(B)** Overview of different forms of cell death of HeLa cells after treatment assessed by flow cytometry. Pre-treatment of histones with aggNETs increases viability of HeLa cells. **(C)** Detailed analysis of the different forms of cell death. Viability of HeLa cells incubated with aggNET-treated histones is significantly increased compared to histone treatment due to a reduction in early apoptosis, apoptosis and primary (1°) necrosis. Standard error of mean was calculated from three independent experiments. $^{***}p \leq 0.001$, $^{**}p \leq 0.01$, and $^{*}p \leq 0.05$ as determined by Two-way ANOVA with Bonferroni post testing. The gating strategy for flow cytometer analysis is depicted in **Figure S2**.

## DISCUSSION

Here, we show for the first time that aggNETs sequester and degrade histones, and thus attenuate their cytotoxic effect on epithelial cells. This process was executed by at least two aggNET-borne serine proteases, NE and PR3. We already have demonstrated the ability of aggNETs to resolve inflammation by the proteolytical degradation of inflammatory cytokines and chemokines (15, 16). NE is established to degrade various proteins, such as immunoglobulins and extracellular matrix components (17, 24). The degradation of histones by NE and PR3 was inhibited by Sivelestat or Elafin, respectively. Importantly, the degradation of histones by aggNETs was resistant to the two inhibitors. Interestingly, a decreased inhibitory capacity of the natural proteinous inhibitors α-1 anti-trypsin and ß2-macroglobulin for membrane-associated NE was already reported before the first description of NET formation (25).

The cytotoxic effect of histones on epithelial cells described here, confirms already existing literature (9, 12). This is especially true when the histone release is exaggerated and not properly

controlled. Here, we analyze this cytotoxic effect in more detail using a four color staining method to discriminate between different states of apoptosis and necrosis as described previously (22, 23). The addition of histones to HeLa cells induced profound apoptosis and necrosis (>90% of the cells). This can be partially rescued pre-treating the histones with aggNETs. This procedure increased viability to 50%. To examine if toxic proteins/peptides are released from the aggNETs, we also co-cultured the cells with aggNET-SN only; the cell viability did not differ from medium controls. It is conceivable that some of the small histone-derived peptides, too small to be detected in PAGE, retain residual cytotoxic activities.

Extracellular histones are described as major mediators of death in sepsis due to their contribution to endothelial injury and dysfunction, hemorrhage, thrombosis and organ failure (9). Released histones potentially act as damage-associated molecular pattern molecules (DAMPs) (26) and signal through toll-like receptors (TLR) 2 and 4 leading to a massive pro-inflammatory cytokine production (27). Moreover, histones are shown to enhance plasma thrombin generation and the blood clotting process by involvement of the platelet TLR2 and TLR4 (13). Histones are further released during trauma or severe cellular stress mediating their cytotoxicity by triggering an increased calcium flux in immune and endothelial cells (12).

Histones, released during NET formation, were described in the circulation of patients suffering from autoimmune diseases such as systemic lupus erythematosus (28) or rheumatoid arthritis (29) and were discussed to cause NET-associated tissue destruction. Histones not only act as autoantigens but also prevented the degradation of DNA by the formation of DNA-histone complexes (28). Here we describe that aggNETs degrade and detoxify histones and thus contribute to the resolution of histone-induced inflammatory reactions. If this also takes place *in vivo* and how it can be further enhanced to completely rescue cells from histone-mediated cytotoxicity, needs further investigation. We conclude that histones are targeted by aggNETs for degradation. This leads to a decreased cytotoxicity of histones and, therefore, fosters the resolution of inflammation.

## ETHICS STATEMENT

All analyses of human material were performed in full agreement with institutional guidelines and with the approval of the Ethical committee of the Universitätsklinikum Erlangen (permit # 193 13B). Participants gave written informed consent.

## AUTHOR CONTRIBUTIONS

JK planned and performed experiments, conducted data analysis, and wrote the manuscript. ML, GS, MH, and LM supervised the project, planned experiments, performed data analysis, and wrote the manuscript. All authors read and approved the manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.02176/full#supplementary-material

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# Stimulation of Mononuclear Cells Through Toll-Like Receptor 9 Induces Release of Microvesicles Expressing Double-Stranded DNA and Galectin 3-Binding Protein in an Interferon-α-Dependent Manner

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**Background:** Microvesicles (MVs) expressing the type 1 interferon (IFN)-inducible protein galectin-3 binding protein (G3BP) may play a pathogenic role in systemic lupus erythematosus (SLE). Co-expression of double-stranded DNA (dsDNA) on such MVs may render them immunogenic and targets for anti-dsDNA antibodies. Little is known about the mechanisms underlying generation of this MV population. In this study, we investigated how Toll-like receptors (TLRs), IFN-α, and T cells are involved in this process in healthy subjects.

**Methods:** Peripheral blood mononuclear cells (PBMCs) isolated from 12 healthy donors were stimulated *in-vitro* for 24 h with a series of TLR-agonists or the T cell activating antibody OKT3 or were subjected to apoptosis by incubation with staurosporine. MVs in the supernatants were subsequently isolated by differential centrifugation and were quantified and characterized with respect to expression of G3BP and dsDNA by flow cytometry.

**Results:** Stimulation of PBMCs with the TLR9-agonist and strong IFN-α inducer ODN2395 significantly increased the release of MVs expressing G3BP. The production of MVs with this phenotype was markedly enhanced by co-stimulation of T cells. Furthermore, dependency on IFN-α in the generation of G3BP-expressing MVs was indicated by a marked reduction following addition of the IFN-α inhibitor IFN alpha-IFNAR-IN-1 hydrochloride.

**Conclusion:** Release of G3BP-expressing MVs from healthy donor PBMCs is induced by stimulation of TLR9 in an IFN-α-dependent manner and is enhanced by co-stimulation of T cells.

**Keywords: peripheral blood mononuclear cells, T cells, microvesicles, Toll-like receptor 9 agonist, type 1 interferon, galectin-3 binding protein, dsDNA, systemic lupus erythematosus**

## BACKGROUND

Extracellular vesicles (EVs) are double-layered membrane vesicles that may be released by cells in response to activation or during apoptosis (1, 2). Accumulating evidence shows that EVs are not inert, but usually carry an orchestrated cargo of proteins and nucleic acids with diverse physiological roles in health and disease (3, 4). These include diverse paracrine functions, with extracellular RNAs playing a central role (5), but it is recognized that also other mechanisms should be explored (6). There is a growing interest and understanding of the role of EVs in the context of systemic autoimmune disease, in particular systemic lupus erythematosus (SLE) (7).

EV studies in SLE have mainly focused on so-called microparticles that comprise 0.1–1 μm microvesicles (MVs) and the somewhat larger apoptotic bodies (8). SLE MVs carry double-stranded DNA (dsDNA), whereby they may become targets for binding of anti-dsDNA antibodies (9). Proteomics show that circulating MVs from SLE patients hold a characteristic signature of increased expression of galectin-3 binding protein (G3BP), immunoglobulin G (IgG), and several other proteins (10, 11). Complementary to these findings are flow cytometric analyses showing elevated blood levels of MVs with surface-bound IgG (12) or G3BP (13) in SLE patients. The origin and pathogenic relevance of this subset of MVs remain obscure. However, defective removal of cellular remnants and immune complexes (ICs) are well-established elements of SLE pathogenesis (14), and specific roles of dsDNA-loaded MVs in this context have been suggested (15, 16). In SLE patients with nephritis, deposits of IgG in the glomerular basement membrane (GBM) colocalize with chromatin forming electron dense structures (EDS) (17) that also contain G3BP (13).

G3BP is a type 1 interferon (IFN)-inducible protein that belongs to the scavenger receptor cysteine-rich (SRCR) superfamily (18) and binds to several components of the GBM, including nidogen, collagen IV, and fibronectin (19)–a property which, in theory, renders G3BP-expressing MVs glomerulophilic.

The increased production of IFN-α frequently found in SLE patients with active disease is thought to prime the immune system toward breach of self-tolerance and persistent autoimmune reactions and appears to be linked to Toll-like receptor (TLR)7 and TLR9 ligation by nucleic acid-containing ICs (20). The notion has been carried forward that ICs are presented to the immune system in the context of chromatin-loaded vesicles formed during apoptosis (16), but the role of smaller MVs formed during cellular activation has not been investigated.

Given that G3BP is type 1 IFN-inducible (21), we speculated that MVs expressing dsDNA and G3BP may be released from mononuclear cells as a result of TLR- and type 1 IFN-mediated activation. Further, since activated T cells may enhance TLR-mediated IFN-α production (22), T cells may play a particular role in this type of MV generation.

In this study, we tap into this hypothesis by stimulating peripheral blood mononuclear cells (PBMCs) from healthy subjects with different TLR ligands, a T cell stimulator and a type-1 IFN inhibitor to quantify the generation of dsDNA- and G3BP-expressing MVs.

## MATERIALS AND METHODS

### Blood Donors

Blood from anonymous healthy donors was obtained from the Blood Bank at Copenhagen University Hospital, Rigshospitalet. The study was approved by the Scientific-Ethical Committee of the Capital Region of Denmark (protocol no. H-15004075).

### Isolation and Staining of Platelets

Three milliliter of blood was collected in Multiplate® Hirudin blood tubes (Roche Diagnostics GmbH, Mannheim, Germany) by venous puncture. The blood was centrifuged at 1,800 × g for 10 min at 21°C, platelet-rich plasma was carefully aspirated, and 1 mL was transferred to Eppendorf tubes (Corning, New York City, USA) and centrifuged at 3,000 × g for 10 min at 21°C to pellet platelets. The platelet-poor plasma was carefully aspirated and discarded, and platelets were gently resuspended in 300 μL phosphate-buffered saline (PBS) (Thermo Fisher Scientific, Waltham, USA) filtered through a 0.2 μm filter (Sartorius, Göttingen, Germany). Five microliter of the platelet-isolate was pipetted into 37.5 μL filtered PBS in FACS tubes (Corning) followed by addition of 5 μL APC-conjugated anti-CD61 antibody (BD, Franklin Lakes, USA) and 2.5 μL calcein-acetoxymethyl ester (calcein-AM) (Sigma-Aldrich, St. Louis, USA) (2.5 μg/ml in filtered PBS) to a final volume of 50 μL. The tubes were incubated for 1 h at room temperature (RT) in the dark. After incubation, 100 μL TruCount beads (BD) and 125 μL Megamix-Plus side-scatter (SSC) beads (Biocytex, Marseille, France) were added to the tubes. The volume was adjusted to 300 μL with filtered PBS before analysis. The TruCount bead solution was prepared by dissolving the lyophilized beads in 500 μL filtered PBS. Samples were analyzed on a FACSCanto II flow cytometer (BD) at low flow-rate and with lowest SSC threshold (=200).

### Isolation of PBMCs

Blood was collected in Vacutainer® EDTA tubes (Greiner Bio-one GmbH, Kremsmünster, Austria) by venous puncture and centrifuged at 1,800 × g for 10 min at 21°C. Platelet-rich plasma was aspirated, and the PBMCs were poured onto a density gradient medium (Lymphoprep™; Alere Technologies, Oslo, Norway), centrifuged at 1,172 × g for 30 min at 24°C, washed twice in sterile PBS, and finally resuspended in sterile medium consisting of RPMI-1640 GlutaMAX medium (Lonza, Basel, Schweiz) supplemented with 20% heat-inactivated fetal calf serum (hFCS) (Sigma-Aldrich) and 0.1% gentamicin (BI, Kibbutz Beit Haemek, Israel). PBMCs were subsequently counted using the NucleoCounter® NC-100™ system (ChemoMetec, Allerød, Denmark) according to the manufacturer's instructions. The PBMC-isolate was divided into 500 μL aliquots in cryotubes (DACOS, Esbjerg, Denmark), followed by addition of 500 μL sterile medium supplemented with 30% hFCS and 20% dimethylsulfoxid (DMSO) (Merck kGaA, Darmstadt, Germany), yielding a final concentration of 25% hFCS and 10% DMSO. The cryotubes were inverted, placed in CoolCell® freezing containers

(BioCision, San Rafael, USA), and stored at −80°C for at least 24 h, before they were cryopreserved.

## Stimulation of PBMCs

The cryopreserved PBMCs were thawed at RT, washed and resuspended in sterile medium supplemented with 20% hFCS (Sigma-Aldrich) and 0.1% gentamicin (BI). Their viability was confirmed using the NucleoCounter® NC-100™ system according to the manufacturer's instructions. The cells were plated into 48-well plates with UpCell™ surface (Nunc, Roskilde, Denmark) at ∼600,000 PBMCs per well and were rested for 30 min at 37°C and 5% $CO_2$ before incubation for 24 h at 37°C and 5% $CO_2$ with the following components or combinations hereof: staurosporine for induction of apoptosis (Abcam, Cambridge, UK) (2.5 μM); the TLR3-agonist poly(A:U) (Invivogen, San Diego, USA) (20 μg/mL); the TLR4-agonist lipopolysaccharide (LPS) (Invivogen) (1.25 μg/mL); the TLR7-agonist gardiquimod (Invivogen) (1.5 μg/mL); the TLR9-agonists ODN2006 (Invivogen) (12 μg/mL) or ODN2395 (Invivogen) (12 μg/mL) (23); the inhibitor of the interaction between IFN-α and the IFN-α receptor (IFNAR) IFN alpha-IFNAR-IN-1 hydrochloride (IN-1) (MedchemExpress, Sollentuna, Sweden) (32 μM) (24); the T cell stimulating anti-CD3 antibody OKT3 (Invitrogen, Carlsbad, USA) (1 μg/mL) (25).

## Preparation of Culture Supernatants

After incubation with stimuli the plates were left at RT for 15 min. Adhered cells were gently loosened and transferred to FACS tubes. The cell suspensions were centrifuged at 458 × g for 10 min at 24°C to pellet PBMCs. The cell-free supernatants were then harvested, aliquoted into cryotubes, and snap-frozen in liquid nitrogen. Samples were stored at −80°C until analysis.

## Isolation of MVs From Culture Supernatants

The frozen cell-free supernatants were thawed at RT, transferred to Eppendorf tubes, and centrifuged at 3,000 × g for 10 min at 21°C to pellet larger particles and potential cell residues. The supernatants were aspirated down to 50 μL and transferred to new tubes (200 μL in each). For some experiments, DNase solution (Stemcell Technologies, Vancouver, Canada) was added to the tubes to a final concentration of 0.1 mg/ml (∼200 U/mL) and the tubes were then incubated for 1 h at 37°C and 5% $CO_2$. The samples were ultracentrifuged at 20,000 × g for 30 min at 21°C to pellet MVs. Subsequently, 175 μL supernatant was aspirated and discarded, and MVs were then resuspended in 175 μL PBS filtered through 0.2 μm pores, followed by another ultracentrifugation step. The supernatant was aspirated as before and discarded, and MVs were resuspended in 70 μL filtered PBS to a final volume of 95 μL (MV-isolate).

## Detection and Staining of MVs

We used calcein as a general marker of MVs (26). Any Fcγ-receptors on MVs were blocked by adding 5 μL commercial Fc blocker (BD) to MV-isolates for 15 min at RT. After this incubation, 5 μL MV-isolate was pipetted into 22.5 μL filtered PBS in FACS tubes followed by addition of 5 μL mouse anti-human G3BP antibody of IgG2b isotype (clone: 2D8E11) (Proteintech, Manchester, UK) (1 μg/mL in filtered PBS) or isotype control (clone: MG2b-57) (Biolegend, San Diego, USA), 5 μL mouse anti-dsDNA antibody of IgG2a isotype (clone: HYB 331-01) (SSI, Copenhagen, Denmark) (0.5 μg/mL in filtered PBS), or isotype control (clone: MG2a-53) (Biolegend). Next, 5 μL APC-conjugated goat anti-mouse IgG2b antibody (Southern Biotech, Birmingham, USA) (0.5 μg/mL in filtered PBS), 5 μL BV510-conjugated rat anti-mouse IgG2a antibody (BD) (0.5 μg/mL in filtered PBS), and 2.5 μL calcein-AM (2.5 μg/mL in filtered PBS) were added, yielding a final volume of 50 μL. Unstained and single-stained controls were included. The tubes were incubated for 1 h at RT in the dark. After incubation, 100 μL TruCount beads (BD) were added to the tubes and the volume was adjusted to 300 μL with filtered PBS before acquisition on a FACSCanto II flow cytometer (BD) at low flow-rate and with lowest SSC threshold (=200). The TruCount bead solution was prepared by dissolving the lyophilized beads in 500 μL filtered PBS. Each sample was run for 4 min or until a minimum of 1,000 TruCount bead events were recorded.

## Quantification and Size Determination of MVs

The absolute count of MVs (MVs/μL) was calculated with TruCount beads as reference, using the formula:

[[(no. of MV events within gates of interest)/(no. of collected bead events)] × [(total no. of beads)/(test volume)]] × (dilution factor).

Megamix-Plus SSC beads–a SSC optimized mixture of polystyrene beads with size references of 0.16, 0.2, 0.24, and 0.5 μm–were utilized to define a SSC specified MV gate. Due to the higher refractive index (RI) of polystyrene relative to that of MVs (lipid vesicles), these size references are not directly translatable, as described by van der Pol et al. and others (27–30). To allow for such discrepancy, we estimated lipid vesicle equivalents based on Mie theory (31–33) by taking the RI of the particles and surrounding medium, the collection angle of the scattered light, and the illumination wavelength and intensity into account. All estimations were made with the free software Mieplot (www.philiplaven.com/mieplot.htm). The resulting plots (**Supplementary Figure 1**) depict that the SSC light of 0.2 and 0.24 μm polystyrene beads corresponds to that of 0.5 and 1.0 μm lipid vesicles, respectively, and the SSC light of 0.5 μm polystyrene beads corresponds to that of 2.7 μm lipid vesicles.

## Transmission Electron Microscopy

MV-isolates were adsorbed onto carbon-coated grids for 1 min. The excess liquid was removed with filter paper, and the grids were then washed in double-distilled water prior to staining with 3% uranyl acetate solution for 1 min. Using the principle of negative staining, the samples were analyzed on a CM100 transmission electron microscope (Philips, Eindhoven, Netherlands).

## Statistical Analysis

Wilcoxon signed-rank test was used for comparisons of concentrations and ratios between paired samples. In cases where

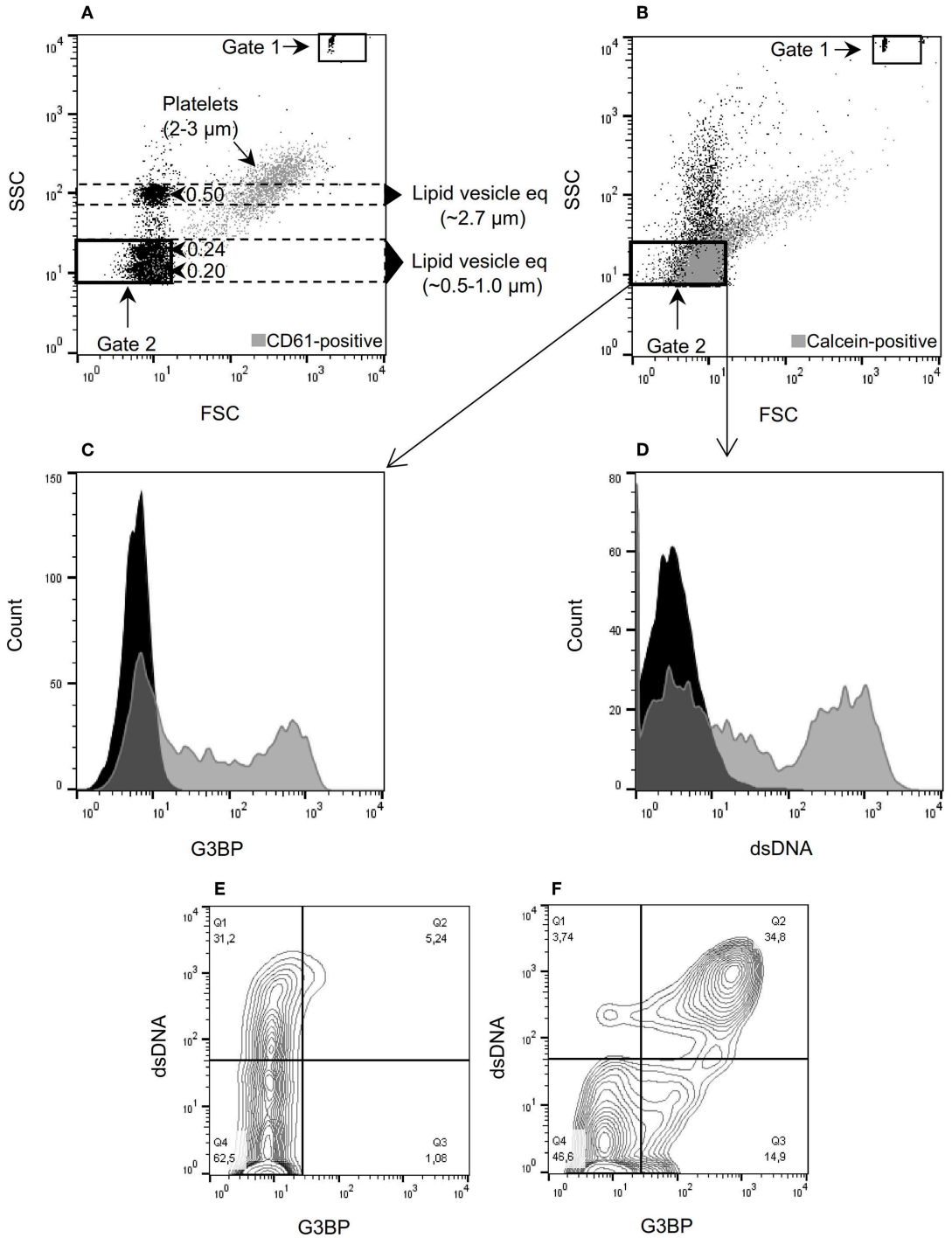


**FIGURE 1 |** Microvesicle staining, gating, and quantification. **(A)** Megamix-Plus side-scatter (SSC) beads (polystyrene) with size references of 0.16, 0.2, 0.24, and 0.5 µm were applied to define a flow cytometric SSC-based gate for microvesicles (MVs) (defined as lipid vesicles). Lipid vesicle equivalents (eq) of the indicated sizes were estimated, taking the different refractive indices of polystyrene and lipid vesicles into account. Gate 1 contains TruCount beads used for quantification of MVs. Gate 2 corresponds to the MV gate used throughout the study. For comparison, normal platelets stained with anti-CD61 antibody were added to the sample (gray events). **(B)** Forward-scatter (FSC)/SSC characteristics of MVs isolated from culture supernatants and stained with calcein (gray events). **(C)** MVs contained in culture supernatants from peripheral blood mononuclear cells (PBMCs) incubated with the TLR9-agonist ODN2395. The MVs were incubated with calcein and anti-G3BP antibody (gray) or isotype control (black). **(D)** Corresponding histogram after staining with anti-dsDNA antibody (gray) or isotype control (black). **(E)** Contour plot of MVs released from non-stimulated PBMCs and stained for G3BP (x-axis) and dsDNA (y-axis). **(F)** Corresponding contour plot of MVs released from PBMCs stimulated with ODN2395. Events within gate 2 are shown in **(C)** through **(F)**.

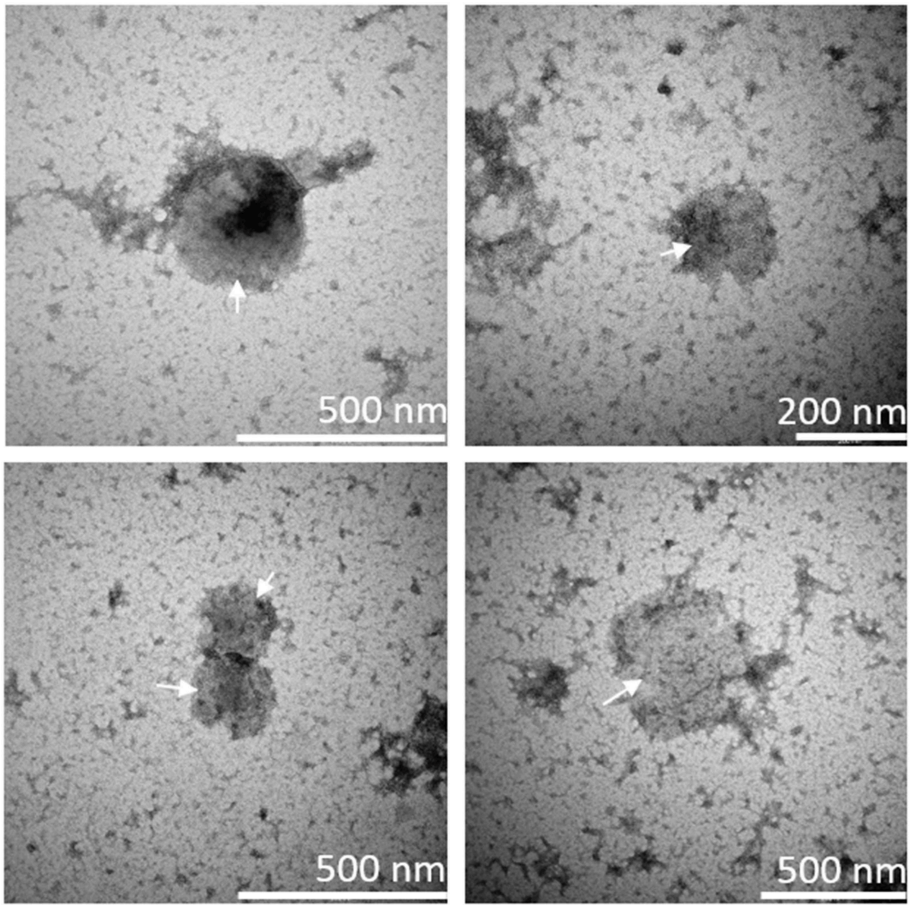


**FIGURE 2 |** Presence of microvesicles in culture supernatant. Microvesicles **(**MVs) in culture supernatant from ODN2395-stimulated peripheral blood mononuclear cells were visualized by means of transmission electron microscopy using the negative stain principle. Arrows indicate round-shaped particles within the size range of MVs as indicated by the scale bars in the lower right corners.

the background (non-stimulated controls) was subtracted, the test was used to assess if the net values differed from zero. The statistical analysis was performed in GraphPad Prism software 8 (GraphPad Software Inc., San Diego, USA). $P < 0.05$ were considered statistically significant.

# RESULTS

## Flow Cytometric Measurement of MVs

MVs isolated from a total of 12 healthy donors [10 women and 2 men, median age 26 years (range 22–63)] were analyzed using the flow cytometry gating shown in **Figure 1**. TruCount beads (**Figures 1A,B**, gate 1) were used for quantification and Megamix-Plus SSC beads (0.16, 0.2, 0.24, and 0.5 μm) were used as size reference to define the MV gate (**Figures 1A,B**, gate 2). Normal platelets (**Figure 1A**, gray events) of ∼2–3 μm in diameter (34) served to validate the estimated lipid vesicle equivalents. In agreement with the predictions, the SSC light of platelets showed considerable overlap with the 0.5 μm bead population (**Figure 1A**, y-axis). The majority of detectable calcein-positive events in the differentially centrifuged culture supernatants (**Figure 1B**, gate 2, gray events) localize within the predicted MV gate, supporting that most of these events are in the size range of MVs. Treatment of culture supernatants with detergent (1% Triton X-100) prior to isolation of MVs abolished the signal from calcein, confirming the lipid nature of calcein-positive events (**Supplementary Figure 2A**). Moreover, MVs were only detectable in supernatants from setups containing added PBMCs, confirming that the signal from calcein within the MV gate is derived completely from the experimental cells and not from artifacts or residual MVs potentially present in the hFCS (**Supplementary Figure 2B**). We also evaluated the MV assay for coincident events which might cause false colocalization signals, but such phenomenon was not observed (**Supplementary Figure 2C**).

## Identification of MVs With Transmission Electron Microscopy

The presence of MVs in culture supernatants was confirmed by use of transmission electron microscopy. Specifically, culture supernatants from ODN2395-stimulated PBMCs were investigated; round-shaped particles within the MV-size range were identified, showing the presence of MVs in these supernatants (**Figure 2**).

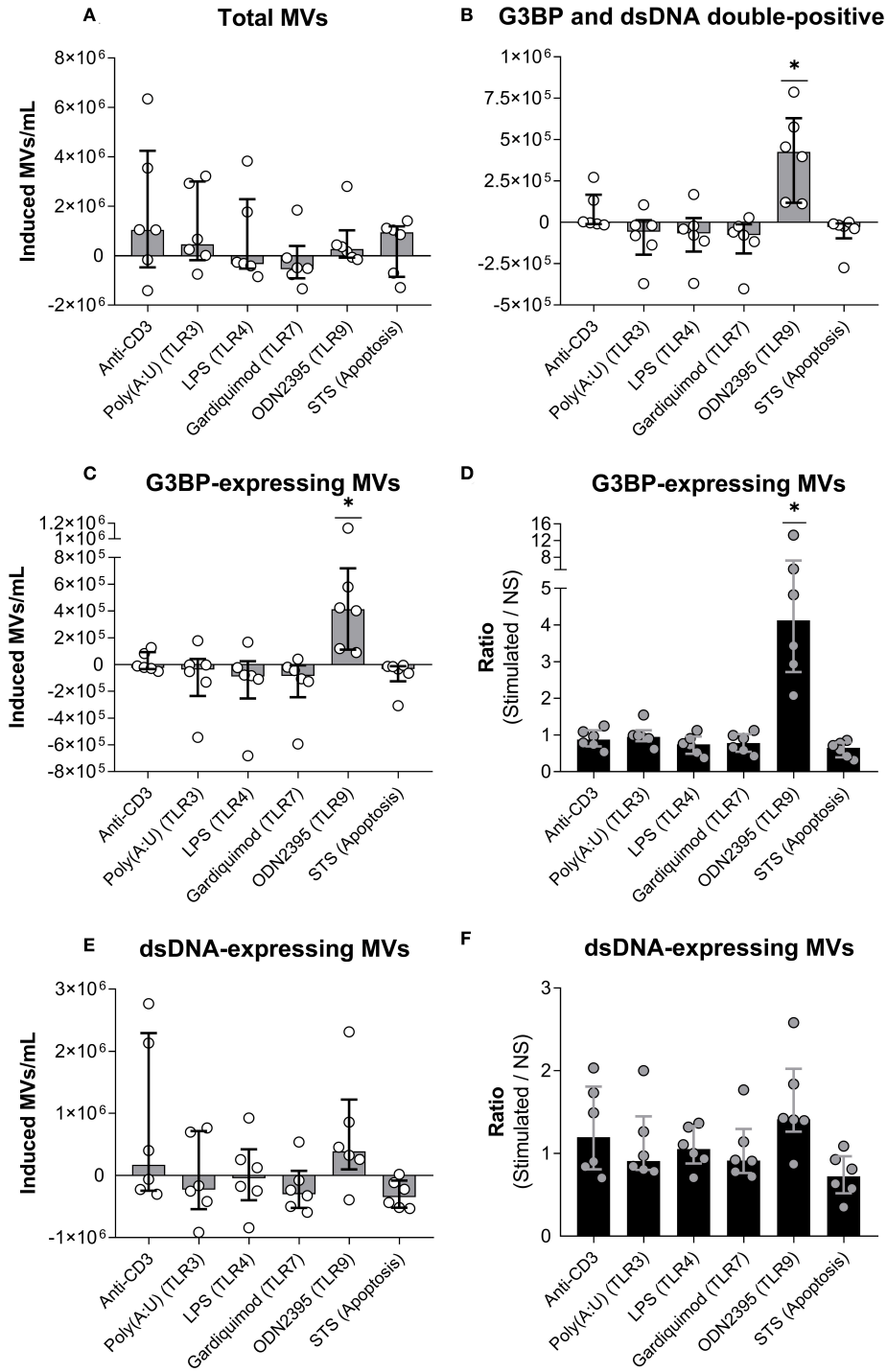


**FIGURE 3 |** Release of G3BP- and/or dsDNA-expressing microvesicles from peripheral blood mononuclear cells. **(A)** Peripheral blood mononuclear cells from healthy donors were incubated for 24 h with either anti-CD3 antibody (OKT3) ($n = 6$), the TLR3-agonist poly(A:U) ($n = 6$), the TLR4-agonist LPS ($n = 6$), the TLR7-agonist gardiquimod ($n = 6$), or the TLR9-agonist ODN2395 ($n = 6$), or were treated with staurosporine (STS) to induce apoptosis ($n = 6$). Microvesicles (MVs) released into the culture supernatants were subsequently isolated by differential centrifugation. Isolated MVs were quantified and characterized with respect to expression of G3BP and dsDNA by flow cytometry. The change in the concentration of MVs in culture supernatants, induced by the various stimuli, is shown. Columns and error bars represent median values and interquartile range after subtraction of background (non-stimulated controls). **(B)** Corresponding quantifications of G3BP and dsDNA double-positive MVs. **(C)** Changes in the concentration of the G3BP-expressing MV population *in toto*. **(D)** The fold change in the concentration of G3BP-expressing MVs, expressed as the ratio of MV count in stimulated samples over that of non-stimulated samples (NS). **(E)** Changes in the concentrations of the dsDNA-expressing MV population *in toto*. **(F)** The fold change in the concentration of dsDNA-expressing MVs. *$P < 0.05$. NS, non-stimulated.

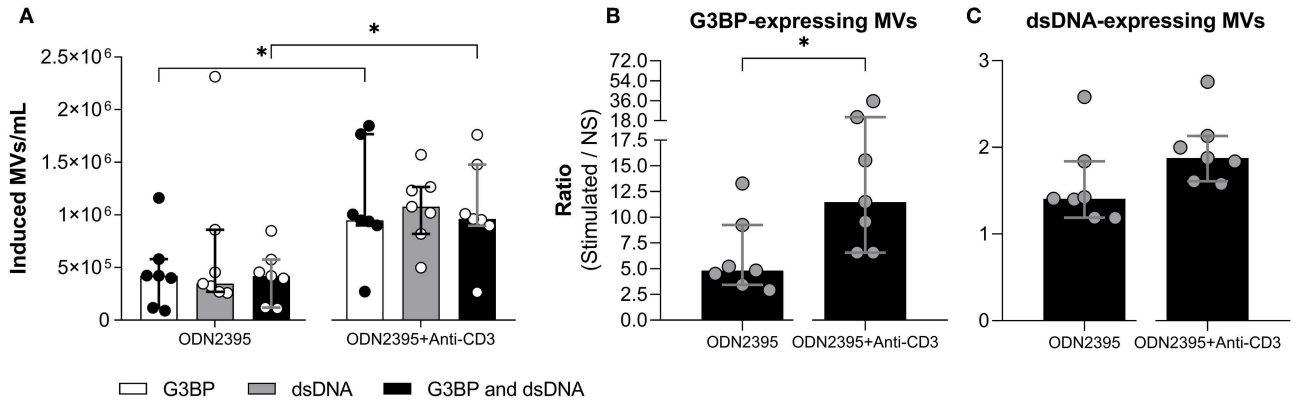


**FIGURE 4 |** Effect of T cell stimulation on release of microvesicles from peripheral blood mononuclear cells. Peripheral blood mononuclear cells from healthy donors ($n = 7$) were incubated for 24 h with the TLR9-agonist ODN2395 in presence or absence of anti-CD3 antibody (OKT3). Microvesicles (MVs) released into the culture supernatants were subsequently isolated by differential centrifugation. Isolated MVs were quantified and characterized with respect to expression of G3BP and dsDNA by flow cytometry. **(A)** Concentration of MVs expressing G3BP (white columns), dsDNA (gray columns), or both (black columns) after subtraction of concentrations observed in non-stimulated cultures. **(B)** The corresponding fold change, expressed as the ratio between G3BP-expressing MVs in supernatants from stimulated and non-stimulated cultures (NS). **(C)** The corresponding fold change in the dsDNA-expressing MV population. Columns and error bars represent median values and interquartile range. *$P < 0.05$. NS, non-stimulated.

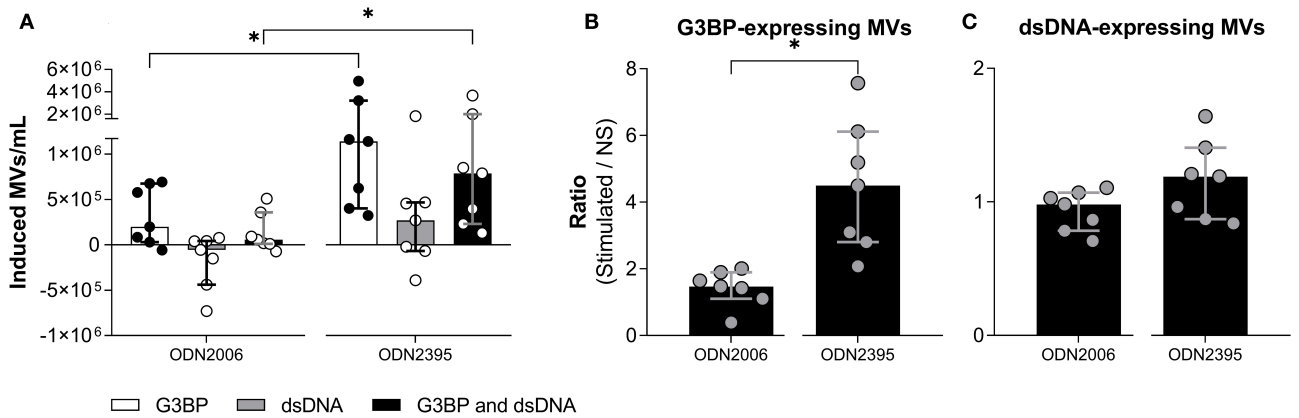


**FIGURE 5 |** Effect of different TLR9-agonists on release of microvesicles from peripheral blood mononuclear cells. Peripheral blood mononuclear cells from healthy donors ($n = 7$) were incubated for 24 h with ODN2006, a weak inducer of IFN-α, or ODN2395, a strong inducer of IFN-α. Microvesicles (MVs) released into the culture supernatants were subsequently isolated by differential centrifugation. Isolated MVs were quantified and characterized with respect to expression of G3BP and dsDNA by flow cytometry. **(A)** Shown is the consequent increase in the concentration of MVs expressing G3BP (white columns), dsDNA (gray columns), or both (black columns). **(B)** The concentration of G3BP-expressing MVs in cultures of stimulated cells relative to that in non-stimulated cultures (NS). **(C)** The corresponding relative concentration of dsDNA-expressing MVs. Columns and error bars represent median values and interquartile range. *$P < 0.05$. NS, non-stimulated.

## TLR-Mediated Release of dsDNA- and G3BP-Expressing MVs From Mononuclear Cells

Stimulation of PBMCs with the TLR9-agonist ODN2395 lead to release of MVs with distinct expression of G3BP (**Figure 1C**) and surface-bound dsDNA (**Figure 1D**) into the culture supernatants. Notably, about 1/3 of MVs present in supernatants from unstimulated PBMCs bore dsDNA but not G3BP (**Figure 1E**). Stimulation with ODN2395 induced co-expression of G3BP (**Figure 1F**).

We stimulated PBMCs with a series of TLR-agonists or the T cell activating antibody OKT3, or induced apoptosis by incubation with staurosporine (**Figure 3**). None of the stimuli significantly affected the total number of MVs released, as shown in **Figure 3A**, where the number of MVs in non-stimulated cultures have been subtracted (allowing occurrence of negative values). However, as the only stimulus, ODN2395 induced a significant increase in the number of G3BP and dsDNA double-positive MVs (**Figure 3B**) and, accordingly, in the total number of G3BP-expressing MVs (**Figure 3C**). The content of G3BP-expressing MVs was thus a median of four times higher in OD2395-stimulated cultures than in non-stimulated cultures (**Figure 3D**). None of the other stimuli examined affected the release of G3BP-expressing MVs.

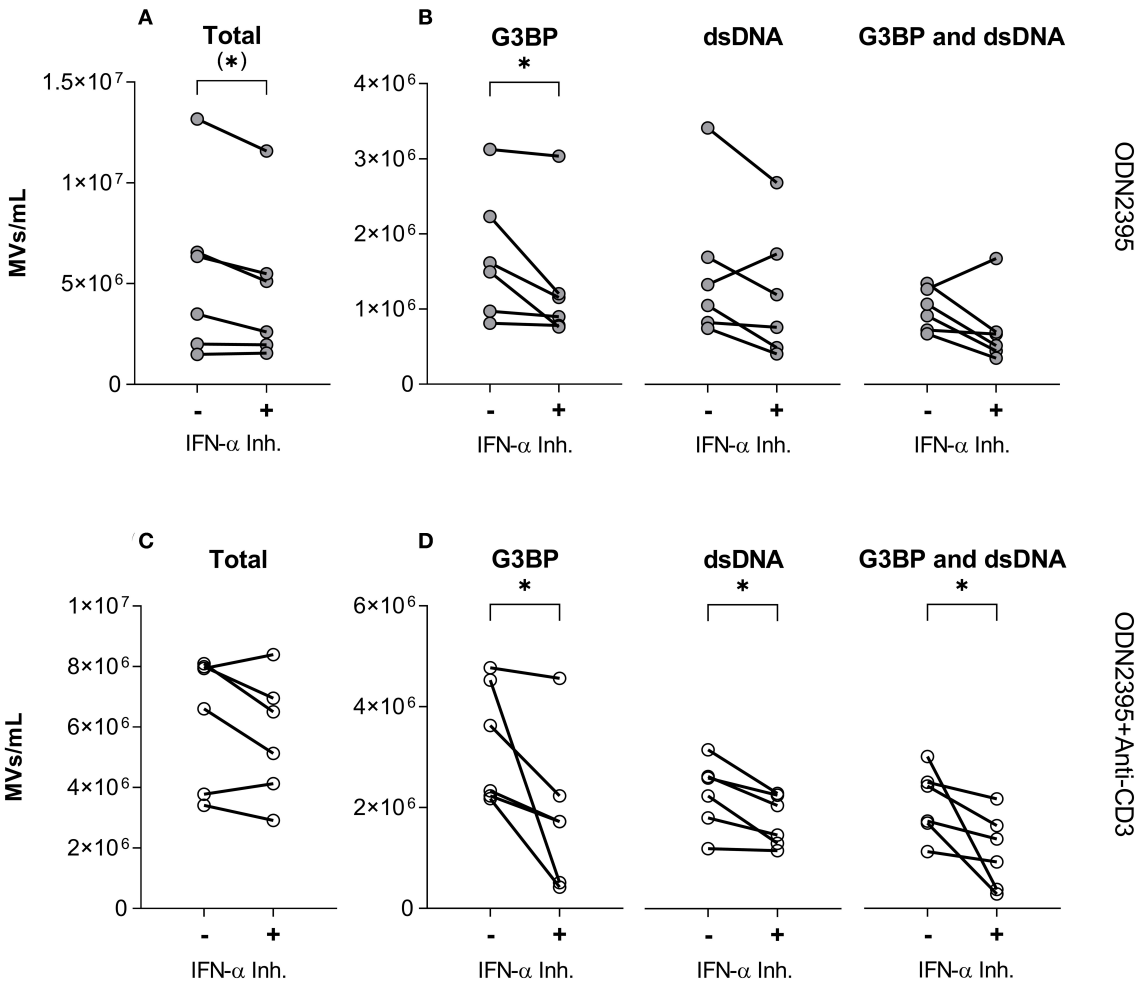


**FIGURE 6 |** Effect of IFN-α inhibition on TLR9-induced release of microvesicles from peripheral blood mononuclear cells. Peripheral blood mononuclear cells from healthy donors ($n = 6$) were incubated for 24 h with the TLR9-agonist ODN2395, alone (–) or in combination with the IFN-α inhibitor IFN alpha-IFNAR-IN-1 hydrochloride (+). Released microvesicles (MVs) were quantified, and expression of G3BP and dsDNA by the MVs was analyzed by flow cytometry. **(A)** Concentration in the culture supernatant of the total MV population and **(B)** subpopulations of G3BP-expressing, dsDNA-expressing, and G3BP and dsDNA double-positive MVs. **(C)** Corresponding concentration of the total MV population and **(D)** subpopulations after co-stimulation of T cells with anti-CD3 antibody. *$P < 0.05$.

Stimulation with ODN2395 induced a non-significant increase in the number of dsDNA-expressing vesicles released into the supernatant (**Figures 3E,F**). Specificity of the staining for dsDNA was confirmed by the observation that preincubation of culture supernatants from ODN2395-treated PBMCs with DNase markedly reduced the binding of the detecting anti-dsDNA antibody to MVs (data not shown).

## Influence of T Cells on TLR9-Mediated Release of MVs

As indicated above, cross-binding of CD3 on T cells *per se* did not influence MV release from mononuclear cells. To investigate whether cross-binding of CD3 had any influence on the TLR9-mediated MV release, we stimulated PBMCs with ODN2395 alone or in combination with anti-CD3 antibody (**Figure 4**). The co-stimulation markedly enhanced the ODN2395-induced release of G3BP-expressing MVs *in toto* and of G3BP and dsDNA double-positive MVs (**Figures 4A,B**), whereas the release of dsDNA-expressing MVs *in toto* was not affected significantly (**Figures 4A,C**).

## Effect of Different TLR9-Agonists on MV Release and Phenotype

Since ODN2395 is a potent inducer of IFN-α, we examined how the effect of another TLR9-agonist, ODN2006, which is known to be a weak IFN-α-inducer (23), affected MV release and phenotype studied. Notably, ODN2006 was a much weaker stimulus for release of G3BP-expressing MVs than ODN2395 (**Figures 5A,B**), suggesting that acquisition of this phenotype depended, at least in part, on secretion of IFN-α. The release of dsDNA-expressing MVs *in toto* did not differ significantly between the two stimuli (**Figures 5A,C**).

### Effect of IFN-α Inhibition on TLR9-Induced Release of MVs

To test directly if IFN-α was involved in generation of G3BP- or dsDNA-expressing MVs, we employed the IFN-α inhibitor IN-1 (**Figure 6**). Despite having little effect on the overall release of MVs from PBMCs stimulated with ODN2395 (**Figure 6A**), this inhibitor significantly reduced the release of G3BP-expressing MVs (**Figure 6B**). A similar effect pattern was observed after co-stimulation of the PBMCs with anti-CD3 antibody (**Figures 6C,D**), but in this situation the release of dsDNA- and double-positive MVs was also reduced significantly by IN-1 (**Figure 6D**).

## DISCUSSION

The purpose of this study was to investigate the mechanisms underlying release of MVs from normal mononuclear cells, and to characterize the released MVs with respect to expression of dsDNA and G3BP. Insight into these mechanisms may enhance our understanding of MV release, in general, and, since G3BP-expressing MVs may deposit on the GBM, it may also help to understand how nephritis develops in SLE (35).

Our main finding was that incubation of PBMCs with the TLR9-agonist ODN2395 caused qualitative changes in MVs released from cultivated PBMCs, while the total number of MVs released were largely unchanged. Thus, ODN2395 induced a substantially increased co-expression of G3BP and dsDNA on the MV surface. A surface-localized signal from both the former and latter is supported by the vesicles' ability to retain calcein, suggesting low vesicular permeability. By contrast, the TLR3-agonist poly(A:U), the TLR4-agonist LPS and the TLR7-agonist gardiquimod had no effect on the total MV release, nor on the MV phenotypes studied. TLR9 binds hypomethylated CpG-rich DNA (36), suggesting that ICs containing such DNA may be a physiological stimulus for release of G3BP-expressing MVs. However, the TLR9-agonist ODN2006 did not induce release of G3BP-expressing MVs. This discrepancy may be related to the ability of ODN2395 to induce production of IFN-α in contrast to ODN2006 (23). In accordance with this notion, we observed a marked reduction in the frequency of G3BP-expressing MVs in presence of the IFN-α inhibitor IN-1. The enhancement of MV expression of G3BP by IFN-α may be relevant to SLE pathogenesis, in light of the exaggerated production of IFN-α by plasmacytoid dendritic cells (pDCs) in this disease (20).

In contrast with the markedly increased G3BP-expression by MVs after stimulation with ODN2395, this TLR-agonist had little effect on the expression of dsDNA by the MVs; nor was this expression differentially regulated by the two TLR9-agonists used in this study.

To examine the effect of T cell stimulation on MV-production and phenotype, we included an anti-CD3 antibody as stimulus. Interestingly, this stimulus markedly enhanced the ODN2395-induced generation of G3BP-expressing MVs and of G3BP and dsDNA double-positive MVs. These effects of T cells may rely on cytokine production by the T cells, in keeping with previous findings that *in-vitro* activated T cells from healthy donors and SLE patients enhance the secretion of IFN-α from pDCs stimulated with the TLR9-agonist ODN2216 (22). However, T cell TLR9 and the T cell receptor (with CD3 as co-receptor) have previously been demonstrated to act in concert (37) and we cannot exclude that the increased number of G3BP-expressing MVs released following co-stimulation via CD3 originate from T cells *per se*. We have previously shown that a significant proportion of MVs isolated from the blood of healthy donors and SLE patients express CD3, indicating that they have been released by T cells (38).

G3BP has been shown to bind to several proteins, including collagen IV, nidogen, and fibronectin (19), all of which have been demonstrated in the GBM. It also binds to galectin-1 and galectin-3 with high affinity (39), and both these galectins are expressed by many immune cells, including T cells, B cells and macrophages (40, 41). Under physiological circumstances, expression of G3BP by MVs may therefore serve an immunoregulatory function. Moreover, G3BP has a scavenging function and may thus facilitate clearance of MVs (18). In SLE, however, G3BP-expressing MVs may deposit in kidney glomeruli, where overexpression of galectin-3 has been observed (42). The dsDNA co-expressed by the MVs is likely to become target for anti-dsDNA antibodies and complement activation may ensue. To this end, bound IgG (12) and complement fragments (38) have been demonstrated on circulating MVs from SLE patients. It has thus been speculated that G3BP and dsDNA double-positive MVs may deposit in the GBM and contribute to the kidney damage observed in SLE (8).

In conclusion, we show that stimulation through TLR9 induce G3BP-expression of MVs released from healthy donor PBMCs in an IFN-α-dependent manner, and that a substantial proportion of the MVs co-express dsDNA. The excessive production of IFN-α and anti-dsDNA antibodies in SLE and overexpression of galectin-3 in the patients' kidney glomeruli suggest that G3BP and dsDNA co-expressing MVs hold a strong pathogenic potential in this disease.

## ETHICS STATEMENT

The study was approved by the Scientific-Ethical Committee of the Capital Region of Denmark (protocol no. H-15004075).

## AUTHOR CONTRIBUTIONS

SJ, CTN, and CHN created the research concept and supervised the research and the preparation of the manuscript. NR, CTN, SJ, and CHN designed the research. NR conducted the experiments, analyzed data, and wrote the manuscript.

## FUNDING

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.02391/full#supplementary-material

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# Long Non-coding RNAs Genes Polymorphisms and Their Expression Levels in Patients With Rheumatoid Arthritis

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Long non-coding RNAs (lncRNAs) are increasingly recognized to play important roles in multiple autoimmune diseases. This study aimed to evaluate the association of four lncRNAs (*ANRIL*, *lnc-DC*, *MALAT1*, *ZFAS1*) genes single nucleotide polymorphisms (SNPs) with susceptibility to rheumatoid arthritis (RA) patients, as well as their expression levels. Seventeen SNPs of the four lncRNAs were genotyped in a cohort of 660 RA patients and 710 controls using improved multiple ligase detection reaction (iMLDR). The lncRNAs expressions in peripheral blood mononuclear cells (PBMCs) from 120 RA patients and 120 controls were detected by qRT-PCR. No significant differences were found for the allele and genotype frequencies distribution of *ANRIL* SNPs (rs1412830, rs944796, rs61271866, rs2518723, rs3217992), *lnc-DC* SNPs (rs7217280, rs10515177), *MALAT1* SNPs (rs619586, rs4102217, rs591291, rs11227209, rs35138901), *ZFAS1* SNPs (rs237742, rs73116127, rs6125607, rs6125608) between RA patients and normal controls (all $P > 0.05$). The genotype effects of dominant and recessive models were also evaluated, but no significant association was found. In addition, our results demonstrated that the rs944796 G allele, rs2518723 T allele, rs3217992 T allele frequencies were significantly associated with anti-CCP in RA patients (all $P < 0.05$). The haplotype CGTA frequency for *ZFAS1* was significantly higher in RA patients ($P = 0.036$). Compared with normal controls, the expression levels of ANRIL, lnc-DC, MALAT1, ZFAS1 in PBMCs were significantly reduced in RA patients (all $P < 0.001$). Moreover, ZFAS1 expression was negatively associated with CRP in RA patients ($P = 0.002$). In summary, *ANRIL*, *lnc-DC*, *MALAT1*, and *ZFAS1* genes SNPs were not associated with RA susceptibility, while altered ANRIL, lnc-DC, MALAT1, ZFAS1 levels in RA patients suggested that these lncRNAs might play a role in RA.

**Keywords: ANRIL, lnc-DC, MALAT1, ZFAS1, single nucleotide polymorphisms, rheumatoid arthritis**

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## INTRODUCTION

Rheumatoid arthritis (RA) is known as a common autoimmune, inflammation disease characterized by systemic manifestations of immune and inflammatory response including marginal bone erosion, inflammatory joint fluid, synovitis, and destruction of articular cartilage (1, 2). Several researches have indicated that the incidences of RA in different ethnic groups, geographical areas are different, and the RA prevalence is approximately 1% around the world (3, 4). It has been revealed that genetic susceptibilities, abnormal metabolic enzymes, aberrant immune response are involved in RA development (5, 6). Latterly, a number of single nucleotide polymorphisms (SNPs), the majority of which are located in the non-coding intervals, are gradually identified to be associated with the susceptibility of this disease according to genome-wide association studies (7, 8).

Long non-coding RNAs (lncRNAs), which are defined as RNAs longer than 200 nucleotides in length, have no or little protein-coding capacity (9). LncRNAs are reported to be involved in a variety of autoimmunity- and inflammation- related processes, and regulate gene expression in multiple mechanisms including alternative splicing, epigenetics, small RNA sponging (10, 11). Increasing studies have been performed to explore the potential role of lncRNAs on the pathogenesis of autoimmune diseases, such as RA and systemic lupus erythematosus (SLE) (12–14). Our recent study demonstrated that the lnc0640, lnc5150 expression levels were alternated among RA patients, and *lnc0640* rs13039216 TT genotype was statistically associated with RA susceptibility (14). Another previous study suggested that aberrant lncRNA expression level in peripheral blood mononuclear cells (PBMCs) could be a potential biomarker for RA diagnosing (13).

Recently, lncRNA ANRIL (antisense non-coding RNA in the INK4 locus) had attracted attention in autoimmune diseases, as it had been implicated in regulation of immune, inflammatory response (15). ANRIL expression was found to be regulated through STAT1 signaling pathway, which participated in immune regulation by induction of the pro-inflammatory cytokine TNF-γ (16). In addition, another study indicated that ANRIL expression level in PBMCs was decreased in RA by lncRNA array (13). Dendritic cell (DC) was a specific antigen presenting cell which link the innate and adaptive immune responses, and was thought to drive the activation of self-peptide-reactive inflammatory T cells, follicular helper T cells and consequently B cells for secreting autoantibodies in RA (17). Lnc-DC was a specialized, highly expressed lncRNA in DCs, and had the ability to regulate Th17 differentiation, DCs to stimulate T cell activation, and the production of interleukin 12 (IL-12) (9). LncRNA MALAT1 (metastasis-associated lung adenocarcinoma transcript-1) had been shown to play a role in the development of autoimmune diseases. SLE patients had increased MALAT1 level in PBMCs compared with normal individuals, and knockdown of MALAT-1 significantly suppressed IL-21 level in monocytes (18). In RA, Pan et al. found that knockdown of MALAT1 could inhibit the apoptosis of fibroblast-like synoviocytes (FLS) and lead to the activation of phosphoinositide 3-kinase (PI3K)/AKT signaling pathway (19). In another study, lncRNA ZFAS1 (zinc finger antisense 1) was shown to participate in RA-FLS migration and invasion by interacting with miR-27a and suppressing miR-27a expression, and ZFAS1 expression level was statistically evaluated in FLS of RA patients (20).

These studies demonstrated that ANRIL, lnc-DC, MALAT1, ZFAS1 might be involved in the occurrence and development of RA. However, no studies regarding the relationship between these lncRNAs genetic variation and RA have been reported. Thus, in the present study, we explored the associations of these lncRNAs genes SNPs with RA risk, as well as these lncRNAs expressions in PBMCs of RA patients and normal controls.

## MATERIALS AND METHODS

### Patients and Normal Controls

In this study, case-control studies were performed in unrelated ethnic Han Chinese population. A total of 1,370 subjects including 660 RA patients and 710 normal controls were consecutively enrolled to investigate the association between *ANRIL*, *lnc-DC*, *MALAT1*, *ZFAS1* genes polymorphisms and RA susceptibility. Then, 120 RA patients and 120 normal controls were included to detect these lncRNAs expression levels. RA patients were selected from the First Affiliated Hospital of University of Science and Technology of China, and the First Affiliated Hospital of Anhui Medical University. The diagnosis of these patients was according to the 1987 American College of Rheumatology revised criteria (21). The normal controls, who were recruited from the healthy blood donors in the same region, did not have no a history of RA, or other inflammatory/autoimmune diseases, cancer. Disease Activity Score 28 (DAS 28) was used to evaluate RA disease activity (22). The demographic data of all subjects were collected, and the following clinical data of RA patients were retrieved from the medical records: complements 3 (C3), complements 4 (C4), erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), anti-cyclic citrullinated peptide (anti-CCP), and rheumatoid factor (RF). After informed consent was obtained, peripheral blood samples and data were collected from RA patients and normal controls. This study protocol was approved by the Medical Ethics Committee of Anhui Medical University.

### SNP Selection, DNA Extraction, and Genotyping

The genetic and location information were obtained from two public databases, LNCipedia.org (v4.0) and Genome Browser Gateway (UCSC). We selected the tagSNPs with a minor allele frequency (MAF) $\geq$ 0.05 in CHB capturing all the common SNPs located in the chromosome locus transcribed into these lncRNAs (*ANRIL*, *lnc-DC*, *MALAT1*, *ZFAS1*) and their flanking 2,000 bp region through using genotype data of Han Chinese in Beijing from Ensembl genome browser 85 and CHBS_1000 g. The selection was conducted through linkage disequilibrium (LD) analysis with $r^2$ threshold $> 0.8$ by using Haploview 4.0 software (Cambridge, MA, USA). In addition, the existing studies about these lncRNA genes polymorphisms were also reviewed. Finally, we selected six tagSNPs (rs1412830, rs7044859, rs944796, rs61271866, rs2518723, rs3217992) in *ANRIL, two* tagSNPs

**TABLE 1 |** Genotypes and alleles frequencies of lncRNAs genes polymorphisms in RA patients and normal controls.

| SNP | Analyze model | | RA (*N* = 660) *n* (%) | Control (*N* = 710) *n* (%) | Adjustment with sex and age | |
|---|---|---|---|---|---|---|
| | | | | | *P* value* | *OR* (95% *CI*) |
| ***ANRIL*** | | | | | | |
| rs1412830 | Genotypes | TT | 13 (1.97) | 3 (0.42) | 0.017 | 0.214 (0.060–0.761) |
| | | CT | 119 (18.03) | 139 (19.58) | 0.564 | 1.084 (0.824–1.425) |
| | | CC | 528 (80.00) | 568 (80.00) | Reference | |
| | Alleles | T | 145 (10.98) | 145 (10.21) | 0.511 | 0.922 (0.722–1.176) |
| | | C | 1,175 (89.02) | 1,275 (89.79) | Reference | |
| | Dominant model | CC | 528 (80.00) | 568 (80.00) | 0.994 | 1.001 (0.766–1.307) |
| | | TT+CT | 132 (20.00) | 142 (20.00) | Reference | |
| | Recessive model | TT | 13 (1.97) | 3 (0.42) | 0.016 | 0.211 (0.059–0.750) |
| | | CC+CT | 647 (98.03) | 707 (99.58) | Reference | |
| rs944796 | Genotypes | GG | 11 (1.67) | 31 (4.37) | 0.013 | 2.452 (1.211–4.962) |
| | | GC | 238 (36.06) | 230 (32.39) | 0.236 | 0.872 (0.695–1.094) |
| | | CC | 411 (62.27) | 449 (63.24) | Reference | |
| | Alleles | G | 260 (19.70) | 292 (20.56) | 0.572 | 1.055 (0.875–1.272) |
| | | C | 1,060 (80.30) | 1,128 (79.44) | Reference | |
| | Dominant model | CC | 411 (62.27) | 449 (63.24) | 0.598 | 1.061 (0.851–1.324) |
| | | GG+GC | 249 (37.73) | 261 (36.76) | Reference | |
| | Recessive model | GG | 11 (1.67) | 31 (4.37) | 0.008 | 2.574 (1.278–5.185) |
| | | CC+GC | 649 (98.33) | 679 (95.63) | Reference | |
| rs61271866 | Genotypes | AA | 25 (3.79) | 26 (3.66) | 0.882 | 0.958 (0.542–1.692) |
| | | TA | 185 (28.03) | 214 (30.14) | 0.437 | 1.099 (0.867–1.392) |
| | | TT | 450 (68.18) | 470 (66.20) | Reference | |
| | Alleles | A | 235 (17.80) | 266 (18.73) | 0.529 | 0.940 (0.774–1.141) |
| | | T | 1,085 (82.20) | 1,154 (81.27) | Reference | |
| | Dominant model | TT | 450 (68.18) | 470 (66.20) | 0.498 | 0.924 (0.736–1.160) |
| | | AA+TA | 210 (31.82) | 240 (33.80) | Reference | |
| | Recessive model | AA | 25 (3.79) | 26 (3.66) | 0.811 | 0.933 (0.531–1.641) |
| | | TT+TA | 635 (96.21) | 684 (96.34) | Reference | |
| rs2518723 | Genotypes | TT | 111 (16.82) | 133 (18.73) | 0.312 | 1.177 (0.858–1.613) |
| | | CT | 326 (49.39) | 353 (49.72) | 0.535 | 1.079 (0.848–1.372) |
| | | CC | 223 (33.79) | 224 (31.55) | Reference | |
| | Alleles | T | 548 (41.52) | 619 (43.59) | 0.256 | 1.092 (0.983–1.271) |
| | | C | 772 (58.48) | 801 (56.41) | Reference | |
| | Dominant model | CC | 223 (33.79) | 224 (31.55) | 0.393 | 0.906 (0.721–1.137) |
| | | TT+CT | 437 (66.21) | 486 (68.45) | Reference | |
| | Recessive model | TT | 111 (16.82) | 133 (18.73) | 0.413 | 0.890 (0.673–1.177) |
| | | CC+CT | 549 (83.18) | 577 (81.27) | Reference | |
| rs3217992 | Genotypes | TT | 160 (24.24) | 152 (21.41) | 0.118 | 0.783 (0.576–1.064) |
| | | CT | 338 (51.21) | 362 (50.99) | 0.368 | 0.889 (0.687–1.149) |
| | | CC | 162 (24.55) | 196 (27.61) | Reference | |
| | Alleles | T | 658 (49.85) | 666 (46.90) | 0.123 | 0.889 (0.765–1.032) |
| | | C | 662 (50.15) | 754 (53.10) | Reference | |
| | Dominant model | CC | 162 (24.55) | 196 (27.61) | 0.206 | 1.170 (0.917–1.493) |
| | | TT+CT | 498 (75.45) | 514 (72.39) | Reference | |
| | Recessive model | TT | 160 (24.24) | 152 (21.41) | 0.199 | 0.846 (0.656–1.092) |
| | | CC+CT | 500 (75.76) | 558 (78.59) | Reference | |
| ***Lnc-DC*** | | | | | | |
| rs7217280 | Genotypes | AA | 3 (0.45) | 4 (0.56) | 0.849 | 1.160 (0.253–5.314) |

*(Continued)*

**TABLE 1 |** Continued

| SNP | Analyze model | | RA (*N* = 660) *n* (%) | Control (*N* = 710) *n* (%) | Adjustment with sex and age | |
|---|---|---|---|---|---|---|
| | | | | | *P* value* | *OR* (95% *CI*) |
| | | GA | 52 (7.88) | 77 (10.85) | 0.084 | 1.388 (0.957–2.014) |
| | | GG | 605 (91.67) | 629 (88.59) | Reference | |
| | Alleles | A | 58 (4.39) | 85 (5.99) | 0.062 | 1.385 (0.984–1.951) |
| | | G | 1,262 (95.61) | 1,335 (94.01) | Reference | |
| | Dominant model | GG | 605 (91.67) | 629 (88.59) | 0.085 | 0.727 (0.506–1.045) |
| | | AA+GA | 55 (8.33) | 81 (11.41) | Reference | |
| | Recessive model | AA | 3 (0.45) | 4 (0.56) | 0.881 | 1.123 (0.245–5.146) |
| | | GG+GA | 657 (99.55) | 706 (99.44) | Reference | |
| rs10515177 | Genotypes | GG | 4 (0.61) | 5 (0.70) | 0.870 | 1.118 (0.294–4.249) |
| | | GA | 94 (14.24) | 117 (16.48) | 0.330 | 1.159 (0.861–1.560) |
| | | AA | 562 (85.15) | 588 (82.82) | Reference | |
| | Alleles | G | 102 (7.73) | 127 (8.94) | 0.251 | 1.173 (0.893–1.540) |
| | | A | 1,218 (92.27) | 1,293 (91.06) | Reference | |
| | Dominant model | AA | 562 (85.15) | 588 (82.82) | 0.327 | 0.864 (0.645–1.157) |
| | | GG+GA | 98 (14.85) | 122 (17.18) | Reference | |
| | Recessive model | GG | 4 (0.61) | 5 (0.70) | 0.896 | 1.093 (0.288–4.151) |
| | | AA+GA | 656 (99.39) | 705 (99.30) | Reference | |
| ***MALAT1*** | | | | | | |
| rs619586 | Genotypes | GG | 6 (0.91) | 4 (0.56) | 0.350 | 0.544 (0.151–1.951) |
| | | GA | 111 (16.82) | 113 (15.92) | 0.628 | 0.931 (0.698–1.243) |
| | | AA | 543 (82.27) | 593 (83.52) | Reference | |
| | Alleles | G | 123 (9.32) | 121 (8.52) | 0.464 | 0.906 (0.697–1.179) |
| | | A | 1,197 (90.68) | 1,299 (91.48) | Reference | |
| | Dominant model | AA | 543 (82.27) | 593 (83.25) | 0.517 | 1.098 (0.827–1.458) |
| | | GG+GA | 117 (17.73) | 117 (16.48) | Reference | |
| | Recessive model | GG | 6 (0.91) | 4 (0.56) | 0.359 | 0.550 (0.153–1.973) |
| | | AA+GA | 654 (99.09) | 706 (99.44) | Reference | |
| rs4102217 | Genotypes | CC | 20 (3.03) | 13 (1.83) | 0.306 | 0.688 (0.337–1.408) |
| | | CG | 154 (23.33) | 205 (28.87) | 0.020 | 1.340 (1.047–1.713) |
| | | GG | 486 (73.64) | 492 (69.30) | Reference | |
| | Alleles | C | 194 (14.70) | 231 (16.27) | 0.257 | 1.128 (0.916–1.388) |
| | | G | 1,126 (85.30) | 1,189 (83.73) | Reference | |
| | Dominant model | GG | 486 (73.64) | 492 (69.30) | 0.053 | 0.791 (0.624–1.003) |
| | | CC+CG | 174 (26.36) | 218 (30.70) | Reference | |
| | Recessive model | CC | 20 (3.03) | 13 (1.83) | 0.216 | 0.638 (0.313–1.300) |
| | | GG+CG | 640 (96.97) | 697 (98.17) | Reference | |
| rs591291 | Genotypes | TT | 124 (18.79) | 132 (18.59) | 0.496 | 1.113 (0.818–1.513) |
| | | CT | 298 (45.15) | 347 (48.87) | 0.125 | 1.207 (0.949–1.534) |
| | | CC | 238 (36.06) | 231 (32.53) | Reference | |
| | Alleles | T | 546 (41.36) | 611 (43.03) | 0.378 | 1.071 (0.920–1.246) |
| | | C | 774 (58.64) | 809 (56.97) | Reference | |
| | Dominant model | CC | 238 (36.06) | 231 (32.53) | 0.153 | 0.848 (0.677–1.063) |
| | | CT+TT | 422 (63.94) | 479 (67.46) | Reference | |
| | Recessive model | TT | 124 (18.79) | 132 (18.59) | 0.979 | 0.996 (0.757–1.310) |
| | | CT+CC | 536 (81.21) | 578 (81.41) | Reference | |
| rs11227209 | Genotypes | GG | 3 (0.45) | 3 (0.42) | 0.880 | 0.883 (0.176–4.420) |
| | | CG | 71 (10.76) | 79 (11.13) | 0.773 | 1.052 (0.747–1.480) |
| | | CC | 586 (88.79) | 628 (88.45) | Reference | |

*(Continued)*

**TABLE 1** | Continued

| SNP | Analyze model | | RA (*N* = 660) *n* (%) | Control (*N* = 710) *n* (%) | Adjustment with sex and age | |
|---|---|---|---|---|---|---|
| | | | | | *P* value* | *OR* (95% *CI*) |
| | Alleles | G | 77 (5.83) | 85 (5.99) | 0.866 | 1.028 (0.748–1.412) |
| | | C | 1,243 (94.17) | 1,335 (94.01) | Reference | |
| | Dominant model | CC | 586 (88.79) | 628 (88.45) | 0.799 | 0.957 (0.684–1.339) |
| | | CG+GG | 74 (11.21) | 82 (11.55) | Reference | |
| | Recessive model | GG | 3 (0.45) | 3 (0.42) | 0.874 | 0.878 (0.176–4.393) |
| | | CG+CC | 657 (99.55) | 707 (99.58) | Reference | |
| rs35138901 | Genotypes | CC | 4 (0.61) | 2 (0.28) | 0.469 | 0.532 (0.097–2.933) |
| | | CT | 93 (14.09) | 115 (16.20) | 0.252 | 1.191 (0.883–1.606) |
| | | TT | 563 (85.30) | 593 (83.52) | Reference | |
| | Alleles | C | 101 (7.65) | 119 (8.38) | 0.483 | 1.104 (0.837–1.445) |
| | | T | 1,219 (92.35) | 1,301 (91.62) | Reference | |
| | Dominant model | TT | 563 (85.30) | 593 (83.52) | 0.312 | 0.859 (0.639–1.154) |
| | | CT+CC | 97 (14.70) | 117 (16.48) | Reference | |
| | Recessive model | CC | 4 (0.61) | 2 (0.28) | 0.450 | 0.518 (0.094–2.852) |
| | | CT+TT | 656 (99.39) | 708 (99.72) | Reference | |
| ***ZFAS1*** | | | | | | |
| rs237742 | Genotypes | TT | 91 (13.79) | 104 (14.65) | 0.994 | 0.999 (0.717–1.391) |
| | | CT | 322 (48.79) | 320 (45.07) | 0.212 | 0.863 (0.685–1.088) |
| | | CC | 247 (37.42) | 286 (40.28) | Reference | |
| | Alleles | T | 504 (38.18) | 528 (37.18) | 0.590 | 0.958 (0.821–1.119) |
| | | C | 816 (61.82) | 892 (62.82) | Reference | |
| | Dominant model | CC | 247 (37.42) | 286 (40.28) | 0.309 | 1.121 (0.900–1.395) |
| | | CT+TT | 413 (62.58) | 424 (59.72) | Reference | |
| | Recessive model | TT | 91 (13.79) | 104 (14.65) | 0.611 | 1.083 (0.797–1.470) |
| | | CT+CC | 569 (86.21) | 606 (85.35) | Reference | |
| rs73116127 | Genotypes | AA | 1 (0.15) | 3 (0.42) | 0.384 | 2.739 (0.283–26.506) |
| | | GA | 109 (16.52) | 133 (18.73) | 0.294 | 1.162 (0.878–1.538) |
| | | GG | 550 (83.33) | 574 (80.85) | Reference | |
| | Alleles | A | 111 (8.41) | 139 (9.79) | 0.211 | 1.182 (0.910–1.535) |
| | | G | 1,209 (91.59) | 1,281 (90.21) | Reference | |
| | Dominant model | GG | 550 (83.33) | 574 (80.85) | 0.253 | 0.850 (0.643–1.123) |
| | | AA+GA | 110 (16.67) | 136 (19.15) | Reference | |
| | Recessive model | AA | 1 (0.15) | 3 (0.42) | 0.398 | 2.661 (0.275–25.738) |
| | | GG+GA | 659 (99.85) | 707 (99.58) | Reference | |
| rs6125607 | Genotypes | TT | 74 (11.21) | 48 (6.76) | 0.007 | 0.576 (0.387–0.857) |
| | | CT | 277 (41.97) | 310 (43.66) | 0.978 | 1.003 (0.801–1.256) |
| | | CC | 309 (46.82) | 352 (49.58) | Reference | |
| | Alleles | T | 425 (32.20) | 406 (28.59) | 0.040 | 0.843 (0.716–0.992) |
| | | C | 895 (67.80) | 1,014 (71.41) | Reference | |
| | Dominant model | CC | 309 (46.82) | 352 (49.58) | 0.407 | 1.095 (0.884–1.356) |
| | | TT+CT | 351 (53.18) | 358 (50.42) | Reference | |
| | Recessive model | TT | 74 (11.21) | 48 (6.76) | 0.005 | 0.576 (0.393–0.844) |
| | | CC+TC | 586 (88.78) | 662 (93.23) | Reference | |
| rs6125608 | Genotypes | GG | 9 (1.36) | 11 (1.55) | 0.716 | 1.181 (0.483–2.890) |
| | | GA | 125 (18.94) | 158 (22.25) | 0.153 | 1.213 (0.931–1.582) |
| | | AA | 526 (79.70) | 541 (76.20) | Reference | |
| | Alleles | G | 143 (10.83) | 180 (12.68) | 0.135 | 1.195 (0.946–1.509) |
| | | A | 1,177 (89.17) | 1,240 (87.32) | Reference | |
| | Dominant model | AA | 526 (79.70) | 541 (76.20) | 0.147 | 0.826 (0.638–1.069) |
| | | GG+GA | 134 (20.30) | 169 (23.80) | Reference | |
| | Recessive model | GG | 9 (1.36) | 11 (1.55) | 0.780 | 1.136 (0.465–2.775) |
| | | AA+GA | 651 (98.64) | 699 (98.45) | Reference | |

** After FDR correction, no P value was statistically significant (all P > 0.05).*

**TABLE 2 |** The positive findings of associations between *ANRIL* gene polymorphisms and anti-CCP of RA patients.

| SNP | Allele | Clinical features | Group | Alleles *n* (%) | | *P* value |
|---|---|---|---|---|---|---|
| | *(M/m)* | | | *M* | *m* | |
| rs944796 | C/G | Anti-CCP | Positive | 853 (79.28) | 223 (20.72) | **0.039** |
| | | | Negative | 143 (86.14) | 23 (13.86) | |
| rs2518723 | C/T | Anti-CCP | Positive | 615 (57.16) | 461 (42.84) | **0.039** |
| | | | Negative | 109 (65.66) | 57 (34.34) | |
| rs3217992 | C/T | Anti-CCP | Positive | 553 (51.39) | 523 (48.61) | **0.039** |
| | | | Negative | 71 (42.77) | 95 (57.23) | |

*Bold value means P < 0.05.*

**TABLE 3 |** Haplotype analysis of lncRNA genes in RA patients and controls.

| Haplotype | RA patients [*n*(%)] | Controls [*n*(%)] | *P* value | *OR* (95% CI) |
|---|---|---|---|---|
| ***ANRIL* rs1412830- rs944796- rs61271866- rs2518723- rs3217992** | | | | |
| CCATC | 91.88 (7.0) | 125.21 (8.8) | 0.077 | 0.776 (0.586–1.028) |
| CCTCC | 105.84 (8.0) | 108.60 (7.6) | 0.692 | 1.058 (0.800–1.400) |
| CCTCT | 614.30 (46.5) | 621.58 (43.8) | 0.109 | 1.135 (0.972–1.324) |
| CCTTC | 116.57 (8.8) | 128.95 (9.1) | 0.847 | 0.974 (0.749–1.268) |
| CGTTC | 190.02 (14.4) | 220.98 (15.6) | 0.419 | 0.916 (0.742–1.132) |
| TCATC | 122.50 (9.3) | 135.90 (9.6) | 0.824 | 0.971 (0.751–1.256) |
| ***Lnc-DC* rs7217280- rs10515177** | | | | |
| AG | 58.00 (4.4) | 85.00 (6.0) | 0.061 | 0.722 (0.512–1.017) |
| GA | 1,218.00 (92.3) | 1,293.00 (91.1) | 0.250 | 1.173 (0.893–1.540) |
| GG | 44.00 (3.3) | 42.00 (3.0) | 0.573 | 1.131 (0.736–1.738) |
| ***MALAT1* rs619586- rs4102217- rs591291- rs11227209- rs35138901** | | | | |
| ACTCT | 192.89 (14.6) | 227.78 (16.0) | 0.297 | 0.895 (0.727–1.103) |
| AGCCT | 769.36 (58.3) | 804.34 (56.6) | 0.393 | 1.069 (0.918–1.244) |
| AGTCC | 96.61 (7.3) | 115.61 (8.1) | 0.419 | 0.890 (0.672–1.180) |
| AGTCT | 133.58 (10.1) | 147.87 (10.4) | 0.796 | 0.968 (0.756–1.239) |
| GGTCT | 45.92 (3.5) | 32.65 (2.3) | 0.065 | 1.531 (0.971–2.413) |
| GGTGT | 75.01 (5.7) | 83.88 (5.9) | 0.799 | 0.959 (0.696–1.322) |
| ***ZFAS1* rs237742- rs73116127- rs6125607- rs6125608** | | | | |
| CACA | 111.00 (8.4) | 136.66 (9.6) | 0.259 | 0.860 (0.661–1.118) |
| CGCA | 137.00 (10.4) | 170.49 (12.0) | 0.170 | 0.846 (0.667–1.074) |
| CGCG | 143.00 (10.8) | 177.70 (12.5) | 0.164 | 0.847 (0.670–1.070) |
| CGTA | 425.00 (32.2) | 403.76 (28.4) | **0.036** | 1.191 (1.012–1.402) |
| TGCA | 504.00 (38.2) | 527.90 (37.2) | 0.622 | 1.040 (0.891–1.214) |

*Frequency < 0.03 in both controls and RA patients has been dropped. Bold value means P < 0.05.*

(rs7217280, rs10515177) in *lnc-DC,* five tagSNPs (rs619586, rs4102217, rs591291, rs11227209, rs35138901) in *MALAT1*, four tagSNPs (rs237742, rs73116127, rs6125607, rs6125608) in *ZFAS1* for genotyping in the present study.

The genomic DNA was extracted from the peripheral blood leukocytes by the Flexi Gene-DNA Kit (Qiagen, Valencia, CA). Improved multiple ligase detection reaction (iMLDR) genotyping assay, with technical support from the Center for Genetic & Genomic Analysis, Genesky Biotechnologies (Inc., Shanghai), was used for genotyping. Those individuals with 100% genotyping success rate for the above SNPs were included for final analysis.

## Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

PBMCs were isolated from 5 ml anticoagulated peripheral blood, and stored at −80°C until processed. Total RNA in PBMCs was extracted with TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) and the concentration of RNA was quantified using NanoDrop 2000 spectrophotometer (Thermo Scientific, USA). Then, the PrimeScriptTM RT reagent Kit (Takara Bio Inc., Japan) was used to reverse-transcribed total RNA into cDNA.

The expression levels of ANRIL, lnc-DC, MALAT1, ZFAS1 in PBMCs were detected by qRT-PCR with SYBR Green (SYBR Premix Ex Taq II, Takara Bio Inc., Japan). This experiment was performed on QuantStudio 12K Flex Real-Time PCR System (Applied Biosystems, Foster City, CA, USA), and according to the following cycle conditions: 95°C for 1 min, followed by 42 cycles at 95°C for 10 s, 60°C for 30 s and 72°C for 1 min. The relative expression levels of lncRNAs were calculated by comparison with housekeeping gene β-actin in the same sample as internal control, and expressed using $2^{-\Delta\Delta Ct}$ method normalized to endogenous control (23).

## Statistical Analysis

Statistical analysis was performed with the SPSS 23.0 (SPSS Inc., IL, USA). We performed Hardy-Weinberg equilibrium test by Chi-square ($\chi^2$) among normal controls. For the associations of genotype, allele distribution frequencies of each SNP with RA were estimated by logistic regression analyses. The lncRNAs levels were shown as median value and interquartile range, and the differences in lncRNAs levels between two groups, three groups were analyzed by Mann-Whitney *U*-test, Kruskal-Wallis *H*-test, respectively. The correlations between lncRNAs levels and several experimental indexes of RA patients were analyzed by Spearman rank correlation coefficient test. Dominant model, recessive mode was used for statistical analysis, and haplotype

analysis was conducted with the SHEsis software (24). *P* value (two-sided) <0.05 was considered as statistically significant. False discovery rate (FRD) was used for multiple testing in SNP analysis.

# RESULTS

## Association of lncRNAs Genes Polymorphisms With RA Susceptibility

We included 546 females and 114 males in RA patients for genotyping with a median age of 51, while there were 574 females and 136 males with a median age of 49 in normal controls. The observed genotype frequencies of rs7044859 was not conform to Hardy-Weinberg equilibrium, thus we excluded this SNP in finally analysis. The results of allele and genotype frequencies of all SNPs were summarized in **Table 1**.

In *ANRIL* gene, the rs1412830 TT genotype frequency was significantly increased in RA patients in comparison to normal controls, while the rs944796 GG genotype frequency was significantly decreased (TT vs. CC: $P = 0.017$; GG vs. CC: $P = 0.013$, respectively). In addition, an increased risk of rs1412830 variant, as well as a decreased risk of rs944796, was observed under the recessive model (TT vs. CC+CT: $P = 0.016$; GG vs. CC+GC: $P = 0.008$, respectively). However, these significant associations were disappeared after multiple testing by FDR correction (all $P > 0.05$). Comparing the genotype and allele frequencies of the ZFAS1 rs6125607 polymorphism among RA patients and normal controls, we found that TT genotype and T allele frequencies were significantly higher in RA patients than normal controls (TT vs. CC: $P = 0.007$; T vs. C: $P = 0.040$, respectively), and an increased risk of rs6125607 polymorphism existed in recessive model (TT vs. CC+TC: $P = 0.005$). After FDR correction, these differences were not statistically significant (TT vs. CC: $P = 0.181$; T vs. C: $P = 0.496$, TT vs. CC+TC: $P = 0.080$, respectively). Similarly, no significant associations between *lnc-DC*, *MALAT1* genes polymorphism and RA susceptibility were found (**Table 1**).

To examine the potential genetic association between the genotype, allele frequencies of *ANRIL, lnc-DC, MALAT1, ZFAS1* genes and anti-CCP, RF in RA patients, we performed a case-only analysis (**Table S1**). In *ANRIL* gene, the rs944796 G allele, rs2518723 T allele frequencies were significantly increased in RA patients with anti-CCP-positive when compared to patients with anti-CCP-negative (all $P < 0.05$), while rs3217992 T allele frequency was reduced ($P = 0.039$) (**Table 2**). No significant differences existed in allele and genotype frequencies of *lnc-DC, MALAT1, ZFAS1* genes.

## Haplotype Analysis

Six main haplotypes (CCATC, CCTCC, CCTCT, CCTTC, CGTTC, TCATC) for *ANRIL*, three main haplotypes (AG, GA, GG) for lnc-DC, six main haplotypes (ACTCT, AGCCT, AGTCC, AGTCT, GGTCT, GGTGT) for *MALAT1* and five main haplotypes (CACA, CGCA, CGCG, CGTA, TGCA) for *ZFAS1* were detected by SHEsis software (**Table 3**). The results demonstrated that the haplotype CGTA frequency was significantly higher in RA patients than normal controls ($OR = 1.191$, 95% CI: 1.012–1.402, $P = 0.036$).

## LncRNAs Expression Levels in PBMCs From RA Patients and Normal Controls

We further analyzed the association of ANRIL, lnc-DC, MALAT1, ZFAS1 levels with RA patients by qRT-PCR. As shown in **Table 4**, the expression levels of ANRIL, lnc-DC, MALAT1, ZFAS1 in PBMCs were significantly reduced in RA patients than normal controls (all $P < 0.001$). However, the differences in these lncRNAs levels between anti-CCP-positive RA patients and anti-CCP-negative RA patients, as well as RA patients with RF-positive and RF-negative RA patients, were not statistically significant.

The correlation of ANRIL, lnc-DC, MALAT1, ZFAS1 expression levels with clinical parameters, disease activity of RA patients were also analyzed, and the results shown that the expression level of ZFAS1 was negatively associated with CRP in RA patients ($P = 0.002$). However, there were no significant correlations of these lncRNAs levels with DAS28 of RA patients (**Table 5**). The potential influence of main medical therapies including glucocorticoids, disease-modifying antirheumatic drugs (DMARDs), biologics on lncRNAs expression levels in RA patients were assessed in this study. Similarly, no significant association was found (**Table 6**).

## Associations Between lncRNAs Genes Polymorphisms With Their Levels in RA Patients

To examine the associations between the respective genotype frequencies of these lncRNAs genes with their expression levels in RA patients, we included 65 patients for analysis. However, there were no significant differences regarding these lncRNAs expression levels between their disparate genotypes of RA patients (**Table 7**).

# DISCUSSION

To date, the exact pathogenic mechanism of RA remains largely unknown, although several pro-inflammatory cytokines including tumor necrosis factor-α (TNF-α), IL-6, IL-1b have been reported to related to the occurrence of RA (25–27). Previous studies have shown that lncRNAs had distinct and specific roles in the activation and differentiation modulating of immune cell, and lncRNAs played an important role in autoimmune diseases (28). A study detected lncRNA transcription in CD14+ monocytes isolated from peripheral blood cells of RA patients before and after anti-IL-6R (tocilizumab) or anti-TNF-α (adalimumab) therapy by a microarray-based experiment. They observed that 7,419 lncRNAs expression levels were altered by either IL-6 or TNF-α, 85 of which exhibited were significant changed (29). These results suggested that lncRNAs were very important in the molecular pathophysiology of RA. In the present study, our results demonstrated that lower expression levels of ANRIL, lnc-DC, MALAT1, ZFAS1 existed in RA patients, and *ANRIL*, *lnc-DC*, *MALAT1*, *ZFAS1* genes were not related to RA susceptibility.

**TABLE 4 |** Comparison of lncRNAs expression level in PBMCs between different subgroups.

| Group | Number | ANRIL | *P* value | Lnc-DC | *P* value | MALAT1 | *P* value | ZFAS1 | *P* value |
|---|---|---|---|---|---|---|---|---|---|
| RA patients | 120 | 0.605 (0.382, 0.849) | <0.001 | 0.378 (0.269, 0.586) | <0.001 | 0.409 (0.257, 0.533) | <0.001 | 0.458 (0.352, 0.646) | <0.001 |
| Normal controls | 120 | 0.853 (0.612, 1.147) | | 0.818 (0.537, 1.166) | | 0.932 (0.627, 1.228) | | 0.870 (0.625, 1.161) | |
| RA patients with anti-CCP-positive | 99 | 0.603 (0.404, 0.868) | 0.866 | 0.387 (0.264, 0.591) | 0.920 | 0.418 (0.250, 0.543) | 0.681 | 0.469 (0.362, 0.635) | 0.926 |
| RA patients with anti-CCP-negative | 21 | 0.619 (0.350, 0.823) | | 0.358 (0.284, 0.577) | | 0.372 (0.270, 0.489) | | 0.391 (0.337, 0.676) | |
| RA patients with anti-RF-positive | 101 | 0.629 (0.404, 0.870) | 0.210 | 0.344 (0.268, 0.561) | 0.453 | 0.415 (0.251, 0.575) | 0.563 | 0.469 (0.352, 0.642) | 0.997 |
| RA patients with anti-RF-negative | 19 | 0.510 (0.360, 0.675) | | 0.436 (0.273, 0.614) | | 0.379 (0.306, 0.474) | | 0.444 (0.349, 0.682) | |

**TABLE 5 |** Association of lncRNAs expression levels with clinical parameters, disease activity of RA patients.

| Parameters | Number | ANRIL | | Lnc-DC | | MALAT1 | | ZFAS1 | |
|---|---|---|---|---|---|---|---|---|---|
| | | $r_s$ | *P* value | $r_s$ | *P* value | $r_s$ | *P* value | $r_s$ | *P* value |
| C3 | 107 | −0.035 | 0.719 | −0.054 | 0.583 | 0.077 | 0.431 | −0.100 | 0.305 |
| C4 | 106 | −0.122 | 0.213 | −0.027 | 0.781 | 0.094 | 0.339 | −0.020 | 0.840 |
| ESR | 118 | 0.035 | 0.705 | 0.034 | 0.712 | 0.069 | 0.457 | −0.090 | 0.334 |
| CRP | 118 | −0.038 | 0.682 | −0.094 | 0.313 | −0.178 | 0.054 | −0.278 | 0.002 |
| DAS28 | 118 | 0.071 | 0.444 | 0.139 | 0.132 | −0.078 | 0.399 | −0.036 | 0.695 |

**TABLE 6 |** Association of these lncRNAs expression levels with medical therapy of RA patients.

| Group | Number | ANRIL level | *P* value | Lnc-DC level | *P* value | MALAT1 level | *P* value | ZFASA level | *P* value |
|---|---|---|---|---|---|---|---|---|---|
| Glucocorticoids | | | 0.302 | | 0.764 | | 0.340 | | 0.500 |
| NA | 30 | 0.516 (0.344, 0.948) | | 0.445 (0.256, 0.608) | | 0.450 (0.259, 0.581) | | 0.452 (0.355, 0.597) | |
| ≤7.5 mg/d | 28 | 0.573 (0.323, 0.831) | | 0.325 (0.250, 0.512) | | 0.437 (0.240, 0.561) | | 0.464 (0.299, 0.614) | |
| >7.5 mg/d | 62 | 0.634 (0.451, 0.834) | | 0.348 (0.278, 0.580) | | 0.389 (0.374, 0.703) | | 0.462 (0.374, 0.703) | |
| DMARDs | | | 0.532 | | 0.232 | | 0.366 | | 0.257 |
| No | 39 | 0.619 (0.428, 0.901) | | 0.414 (0.269, 0.614) | | 0.404 (0.226, 0.539) | | 0.410 (0.349, 0.575) | |
| Yes | 81 | 0.587 (0.350, 0.835) | | 0.338 (0.265, 0.540) | | 0.418 (0.273, 0.533) | | 0.471 (0.361, 0.679) | |
| Biologics | | | 0.489 | | 0.423 | | 0.211 | | 0.095 |
| No | 111 | 0.603 (0.399, 0.838) | | 0.377 (0.266, 0.570) | | 0.408 (0.256, 0.533) | | 0.455 (0.345, 0.618) | |
| Yes | 9 | 0.760 (0.291, 1.120) | | 0.461 (0.289, 0.687) | | 0.432 (0.395, 0.588) | | 0.632 (0.392, 0.902) | |

*ANRIL* gene was located in the chromosome 9p21 region, and it was the well-defined genetic risk locus related to several diseases such as coronary artery disease (CAD), diabetes, and breast cancer (30–32). Our results implied that *ANRIL* rs1412830, rs944796 variant might associated with RA susceptibility, while the significant associations were disappeared after multiple testing. However, we found that *ANRIL* rs944796 G, rs2518723 T, rs3217992 T allele frequencies were significantly associated with anti-CCP in RA patients, this suggested to us that *ANRIL* gene variation might be involved in the RA development. In addition, disease-associated SNPs resided in this region had been reported to change the expression of ANRIL, demonstrating that altered ANRIL expression might be involved in predisposition to these disorders (33). Two SNPs (rs10757278 and rs1333045) in *ANRIL*, which had been highlighted as potential causal variants for the association with CAD, were reported to be associated with abnormal ANRIL expression level in Peripheral blood (34, 35). Moreover, our results demonstrated that compared with normal controls, ANRIL expression level was significantly decreased in PBMC from RA patients. We further explored the influence of the five SNPs on ANRIL level in PBMC from RA patients, unfortunately, there were no significant differences regarding ANRIL level between disparate genotypes of these SNPs.

In a previous study, the authors discovered a new lncRNA (named lnc-DC) located on chromosome 17 region, which near signal transducer and activator of transcription 3 (*STAT3*) gene (36). There were increasing researches to explore the contribution of lnc-DC in autoimmune diseases. Shaker et al. found that serum level of lnc-DC in multiple sclerosis (MS) patients were significantly increased, and serum lnc-DC level maybe used to as a potential novel biomarkers for MS diagnosis (37). One of our recent studies shown that the lnc-DC expression level was significantly decreased in PBMCs from SLE patients than controls, while *lnc-DC* rs10515177 variant was not associated with SLE susceptibility (38). Similarly, the lnc-DC expression level was significantly lower in PBMC from

**TABLE 7 |** Association between lncRNA levels with their respective genotype in RA patients.

| *ANRIL* SNPs | Genotype | Number | *ANRIL* level | *P* value |
|---|---|---|---|---|
| rs1412830 | CC | 49 | 0.542 (0.404, 0.742) | 0.210 |
| | CT | 14 | 0.418 (0.267, 0.672) | |
| | TT | 2 | 0.913 (0.504, 1.322) | |
| rs944796 | CC | 36 | 0.585 (0.371, 0.798) | 0.437 |
| | GC | 27 | 0.493 (0.360, 0.786) | |
| | GG | 2 | 0.325 (0.088, 0.562) | |
| rs61271866 | TT | 44 | 0.562 (0.404, 0.764) | 0.251 |
| | TA | 19 | 0.497 (0.274, 0.641) | |
| | AA | 2 | 0.913 (0.504, 1.323) | |
| rs2518723 | CC | 21 | 0.603 (0.414, 0.975) | 0.329 |
| | CT | 28 | 0.520 (0.339, 0.646) | |
| | TT | 16 | 0.476 (0.335, 0.818) | |
| rs3217992 | CC | 21 | 0.459 (0.296, 0.647) | 0.219 |
| | CT | 29 | 0.582 (0.472, 0.839) | |
| | TT | 15 | 0.587 (0.404, 0.955) | |
| ***Lnc-DC* SNPs** | **Genotype** | **Number** | **Lnc-DC level** | ***P* value** |
| rs7217280 | GG | 63 | 0.378 (0.282, 0.603) | 0.649 |
| | GA | 2 | 0.458 (0.431, 0.484) | |
| | AA | 0 | | |
| rs10515177 | GG | 58 | 0.383 (0.289, 0.600) | 0.703 |
| | GA | 7 | 0.431 (0.269, 0.629) | |
| | AA | 0 | | |
| ***MALAT1* SNPs** | **Genotype** | **Number** | **MALAT1 level** | ***P* value** |
| rs619586 | AA | 55 | 0.415 (0.253, 0.530) | 0.167 |
| | GA | 10 | 0.285 (0.128, 0.473) | |
| | GG | 0 | | |
| rs4102217 | GG | 48 | 0.337 (0.214, 0.486) | 0.064 |
| | CG | 13 | 0.477 (0.381, 0.634) | |
| | CC | 4 | 0.463 (0.380, 0.851) | |
| rs591291 | CC | 24 | 0.416 (0.257, 0.502) | 0.905 |
| | CT | 29 | 0.415 (0.199, 0.508) | |
| | TT | 12 | 0.389 (0.155, 0.617) | |
| rs11227209 | CC | 59 | 0.415 (0.245, 0.530) | 0.267 |
| | CG | 6 | 0.285 (0.181, 0.473) | |
| | GG | 0 | | |
| rs35138901 | TT | 56 | 0.418 (0.257, 0.526) | 0.068 |
| | CT | 9 | 0.193 (0.161, 0.454) | |
| | CC | 0 | | |
| ***ZFAS1* SNPs** | **Genotype** | **Number** | **ZFAS1 level** | ***P* value** |
| rs237742 | CC | 31 | 0.471 (0.376, 0.584) | 0.392 |
| | CT | 30 | 0.394 (0.300, 0.605) | |
| | TT | 4 | 0.423 (0.359, 0.783) | |
| rs73116127 | GG | 51 | 0.444 (0.362, 0.6001) | 0.342 |
| | GA | 14 | 0.377 (0.325, 0.529) | |
| | AA | 0 | | |
| rs6125607 | CC | 25 | 0.398 (0.351, 0.516) | 0.375 |
| | CT | 31 | 0.434 (0.316, 0.618) | |
| | TT | 9 | 0.523 (0.419, 0.655) | |
| rs6125608 | AA | 50 | 0.438 (0.353, 0.600) | 0.998 |
| | GA | 14 | 0.428 (0.344, 0.652) | |
| | CC | 1 | 0.444 | |

*(Continued)*

**TABLE 7 |** Continued

*Median (interquartile range).*

RA patients than normal controls in the present study. We also explore the potential association of two SNP (rs7217280, rs10515177) in *lnc-DC* with RA susceptibility, however, no significant relationship was found. Our study provided the first evidence that lnc-DC might be involved in the development of RA, and the specific roles of *lnc-DC* genetic variation in pathophysiology of RA need to be further explored.

MALAT1 was expressed on chromosome 11q13, and widely expressed in multiple normal tissues such as reproductive, endocrine and immune systems with an important role in autoimmune diseases including RA, SLE, MS (18, 19, 37, 39). Quercetin is a dietary antioxidant, which has been shown to be effective in the treatment of arthritis in pre-clinical studies, and Pan et al. tried to analyze the mechanisms responsible for the quercetin-induced FLS apoptosis in RA patients (19). Their data indicated that quercetin induced FLS apoptosis in RA patients via upregulating MALAT1, and MALAT1 promoted apoptosis by inhibiting the activation of the phosphoinositide 3-kinase (PI3K)/AKT pathway. In this study, decreased MALAT1 expression level in PBMC from RA patients was firstly reported, while *MALAT1* genetic variation was not correlated with RA risk. Our findings provided new evidence that MALAT1 might be involved in RA development.

ZFAS1, located at chromosomal band 20q13.13, was reported as an important player to regulate the development of human cancers including glioma, lung, ovary, gastric, and breast cancer (40–42). In addition, ZFAS1 was found to promote chondrocytes proliferation, migration, and inhibit apoptosis and matrix synthesis in osteoarthritis (OA), and ZFAS1 expression level was downregulated in OA chondrocytes in comparison to mild chondrocytes (43). Another study by Xiao et al. also found more than five times ZFAS1 level in the healthy appearing area of cartilage compared with the pathology area in human knee osteoarthritis (44). Similarly, our results demonstrated that the expression of ZFAS1 in PBMCs was significantly reduced in RA patients than normal controls, and associated with CRP in RA. In the present study, we also analyzed the potential relationship between rs237742, rs73116127, rs6125607, rs6125608 variants in *ZFAS1* and genetic susceptibility to RA, and no difference achieved statistical significance. However, haplotype analysis implied that the haplotype CGTA frequency for *ZFAS1* was significantly higher in RA patients than normal controls. These founding would help improve our understanding of the roles of *ZFAS1* genetic variants in the pathogenesis of RA.

In conclusion, our study provided the first evidence that *ANRIL*, *lnc-DC*, *MALAT1*, *ZFAS1* genes polymorphisms might not be associated with RA susceptibility in the Chinese population. However, several SNPs in *ANRIL* were related to anti-CCP in RA. In addition, alternations of ANRIL, lnc-DC, MALAT1, ZFAS1 levels and significant correlations of ZFAS1 level with CRP in RA patient demonstrated that these lncRNAs might be regarded as an auxiliary biomarker for RA diagnosis, as well as used to distinguish RA serotypes.

However, some limitations existed in our study should be acknowledged. Firstly, this study does not eliminate the potential influence of ethnic background, environmental factor. Secondly, we are not able to assess the associations between these lncRNAs levels and disease severity, clinical variables, and therapeutic schedule of RA patients over a long period in this case-control study. Finally, our sample size may not be sufficient, and lead to the low power of this study. Hence, replication studies with larger sample size, different ethnic populations are awaited to further explore the exact role of these lncRNAs in RA.

## ETHICS STATEMENT

This study was approved by the Ethical Committee of Anhui Medical University (Hefei, Anhui, China). All the study subjects provided informed consent to participate in this study. All studies on humans described in the present manuscript were carried out with the approval of the responsible ethics committee and in accordance with national law and the Helsinki Declaration of 1975 (in its current, revised form).

## AUTHOR CONTRIBUTIONS

T-PZ, H-FP, and D-QY designed the study. T-PZ and B-QZ conducted the experiment. S-ST and Y-GF performed the statistical analyses. T-PZ drafted the manuscript. X-ML participated in the collection of samples. H-FP and D-QY contributed to manuscript revision. All the authors approved the final submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.02529/full#supplementary-material

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REVIEW
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# Triggers of Autoimmunity: The Role of Bacterial Infections in the Extracellular Exposure of Lupus Nuclear Autoantigens

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Infections are considered important environmental triggers of autoimmunity and can contribute to autoimmune disease onset and severity. Nucleic acids and the complexes that they form with proteins—including chromatin and ribonucleoproteins—are the main autoantigens in the autoimmune disease systemic lupus erythematosus (SLE). How these nuclear molecules become available to the immune system for recognition, presentation, and targeting is an area of research where complexities remain to be disentangled. In this review, we discuss how bacterial infections participate in the exposure of nuclear autoantigens to the immune system in SLE. Infections can instigate pro-inflammatory cell death programs including pyroptosis and NETosis, induce extracellular release of host nuclear autoantigens, and promote their recognition in an immunogenic context by activating the innate and adaptive immune systems. Moreover, bacterial infections can release bacterial DNA associated with other bacterial molecules, complexes that can elicit autoimmunity by acting as innate stimuli of pattern recognition receptors and activating autoreactive B cells through molecular mimicry. Recent studies have highlighted SLE disease activity-associated alterations of the gut commensals and the expansion of pathobionts that can contribute to chronic exposure to extracellular nuclear autoantigens. A novel field in the study of autoimmunity is the contribution of bacterial biofilms to the pathogenesis of autoimmunity. Biofilms are multicellular communities of bacteria that promote colonization during chronic infections. We review the very recent literature highlighting a role for bacterial biofilms, and their major components, amyloid/DNA complexes, in the generation of anti-nuclear autoantibodies and their ability to stimulate the autoreactive immune response. The best studied bacterial amyloid is curli, produced by enteric bacteria that commonly cause infections in SLE patients, including *Escherichia coli* and *Salmonella spps.* Evidence suggests that curli/DNA complexes can trigger autoimmunity by acting as danger signals, molecular mimickers, and microbial chaperones of nucleic acids.

**Keywords: autoantigens, autoantibodies, extracellular DNA, bacterial infections, lupus (SLE)**

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## INTRODUCTION

Nucleic acids and the proteins that bind to nucleic acids are the main autoantigens (autoAgs) in the autoimmune disease systemic lupus erythematosus (SLE) (1). In SLE patients, autoantibodies (autoAbs) are found against lupus specific nuclear antigens, such as double-stranded DNA (dsDNA) and the Smith antigen (Sm), a non-histone nuclear RNA complex with ribonucleoprotein present in spliceosomes. Other SLE autoAbs bind different nucleic acid constituents, nucleosomes, ribosomes, and ribonucleoproteins such as Ro60 and La, and are shared with other autoimmune diseases (2). Of note, nucleic acids are not only autoAgs recognized by autoAbs in SLE, but they also represent conserved pathogen-associated molecular patterns (PAMPs) (3) of viruses and bacteria (4–8) and host nucleic acids are damage-associated molecular patterns (DAMPs) (9–14). The immune system has evolved pattern recognition receptors (PRRs) to detect the inappropriate presence of these macromolecules in the cytosolic and extracellular spaces. The compartmentalization of endogenous nucleic acids and PRRs usually prevents the inappropriate stimulation of the immune system by these potent danger signals in absence of infections (15).

The main PRRs that have been found to be involved in the pathogenesis of lupus are toll-like receptors (TLR) 7 and 9, which, respectively, recognize dsRNA and DNA rich in hypomethylated CpGs (16, 17). TLR7 and TLR9 are localized within the endosomes (18), suggesting that the origin of their ligands is extracellular and prompting the question of the source of the nucleic acids being detected. More recently, an interest has been sparked for intracellular DNA sensors, including cGAS, suggesting that nucleic acids may also be stimulating the immune system in the cytoplasm (19). Nevertheless, as autoAgs, nucleic acids have to operate in the extracellular compartment to engage B cells and autoAbs, leaving the research field wondering about source and nature of the extracellular nuclear autoAgs.

There is abundant evidence—mostly in murine models of lupus—that genetic defects in cell death and clearance of dead cells (efferocytosis) lead to release of lupus autoAgs, the combination of which can trigger autoimmunity in the right genetic background (20–23). These genetic defects, however, are rarely found in SLE patients (24), indicating the need to search for alternative causes of release of nucleic acids in the extracellular compartment.

Infections in general are thought to play a role in the development of autoimmune disease, contributing to abnormal immune responses through molecular mimicry, epitope spreading, and bystander activation (25–27). While there are multiple theories in which infection by a pathogen is thought to lead to lupus autoimmunity, a common thread that ties the many mechanisms together is that infections can lead to exposure of the immune system to nucleic acids that the host otherwise would not be exposed to (28, 29). Infections can be a dangerous and powerful source of extracellular nuclear antigens because they expose nucleic acids derived from bacteria or viruses, especially from the bacterial biofilms, which are very rich in bacterial DNA and amyloids carrying extracellular DNA (30–32). Moreover, infections can release host nucleic acids in the extracellular compartment because of the different types of cell death that can occur during infection, either as a direct cytotoxicity of the pathogen or as a consequence of normal immune responses, notably pyroptosis (33) and the extrusion of neutrophil extracellular traps (NETs) (11, 34). The interplay between infections, biofilms and cell death continues to be the focus of much discussion in the field (35–38).

Circulating extracellular nucleic acids can be found in healthy individuals and were first described in 1948 (39). Their role was not associated with autoimmunity until 1966, when free DNA was found in SLE patients (40). Since then, novel techniques have shown that microorganism-derived and host-derived nucleic acids can be immunostimulatory, inducing the production of type I Interferons (I-IFNs) through both TLR-dependent and independent pathways (19, 41–45). In this review, we present findings from recent literature highlighting a role for bacterial infections and bacterial biofilms in the extracellular exposure of nuclear autoAgs, and their ability to stimulate the autoreactive immune responses in SLE (**Figure 1**).

## INFECTIONS ARE AN IMPORTANT CAUSE OF MORBIDITY AND MORTALITY IN SLE

Infections are the leading cause of both morbidity and mortality in SLE patients, accounting for up to 55% of deaths in SLE (46–48). A large study that analyzed more than 30,000 SLE patients found that infections were a major burden, with many subsequent deaths correlated with immunosuppressive drugs and lupus nephritis (49, 50). It remains unclear whether immunosuppressive drugs and the severity of the autoimmune disease that requires such drugs predispose to infections or infections augment disease severity, or rather whether the two entities create a pathogenic vicious circle. Although lupus disease develops from an interplay between genetic and environmental factors, infectious agents have been proposed as triggers of lupus disease development due to compelling evidence of shared production of SLE-related autoAbs like anti-Sm Abs in infectious mononucleosis and cross-reactivity between SLE autoAbs and Epstein-Barr virus (EBV) proteins, which suggest the occurrence of molecular mimicry (27, 51). The most common type of infection in lupus patients is bacterial, accounting for 80% of all infections in lupus, followed by viral infections (52, 53). Viral infections are also very common in SLE patients and have been hypothesized to play a pathogenic role in SLE. A large body of literature supports a role for EBV (54, 55) and parvovirus (56–60), and most recently human papilloma virus (61, 62) has also been implicated. In this review, we focus on the perspectives of bacterial infections in lupus because of their higher incidence in SLE patients, bridging new research on biofilms and sensing of extracellular nucleic material with its implications in autoimmunity. We recommend recent reviews with excellent focus on viral infections (54, 55, 63, 64).

Among the bacterial infections, urinary tract infections (UTIs), soft tissue infections, bloodstream infections, and pneumonia are more common in SLE patients than in the general

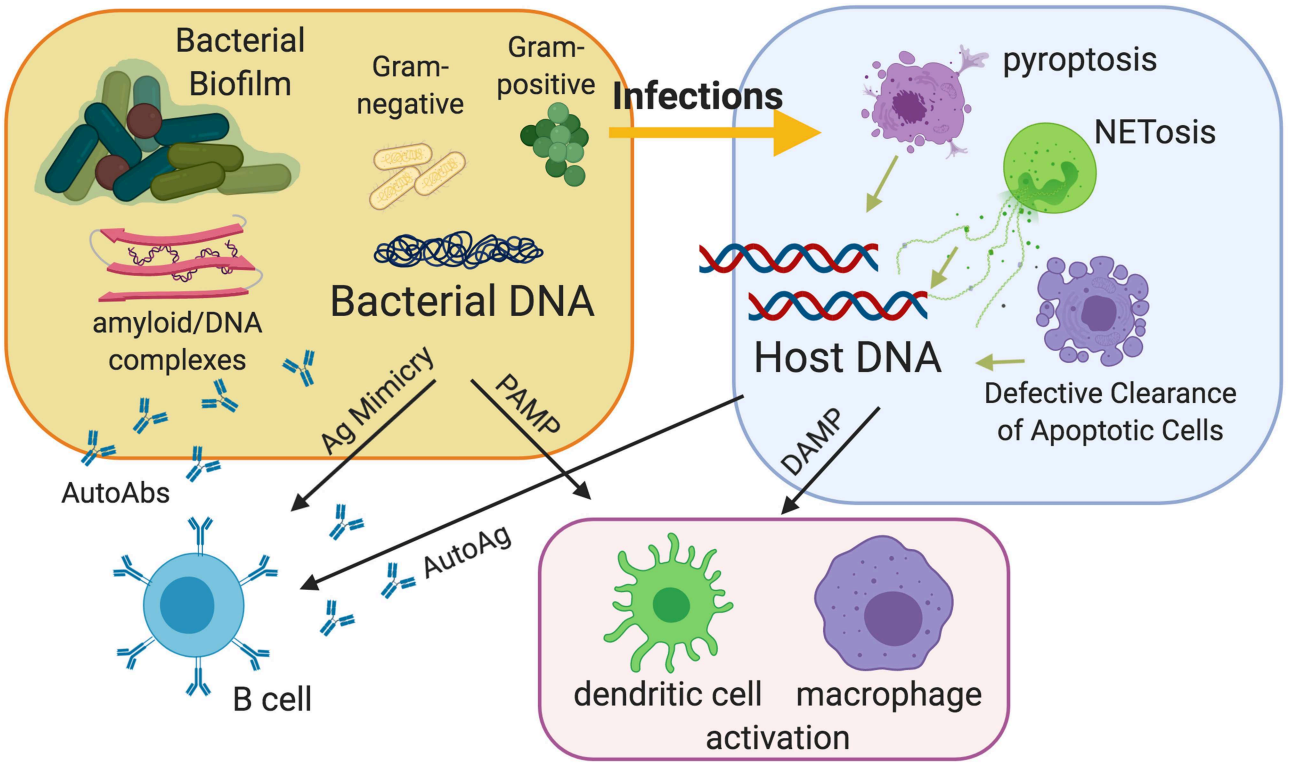


**FIGURE 1 |** Model of the contribution of bacterial infections and bacterial biofilms to the pathogenesis of autoimmunity. Infections can be a source of extracellular nuclear antigens because they expose nucleic acids derived from bacteria and especially from bacterial biofilms, which are very rich in bacterial DNA and amyloids carrying extracellular DNA. Amyloid curli/DNA complexes can trigger autoimmunity by acting as danger signals to activate innate immunity and as molecular mimickers to activate autoreactive B cells. Moreover, infections can release host nucleic acids because of the different types of cell death that can occur during infection, notably pyroptosis and the extrusion of neutrophil extracellular traps (NETs). Defective clearance of apoptotic cells and subsequent post-apoptotic necrosis may also be a source of extracellular nucleic acids.

population (65, 66), either because of the immunosuppressive therapy or inherent immune abnormalities. *Streptococcus pneumoniae*, *Escherichia coli,* and *Staphylococcus aureus* are the most frequently associated pathogens in these infections (65, 66). Moreover, common pathogens, including *Salmonella enterica* serovar Typhimurium and *Salmonella enterica* serovar Enteritidis, appear to behave more aggressively in SLE patients; instead of causing localized gastroenteritis, *Salmonella* infection in SLE patients results in bacteremia and complications in soft tissues with high mortality rates (66–69). Additionally, SLE patients with bloodstream infections have a higher risk of developing severe flares (27, 70), making it difficult to distinguish cause and effect of the flare (27, 71–73). These results beg the question of whether infections can trigger SLE onset, or whether they are only associated with flares after the disease has started, and a definitive answer is yet to be found. Clinical studies have shown that patients with SLE who had infection-related hospitalizations suffer a profoundly increased risk of end-stage renal disease, suggesting that infections have an effect on SLE disease activity (74, 75). A study of 7,326 patients newly diagnosed with SLE showed that the occurrence of three or more infection-related hospitalizations greatly increased risk of end-stage renal disease (ESRD), indicating an effect of infections on SLE disease activity (75). The risk of infection-related hospitalizations was independently associated with ESRD following stratified analysis that adjusted for chronic kidney diseases (CKD) and other confounding factors. In the same article, the infections that had a higher hazard risk of ESRD were septicemia-bacteremia, followed by pneumonia and UTI, with soft tissue infections at the fourth place, indicating that the infections leading to ESRD were both systemic and localized to the kidney. UTIs were classified as any genitourinary infection, including pyelonephritis, UTIs and perinephric abbesses, and only patients who had infections as reason for requiring hospitalization were enrolled in the study, therefore minimizing the inclusion of iatrogenic infections such as catheter-induced ones. These data suggest a role as a promoter of lupus severity for a generalized activation of the immune system that is induced by severe bacterial infections, even when the stimulation derives from localized infection. In another study, the incidence of invasive pneumococcal infections in SLE patients was found to be 13 times higher than the incidence in the general population, an association that did not correlate with the use of immunosuppressants (76). Although the frequency of infection before lupus onset has not been thoroughly documented in the literature, some case reports suggest that it is increased, especially

in pediatric lupus (77, 78), suggesting that infections accelerate SLE onset in predisposed individuals.

## MICROBIOME AND LUPUS

Recently, the symbiotic microbiota in our body have gained much attention as an influential variable conditioning human health and disease (79, 80). The gut microbiota have been subject of intense investigation because of the intriguing findings that gut dysbiosis has local and systemic effects on the immune system (81–84), but microbiota also reside beyond the gut, colonizing mucosal tissues and specific niches, from the skin to oral cavity, vagina, or the bladder, where they are expected to exercise major effects as well (85). Studies focused on lupus specifically found a reduction in species diversity in the gut microbiota that is associated with specific enteric bacteria in SLE patients (86–88) or their first-degree relatives (89) and was present in cohorts from different continents with different ethnicities (90). DNA from Enterococcus gallinarum was found in the liver of SLE patients, and colonization of autoimmune-prone mice with these bacteria induced autoantibodies and decreased survival (91). *Ruminococcus gnavus* of the *Lachnospiraceae* family is another pathobiont reported to be overrepresented in SLE gut dysbiosis and has been shown to elicit specific Ab responses correlating with anti-DNA autoAb levels, SLE activity and lupus nephritis in particular. *Ruminococcus gnavus* specific lipoglycans are proposed as novel immunodominant antigens as well as innate stimuli in SLE through the binding of TLR2 (83).

Studies exploring the role of gut microbiota on disease progression in lupus-prone mice further corroborate the importance of bacteria and infection on lupus disease development (87, 92). Consistent with findings that germ-free conditions do not influence disease outcome (93), treating lupus-prone mice with antibiotics from the time of weaning also did not impact disease activity. However, when antibiotic treatment initiation was delayed until after disease onset, SLE autoimmunity was attenuated. Lupus disease progression was thought to be attenuated by targeting Clostridial strains (i.e., *Lachnospiraceae*) found to be increased in both lupus mice and feces of human SLE patients, while allowing for beneficial commensals found in healthy individuals (i.e., *Lactobacillus* spp.) to thrive. Treatment with vancomycin, which targets Gram-positive bacteria and thus spares Lactobacilli, also reduced the translocation of LPS across the intestinal barrier, further suggesting that microbial translocation from barrier dysfunction may be an environmental trigger in SLE (94). Beside antibiotics, another variable that affects the microbiome of experimental mice and influences the severity of lupus in susceptible strains is the diet (95). An example, especially important for researchers working with mouse models of lupus, is the acidification of water that was found to decrease the levels of autoantibodies and delay the onset of nephritis in lupus-prone mice (96) while augmenting the presence of *Lactobacillus reuteri*, belonging to the phylum of Firmicutes. These results are in agreement with the lower Firmicutes/Bacteroidetes ratio that was found in SLE patients, in other inflammatory diseases, and in elderly people (86, 97–99), suggesting that bacteria belonging to the Firmicutes phylum, such as Lactobacilli ssp, are important to maintain immunological tolerance (92, 94).

## INFECTIONS AS ENVIRONMENTAL PATHOGENIC FACTOR

The association between infections and autoimmunity raises the question of why autoimmune diseases are not more common in the human population, which normally is exposed to a wide variety of bacteria and infections. Indeed, humoral autoimmunity is relatively common in the context of infections. For example, antinuclear antibodies (ANA) and rheumatoid factor (RF) are found in acute or chronic infections (100–102); the major difference is that in these circumstances, the autoantibodies are transient and do not induce a chronic defined autoimmune disease. Coupled with conflicting studies showing that the lupus-prone MRL/lpr strain of mice, which harbors a strong genetic drive for autoimmunity through the lack of the apoptotic receptor Fas, can still develop SLE-like disease in germ-free conditions, these data suggest that genetics do play an important part in disease manifestation (93). A different strain of lupus-prone mice showed instead a milder autoimmunity under germ-free conditions (103). To account for all of these findings, we envision that, in susceptible humans and mice, a genetic predisposition for immune dysfunction may increase the host exposure to nucleic acid material during infections and trigger a lymphocyte repertoire already prone to autoreactivity in the presence of specific HLA haplotypes, while in non-autoimmune-prone humans and mice, infections normally result in less prolonged exposure of a repertoire more self-tolerant to nucleic acid material. This is supported by reports of high levels of circulating endotoxin and more frequent bacteremia in SLE patients (68, 69, 89, 104).

## EXPOSURE TO EXTRACELLULAR NUCLEAR AUTOAGS IN SLE

Bacterial infections can expose the immune system to nuclear material—and nucleic acids in particular—through two main processes: induction of release of host nuclear autoAgs by triggering cell death directly or as a result of the immune response against the pathogen, and the release of bacterial DNA due to bacterial death or active extrusion. The endogenous DNA, such as mitochondrial DNA (11, 105), can act as DAMP and be recognized by autoreactive B cells. Similarly, bacterial DNA, possibly associated with other bacterial molecules, can elicit autoimmunity by acting as PAMP and stimulating autoreactive B cells through molecular mimicry.

Because DNA is a major autoAg in SLE, many studies have attempted to determine whether an excess of circulating DNA may distinguish SLE patients from healthy subjects. Circulating extracellular nucleic acids were originally detected in the serum (40, 106) and then in plasma to avoid the *in vitro* artifacts due to release of cellular DNA caused by the procedure of *in vitro* coagulation (107). Initially, no differences were noted in

SLE patients when compared to healthy individuals, except for SLE patients with vasculitis. SLE patients with vasculitis had higher levels of circulating DNA, suggesting that tissue damage affecting endothelia may result in the release of extracellular DNA at the site of damage (108). This was corroborated with studies showing very high levels of plasma DNA in patients who recently underwent major surgery or experienced traumatic bodily injury and, together, suggests that cell death is the source of the extracellular DNA (107, 109, 110). This concept was successfully replicated in mice, when an injection of necrotic cells induced a rapid increase of plasma DNA levels (110).

Concurrently, testing the pristane-induced model of murine lupus in mice lacking caspase-activated DNase (CAD), which results in a lack of nuclear fragmentation during caspase-dependent apoptosis, resulted in the prevention of the development of lupus by diminishing the amount of available extracellular DNA (111). Interestingly, the opposite was true when the CAD impairment was in spontaneous genetically driven lupus models, since the absence of CAD resulted in higher levels of autoAbs in triple congenic B6.Sle1,2,3 spontaneous lupus mice (112). These results suggest that in induced autoimmunity, chromatin fragmentation is essential for the presentation of nuclear autoantigens, while in mice genetically predisposed to autoimmunity the absence of nuclear modifications occurring during apoptosis promotes B cell autoreactivity, possibly by preventing the induction of self-tolerance toward DNA (112).

More recently, microparticles derived from apoptotic cells and tissue damage have been shown to be a source of these extracellular host nucleic acids and found to be present in higher numbers in the blood of SLE patients in many studies, although without full consensus (113, 114), as often seen in human studies possibly due to broad patient heterogeneity. Similar inconsistencies apply to more recent quantifications of circulating free DNA (cfDNA), which was reported to be significantly higher in SLE patients compared to controls, in correlation (115) or not (116) with high SLEDAI scores, confirming that levels of DNA, either free or bound to autoAbs or contained in microparticles, are increased in SLE patients, although the cause and pathogenic role remains to be understood.

Novel techniques allowing for plasma DNA sequencing have revealed that most circulating cell-free host DNA molecules have a size distribution that suggests a nucleosomal origin (117). These techniques are used in the clinic for non-invasive prenatal genetic testing (118) or cancer liquid biopsies, which can detect asymptomatic tumors and cancer-associated mutations (119, 120). Massive parallel sequencing revealed a spectrum of abnormalities in plasma DNA from SLE patients, including hypomethylation and fragment size shortening, abnormalities that positively correlate with levels of anti-dsDNA autoAbs and SLEDAI scores; interestingly, the abnormal DNA was bound to anti-dsDNA IgGs, suggesting that either these short sequences are specific autoAgs or they are increased because binding to Abs protected them from degradation (121). The same techniques can be used to test microbial DNA in the blood during infections and sepsis, and very recently an analytical and clinical validation of a next-generation sequencing test, which can identify and quantify microbial cell-free DNA in the plasma of patients with and without sepsis, has demonstrated the feasibility to detect in plasma the circulating free DNA of 1,250 clinically relevant bacteria, DNA viruses, fungi, and eukaryotic parasites (122). The abovementioned sequencing of plasma DNA from SLE patients used libraries of host DNA (121), leaving open the question of whether sequences of bacterial DNA are present as well, and can account for the higher levels of cfDNA found in SLE patients compared to healthy controls (115). It would be important to use these novel techniques to determine the host vs. microbial nature of circulating DNA in SLE patients.

## INDUCTION OF PYROPTOSIS AND OTHER TYPES OF CELL DEATH BY BACTERIAL INFECTIONS CAN RELEASE NUCLEAR AUTOAGS TO FUEL AUTOIMMUNITY

Cell death is a natural and necessary process, and efficient recognition and clearance of products is important to avoid eliciting an immune response. Whether occurring by the programmed and regulated apoptosis or via inflammatory forms of necrosis, the accumulation of cell debris from inefficient clearance of dead bodies was proposed to cause breakdown of self-tolerance (21, 123–125). Originally, it was hypothesized that genetic defects in efferocytosis could be an underlying cause of lupus. Seminal papers reported evidence of defective phagocytosis (124, 126, 127), but genetic studies have so far identified only a few polymorphisms in genes regulating efferocytosis, or phagocytosis in general, that are linked to higher risk of developing human SLE (128, 129). Therefore, these results suggest that the defects in phagocytosis are either limited to a few patients or are not genetically determined but rather may be secondary to immunosuppressive therapies, infections or a prolonged inflammatory state.

An exception may be the specific defective clearance of nucleic acids due to loss of function of DNase 1L3. Indeed, recent studies have identified families with a high incidence of aggressive SLE and strong anti-dsDNA reactivity, in which there were children with homozygosity for a mutation in the *DNASE1L3* gene (130–133). DNASE1L3, a homologous to DNASE1, is a secreted DNase that can digest DNA in chromatin present in microparticles released from apoptotic cells (134). An SLE-associated *DNASE1L3* polymorphism (R206C) was also shown to have reduced DNase activity (135, 136). Collectively, these reports suggest that in a so far limited subset of SLE patients, the exposure of extracellular nucleic acids has a genetic cause. It remains to be determined whether the loss of function of *DNASE1L3* also affects host defense. Indeed, genetic defects in phagocytosis predispose to infections and generate a vicious circle that increases the exposure to extracellular nucleic acids. For example, the monogenic lupus due to the complement C1q deficiency is thought to be in part due to the defective clearance of immune complexes and defective uptake of dying cells, with a subsequent presence of excess extracellular host DNA (137). Nevertheless, C1q can also bind many bacterial species in an Ab-dependent and—independent manner, and C1q deficiency

renders patients susceptible to bacterial infections, especially early in life (138–140). Therefore, we can speculate that an increased bacterial burden may play a pathogenic role in the development of SLE in C1q deficient patients, as in other forms of immune dysfunction, making these patients more susceptible to lupus.

It is fair to remember that mice with deficiencies in receptors necessary for efferocytosis develop SLE-like diseases including splenomegaly and glomerulonephritis and generate high levels of hallmark antinuclear antibodies (141). Mutations in BAI, MerTK, MFG-E8, Scavenger Receptor, and TIM-4 receptors involved in efferocytosis have all resulted in SLE-like disease in mice (127, 142–144). Nevertheless, caution is important when considering the direct translation of observations in mice to the human population, which is complicated by the fact that mice used in immunology are kept in specific-pathogen-free (SPF) conditions, and therefore are not exposed to the same degree of bacterial challenges that most humans see. This difference has profound consequences on the development of the immune system, as highlighted by a recent study that compared mice housed in SPF conditions with mice co-housed with pet store mice and found that the lack of pathogen experience has major effects on the cellular composition of the innate and adaptive immune systems, especially failing to elicit effector-differentiated and mucosally distributed memory T cells (145). Therefore, any conclusion on the role of genetic defects of apoptosis and efferocytosis on autoimmune outcomes warrants investigation on how natural infections may influence these murine models of autoimmunity.

Although apoptosis is most broadly recognized, a pathway of programmed cell death that is stimulated by microbial infections is pyroptosis. Pyroptosis is canonically dependent on the protease caspase 1, making this process inherently inflammatory. When caspase 1 is activated, gasdermin-D rapidly forms pores in the plasma membrane, allowing for osmotic lysis and release of inflammatory cytokines and cell contents, in contrast to the non-inflammatory apoptosis. When LPS is recognized in the cytoplasm by caspase 4 or 5 in humans (caspase 11 in mice), caspase 1-independent pyroptosis is also initiated. Both types of pyroptosis lead to the release of potent inducers of inflammasome activation and consequent inflammation. Additionally, both nuclear and mitochondrial DNA are released by pyroptotic cells (146–149).

Together with viral infections (150–152), many bacterial infections have been shown to trigger pyroptosis, and much of the tissue damage associated with such infections is caused by the induction of pyroptosis and the consequent released of DAMPs (153). Many bacterial PAMPs can trigger cytoplasmic PRRs like AIM2 and NLRPs, which are upstream of the inflammasome and the downstream caspases, and their activation leads to secretion of caspase 1-dependent cytokines IL-1b and IL-18 (154). Many bacterial models have been reported to activate the inflammasome and induce pyroptosis, from Salmonella ssp (155, 156) to Francisella novicida (157), Streptococcus pneumoniae (158), and Listeria monocytogenes (159). Moreover, infection by Uropathogenic E. coli (UPEC) was shown to induce pyroptosis in bladder urothelial cells and release of IL-1β and IL-18 in the form of exosomes. As a consequence, mast cells migrate in the site of infection and worsen the damage to the barrier function of bladder urothelium (160).

An important inflammatory protein released by pyroptotic cells is high-mobility group box 1 (HMGB1), a nuclear DNA binding protein ubiquitously expressed in eukaryotic cells (161, 162). Circulating anti-HMGB1 antibodies are present in SLE patients and increased extracellular expression of HMGB1 is found in cutaneous lupus lesions (163, 164). *In vitro*, HMGB1, when complexed with DNA, can stimulate TLR9 and subsequent production of type I IFN by dendritic cells (165). Additionally, these HMGB1-DNA complexes can activate B cells via the receptor for advanced glycation end-products (RAGE), supporting the role of HMBG1 in promoting the formation of autoreactive B cells. Finally, our group has found that HMBG1 levels in the urine correlate with the SLEDAI and the occurrence of lupus nephritis. The highest levels were observed in class V membranous nephritis, in which they correlated with complement deposition, suggesting that the release of HMGB1 in the urine is not only due to passive excretion secondary to elevated proteinuria, but is likely linked to a mechanism inherent to class V disease (166).

Other cytokines released by pyroptosis include the caspase 1-dependent IL-1b and IL-18, both thought to play a role in promoting autoimmune disease (167–170). Moreover, many studies are reporting increased cytokines linked to pyroptosis in both human and murine SLE, contributing to lupus manifestations including nephritis. Microarray analysis of kidney tissue from SLE patients revealed an increase of inflammasome-associated transcripts (171) and low serum levels of IL-1 receptor antagonist in SLE patients suffering from renal flares suggest a pathogenic role for IL-1 in lupus nephritis (172). This enhanced pyroptosis may be due to polymorphisms in the IL-18 gene, which have been linked to SLE (173, 174) and found to lead to heightened expression of IL-18 and development of kidney disease (175, 176). These findings were further supported by the detection of heightened levels of sera and urine IL-18 in SLE patients, especially those with active lupus nephritis (177, 178). As mentioned above, bacterial infections are well-known triggers of pyroptosis (33), and common pathogens in SLE patients including E. coli and Salmonella are models of pyroptosis (155, 156, 158, 160, 179), and therefore the increased levels of this category of cell death may be due to subclinical infections causing tissue damage without generalized signs of disease manifestation. Together, these findings strongly suggest that infectious pyroptosis may play a pathogenic role in releasing host nuclear autoAgs in SLE.

NETosis is a form of cell death that specifically releases extracellular nuclear autoAgs and is triggered by bacterial infections as a weapon of host defense (180). Neutrophils are the first cells to migrate to the site of infection where they release chromatin relaxed in extracellular fibers, which can entrap Gram-positive and Gram-negative bacteria (181). NETosis is a direct antibacterial mechanism, blocking the pathogens, and it also stimulates the innate and adaptive immune response, with increased phagocytosis and production of I IFNs (125). An excess in NET formation can promote tissue damage during sepsis and many inflammatory conditions,

like diabetes (182), atherosclerosis (183), and SLE (184, 185). SLE patients showed enhanced NETosis and post-translational modifications of cellular proteins, such as histone acetylation and citrullination, that can be auto-immunogenic (186, 187). NETosis also releases oxidized mitochondrial DNA, which is proinflammatory and interferogenic (11, 185), suggesting a pivotal role for NETosis in mediating the release of extracellular nuclear autoAgs in lupus. It remains to be determined whether clinical or subclinical infections are fueling NETosis, and whether genetic or environmental factors cause the increased propensity of NETting in SLE patients.

## BIOFILMS

Up to 80% of bacterial infections in humans are associated with biofilms (188) that bacteria build to protect themselves from environmental or immune stress (189, 190). Biofilms, a term coined by Bill Costerton in 1978 to describe a sessile, attached community of microbial cells embedded in microbe-produced extracellular matrix, was first described by Anton Von Leeuwenhoek—the pioneer of the microscope—in the 17th century (191, 192). Since then, biofilms have been defined as an aggregation of microbial cells that are firmly attached or enclosed in an extracellular matrix produced by the microbes themselves (193). Biofilms have been in the public health spotlight due to the increased recognition of their role in a number of infectious disease processes, including common infections such as UTIs, otitis media, periodontitis, and a broad spectrum of colonization of indwelling medical devices (194, 195). We are just beginning to understand the effects of biofilms on the immune system (196, 197). Very recent evidence supports a role for biofilm-forming infections in SLE pathogenesis. Indeed, Abs against periodontogenic bacteria, which produce biofilms in the oral cavity, were found to correlate with anti-dsDNA Abs and higher SLE disease activity (198), indicating a correlation between immune response to biofilm and autoreactivity. SLE patients were found to have higher prevalence of periodontal disease at younger age than healthy controls, with severe forms of periodontitis and changes in the oral microbiota characterized by decreased species diversity and higher bacterial loads, which were linked to increasing local production of pro-inflammatory cytokines (199, 200), highlighting a role for the oral microbiome in the pathogenesis of lupus.

While the primary matrix material in biofilms is extracellular polymeric substances (EPS), more than 40% of bacteria produce amyloids, proteins with a conserved quaternary β-sheet structure, which are a major structural component of the biofilm and provide the scaffold to support the biofilm tridimensional structure (32, 197, 201). The best studied bacterial amyloid is curli, produced by enteric Gram-negative bacteria that commonly cause infections in SLE patients, including *Escherichia coli* and *Salmonella spps* (197, 202). Pathological amyloids, which are generally associated with neurodegenerative disease, such as Alzheimer's or Parkinson's disease, are the result of protein misfolding and are cytotoxic for the host that produces them. In contrast, in the context of biofilm formation, bacterial amyloids such as curli are actively produced by bacteria while generating the biofilm through a finely regulated process: the main subunit protein of curli, CsgA, is synthetized by the enteric bacteria and transported to the bacterial surface, where it is polymerized into an amyloid fiber through the operons *csgBAC* and *csgDEFG* (196).

Interestingly, Robertson and Pisetsky reported in 1992 that patients with *Escherichia coli* bacteremia were positive for anti-DNA Abs and subsequently demonstrated that immunization with bacterial DNA led to or accelerated lupus-like autoimmunity in mouse models (101, 203). Our group recently reported that curli amyloids form a complex with bacterial DNA. Such binding accelerates the fibrillation of the amyloid and protects the DNA from degradation by DNases (30). Biofilms contain significant amounts of extracellular bacterial DNA, either actively extruded by live bacteria or released by bacteria upon death (204–206), some of which is bound to curli amyloids. We found that curli can fibrilize with eukaryotic DNA as well, suggesting that bacterial amyloids can not only expose the immune system to bacterial DNA, but also bind and render the host DNA immunogenic (30).

The idea that DNA complexed with a protein antigen can induce SLE-like disease has been shown by both our group and others before us (207). Di Domizio et al. showed that albumin aggregated *in vitro* into amyloid and in complex with DNA could trigger autoantibodies in a pDC dependent manner when injected in mice in presence of Complete Freund's adjuvant (208, 209), suggesting that amyloid/DNA complexes can induce autoimmunity. We discovered that injection of natural curli/DNA complexes purified from biofilms generated *in vitro* by *Salmonella* Typhimurium accelerates the development of anti-dsDNA autoAbs and anti-chromatin autoAbs in lupus-prone NZBxW/F1 mice, with the levels quickly rising by the second week of injections, without the need of any added adjuvant (30). This rapid development of autoAbs in response to curli/DNA complexes is also seen in C57BL/6 wild-type mice, suggesting a strong stimulation toward autoimmunity during infections.

Additionally, systemic infection with curli-expressing bacteria, either the commensal *E. coli* or the virulent *S.* Typhimurium, induces the production of high autoantibody titers in lupus-prone NZBxW/F1 mice. Lupus-prone mice exposed to mutant *S.* Typhimurium that cannot produce curli—and therefore cannot generate biofilms—still developed autoantibodies (30, 196), albeit at a much lower level than those infected with *Salmonella* that could produce curli. Mice exposed to curli-deficient mutant *E. coli* did not produce autoAbs at all, suggesting that exposure to curli amyloid or infection with bacteria that can make biofilms containing curli/DNA complexes stimulate the development of autoantibodies in susceptible mice (30).

Looking at the response of immune cells to curli/DNA complexes, we found that these molecules are powerful stimulators of both the innate and adaptive immune systems, inducing activation of conventional dendritic cells and macrophages *in vitro* and *in vivo,* increasing activation markers in T and B cells, and inducing the production of pro-inflammatory cytokines, like TNFα, IL-12, and IL-6, and pathogenic type I IFNs (30, 32). The mechanism of how

curli/DNA elicits an autoimmune response can be explained by the ability of the amyloid to complex securely with DNA. The immunogenic curli/DNA complexes stimulate immune cells by binding to TLR2 with the β-sheet structure of curli, allowing for internalization, after which the DNA portion of the complex binds to the endosomal TLR9. Synchrotron small-angle X-ray scattering (SAXS) showed that curli organizes DNA into a columnar lattice with an inter-DNA spacing compatible with the steric size of TLR9 and maximizes TLR9 binding to DNA, leading to the amplified type I IFN response observed *in vitro* and *in vivo* (32). The role of DNA in the curli/DNA complex as a PAMP is further supported by the result that *in vitro* fibrillization of the curli monomers CsgAR4-5 polymerized into amyloids in the presence of bacterial DNA induced in dendritic cells significantly more IL-6 and IL-12 than CsgAR4-5 alone or DNA alone (30), suggesting that curli and DNA synergize to activate innate immunity.

The TLR2/TLR9 stimulation by curli/DNA complexes results in the production of type I IFNs and subsequent production of autoAbs. The autoAb production in response to curli-DNA complexes was attenuated in mice deficient for TLR2 or TLR9, suggesting that both TLR2 and TLR9 are necessary to shape the autoimmune response (32, 210). Curli/DNA was also shown to activate the inflammasome via NLRP3, extending the possible PRRs involved in their pro-inflammatory effects (210). The findings that an amyloid component of bacterial biofilms forms complexes with DNA and can potently activate a type I IFN immune response further supports the link between bacterial infections and SLE disease and highlights the important role that biofilms may play in progressing the generation of autoAbs against nucleic acids.

Curli amyloid from enteric biofilms are not the only actively produced bacterial amyloids, as homologs are found in four other phyla, i.e., *Bacteroidetes*, *Proteobacteria*, *Firmicutes*, and *Thermodesulfobacteria*, many of which are found in the human gut (211). Other bacterial species, including *Mycobacterium tuberculosis,* produce amyloids that do not share sequence homology with curli but bear the same quaternary structure and ability to strengthen the biofilm (212). Of particular relevance to autoimmunity, Gram-positive *Staphylococcus aureus* produces amyloids called phenol-soluble modulins (PSMs) (213), which fibrilize with bacterial DNA to stabilize the biofilm structure. It would be interesting to investigate the ability of PSMs and Gram-positive *Staphylococcus aureus* to stimulate autoimmunity, as we have shown for curli/DNA complexes from Gram-negative bacteria. All together, these results suggest that bacterial amyloids can act as chaperones to expose bacterial DNA to the immune system and stimulate autoimmunity in genetically predisposed individuals. Because we found that curli can bind eukaryotic DNA as well (30), we further speculate that these microbial PAMPs can also chaperone and add immunogenicity to host DNA, forming PAMP/DAMP/autoAg complexes, formidable stimulators of autoimmunity.

## INFECTIONS TRIGGER AUTOIMMUNITY VIA MOLECULAR MIMICRY

Molecular mimicry between molecules of infectious agents and SLE-related autoAgs has been proposed as a mechanism of how SLE is triggered in a susceptible genetic background and how it leads to the breakdown of self-tolerance (214). Notably, the development of antinuclear antibodies specific for nucleic acids, arguably the hallmark of SLE, has been linked with bacterial infections in both humans and mice. We propose that curli amyloids expose bacterial DNA to autoreactive B cells and stimulate the production of anti-dsDNA autoAbs through a process of molecular mimicry. The injection of bacterial DNA induced anti-dsDNA autoAbs by the same mechanism (215), and the report that mammalian DNA did not elicit the same result can be explained by the fact that genomic mammalian DNA is not as immunogenic as bacterial DNA, especially if the latter is complexed to a TLR2 ligand like curli or another amyloid (32). Other examples of molecular mimicry were reported in SLE. Sera from human SLE patients have shown anti-dsDNA antibodies with similarity to peptides from burkholderia bacteria, and the relationship was substantiated when purified anti-dsDNA antibodies were shown to react with *Burkholderia fungorum* bacterial lysates (216). A common anti-dsDNA idiotype in humans was also found in high amounts in patients infected with *Klebsiella pneumoniae* (217). The interaction of anti-dsDNA antibodies to bacteria was also found in mice, where anti-dsDNA antibodies produced by lupus-prone mice reacted with endogenous murine flora (103, 218).

A proof-of-concept study exploring the bacterial RNA binding protein Ro60 further points to bacteria exposing homolog of nuclear autoantigens as a trigger for autoantibody production. The earliest autoantibodies in lupus are directed toward Ro60 (219, 220), and the presence of Ro60 orthologs in both lupus patients and healthy controls suggests that cross-reactivity may occur in susceptible individuals. Anti-Ro antibodies are pathogenic in lupus and are best known for leading to cardiac conduction defects and cutaneous lesions due to their trans-placental spread in neonatal lupus erythematosus (221, 222). The spontaneous development of anti-Ro60 antibodies can be induced in germ-free wild-type C57Bl/6 mice when monocolonized by a common gut commensal that produces a Ro60 ortholog (223). Within 3–5 months of monocolonization, sera are positive for anti-human Ro60 IgG. This spontaneous production of antibodies was equivalent to mice that were monocolonized by the same strain but had induced barrier inflammation and dysfunction from treatment with oral 0.1% imiquimod or 1–2% dextran sulfate sodium salt. Monocolonization with a different gut commensal does not result in the production of anti-human Ro60 IgG antibodies. Together, this model suggests that there is selective cross-reactivity between a Ro60 ortholog from commensal bacteria and human Ro60, further emphasizing how infection may play a role in triggering autoimmunity in lupus (223). This supports the concept that cross-reactivity may occur in susceptible individuals with colonization by autoantigen ortholog-producing bacteria.

Additionally, candidate antigens for the pathogenic Th cells that allow for the expansion of autoreactive B cells include those with sequences that resemble both microbial proteomes and self proteins (224, 225). The role for Th cells is well-established in SLE, and autoreactive B cells have been shown to present variable region-derived idiotype peptides on their MHC class II molecules to ideotype-specific T helper cells (226–229). Systemic autoimmune disease can be established in mice by prolonged idiotype-driven T helper cell and B cell collaboration (224, 230–235). Interestingly, an analysis of the seemingly dissimilar specificities of the T helper cells from lupus-prone mice showed a high rate of matches with microbial proteomes. Additionally, these T helper cells also developed responses toward related sequences that resembled self histones, suggesting that there is molecular mimicry between microbial peptides, idiotypes, and self proteins carrying DNA (224).

## CONCLUSIONS

In summary, the review of the recent literature presented here highlights an unmet need for studying how bacterial infections contribute to the pathogenesis of lupus and to the extracellular exposure of nuclear autoantigens in particular. Infections are a major cause of morbidity and mortality in SLE, and incomplete evidence suggests that they may accelerate SLE onset in predisposed individuals and increase disease severity in patients. Recent studies have discovered a disturbance in the microbiota profile in SLE patients and associations between pathobionts and lupus, its severity, and specific end-organ damage. These altered microbiota and repetitive infections can expose the immune system to extracellular nuclear autoAgs through host cell death and to their molecular mimics through bacterial death and extrusion of bacterial DNA. Robust experimental data supports the widely accepted working hypothesis of the complex involvement of TLRs and other PRRs in lupus pathogenesis, which is thought to promote DNA autoantibody production through activating innate and adaptive immunity. Additionally, bacterial DNA and ribonucleoproteins like Ro60 can mimic nuclear self-Ags and stimulate BCRs of autoreactive B cells in lupus autoimmunity. Furthermore, the fact that TLRs may recognize bacterial amyloid, and that bacterial biofilms contain extracellular DNA, which is bound in part to bacterial amyloid, and that they together can mimic host DNA, suggests a novel mechanism by which bacterial infections can trigger autoantibody production. The data presented here provide concrete support for bacterial infections as candidates for the extracellular exposure of lupus nuclear autoantigens, highlighting a role for bacterial biofilms in the generation of nuclear autoantigens and the stimulation of the autoreactive immune response.

## AUTHOR CONTRIBUTIONS

CQ drafted the review and RC and SG revised it. All the authors read and approved the final version.

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# Cleavage of HMGB1 by Proteolytic Enzymes Associated with Inflammatory Conditions

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Extracellular HMGB1 acts as an alarmin in multiple autoimmune diseases. While its release and functions have been extensively studied, there is a substantial lack of knowledge regarding HMGB1 regulation at the site of inflammation. Herein we show that enzymes present in arthritis-affected joints process HMGB1 into smaller peptides *in vitro*. Gel electrophoresis, Western blotting and mass spectrometry analyses indicate cleavage sites for human neutrophil elastase, cathepsin G, and matrix metalloproteinase 3 within the HMGB1 structure. While human neutrophil elastase and matrix metalloproteinase 3 might alter the affinity of HMGB1 to its receptors by cleaving the acidic C-terminal tail, cathepsin G rapidly and completely degraded the alarmin. Contrary to a previous report we demonstrate that HMGB1 is not a substrate for dipeptidyl peptidase IV. We also provide novel information regarding the presence of these proteases in synovial fluid of juvenile idiopathic arthritis patients. Correlation analysis of protease levels and HMGB1 levels in synovial fluid samples did not, however, reveal any direct relationship between the recorded levels. This study provides knowledge of proteolytic processing of HMGB1 relevant for the regulation of HMGB1 during inflammatory disease.

**Keywords: high mobility group box 1, neutrophil elastase, cathepsin G, matrix metalloproteinase-3, juvenile idiopathic arthritis, proteolytic cleavage**

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## INTRODUCTION

High Mobility Group Box 1 protein (HMGB1) is a prototypical alarmin which is secreted by activated immune cells and passively released by damaged cells. Its extracellular inflammatory properties have been studied in multiple inflammatory diseases (1, 2). Intra-articular administration of recombinant HMGB1 into the knee joints of mice induces destructive arthritis, while administration of monoclonal anti-HMGB1 antibody or the antagonistic box A peptide can ameliorate inflammatory symptoms (3–6). Administration of HMGB1-neutralizing agents also provides significant protection in animal models of experimental sepsis, drug-induced liver injury, ischemia reperfusion injury and trauma (1, 7). HMGB1 contains conserved cysteine residues at position 23, 45, and 106, and the redox state of these cysteines determine how HMGB1 functions as a proinflammatory mediator (8).

HMGB1 has a highly conserved sequence consisting of three distinct structures: the DNA binding domains box A and box B, as well as an acidic tail at the C-terminal that is comprised of glutamic and aspartic acid residues. Dynamic interaction of the C-tail with amino acid residues within box A, box B, and within the linker region between box B and the C-tail affects both the stability of the protein and the DNA binding properties of the A and B boxes. HMGB1 in which the C-tail interacts with box A and box B has a more stable structure as compared to the "unbound" form (9–11).

Not only is full-length HMGB1 biologically active with potentially harmful, inflammatory effects but its peptides can also have inflammatory features. Studies with recombinant peptides show that box B harbors the cytokine-inducing property through its interaction with MD2/TLR4 (12), while box A on the other hand has the ability to inhibit proinflammatory activity of the full-length protein (13, 14) (**Figure 1**). Earlier reports of the anti-bacterial properties of HMGB1 attributed this feature to the acidic C-terminal tail (15). Full-length HMGB1, as well as box A and box B, can form complexes with other molecules, for example LPS and IL-1β, and thereby augment their inflammatory features (16). However, the existence of functional HMGB1 peptides *in vivo* is not as yet proven.

While HMGB1 release and its functions in inflammatory diseases have been extensively studied, less is known regarding how extracellular HMGB1 activity is regulated during disease. A recent study reports that haptoglobin, an acute phase extracellular hemoglobin-binding protein, binds circulating HMGB1 and thereby protects against sepsis (17). Thrombomodulin was earlier demonstrated to inhibit the inflammatory features of HMGB1 through interaction of its lectin-like domain with HMGB1 (18–20).

To date there are no studies focusing on the downregulation of HMGB1 at the focal site of inflammation. We hypothesized that proteolytic cleavage of HMGB1 could play important functions in the regulation of HMGB1 activity. Firstly, protein degradation would promote clearance of HMGB1 from the site of inflammation. Secondly, partial degradation could also result in certain receptor binding sites being more accessible and thereby improve the binding of HMGB1 to its receptors (21). Thirdly, it is also possible that enzymatic processing of HMGB1 could result in antagonistic HMGB1-derived peptides being formed with similar functions as "free" box A (22).

We set out to investigate proteolytic regulation of HMGB1 by proteases associated with chronic inflammatory disease, using JIA as a model disease. Four proteases associated with arthritis, neutrophil-derived human elastase (HNE) and Cathepsin G (CG), fibroblast-derived matrix metalloproteinase 3 (MMP-3) and the serine protease dipeptidyl peptidase-IV (DPP-IV), were investigated for their ability to process HMGB1. We characterized the resulting peptide patterns and correlated levels of HMGB1 with HNE, CG, MMP3 and DPP-IV levels in JIA joints.

## MATERIALS AND METHODS

### Prediction of Protease Cleavage Sites in HMGB1 and Selection of Proteases Investigated

For an initial prediction of proteases with the potential to cleave HMGB1 we used the online software Protease specificity prediction server PROSPER (PROSPER, Monash University; https://prosper.erc.monash.edu.au/). PROSPER performs *in silico* prediction of protease substrates and cleavage sites predictions covering 24 proteases and the four major protease families (23)). The sequence of HMGB1 (UniProtKB-P09429, HMGB1_HUMAN) was uploaded to the online server. Based on PROSPER-identified proteases together with literature studies regarding proteases associated with inflammatory conditions in general and in arthritis in particular, we selected the proteases HNE, CG, MMP3 and DPP-IV for further investigation.

### Recombinant Protein Production

Human HMGB1 cDNA was cloned into the pETM-11 vector and expressed in *Escherichia coli* strain BL21 (DE3) cells. The protein expressed a 6-residue N-terminal histidine tag with a tobacco etch virus (TEV) cleavable linker and was purified by FPLC using Ni-sepharose affinity chromatography (HisTrap HP,

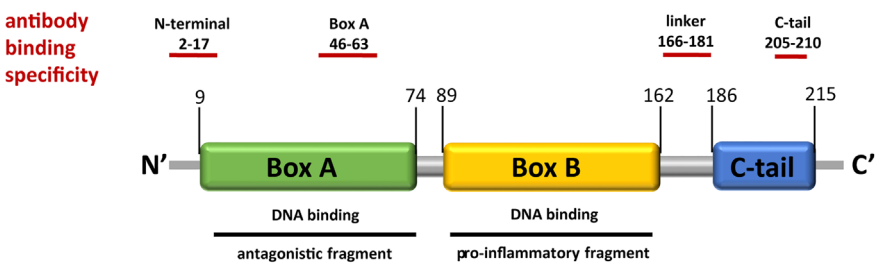



**FIGURE 1** | HMGB1 structure indicating functional domains and antibody epitopes. HMGB1 is a 25-kDa protein with a helical structure and consists of two DNA binding domains, box A and box B, as well as an acidic C-tail which can interfere with DNA binding. Different regions of the protein are ligands to immune receptors. While box B is known to have cytokine-like activity, free box A has been shown to act as an antagonist. Several antibodies have been developed to target different epitopes of HMGB1, those used in this study are indicated above.

GE Healthcare, Uppsala, Sweden) in an ÄKTA explorer (GE Healthcare). The histidine tag was cleaved using TEV protease (Sigma-Aldrich, Stockholm, Sweden) at a ratio of 1:20. Proteolytic TEV cleavage leaves a GA scar at the N-terminal. Endotoxins were removed using Triton-X114 two phase extraction. Protein purity was confirmed using SDS-PAGE gel electrophoresis analysis (21).

## Enzymatic Reactions

CG (Cat: 219373), HNE (Cat: 324681) and MMP3 (catalytic domain, Cat: 444217) were obtained from Merck (Millipore, Billerica, MA, USA). DPP-IV (Cat: D4943) was obtained from Sigma (Sigma-Aldrich) or from BioVision (Cat: 4709, AH Diagnostics, Stockholm, Sweden). Enzyme reconstitution and reaction buffers were prepared according to the supplier's recommendations. CG and recombinant HMGB1 were mixed at a molar ratio of 1:80 in phosphate-buffered saline (PBS), pH 7.4 and the reaction was carried out at 37°C. HNE and recombinant HMGB1 were mixed at a molar ratio of 1:50 in 100 mM Tris-HCl, 500 mM NaCl, pH 7.5 and the reaction was carried out at 25°C. MMP3 and recombinant HMGB1 were mixed at a molar ratio of 1:22 in 10 mM $CaCl_2$, 1 µM $ZnCl_2$, pH 7.5 and the reaction was carried out at 37°C. DPP-IV and recombinant HMGB1 were mixed in 50 mM Tris-HCl, 1 mM EDTA, pH 7.4 at a molar ratio of 1:28 and the reaction was carried out at 37°C. Reactions were performed in sterile 1.5-ml tubes and heat block was used in order to maintain constant temperature. All enzymatic digestions were performed with a set amount of 2 µg HMGB1. The reactions were stopped by addition of 5 µl of Laemmli sample buffer containing β-mercaptoethanol to 10 µl sample and heat inactivation at 95°C for 10 min.

Activity of DPP-IV was verified in an activity assay according to the manufacturer's instructions (Promega DPP-IV Glo assay, G8350, Promega Biotech AB, Nacka, Sweden).

## SDS-PAGE Gel Electrophoresis

Digested samples were analyzed by SDS-page gel electrophoresis using 4% to 20% gradient Tris-Glycine gels (Bio-Rad Laboratories Inc, CA, US). Gels were stained with Coomassie blue and analyzed using Image Lab 6.0.0 (Bio-Rad Laboratories Inc).

## Western Blotting

SDS-PAGE gels were loaded with sample volumes corresponding to 200 to 500 ng digested HMGB1 and run at 250 V for 25 min and transferred to nitrocellulose blotting membranes (GE Healthcare) at 100 V for 1 h. Membranes were subsequently blocked overnight in 5% dry milk in TBS-T, incubated with selected primary antibodies (1 µg/ml) for 1 h at RT followed by incubation with secondary antibodies coupled to HRP (1:10000 dilutions) for 1 h at room temperature. Chemiluminescence was detected by ChemiDoc MP imaging system (Bio-Rad Laboratories Inc). The following primary antibodies were used: Ab67281 (rabbit polyclonal against HMGB1 amino acids 2 to 17, Abcam, Cambridge, UK), ab 2G7 (mouse monoclonal IgG2b, against HMGB1 amino acids 46 to 63 (24)), Ab K25 (rabbit polyclonal against HMGB1 amino acids 161–188, in house production) and ab #10–22 (rat monoclonal IgG2a against HMGB1 amino acids 205–210, a kind gift from Prof. Nishibori, Okayama University, Japan (25)), (**Figure 1**). The following secondary antibodies were used: polyclonal donkey anti rabbit IgG-HRP (711-035-152, Jackson Immunoresearch Laboratories Inc), polyclonal rabbit anti-mouse IgG-HRP (P0260, Dako Cytomation), polyclonal rabbit anti rat Ig-HRP (P0163, Dako Cytomation).

## Mass Spectrometry Analysis

Protein digestion mixtures were separated by SDS-PAGE (4 µg protein/time point) and specific bands were excised. All bands were excised at the 60-min time point, except fragment I/MMP3 cleavage which was excised at the 30-min time point. In-gel trypsin digestion was performed and thereafter the samples were resolved in 30 µl 0.1% formic acid prior to nanoLC-MS/MS. The resulting peptides were separated on a C18-column and electrosprayed online to a QEx-Orbitrap mass spectrometer (Thermo Finnigan) with 35 min gradient. Tandem mass spectrometry was performed applying higher-energy collisional dissociation (HCD) fragmentation.

MS/MS data were matched to a sequence database (*Homo sapiens* proteome extracted from Uniprot, release December 2017) using the Sequest algorithm, embedded in Proteome Discoverer 1.4 (Thermo Fisher Scientific). This sequence identifies HMGB1 as a 215 amino acid long protein with a methionine in position 1. The search criteria for protein identification were set to at least two matching peptides of 95% confidence level per protein. Compared to standard search settings, peptides down to four amino acids length were accepted. The excised gel bands were delivered to SciLife Lab, MS facility, Uppsala, Sweden, where the digestion, MS analysis and protein identification was performed.

## 3D Modeling of Predicted Cleavage Sites

HMGB1 and its cleavage sites was visualized in 3D using The PyMOL Molecular Graphics System, Version 1.8 Schrödinger, LLC. A solution NMR structure of HMGB1 corresponding to amino acids 1 to 166 (i.e. lacking the C-tail part of the molecule) was used as a model (2YRQ accession in Protein Data Bank, https://www.rcsb.org/).

## Synovial Fluid Samples

Synovial fluid (SF) from 16 juvenile idiopathic arthritis (JIA) patients with active disease was collected at Astrid Lindgren's Children Hospital, Stockholm, Sweden as part of the sample collection JABBA. Median patient age was 11 years (range: 3–18). Mean disease duration was 30.11 months (range: 0–180). Patients were enrolled according to ILAR criteria, and JIA subtype distributions were as follows: oligoarthritis (75%), polyarthritis (12.5%), and undifferentiated arthritis (12.5%). SF was collected in citrate tubes, filtered and centrifuged to obtain cell-free SF and stored at −80°C until use.

Informed consent was given by both parents and children to participate in the study. The study is in accordance with the Helsinki declaration and was approved by the North Stockholm Ethical Committee in Stockholm, Sweden (Dnrs 2009-1139-31-4 and 2010-165-31-2.)

## HMGB1 and Enzyme Detection in Synovial Fluid (SF) of JIA

HMGB1 levels in JIA SF samples were determined using a commercial ELISA according to the manufacturer's instructions (Shino-test, IBL International, Hamburg, Germany). Levels of HNE, MMP3 (duosets # DY9167-05 and # DY513, R&D Systems, Minneapolis, MN, USA), CG (NBP2-60614, Novus Biologicals, Bio-Techne, Stockholm, Sweden) and DPP-IV (RAB 0147, Sigma Aldrich) were measured in SF using ELISA systems according to the manufacturer's instructions.

## Statistical Methods

Due to non-normality of the data Spearman correlation test of recorded levels of HMGB1 and enzymes in SF specimens was performed using GraphPad Prism 6.0.

# RESULTS

## Proteases Predicted to Cleave HMGB1

Analysis of the HMGB1 sequence using the protease specificity prediction server PROSPER revealed a number of proteases with the potential of cleaving HMGB1. 5 serine proteases, 2 cysteine proteases and 3 matrix metalloproteinases were predicted (**Table 1**). HNE, CG and MMP3 were selected for further investigation together with DPP-IV. DPP-IV has previously been reported to cleave HMGB1 (26) but was not predicted by PROSPER.

## Elastase (HNE) Cleaves HMGB1 at Multiple Sites

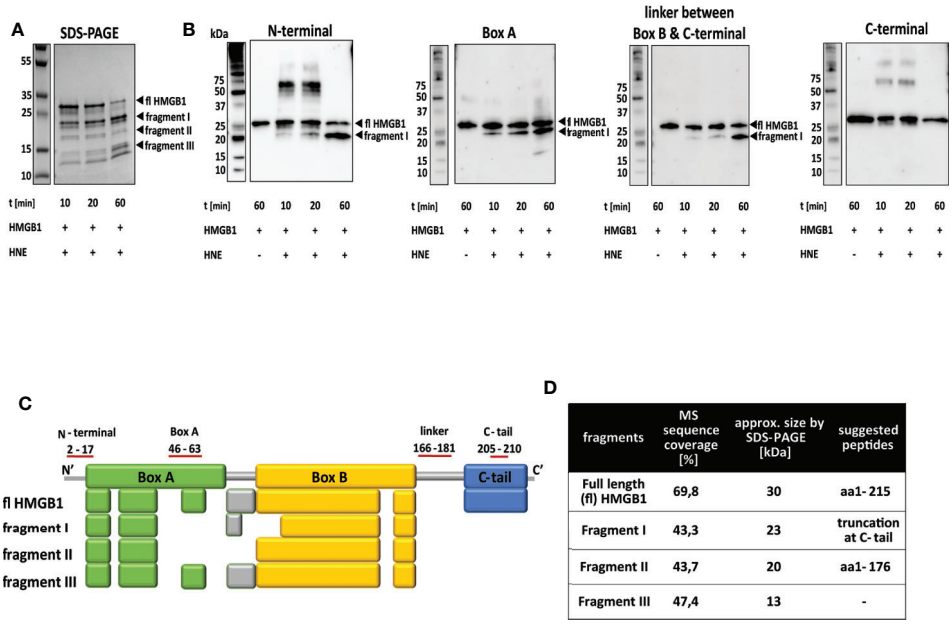
Processing of HMGB1 with HNE resulted in a distinct peptide pattern. During the first minutes of the reaction, a larger fragment with an apparent molecular weight (Mw) of 23 kDa (fragment I, **Figure 2A**) dominated while after 60 min incubation a lower Mw fragment of approximately 13 kDa appeared as the final product of the reaction (fragment III, **Figure 2A**). A constant intermediate product was also detected (fragment II, **Figure 2A**). Analysis of the fragments using antibodies against different HMGB1 epitopes demonstrated a fragment containing the N-terminal region, the box A region and the linker region between box B and the C-tail (**Figure 2B**). The Mw of the peptide corresponded to the major cleavage product evident in SDS-PAGE (**Figure 2A**, fragment I). A complete lack of signal for the C-terminal region at all time points indicates that C-terminal tail truncation is an early event.

In order to investigate the possible cleavage sites, full length HMGB1 and the three fragments were analyzed by mass spectrometry. Analysis of full length HMGB1 created a mapping reference for the fragments with a sequence coverage of 69.8%. It also demonstrated that the N-terminal region could never be detected since cleavage with trypsin, the enzyme used in

**TABLE 1** | Proteases predicted to cleave HMGB1.

| Merops ID | Protease name | Position | P4-P4′ site | N-fragment (kDa) | C-fragment (kDa) |
|---|---|---|---|---|---|
| C01.036 | CK | 39 | VNSF/EFSK | 4.78 | 21.35 |
| C01.036 | CK | 14 | GKMS/SYAF | 1.84 | 24.29 |
| C02.001 | Calpain 1 | 52 | WKTM/SAKE | 6.42 | 19.71 |
| M10.003 | MMP2 | 101 | PPSA/FFLF | 12.25 | 13.87 |
| M10.004 | MMP9 | 11 | KPRG/KMSS | 1.49 | 24.63 |
| M10.004 | MMP9 | 150 | KAAK/LKEK | 18.25 | 7.87 |
| M10.004 | MMP9 | 128 | VAKK/LGEM | 15.65 | 10.48 |
| M10.004 | MMP9 | 145 | QPYE/KKAA | 17.73 | 8.40 |
| M10.004 | MMP9 | 103 | SAFF/LFCS | 12.55 | 13.58 |
| M10.004 | MMP9 | 101 | PPSA/FFLF | 12.25 | 13.87 |
| M10.004 | MMP9 | 20 | AFFV/QTCR | 2.56 | 23.57 |
| M10.005 | MMP3 | 161 | DIAA/YRAK | 19.54 | 6.58 |
| M10.005 | MMP3 | 34 | HPDA/SVNF | 4.24 | 21.88 |
| M10.005 | MMP3 | 120 | HPGL/SIGD | 14.74 | 11.39 |
| S01.001 | Chymotrypsin A | 16 | MSSY/AFFV | 2.09 | 24.04 |
| S01.001 | Chymotrypsin A | 78 | MKTY/IPPK | 9.63 | 16.50 |
| S01.131 | HNE | 20 | AFFV/QTCR | 2.56 | 23.57 |
| S01.133 | CG | 38 | SVNF/SESF | 4.69 | 21.44 |
| S01.133 | CG | 120 | HPGL/SIGD | 14.74 | 11.39 |
| S26.008 | CG | 75 | EREM/KTYI | 9.23 | 16.89 |
| S26.008 | GP 1 | 40 | NFSE/FSKK | 4.91 | 21.22 |
| S26.008 | TPP | 171 | PDAA/KKGV | 20.72 | 5.41 |
| S26.008 | TPP | 207 | EDED/EEED | 25.15 | 3.60 |
| S26.008 | TPP | 35 | PDAS/VNFS | 4.33 | 21.80 |
| S26.008 | TPP | 160 | KDIA/AYRA | 19.47 | 6.65 |
| S26.008 | TPP | 69 | ADKA/RYER | 8.37 | 17.76 |
| S26.008 | TPP | 198 | EDEE/EEED | 25.41 | 3.60 |
| S26.008 | TPP | 64 | EDMA/KADK | 7.86 | 18.27 |

*Proteases predicted to cleave HMGB1 using the protease specificity prediction server PROSPER. Merops ID defines the protease identity and classification in the MEROPS peptidase database (www.ebi.ac.uk/merops). CK; Cathepsin K, MMP; matrix metalloproteinase, HNE; human elastase 2; CG; Cathepsin G, GP 1; glutamyl peptidase 1, TPP; thylakoid processing peptidase.*




| fragments | MS sequence coverage [%] | approx. size by SDS-PAGE [kDa] | suggested peptides |
|---|---|---|---|
| Full length (fl) HMGB1 | 69,8 | 30 | aa1- 215 |
| Fragment I | 43,3 | 23 | truncation at C- tail |
| Fragment II | 43,7 | 20 | aa1- 176 |
| Fragment III | 47,4 | 13 | - |

**FIGURE 2** | HNE cleaves HMGB1 at the C-terminal part and within box A. **(A)** Left panel: SDS-PAGE showing full length HMGB1. Right panel: SDS-PAGE showing the rapid cleavage by HNE over time. A larger fragment (I) appeared early and increased in strength during the studied time frame. A smaller fragment (III) also increased in strength during the studied time frame while an intermediate-sized fragment (II) appeared equal in strength throughout the cleavage reaction. **(B)** Western blotting demonstrating the presence of different protein regions in the HNE-generated HMGB1 fragments. Fragment I contained both the N-terminal and the box A epitopes but not the C-terminal tail epitope. A lower Mw fragment with an apparent size of 15 kDa was detected in Western blotting with the antibody against Box A but with none of the other antibodies used. **(C)** Gel bands of HMGB1 fragments I to III were analyzed by mass spectrometry and the resulting peptides were compared to peptides detected in the full length protein. Colored boxes refer to the functional domains of HMGB1 in which peptides could be identified (Box A: Green, linker region: grey, box B: yellow, C-terminal tail: blue). **(D)** Suggested cleavage sites based on data in **(A–C)** together with literature and database searches.

the mass spectrometry protocol, did not generate any peptide fragments in this region (**Figure 2C**). The lack of C-terminal peptides in fragments I-III confirmed that HNE-mediated C-terminal truncation was an early event (**Figures 2A, B**, fragment I, sequence coverage 43.2%). Multiple peptides spanning over box A and box B were detected in fragments II and III. Sequence coverage was 43.7% and 47.4%, respectively. Comparison of detected peptides with the fragment sizes estimated by SDS-PAGE suggested that there were more than one HMGB1 peptide of similar size in these fragments. The poor resolution of SDS-PAGE makes it challenging to resolve these fragments for separate extraction/isolation (**Figure 2C**, fragments II and III). Cleavage site prediction for HNE and HMGB1 by *PROSPER* indicated one potential cleavage site, at V20 (**Table 1**). This was not evident in our MS analysis. However, the lack of signal for the N-terminal antibody in Western blotting might preclude detection of cleavage in position V20. Earlier published reports suggest HNE cleavage sites at A34/A94/A101/G130/L120/V175/V176 in position P1 of substrates (27–30). Thus, based on Western blotting, mass spectrometry data and literature searches, we suggest that HNE prefers the cleavage site V175 or V176 at position P1 (**Figure 2D**). Cleavage at position V176 would result in a fragment with intact N-terminal of a predicted size of 19.7 kDa. Fragment II could consist of peptides cleaved either at V20 or A34 resulting in peptides with Mws of 17.4 kDa and 15.7 kDa. Fragment III could similarly as fragment II consist of peptides cleaved either at V20 or A34 and potentially at the cleavage sites suggested in literature previously. The calculated fragment sizes agree with the apparent fragment sizes approximated from Western blotting.

A lower Mw fragment with an apparent size of 15 kDa was detected in Western blotting with the antibody against Box A but with none of the other antibodies used. It was faintly visible at 60 min but not at the earlier time points (**Figure 2B**). As MS analysis of fragment II indicated overlaying peptides, potentially cleaved at either V20 or A34 within Box A, it is possible that either of the cleavage sites was more pronounced at later time points resulting in a peptide faintly visible only at the 60-min time point.

## Cathepsin G (CG) Rapidly Degrades HMGB1

The use of PROSPER indicated that the serine protease CG can cleave HMGB1 at three sites, F38, M75, and L120 in P1 positions. We could verify cleavage with CG *in vitro* by SDS-PAGE. However, at a molar ratio as low as 1:80, CG completely degraded HMGB1 within 5 min (**Figure 3A**). This observation was confirmed by Western blotting as no positive signal was evident with any of the epitope-specific antibodies used (**Figure 3B**). Due to the fast kinetics of the reaction and poor resolution

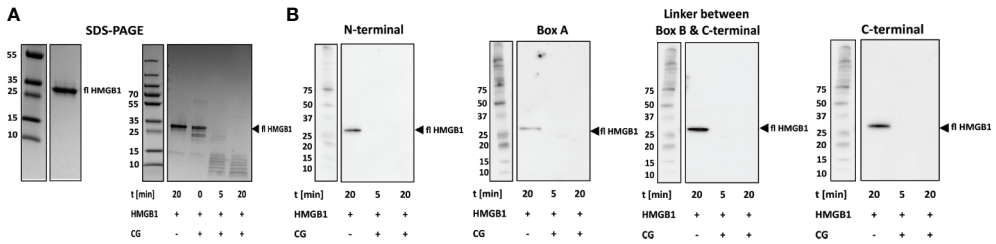
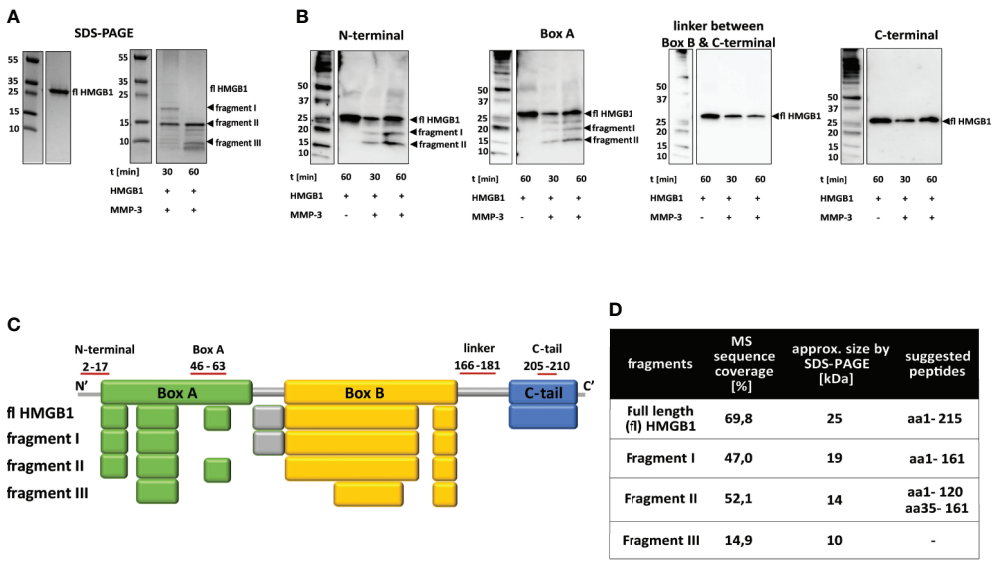



**FIGURE 3** | CG rapidly degrades HMGB1. **(A)** Left panel: SDS-PAGE showing full length HMGB1. Right panel: SDS-PAGE showing the rapid degradation of HMGB1 by CG over time. **(B)** Western blotting with antibodies recognizing epitopes within the N-terminal, the Box A, the linker between Box B and C-terminal and within the C-terminal regions resulted in detection of fragments.

of SDS-PAGE, we decided not to investigate the cleavage sites by mass spectrometry. It is noteworthy that a band of approximately 25 kDa occurs at t = 0, ie immediately after the addition of CG, similar in size to fragment 1 generated by HNE cleavage.

## Matrix Metalloproteinase 3 (MMP3) Cleaves HMGB1 at Multiple Sites

Exposure of HMGB1 to MMP3 processing resulted in the rapid formation of two fragments of approximately 19 and 14 kDa in Mws, visible after 30 min. Several smaller fragments were also visible both after 30 min and after 60 min (**Figure 4A**, fragments I, II, III and other fragments). However, the major reaction product was the 14-kDa fragment (fragment II), which was also evident after 60 min when the 19-kDa fragment (fragment I) had disappeared (**Figure 4A**). Western blotting analysis revealed a fragment of approximately 14 kDa being recognized by both the N-terminal specific antibody and the box A-specific antibody, corresponding to fragment II. Additionally, a fragment of approximately 19 kDa was detected after 30- and 60-min digestion with the N-terminal- and box A-specific antibodies, corresponding to fragment I (**Figure 4B**). None of the fragments I or II could be detected in Western blotting with the antibodies




| fragments | MS sequence coverage [%] | approx. size by SDS-PAGE [kDa] | suggested peptides |
|---|---|---|---|
| Full length (fl) HMGB1 | 69,8 | 25 | aa1- 215 |
| Fragment I | 47,0 | 19 | aa1- 161 |
| Fragment II | 52,1 | 14 | aa1- 120<br>aa35- 161 |
| Fragment III | 14,9 | 10 | - |

**FIGURE 4** | MMP3 cleaves HMGB1 at multiple sites. **(A)** Left panel: SDS-PAGE showing full length HMGB1. Right panel: SDS-PAGE showing cleavage by MMP3 over time. Two distinct fragments of approximately 19 and 14 kDa (I and II) appeared at time point 30 min. At 60 min, fragment I had disappeared while fragment II was still the dominant fragment. **(B)** Western blotting of HMGB1 fragments after processing with MMP3. A fragment corresponding to fragment I in size was detected by the antibodies specific for epitopes in the N-terminal region and in the box A region. In agreement with the SDS-PAGE kinetic pattern, the staining was stronger at the 30-min time point than the 60-min time point. Antibodies specific for epitopes within the B-to-C linker region and the C-terminal region only detected full length HMGB1 and none of the fragments. **(C)** Gel bands of HMGB1 fragments I-III were analyzed by mass spectrometry and the resulting peptides were compared to peptides detected in the full length protein. Colored boxes refer to the functional domains of the HMGB1 in which peptides could be identified (Box A: Green, linker region: grey, box B: yellow, C-terminal tail: blue). **(D)** Suggested cleavage sites based on data in **(A–C)** together with literature and database searches.

specific for the B-to-C linker region and the C-terminal tail region, respectively. This indicates a rapid cleavage within the B-to-C linker region resulting in C-terminally truncated HMGB1. Fragment III (**Figure 4A**) was undetectable by blotting.

Mass spectrometry analysis of fragments I and II confirmed the Western blot results that these fragments do not contain the box B-to-C linker region resulting in a C-terminally truncated HMGB1. Peptide coverage by MS analysis (47%) and fragment size estimated for fragment I by SDS-PAGE supported the conclusion that fragment I contained the N-terminal region, the box A region and the box B region. For fragment II the MS peptide coverage (52.1%) indicated a similar peptide as fragment I. However, this is not in total agreement with the fragment size estimated by SDS-PAGE, which is slightly lower with an approximate Mw of 14 kDa. This might indicate an additional cleavage site. Western blotting data suggests this cleavage site to be within box B. Mass spectrometry analysis of fragment III (peptide coverage 14.9%), indicated an overlay of different smaller peptides within the analyzed fragment, as peptides detected by MS did not correspond to the fragment size estimated by SDS-PAGE (**Figures 4A, C**).

Cleavage prediction using PROSPER indicated three cleavage sites for MMP3; A161, A34, and L120 in position P1. Based on Western blotting, mass spectrometry data, PROSPER predictions and literature searches, we suggest that MMP3 has multiple cleavage sites within HMGB1. The most preferred cleavage site removes the C-terminal tail by cutting at A161 and creates a 19-kDa fragment detected by N-terminal and box A-specific antibodies (fragment I, **Figure 4**). Thus, both HNE and MMP3 have a cleavage preference which results in rapid removal of the C-terminal tail. The smaller fragment recognized by the same antibodies as described above, fragment II, can be a result of cleavage at L120 in position P1 (**Figure 4D**). We cannot exclude the possibility of additional cleavage sites for MMP3 within the HMGB1 structure. If HMGB1 is cleaved at both A34 and A161 in P1 positions then the resulting peptide would be of similar size to the peptide corresponding to amino acids M1-L120, but this was only seen by Western blotting with the box A specific antibody. Moreover, cleavage at E61 would create a peptide spanning from D62 to A161, approximately 10 kDa in size, which would not be recognized by any of the antibodies.

Fragment III contained both box A peptides and box B peptides, indicating that the fragment contained multiple smaller peptides less than 10 kDa in size.

## DPP-IV Does Not Cleave HMGB1

To verify a previous report that HMGB1 is cleaved by DPP-IV and to define the fragments generated, we reproduced the *in vitro* system utilized in the report (26). We could not record any cleavage occurring despite incubation times up to 24 h (**Figures 5A, B**). The activity of the enzyme was verified in an activity assay (**Supplemental Figure 1**). To further verify whether cleavage close to the N-terminal region occurred, as stated in the previous report, we performed Western blotting both with the polyclonal antibody binding to the B-to-C linker region (**Figure 5B**) and with the antibody specific for the N-terminal (**Figure 5C**) and calculated signal ratios obtained by image analysis (**Figure 5C**). The ratio was constant over time, thus further supporting the notion that DPP-IV does not cleave HMGB1.

## HMGB1 Is Detected in Synovial Fluid from Juvenile Idiopathic Arthritis Patients

We recorded the levels of HMGB1 in synovial fluid samples obtained from 16 JIA patients by ELISA in order to verify its presence in a biological fluid from an inflammatory condition. The levels ranged from 6 to 98 ng/ml; average 33.4 ng/ml, in agreement with previous reports (31, 32) (**Figure 6A**).

## HMGB1-Regulating Proteases Are Present in Synovial Fluid of JIA Patients

We confirmed the presence of the neutrophil-derived proteases, HNE and CG, in synovial fluid aliquots obtained from the 16 JIA patients in which we defined HMGB1 levels above (**Figures 6B, C**). HNE could be detected in all samples with levels ranging from 5.4 to 590.6 ng/ml with an average of 278.1 ng/ml. For CG, 2 samples had undetectable levels and the 14 positive samples had levels ranging from 18.7 to 150.8 pg/ml with an average of 60.2 pg/ml. The matrix metalloproteinase MMP3 was detected in all samples with levels ranging from 1.5 to 71.9 μg/ml with an average of 31.5 μg/ml (**Figure 6D**). DPP-IV could also be detected in all SF samples and ranged from 169 to 1536.9 ng/ml with an average of 828.9 ng/ml (**Figure 6E**).

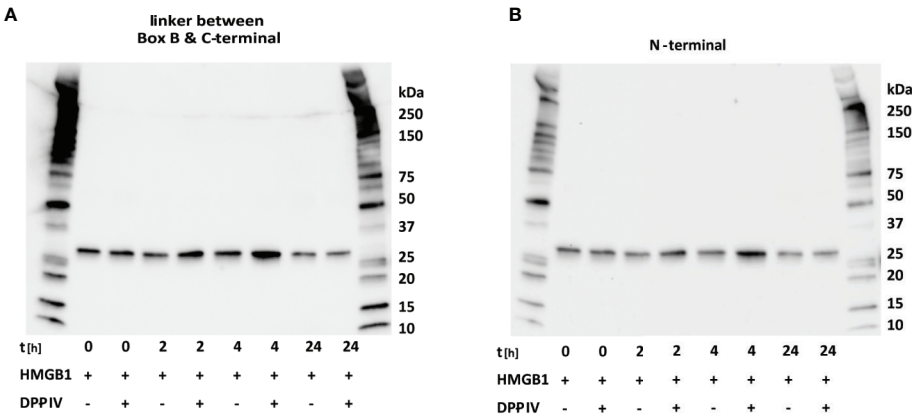
We used the recorded average value for each analyzed protease and their molecular weight (HNE Mw, 28.5 kDa; CG Mw, 28.8 kDa; MMP3 Mw, 54.0 kDa; and DPP-IV Mw, 88.3 kDa) to compare the molar ratios of HMGB1 (Mw, 24.9 kDa) to proteases in synovial fluid. We could demonstrate that HNE, MMP-3, and DPP-IV are present in higher molar ratios in SF whereas the molar ratio for HMGB1:CG is lower in SF than the molar ratios used *in vitro* in this study (**Table 2**). Thus, from a stochiometric aspect, it is plausible that HMGB1 could be enzymatically processed by HNE and MMP-3 in arthritic joints. Considering the very rapid degradation of HMGB1 by CG *in vitro*, it is also plausible that the amount of CG present in SF, although lower that used in our *in vitro* experiments, is sufficient for processing of HMGB1.

## Levels of HMGB1 in Synovial Fluid Do Not Correlate With Levels of HNE, CG, or MMP-3

Correlation of HMGB1 levels with the levels of the respective proteases did not reveal a direct relationship between low levels of HMGB1 and presence of proteases (HNE vs HMGB1 $r = -0.167$, $p = 0.534$, CG vs HMGB1 $r = 0.024$, $p = 0.940$, MMP-3 vs HMGB1 $r = 0.156$, $p = 0.564$, DPP-IV vs HMGB1 $r = -0.277$, $p = 0.299$) (**Figures 6B–E**).

# DISCUSSION

In this study we set out to test our hypothesis that the alarmin HMGB1 can be regulated by proteases associated with inflammatory conditions. Such proteolytic cleavage could either




C

| product detected with N-terminal in relation to full-length HMGB1 | | |
|---|---|---|
| DPP-IV | $t_{[h]}$ | volume ratio |
| - | 0 | 1,697916 |
| + | 0 | 1,664672 |
| - | 2 | 1,438342 |
| + | 2 | 1,519804 |
| - | 4 | 1,435946 |
| + | 4 | 1,555939 |
| - | 24 | 1,50827 |
| + | 24 | 1,58647 |

**FIGURE 5** | DPP-IV does not cleave HMGB1. HMGB1 was incubated with DPP-IV up to 24 h without any detectable sign of processing occurring. **(A)** Western blotting with an antibody recognizing the B-to-C linker region. **(B)** Western blotting with an antibody recognizing the N-terminal epitope aa 2–17. **(C)** Calculated signal ratios for Western blotting results in **(A, B)** reveal equal signals from reactions with DPP-IV and control reactions.

lead to the formation of fragments with altered, enhanced or antagonistic features or direct downregulation of HMGB1 activity through degradation. As a model inflammatory condition, we used chronic inflammatory arthritis. High levels of HMGB1 have been recorded in synovial fluid samples from both RA patients and JIA patients, which was also verified in this study.

Major cellular sources of proteases are neutrophils, the dominant cell type in synovial fluid, and activated synovial fibroblasts. We thus opted to study the ability of HNE, CG and MMP3, all derived from neutrophils or fibroblasts, to cleave HMGB1. Additionally, we investigated the HMGB1-cleaving properties of DPP-IV as this protease has been reported to cleave HMGB1 with implications for diabetes (26), and is detected at increased levels in arthritic synovium (33).

Our results demonstrate that three of the four studied proteases have the ability to cleave HMGB1. In our hands, DPP-IV, the investigated protease reported to cleave HMGB1 in a previous study, did not interact with HMGB1. This is in agreement with the protease prediction analysis we performed. Despite prolonged incubation time, full length HMGB1 incubated with DPP-IV was intact after 24 h using similar conditions as reported by Marchetti *et al*. These results are puzzling, and we do not have a good explanation. The only difference in experimental set up clear to us is the use of the commercially available full length HMGB1 from HMGBiotech used by Marchetti *et al*, and our in house produced full length HMGB1. Full length HMGB1 from HMGBiotech is tag free. Our in-house tag free HMGB1 has a GA scar in the N-terminus. Whether this affects the DDP-IV activity is presently unclear.

Conversely, CG induced a rapid and total fragmentation of HMGB1. Multiple smaller peptides were already evident after 5 min incubation. This result was somewhat expected, as CG cleaves substrates with Glu, Lys, Trp, and Phe in position P1 and with no selectivity of any amino acid in position P1′ (45,50). HMGB1 contains 35 Glu residues, 43 Lys residues, 2 Trp residues and 9 Phe residues. Our results implicate that CG might be a rapid and efficient mediator of HMGB1 removal through degradation during inflammatory conditions.

Digestion of HMGB1 with both HNE and MMP3 resulted in the generation of larger HMGB1 fragments. Interestingly, Both HNE and MMP3 generated fragments lacking the C-terminal

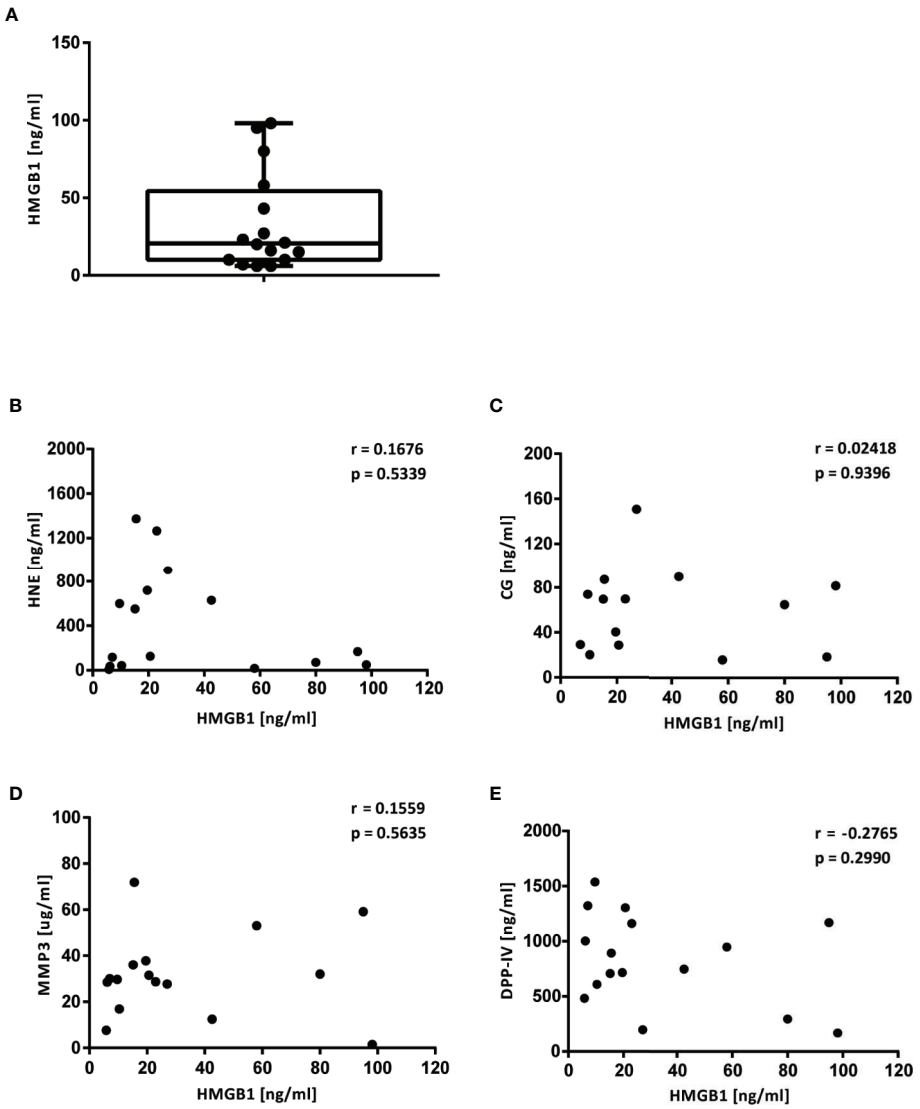



**FIGURE 6** | Levels of HMGB1, HNE, CG, MMP3 and DPP-IV in synovial fluid from JIA patients. Levels of HMGB1 and of the proteases investigated *in vitro* were defined in 16 synovial fluid samples from JIA patients. **(A)** Levels of HMGB1. **(B)** Levels of HNE. **(C)** Levels of CG. **(D)** Levels of MMP3. **(E)** Levels of DPP-IV. Direct correlations between HMGB1 levels and the level of each protease were assessed by Spearman's correlation test.

tail. Earlier studies have demonstrated that the C-terminal tail interacts with both box regions and the linker region of HMGB1 in a dynamic fashion, resulting in tail-bound and tail-unbound conformations (10, 11). Binding of the tail to the boxes modulates the interaction between HMGB1 and DNA and also regulates acetylation of HMGB1. As the receptor-binding domains for RAGE and TLR4 in HMGB1 are located within either the boxes or in the C-terminal linker region (see **Figure 1**), an HMGB1 fragment lacking its C-tail could have altered receptor associations. In support of this are recent findings that only HMGB1 lacking its C-terminal tail binds to TLR2 and to TLR5 (21, 34). Similarly, removal of the N-terminal region of the alarmin IL-33 by HNE or by CG increases the ligand-binding activity of the resulting fragment (35).

**TABLE 2** | Molar ratios of proteases and HMGB1.

| Protease | SF levels (ng/ml) | Mw (kDa) | Protease/HMGB1 ratio in SF | Protease/HMGB1 ratio *in vitro* |
|---|---|---|---|---|
| HNE | 278.1 | 28.5 | 7.3:1 | 1:50 |
| CG | 0.06 | 28.8 | 0.002:1 | 1:80 |
| MMP3 | 31500 | 54.0 | 435:1 | 1:22 |
| DPP-IV | 828.9 | 88.3 | 7:1 | 1:28 |
| *HMGB1* | *33,4* | *24.9* | | |

*Using the recorded average values for each investigated protease and for HMGB1 in synovial fluid samples from JIA patients, molar ratios of protease/HMGB1 were calculated.*

Both HNE and MMP3 digestion of HMGB1 resulted in cleavage at A34, creating a fragment lacking the N-terminal

part of the molecule in addition to lacking the C-terminal tail (**Figure 7**). This could alter the accessibility of box A for receptor interactions. This is of interest as recombinantly produced "free" box A acts as an antagonist to HMGB1 in multiple models of inflammation. To define the exact P1/P1′ positions, point mutated HMGB1 needs to be produced and subjected to cleavage. This was however outside the scope for this study.

It is notable in the Western blotting experiments that incubation of HNE with HMGB1 results in the formation of a large Mw complex (**Figure 2B**, time points 10 and 20 min). The Mw corresponds to a dimer of HNE and HMGB1, most likely formed by covalent bonds as the presence of reducing agents in the sample buffer did not dissolve the complex. It is a known feature of endopeptidases to form covalent complexes with substrates during the catalytic reaction. The covalent bond is subsequently broken and the enzyme regenerated (36). In our study the high molecular weight complex was absent from the 60-min time point.

Our Western blotting analyses were somewhat restricted by the lack of a commercially available box B-specific antibody and neither have we managed to produce such an antibody in-house despite several attempts. Additionally, it has to be noted that we have worked with a recombinant HMGB1 most likely being oxidized as it was produced with a histidine tag, without any reducing agent in the buffers and neither did it induce cytokine production when tested (data not shown).

In order to assess the biological relevance of our *in vitro* findings we first verified the presence of the investigated proteases during an inflammatory condition where HMGB1 is regarded as a pathogenic mediator; arthritis. We could demonstrate that all four investigated proteases were present in JIA synovial fluid. This is the first report of HNE, CG, MMP3 and DPP-IV levels in JIA synovial fluid as well as their co-existence with HMGB1 in a biological fluid.

Based on our findings that HMGB1 is a substrate for 3 proteases readily detected in arthritic joints it might be surprising that HMGB1 itself can be detected in arthritic synovial fluid. A technical reason could be that the antibody pair in the commercial ELISA assay used recognizes not only full length HMGB1 but also fragments of the protein. A more likely reason, however, is the intricate system of protease-binding and inhibiting proteins present in arthritic joints.

In conclusion, we report the novel finding that HMGB1 can be regulated by proteases associated with inflammation and arthritis. Using literature searches, protease specificity prediction servers and by performing *in vitro* studies we report that CG rapidly degrades HMGB1 while HNE and MMP3 processing had a slower kinetics resulting in larger fragments. We propose that HNE primarily cleaves HMGB1 at A170 and A34 in position P1, and that MMP3 primarily cleaves HMGB1 in position A161. Both HNE and MMP3 processing hence results in cleavage in the linker region, removing the C-terminal tail. Such truncation of HMGB1 has previously been reported by us and others to affect HMGB1-receptor interactions. Further processing resulted in fragments lacking the N-terminal region, a feature described for other inflammatory mediators to regulate their activity.

For the first time, we report the presence of HNE, CG, MMP3 and DPP-IV in synovial fluid obtained from JIA patients.

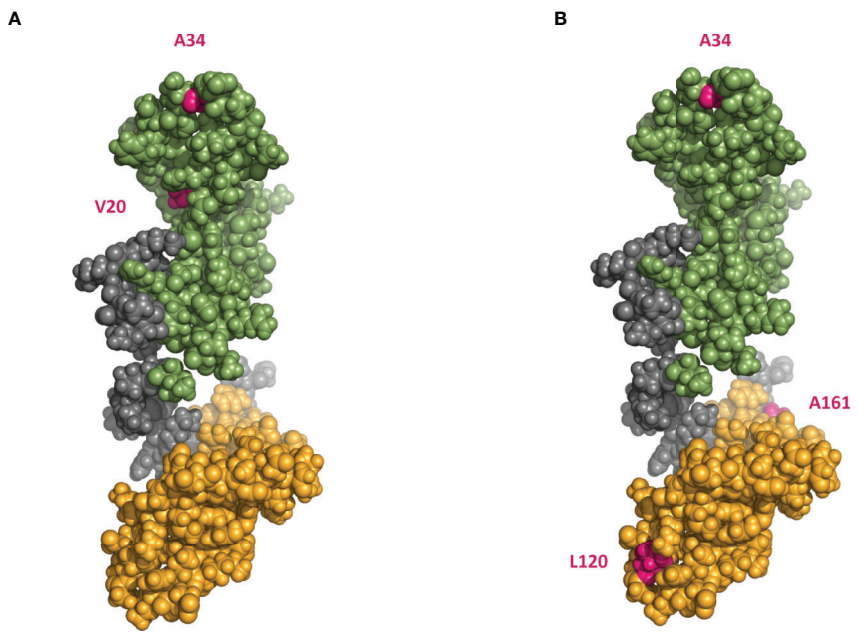



**FIGURE 7** | A 3D model indicating proposed cleavage sites at HMGB1 by HNE and MMP3. **(A)** 3D model of HMGB1 showing predicted HNE cleavage sites at positions V20 and A34 **(B)** 3D model of HMGB1 showing predicted MMP3 cleavage sites at positions A34, L120 and A161 (pink). Box A is marked in green, box B marked in yellow. The model is based on solution structure of the tandem HMG box domain from Human High mobility group protein B1 aa 1–166, #2YRQ in the RCSB Protein Data Bank.

Although the levels of each protease did not correlate with the levels of HMGB1, our study suggests that proteolytic cleavage of HMGB1 can be a downregulatory mechanism of HMGB1 activity during arthritis. Future studies are needed to clarify functional consequences of the observed fragments produced by the investigated enzymes.

## DATA AVAILABILITY STATEMENT

The data sets generated for this study are available on request to the corresponding author.

## ETHICS STATEMENT

This study was carried out in accordance with the recommendations of North Ethical Committee in Stockholm, Sweden with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by North Ethical Committee in Stockholm, Sweden.

## AUTHOR CONTRIBUTIONS

AS, PL, and HH conceived and designed the study. AS, MR, MN, and LK performed the experiments. AS, PL, and HH wrote the paper. MR, MN, and LK reviewed and edited the manuscript. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.448262/full#supplementary-material

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