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## Targeting DAMPs with Nucleic Acid Scavengers to Treat Lupus

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### Abstract

Systemic lupus erythematosus (SLE) is a chronic and often progressive autoimmune disorder marked clinically by a variable constellation of symptoms including fatigue, rash, joint pains, and kidney damage. The lungs, heart, gastrointestinal system, and brain can also be impacted, and individuals with lupus are at higher risk for atherosclerosis, thrombosis, thyroid disease, and other disorders associated with chronic inflammation [1, 2]. Autoimmune diseases are marked by erroneous immune responses in which the target of the immune response is a “self”-antigen, or autoantigen, driven by the development of antigen-specific B or T cells that have overcome the normal systems of self-tolerance built into the development of B and T cells. SLE is specifically characterized by the production of autoantibodies against nucleic acids and their binding proteins, including anti-double stranded DNA, anti-Smith (an RNA binding protein), and many others [3]. These antibodies bind their nuclear-derived antigens to form immune complexes that cause injury and scarring through direct deposition in tissues and activation of innate immune cells [4]. In over 50% of SLE patients, immune complex aggregation in the kidneys drives intrarenal inflammation and injury and leads to lupus nephritis, a progressive destruction of the glomeruli that is one of the most common causes of lupus-related death [5]. To counter this pathology increasing attention has turned to developing approaches to reduce the development and continued generation of such autoantibodies. In particular, the molecular and cellular events that lead to long term, continuous activation of such autoimmune responses have become the focus of new therapeutic strategies to limit renal and other pathologies in lupus patients. The focus of this review is to consider how the innate immune system is involved in the development and progression of lupus nephritis [6] and how a novel approach to inhibit innate immune activation by neutralizing the activators of

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Duke University has applied for patents on the strategy to reduce inflammation via nucleic acid scavengers. All authors are listed as inventors on such patents.

this response, called Damage Associated Molecular Patterns (DAMPs), may represent a promising approach to treat this and other autoimmune disorders.

### Keywords

Damage Associated Molecular Pattern (DAMP); Toll Like Receptors (TLRs); Innate Immune Activation; Nucleic Acid Scavenger; Systemic Lupus Erythematosus

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### Overview

Systemic lupus erythematosus (SLE) is a chronic and often progressive autoimmune disorder marked clinically by a variable constellation of symptoms including fatigue, rash, joint pains, and kidney damage. The lungs, heart, gastrointestinal system, and brain can also be impacted, and individuals with lupus are at higher risk for atherosclerosis, thrombosis, thyroid disease, and other disorders associated with chronic inflammation [1, 2]. Autoimmune diseases are marked by erroneous immune responses in which the target of the immune response is a “self”-antigen, or autoantigen, driven by the development of antigen-specific B or T cells that have overcome the normal systems of self-tolerance built into the development of B and T cells. SLE is specifically characterized by the production of autoantibodies against nucleic acids and their binding proteins, including anti-double stranded DNA, anti-Smith (an RNA binding protein), and many others [3]. These antibodies bind their nuclear-derived antigens to form immune complexes that cause injury and scarring through direct deposition in tissues and activation of innate immune cells [4]. In over 50% of SLE patients, immune complex aggregation in the kidneys drives intrarenal inflammation and injury and leads to lupus nephritis, a progressive destruction of the glomeruli that is one of the most common causes of lupus-related death [5]. To counter this pathology increasing attention has turned to developing approaches to reduce the development and continued generation of such autoantibodies. In particular, the molecular and cellular events that lead to long term, continuous activation of such autoimmune responses have become the focus of new therapeutic strategies to limit renal and other pathologies in lupus patients. The focus of this review is to consider how the innate immune system is involved in the development and progression of lupus nephritis [6] and how a novel approach to inhibit innate immune activation by neutralizing the activators of this response, called Damage Associated Molecular Patterns (DAMPs), may represent a promising approach to treat this and other autoimmune disorders.

### Innate immunity and the pathogenesis of lupus nephritis

While seemingly an adaptive immunity-driven process, SLE progression and pathogenesis also relies on innate immune activity. Innate immunity both triggers the defective adaptive response and is a direct actor with the simultaneous involvement of innate cells, associated signaling, and inflammation all being consequential in SLE and associated lupus nephritis [6, 7]. For instance, clonal expansion of autoreactive B cells and T cells is directed by dendritic cell presentation of autoantigens coupled with appropriate inflammatory co-stimulatory signals [7]. The particularly pro-inflammatory neutrophils that are elevated in

SLE patients produce more cytokines such as type I interferons, that lead to further immune dysregulation, and are prone to the release of neutrophil extracellular traps or NETs [8]. The associated NETosis products have been implicated in the etiology of SLE because the nuclear antigens released by this mechanism are protected from nuclease degradation and are accompanied by inflammatory cytokines that provide costimulatory signals that can assist the break in immune tolerance [9, 10]. Of significance, many clinical studies have found that bacterial and especially viral infections trigger SLE presentation or exacerbate symptoms, pointing to connection between pathogen driven activation of innate immunity, systemic inflammation and the break in immune tolerance [11]. Finally, innate immune cells can directly exacerbate disease. For example, neutrophils, activated by immune complex deposition, release proteases and reactive oxygen species that directly damage renal tissue [12, 13].

### **PAMPs and DAMPs as triggers of inflammation**

Small molecular motifs from microbes like bacterial lipopolysaccharides or viral double stranded RNAs bind to Pattern Recognition Receptors (PRRs) of innate immune cells including neutrophils and monocytes and many non-hematopoietic cells, triggering a pro-inflammatory signaling cascade in response to this “non-self” signal. The pathogen-associated molecular patterns are termed PAMPs. Also recognized by the PRRs, when present in atypical locations or concentrations indicative of cellular damage, are endogenous nucleic acids, proteins, and metabolites from damaged or dying cells, collectively termed damage-associated molecular patterns or DAMPs [14]. The best characterized family of PRRs are toll-like receptors (TLRs). TLR1, 2, 4, 5, 6, and 10 sense proteins, lipids, or glycans and are primarily found on the cellular membrane and while TLRs 3, 7, 8, and 9 are nucleic acid sensing and are usually located on endosomal membranes. Their varied specificities are demonstrated by the recognition of viral double-stranded RNA and RNAs released from necrotic cells by TLR3 [15] and the recognition of bacterial lipopolysaccharides as well as host cell products like fibrinogen, nuclear protein HMGB1, and heparan sulfate fragments by TLR4 [16]. Upon binding these PAMPs and DAMPs, PRRs initiate signaling cascades of cellular adaptors and kinase activity that results in the activation and translocation of various transcription factors, like NF $\kappa$ B, AP-1, and IRFs [17]. These in turn induce the transcription, translation, and/or activation of effectors like cytokines, chemokines, interferons, bioactive amines (like histamine), arachidonic acid metabolites (leukotrienes and prostaglandins) and inflammatory mediators like bradykinins [18, 19]. The cardinal signs of inflammation, namely redness, heat, pain and swelling, are the direct consequence of this signaling cascade in innate immune cells as well as non-hematologic cells including endothelial cells lining the vasculature and epithelial cells within the tissue. The functional outcome of this inflammatory signaling cascade is the recruitment of additional immune effector cells to the site of infection and injury. With infection, innate immune cells reduce the spread of pathogens through direct elimination, consumption of infected cells, and release of systemic inflammatory signals to make the environment less hospitable to the invader (i.e. through elevation of body temperature or sequestration of nutrients like iron). Furthermore, activated innate immune cells present the pathogenic material and appropriate co-stimulatory signals to engage the slower but more

specific adaptive immune response. Inflammation is generally self-limited and its resolution is a tightly controlled process that begins shortly after the initiation of the inflammatory response [20, 21]. Inflammation thus does not simply fade out but instead is balanced by anti-inflammatory signals that accompany the removal of noxious stimuli.

## DAMPs in SLE and lupus nephritis

While important for tissue repair, excess production of DAMPs and activation of PRR-DAMP pathways triggers chronic inflammation and resulting diseases [14]. A central basis of lupus is the loss of tolerance for nuclear DAMPs that have emerged from dying and dead cells not effectively cleared after infection or sterile inflammation [22, 23]. Progression ensues as endocytosed immune complexes containing nucleic acids and pre-formed autoantibodies engage TLR7 (sensor of single stranded RNA) and TLR9 (sensor of CpG DNA), causing NFkB translocation and pro-inflammatory transcription initiation in dendritic cells [24] and proliferation of autoantibody producing B cells [25, 26]. The amplified production of autoantibodies against DNA or DNA associated proteins leads to the deposition of DNA containing immune complexes in the kidney that then further stimulate both innate and adaptive immune cells. The consequent DAMP-stimulated inflammation is implicated in the acceleration of renal scarring and fibrosis leading to renal failure [7].

## cfDNA

A representative DAMP that can serve as an autoantigen is cell-free DNA (cfDNA), or circulating extracellular DNA. DNA is “the central autoantigen in SLE” [27] with antibodies to double stranded DNA considered diagnostic for SLE [28]. While hypomethylated CpG DNA from bacteria is the appropriate ligand, endogenous DNA can stimulate the TLR9 axis in error [29, 30]. The cfDNA in the blood nucleome that triggers and modulates immune response in SLE patients is associated with three categories of cell death: apoptosis, necrosis, and NETosis. It is the impaired clearance of apoptotic cells, secondary necrosis, and increased NETs that leads to the increased DAMPs that then amplifies autoimmunity and inflammation [31]. cfDNA arising from apoptosis is digested by nucleases, producing low molecular weight DNA strands while necrosis and NETosis, which are comparatively much faster mechanisms, produce higher weight, more intact cfDNA [27]. While the most studied cfDNA in the blood nucleome is linear, other topologies such as circular DNAs are present and the extrachromosomal circular DNA found in circulation tends to be longer than linear DNA [32]. Mitochondria contribute to the circular DNA as well as nuclease-digested linear DNA species. Notably, mitochondrial DNA is particularly inflammatory because it has a high content of unmethylated CpG motifs, similar to bacterial DNA [29] and CpG motif containing DNA is enriched in immune complexes from SLE patients [33]. Characterization of circulating cfDNA in SLE patients found that those with higher cfDNA concentration, more fragmentation, and certain characteristic fragment lengths, had worse glomerular filtration rates and more severe lupus nephritis in comparison to SLE patients with cfDNA profiles comparable to healthy individuals [34].

## Other DAMPs

Beyond DNA DAMPs, other molecules such as RNA and intracellular proteins are associated with cell damage and contribute to SLE inflammation. Upon release from nuclear compartments RNAs and histones interact with endosomal and cell surface TLRs respectively [35]. HMGB1, a nuclear protein affecting chromatin structure and transcription, is released through NETosis and necroptosis and interacts with TLRs 2, 4, and 9 to activate the NF- $\kappa$ B pathway and RAGE to activate pro-inflammatory genes [31, 35]. Originating in the cytosol, S100 proteins released during phagocytosis and heat shock proteins from necrotic cells are released as DAMPs and interact with cell surface TLRs 2 and 4 [35]. In addition, while cfDNA and other nucleic acid DAMPs can exist freely and are often modeled as such in various *in vitro* assays, nucleic acids *in vivo* closely associate with other molecules. Nucleic acids are intrinsically protein binding and can associate with extracellular vesicles such as microparticles [36] and apoptotic bodies. Thus, nucleic acid DAMPs exist to a large extent in complexes *in vivo* which further facilitates multiple interactions with TLRs as well as immune complex formation.

## Nucleic acid degradation enzymes

Nucleic acid degradation enzymes have pivotal roles in ligand availability for TLR interaction as documented in a recent comprehensive review by Santa et al. [37]. Cell-free nucleic acids are degraded for clearance by various DNases and RNases. In general, deactivation or mutation of nucleases promotes autoimmune onset as DAMPs then evade degradation and interact with TLRs. Deficiencies in DNASE1L3, an extracellular DNase capable of digesting cfDNA as well as DNA in NETs and microparticles, are particularly associated with SLE. Mutations of this nuclease in humans are associated with pediatric onset of SLE, and knockouts in murine models display strong SLE-like serologic features [37].

## TLR signaling in SLE and lupus nephritis

Crucial to the response to viral pathogens is signaling through TLRs leading to production of interferons and inflammatory cytokines. Such signaling, particularly the induction of type I interferon alpha, is also implicated in chronic inflammatory diseases such as SLE and associated lupus nephritis [38, 39]. Given the central role of TLR signaling in SLE and lupus nephritis pathogenesis, TLR perturbations have been examined to elucidate specific pathways and identify possible interventional targets. Included in these efforts are TLR knockout studies in murine models and polymorphism analysis of TLR pathway genes in SLE patients.

## Mouse studies to dissect the significance of the different TLRs in lupus nephritis

Mouse studies, particularly those utilizing MRL/MP<sup>lpr/lpr</sup> mice, have highlighted the involvement of several TLRs in lupus. In these mice exposure to TLR3 agonists exacerbates renal pathology and *Tlr3* mRNA expression increases with worsening glomerulonephritis

[40-42]. Knockout of TLR7, a sensor for single stranded RNA, partially ameliorates SLE-like pathology in MRL/MP<sup>lpr/lpr</sup> mice [43], and TLR7 deficiency in pristane-induced lupus mice is associated with a decrease in anti-snRNP antibody production, IgG immune complex glomerular deposition, and attenuated glomerulonephritis [44]. Signaling through TLR8, also activated by single stranded RNA, is sufficient in the absence of TLR7 for the loss of B-cell tolerance in an autoantibody knockin mouse strain [45]. The consequences of manipulating TLR9, the sensor of DNA featuring unmethylated CpG motifs, have also been assessed in the MRL/MP<sup>lpr/lpr</sup> mice. Exposure to the TLR9 agonists CpG and bacterial DNA leads to lupus nephritis progression [46] and ablation of TLR9 inhibits anti-dsDNA and antichromatin antibody production [47]. Loss of TLR9, however, exacerbates lupus nephritis [43], possibly because its absence leads to enhanced TLR7 signaling and a net promotion of disease [48].

## TLR pathway polymorphisms linked to SLE

Many genes associated with susceptibility to lupus encode innate immune functions involved in the clearance of cellular debris and immune complexes and the activation of TLR/interferon/NFκB signaling pathways [49]. Aberrantly high DAMP levels can be caused by nuclease deficiency, as mentioned above, and by defects in the clearance of apoptotic cells and antibody complexes. Such defects lead to increased availability of autoantigens and are linked to the development of lupus [50]. For instance, a missense integrin subunit encoded by a mutant *ITGAM* gene leads to impaired phagocytosis of complement-opsonized targets by macrophages, monocytes and neutrophils resulting in reduced clearance [51] and this particular *ITGAM* gene variant is associated with susceptibility to lupus in women of European descent [52]. Lupus risk is also associated with mutations causing elevated expression or, more rarely, altered function of various TLRs. A 3' untranslated region mutation in the *TLR7* gene leading to increased *TLR7* transcripts and TLR7 protein is associated with development of lupus in humans [53, 54]. Based on dosage studies in mice, the X-linked expression of *TLR8* has been theorized to underly the female predominance of SLE [45]. Higher *TLR9* mRNA expression in leukocytes is associated with lupus nephritis [55]. Common polymorphisms of the *TLR9* encoding gene have been found to be associated with increased risk for asthma and Crohn's disease in humans. While studies assessing the association of *TLR9* gene polymorphisms with an increased risk of SLE are conflicting [56, 57], intense TLR9 staining in the kidney is associated with lupus nephritis [57]. A polymorphism causing a premature stop codon in the *TLR5* gene that truncates TLR5 is associated with reduced lupus nephritis [56-58]. Given its primary role in interacting with bacterial flagella, the mechanism by which TLR5 mutations affect SLE pathogenesis requires additional characterization. Factors further downstream of the TLRs in the type I interferon and pro-inflammatory cytokine pathways also feature variants associated with lupus. The gene variants include those encoding the IRF transcription factors and factors regulating NFκB activity [49].

## Current therapeutics and unmet clinical need

Current clinical treatments for SLE utilize immunomodulators and immunosuppressants to regulate immune activity [59] as well as lifestyle modifications to reduce inflammation.

Lifestyle modifications can be made to avoid triggers, minimize damage and inflammation, and manage symptoms. For example, minimizing exposure to sunlight prevents cellular injury and subsequent DAMP release following damage by ionizing UV light. Fibromyalgia and fibromyalgia-like symptoms are common in SLE patients, and the associated fatigue, pain, and cognitive dysfunction can be alleviated through exercise and cognitive rehabilitation [59, 60]. Beyond lifestyle modifications, the antimalarial drug hydroxychloroquine is the current pharmacologic standard of care for SLE [61]. Given the widespread role of TLR signaling in SLE pathogenesis, hydroxychloroquine functions in part by inhibiting acid-dependent receptor processing in endosomes [62] and/or shielding the nucleic acid ligands [63] and thereby muting the activation of TLRs 7 and 9 [64]. Hydroxychloroquine also blocks MHCII-mediated autoantigen presentation and binds internalized nucleic acids, preventing their binding to nucleic acid sensor cyclic GMP-AMP synthase, which if bound mediates type 1 IFN transcription [64]. These mechanisms work in tandem to prevent excessive production of pro-inflammatory cytokines, ameliorating SLE symptoms and slowing lupus nephritis onset [61]. Hydroxychloroquine is generally safe and effective in lupus patients, although side effects include GI distress [65] and dose and duration dependent risk of retinopathy [66, 67]. Furthermore, corticosteroids are still required acutely for management of flares and chronically given at lower doses for many patients. Though potent as an anti-inflammatory and immune suppressant, corticosteroid treatment is accompanied by a wide range of side effects, including metabolic dysregulation [68], bone loss [69], increased risk of infection and other conditions [70]. Thus, additional therapies that can effectively and specifically target innate immune activation with fewer side effects would be welcomed for management of this challenging autoimmune disease process.

## Targeting of DAMPs and TLRs

As described above, DAMPs and PRR signaling is centrally involved in inflammation and has pathologic roles associated with many infectious and inflammatory diseases. Many approaches have been made to pharmacologically intervene in this axis. Given that many of the lupus susceptibility genes are associated with increased activity of TLR/interferon/NF $\kappa$ B pathways [49], targeting TLR signaling in its role in initiating and perpetuating chronic inflammation is a sound focus of therapeutic development. TLR axis inhibition using therapeutics generally focuses on one of two mechanisms: preventing the interaction of DAMPs with TLR receptors, and blocking intracellular signal transduction following pathway activation [71]. Oligonucleotides are one class of molecules that have been studied as inhibitors of DAMP-TLR interactions. Treatment of lupus-prone NZB/W mice with synthetic, guanine-rich nucleic acid sequences that inhibit activation of TLRs 3, 7, 8, and 9 delayed progression of glomerulonephritis and proteinuria in conjunction with decreased production of Th1 cytokines and anti-dsDNA antibodies [72]. In addition, as reviewed by Gao et al. [71], small molecule inhibitors (SMIs) targeting TLRs 7, 8, and 9 and the MyD88 protein that activates IFN transcription factors after TLR-ligand binding have been tested for their abilities to interfere in TLR signaling. As discussed above, hydroxychloroquine is an SMI that is in wide clinical use for SLE treatment. Antibodies targeting specific DAMPs like anti-HMGB1 have been trialed for neutralization of DAMPs in the extracellular space

[73, 74]. These approaches have largely remained in the preclinical phase or have not been approved for clinical use [71].

## Nucleic acid scavengers

Despite the sound biological premise of intervening on the TLR axis to interrupt autoimmune inflammation, most preclinical work inhibiting TLR signaling has not yielded translatable results. The redundancy of TLRs and other PRRs likely limits efficacy when trying to inhibit a single TLR receptor to mitigate the hyperinflammatory response to multifactorial cellular damage and debris [75]. A therapeutic agent that neutralizes a wide range of DAMPs may be more effective as it could theoretically prevent interactions of this complex mixture of proinflammatory ligands with a wide range of TLRs and PRRs, thereby interrupting the feedforward cycle of hyperinflammation that underpins lupus nephritis and other autoimmune conditions.

Molecular scavengers that bind nucleic acid-containing DAMPs have emerged as a potential therapeutic solution to the challenge of targeting multiple TLRs for inhibition. A subset of nucleic acid binding polymers, these nanoparticles are able to bind and neutralize a wide range of DAMPs from activating their respective PRRs. The binding properties of these polymers is enabled by cationic surface groups, most commonly amines, that are positively charged via protonation at physiologic pH. The negatively charged phosphate backbone of DNAs and RNAs bind to these cationic polymers, an ionic interaction that has a wide range of applications. The best studied polymer in this class of nanoparticles is polyamidoamine, or PAMAM. Originally synthesized by Tomalia et al. in 1985 [76] this “starburst dendrimer” was recognized for its biocompatibility and ability to bind and deliver nucleic acids and other agents to cells as described in a recent review [77]. Herein, unless otherwise specified, PAMAM will refer to PAMAM species with all terminal amines as end groups. Nucleic acid binding polymers like PAMAM are used in many commercially available transfection reagents because of their ability to load nucleic acid cargo and cross cellular membranes [78]. Larger generations of PAMAM have container properties, and have been studied extensively as agents for targeted drug delivery [78].

## Pharmacologic utility of nucleic acid scavengers

This property of high affinity binding to nucleic acids attracted our group to test the pharmacologic utility of PAMAM as a universal-reversal agent to RNA aptamers. While aptamers can often be readily inactivated by their antisense “antidote,” which binds and unfolds the functional three-dimensional confirmation of the original aptamer [79-81], Oney et al. demonstrated that PAMAM G3 and certain other cationic polymers could also reverse function for multiple high affinity protein-binding aptamers [82]. Exploiting these nucleic acid binding polymers would preclude the cost-prohibitive clinical development of multiple antisense oligos for each of the many aptamers that eventually may be used clinically. These studies were intriguing because the administration of such nucleic acid binding polymers rapidly neutralized aptamers *in vivo* despite the fact that the aptamers bound their target proteins with low nanomolar affinities. Thus such binding agents were able to reversibly



inhibit high affinity protein-nucleic acid interactions in the blood even when the nucleic acid and protein were preassembled into a complex in circulation in large animals [82].

### **Pre-clinical anti-inflammatory functionality *in vitro* and *in vivo***

Our group then wondered if the ability to disrupt extracellular nucleic acid-protein interactions could be utilized as an intervention in inflammatory disorders and thromboinflammation. As discussed earlier, DAMPs and PAMPs, exemplified by host and pathogen derived DNAs and RNAs, induce inflammatory responses through binding to their target proteins, pattern-recognition receptors (PRRs) including TLRs, just as aptamers bind to their target proteins with high affinity and specificity [83, 84]. Often in injury and disease, excessive inflammation is induced, leading the immune system to cause collateral damage in its attempt to heal or sequester infection. First, our group observed that certain nucleic acid scavengers, like PAMAM G3, can bind and sequester mediators of cellular damage, preventing the immune stimulating interactions of these DAMPs and PAMPs with TLRs, and reducing mortality in a DAMP-mediated murine model of toxic shock-induced liver injury [85]. *In vitro* imaging revealed that PAMAM G3 both reduces uptake of the pro-inflammatory synthetic oligonucleotide CpG 1668 and alters its intracellular distribution away from co-localization with TLR9 [85]. PAMAM G3 was also found to have antithrombotic properties, as the pro-thrombotic DAMPs released by vascular wall injury, and neutrophil and platelet activation can be bound and neutralized by PAMAM's cationic surface groups [86, 87]. PAMAM G3 treatment also reduced wound scarring [88], and cancer metastasis in murine models of pancreatic cancer and breast cancer [89-91]. As well, PAMAM or other scavenger variants have been shown to limit inflammation in models of rheumatoid arthritis [92, 93] and psoriasis [94]. Thus PAMAM-mediated nucleic acid-DAMP scavenging has proven effective at inhibiting a variety of pathological thromboinflammatory insults.

### **Nucleic acid scavengers for potential treatment of SLE, lupus nephritis and cutaneous lupus**

The observation that nucleic acid DAMP scavengers could limit activation of TLRs and other PRRs by prototypical nucleic acid-containing DAMPs led us to evaluate the potential utility of this approach to limit innate immune activation and associated pathologies in chronic autoimmune and inflammatory disorders, such as SLE. As discussed above, autoimmune diseases like SLE are disorders of aberrant immune response to one's own tissues that are also mediated by excessive DAMP-driven inflammation. As well, a DAMP targeting strategy would likely be helpful to a lupus patient with a defect in the clearance of cellular debris or with abnormally elevated TLR activity driven by genetic polymorphisms. *In vitro*, PAMAM G3 and other cationic polymers are able to inhibit the binding of anti-DNA antibodies to DNA [95] and inhibit dendritic cell and B cell responsiveness to nucleic acid TLR agonists without blocking T-cell or anti-viral responses [96]. These *in vitro* results suggested that the nucleic acid DAMP-scavenger approach should be evaluated in animal models of lupus. To determine if PAMAM G3 limits pathological inflammation in lupus-prone mice, we determined the maximal lethal dose of the polymer in NZBW F<sub>1</sub> mice,

100 to 200 mg/kg, and then performed all studies at 5-10 fold below this level at 20 mg/kg [97]. First the DAMP scavenger approach was evaluated in a cutaneous lupus erythematosus (CLE) model using NZBW F<sub>1</sub> mice (Figure 1). Following dermal injury using tape stripping, it was observed that PAMAM G3 (20 mg/kg twice per week) when delivered by subcutaneous administration around the site of injury over 14-21 days reduces skin inflammation [97]. Blinded pathological analyses of PAMAM G3 treated animals reveals significantly reduced disease grades compared to PBS-treated animals. Although these results were encouraging, we noticed in subsequent experiments that delivering PAMAM G3 at this dose occasionally induces skin irritation at sites of injection in mice with the most advanced lupus. Therefore, we sought to evaluate alternative nucleic acid scavenging agents in the CLE model. A linear  $\beta$ -cyclodextrin-containing polymer, termed CDP, had been reported by Mark Davis's laboratory to have a good safety profile including in clinical studies [98, 99]. As we had previously observed that the CDP could limit TLR activation by nucleic acid DAMPs [85], it was evaluated in the same CLE murine tape stripping injury model (Figure 1). Results from these studies indicated that CDP significantly improves skin healing following dermal damage of lupus prone mice and moreover CDP is associated with limited toxicity compared to PAMAM G3 [100].

*In vivo*, long term systemic PAMAM G3 treatment (20 mg/kg i.p., twice per week) slowed the progression of glomerulonephritis in the kidneys of lupus-prone male MRL<sup>lpr</sup> mice [97]. These studies also demonstrated that a 10-week treatment regimen with PAMAM G3 reduces immune complex and complement C3c deposits in the kidneys in the nucleic acid scavenger treated animals at 5 months of age. When this treatment period was extended however, we started to observe some toxicity revealed by weight loss associated with PAMAM G3 administration at this dose. Thus, for prolonged exposure, lower doses of PAMAM G3 or alternative nucleic acid scavengers such as CDP should be evaluated.

To determine if PAMAM G3 or CDP treatment causes immune suppression, mice being systemically treated with either of the DAMP scavengers were challenged with PR8 influenza infection. Such treatment did not make the mice more susceptible to influenza but rather reduced systemic symptoms of infection, ameliorating weight loss and hypothermia, and improving survival [97, 100]. This observation was initially somewhat surprising. However, given that much of the morbidity and mortality associated with influenza infections is thought to result from a cytokine storm elicited from DAMPs and PAMPs released from virus infected cells, the nucleic acid scavengers utilized to reduce DAMP induced inflammation in the setting of lupus could well have potential as antiviral agents.

## Barriers to translation

Despite the promising preclinical studies using PAMAM G3, concerns about its potential toxicity have precluded immediate translation of it as a systemic anti-inflammatory agent. As a rule, the higher the generation of PAMAM, the greater the risk. Studies of cationic PAMAM interaction with lipid bilayers show that cationic PAMAM, especially of larger generations, can form pores in cellular membranes [101, 102]. Cationic PAMAM G4 and larger generations can cause immediate toxicity when administered at high doses intravenously, notably causing thrombosis and hemorrhage. While we have used PAMAM

G3 as an antithrombotic in an injury-induced thrombosis model, intravenous delivery of this polymer can also induce acute toxicity when delivered at high doses (>20 mg/kg) to mice [86]. When dosed i.p., PAMAM G3 at 20 mg/kg induces, on average, a transient 5% dip in weight over the next 24 hours [100]. Within the literature, side effects of treatments (when reported) appear to be strain, age, and insult dependent. In our hands, some strains appear more sensitive to PAMAM treatment and have more weight loss following treatment, while other strains seem to adapt faster to any harmful impact of treatment. Toxicity is also more common in older mice or mice with severe illness. These findings demonstrate the narrow therapeutic window of cationic PAMAM G3. This rather limits the translational potential of this polymer, especially for use in chronic inflammatory illnesses like autoimmune disease such as SLE which require dosing over long spans of time. For this reason, we believe that evaluation of alternative nucleic acid DAMP scavengers may reveal more viable candidates for clinical translation. As mentioned above, Kelly et al. found that the linear  $\beta$ -cyclodextrin-containing polymer, termed CDP, appears to have an improved therapeutic window compared to PAMAM-G3 [100].

## Summary

Scavenging of nucleic acid-containing DAMPs has been shown to induce a significant therapeutic effect in multiple animal models of thromboinflammation including lupus nephritis and CLE models of SLE. Much of this work was performed with first generation scavengers such as PAMAM G3. More recent studies using optimized nucleic acid scavengers suggests DAMP scavengers with wider therapeutic windows are now being identified. Their discovery will almost assuredly lead to the translation of this novel and innovative approach to limiting the induction and pathological progression of inflammation in several disease settings including the treatment of patients with lupus.

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## Abbreviations:

<b>SLE</b>	systemic lupus erythematosus
<b>PRRs</b>	Pattern Recognition Receptors
<b>PAMPs</b>	pathogen-associated molecular patterns
<b>DAMPs</b>	damage-associated molecular patterns
<b>TLRs</b>	Toll-like receptors

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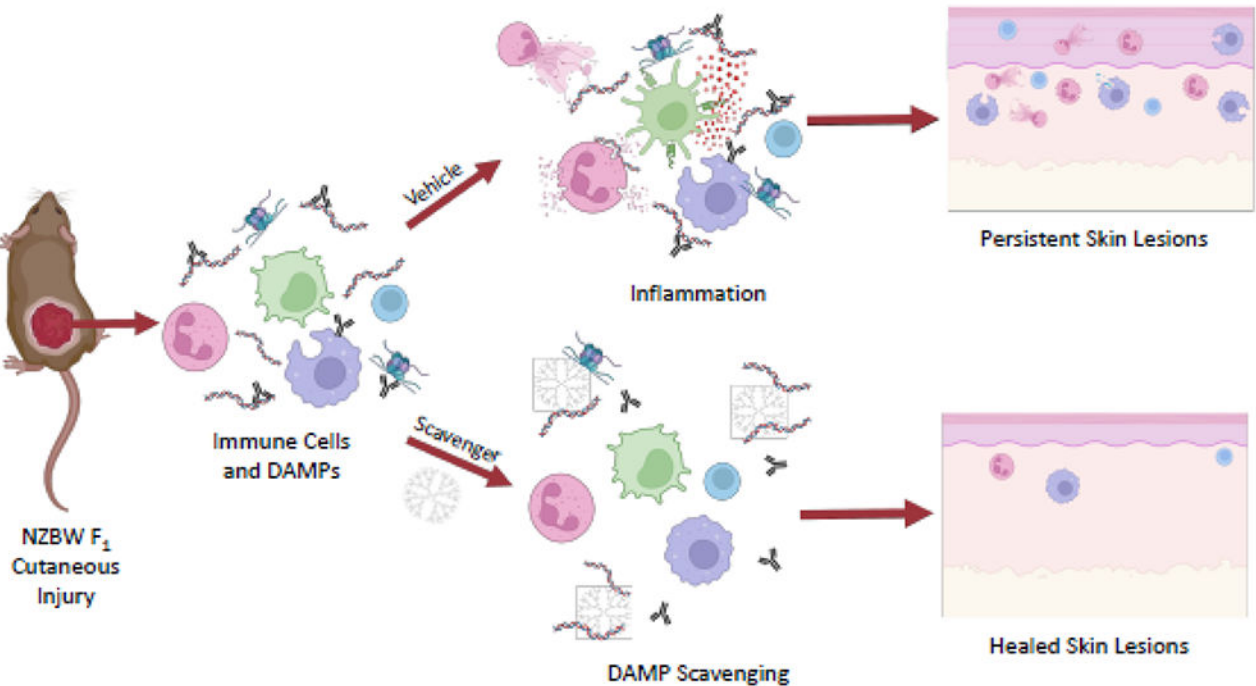
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**Figure 1. Overview of Nucleic Acid DAMP Scavengers Reducing Inflammation and Improving Skin Healing in a Mouse Model of Cutaneous Lupus Erythematosus**

When lupus-prone NZBW F<sub>1</sub> mice undergo tape stripping, they develop hyper-inflamed cutaneous lesions. Nucleic acid DAMPs are released and anti-nuclear antibodies and immune cells such as neutrophils, dendritic cells, macrophages and T cells, flood the wound zone. Mice treated with subcutaneous injections of vehicle (saline) exhibit continued inflammation that leads to persistent leukocyte infiltration of the dermis and epidermis, epidermal hyperplasia, and slowed wound healing (top right). In contrast, the skin of scavenger-treated mice shows reduced inflammation and improved wound healing (bottom right). Figure created with [BioRender.com](https://www.biorender.com).

Abbreviations in figure:

The New Zealand Black White hybrid mouse strain abbreviated as NZBW F<sub>1</sub>.

Damage associated molecular patterns abbreviated as DAMPs.