Nucleic Acid Scavengers as Novel Regulators of Autoimmunity and Viral Infection

by Kara Shumansky

Department of Molecular Genetics and Microbiology
Duke University

Date: November 20, 2017
Approved:

___________________________
Bruce Sullenger, Supervisor

___________________________
Mari Shinohara

___________________________
Stacy Horner

___________________________
Douglas Marchuk

___________________________
Micah A. Luftig

Thesis submitted in partial fulfillment of
the requirements for the degree
Master of Science, in the Department of
Molecular Genetics and Microbiology in the Graduate School
of Duke University

2017
ABSTRACT

Nucleic Acid Scavengers as Novel Regulators of Autoimmunity and Viral Infection

by Kara Shumansky

Department of Molecular Genetics and Microbiology
Duke University

Date November 20, 2017
Approved:

___________________________
Bruce Sullenger, Supervisor

___________________________
Mari Shinohara

___________________________
Stacy Horner

___________________________
Douglas Marchuk

___________________________
Micah A. Luftig

An abstract of a thesis submitted in partial fulfillment of the requirements for the degree of Masters of Science in the Department of Chemistry in the Graduate School of Duke University

2017
Copyright by
Kara Shumansky
2017
Abstract

Nucleic acids released from dead and dying cells can be recognized as damage-associated molecular patterns (DAMPs) or pattern-associated molecular patterns (PAMPs) by the innate immune system. Unregulated activation of the innate immune response by such endogenous molecules can stimulate pathological inflammation resulting autoimmune disease. Therapeutic efforts have been made to block this inflammation directly by targeting a class of pattern recognition receptors (PRRs), known as Toll-like receptors (TLRs) that recognize such DAMPs and PAMPs, or their downstream signaling molecules. Unfortunately, such therapeutic approaches can suppress immune system and its ability to fight off pathogens. There is a great need for novel therapies that can block this aberrant inflammation prior to TLR and immune recognition, thus allowing normal immune function. A novel class of Nucleic Acid Scavenging Polymers (NASPs) were examined for their ability to do just that. Previously shown to act as nucleic acid scavengers with the ability to neutralize agonists of TLRs NASPs are evaluated for their ability to act prior to TLR activation. Thus, not incurring the non-specific immune suppression evident in other autoimmune therapeutic strategies. Furthermore, NASPs do not limit an animal’s ability to combat viral infection, but rather their administration improves survival when animals are challenged with lethal doses of influenza. The studies outlined in this document suggest that molecules
that scavenge extracellular nucleic acids potentially represent promising therapeutic agents to control pathological inflammation associated with a wide range of autoimmune and infectious diseases.
# Contents

Abstract ........................................................................................................................................ iv

List of Figures ................................................................................................................................ ix

1. Introduction ................................................................................................................................. 1
   1.1 Pattern Recognition Receptors .......................................................................................... 1
   1.2 Inflammatory Endogenous Nucleic Acids and TLRs ....................................................... 3
   1.3 Inflammatory Self Nucleic Acids and Autoimmunity ...................................................... 4
      1.3.1 Blocking TLR-Activated Inflammation in Autoimmunity .................................... 5
      1.3.2 Nucleic Acid Scavenging ....................................................................................... 6
      1.3.3 Nucleic Acid Scavenging Polymers ....................................................................... 7
      1.3.4 Scavenging TLR Agonists ..................................................................................... 8
      1.3.5 Nucleic Acid Scavenging in Primary Cells ............................................................ 9

2. Nucleic Acid Scavengers and Viral Infection *In Vitro* .............................................................. 11
   2.1 Introduction ......................................................................................................................... 12
   2.2 Viruses .................................................................................................................................. 13
      2.2.1 Vesicular Stomatitis Virus .................................................................................... 13
         2.2.1.2 VSV G Protein ............................................................................................. 14
      2.2.2 Vaccinia Virus ......................................................................................................... 14
   2.3 NASP: PAMAM-G3 ........................................................................................................... 14
   2.3 Materials and Methods ...................................................................................................... 15
      2.3.1 Ethics Statement .................................................................................................. 15
2.3.2 Mice .......................................................................................................................... 15
2.3.3 Nucleic Acid Scavenging Polymers ........................................................................ 15
2.3.4 Cell Culture ............................................................................................................. 15
  2.3.4.1 Bone Marrow Dendritic Cells ........................................................................ 15
  2.3.4.2 Plasmacytoid Dendritic Cells ........................................................................ 16
2.3.5 Viruses .................................................................................................................... 16
2.3.6 Virus Assays .......................................................................................................... 16
2.3.7 Cytokine ELISA ..................................................................................................... 17
2.3.8 Statistical Analysis ................................................................................................. 17
2.4 Results ........................................................................................................................ 17
  2.4.1 Effects of PAMAM-G3 Dendrimer on the Immune Response to Viral Infection  17
  2.4.2 Mechanism of PAMAM-G3 Differs From Direct TLR Inhibitors ....................... 19
2.5 Discussion .................................................................................................................. 20
3. Nucleic Acid Scavengers and Influenza Infection In Vivo ........................................... 22
  3.1 Background .............................................................................................................. 22
  3.1.1 Influenza Virus ..................................................................................................... 23
    3.1.1.2 Influenza Virus Overview ........................................................................... 23
    3.1.1.2 Influenza Biology .......................................................................................... 24
    3.1.1.3 Mouse Adapted Influenza ......................................................................... 24
  3.2 Materials and Methods ............................................................................................. 25
    3.2.1 Mice ..................................................................................................................... 25
3.2.2 Influenza Infection Studies ................................................................. 25
3.2.3 Virus Microneutralization Assay ....................................................... 26
3.2.4 Antibodies and FACS .................................................................. 26
3.2.5 Statistical Analysis ...................................................................... 27
3.3 Results .............................................................................................. 27
  3.3.1 NASPs Do Not Increase Morbidity Or Mortality In Lupus Prone Mice During PR8 Influenza Infection ................................................................. 27
  3.3.2 NASPs Do Not Suppress The Immune System In Lupus Prone Mice .......... 28
  3.3.3 NASPs Protect Wild Type Mice From Lethal PR8 Influenza Infection .......... 29
  3.3.2 Immune Responses Remain Normal in Wild Type Mice Treated with NASPs during Lethal PR8 Infection ................................................................. 31
3.4 Discussion .......................................................................................... 32
4. Conclusions .......................................................................................... 34
  4.1 Thesis Summary .............................................................................. 34
  4.2 Future Studies ................................................................................ 36
  4.2.1 Mechanism ................................................................................ 36
References............................................................................................... 38
List of Figures

Figure 1: Schematic diagram of TLRs, adapter molecules and downstream products. ..... 2

Figure 2: Working model of nucleic acid mediated inflammation......................................... 4

Figure 3: Cationic NASPs bind the negatively charged backbone of nucleic acids and form stable polypeptides............................................................... 7

Figure 4: Nucleic acid scavenging polymers......................................................................... 8

Figure 5: Aptamer and universal antidote (CDP) in swine. ...................................................... 8

Figure 6: NASPs are able to inhibit TLR 7 and 9 agonists in immune cells......................... 9

Figure 7: NASs block TLR activation by nucleic acid agonists but not LPS in DCs isolated from NZBW F1 animals similarly to wild-type mice .............................................. 10

Figure 8: Effect of Cationic Polymers on B Cell Proliferation Cationic polymers (20mg/ml) selectively inhibit CpG induced B-cell proliferation in vitro from C57BL/6 (B6) and lupus mice (NZBWF1), but do not interfere with non-nucleic acid induced proliferation of B-cells (LPS-induced). ........................................................................ 11

Figure 9: Endogenous pro-inflammatory nucleic acids/nucleic acid-protein complexes versus shielded microbial nucleic acids.............................................................. 13

Figure 10: (A) Polymers do not affect TNFa production post VSV stimulation................. 18

Figure 11: Polymers do not affect IL6 production post VSV stimulation as compared to TLR inhibitors........................................................... 20

Figure 12: PAMAM-G3 treatment does not suppress the immune system of NZBW F1 animals during PR8 influenza infection. .......................................................... 28

Figure 13: PAMAM-G3 treatment does not affect the ability of NZBWF1 mice to mount a germinal center response after a sublethal dose of PR8 influenza treatment ....... 29

Figure 14: PAMAM-G3 treatment protects C57BL6/J mice from lethal PR8 influenza infection... 30
Figure 15: PAMAM-G3 treatment protects C57BL6/J mice from lethal PR8 influenza infection.

Figure 16: PAMAM-G3 treatment does not affect the ability of wild-type mice to mount a germinal center response after lethal dose of PR8 influenza treatment.
1. Introduction

Autoimmune disorders are one of the leading causes of death for young – middle aged women. They account for a group of more than 80 disorders, and can involve almost every organ in the human body. Autoimmune disease is the third leading cause of disease following cancer and heart disease, and affects between 5-8% of the population. Despite the deleterious impacts on health and well being, much is still unknown about the etiology and downstream treatment of such disorders. It is hypothesized that there is failure of one’s own immune system to regulate itself properly, thus failing to be able to discern self from non self.

Most current therapies such as non-selective immunosuppressants, cytotoxic drugs and corticosteroid serve merely to ameliorate the symptoms of the condition and are nonspecific in nature. Thus, they do not treat at the root of the disease, simply the downstream effects. Furthermore, many treatments broadly suppress the immune system leaving those suffering susceptible to a whole host of opportunistic infections. Novel therapeutics are needed to neutralize the disease state without suppressing the immune system.

1.1 Pattern Recognition Receptors

The immune system is able to recognize harmful endogenous and exogenous stimuli called pathogen-associated molecular patterns (PAMPs) through pattern-recognition receptors (PRRS). PRRS enable the immune system to recognize harmful
pathogens in addition to damaged cells, and initiate an inflammatory response. Toll-like receptors (TLRs) are the best categorized PRRs and have been extensively characterized as initiators of innate and adaptive immune responses to pathogenic stimuli. To date, 10 human and 12 murine functional TLRs have been identified. Cell surface TLRs recognize viruses or microbial membrane components such as lipopolysaccharide (LPS). Endosomal TLRs 3, 7, 8, and 9 predominantly recognize nucleic acids. The activation of TLRs results initiates a cascade of inflammatory responses characterized by the activation of transcription factors, such as the nuclear factor of light polypeptide gene enhancer in B cells (NF-κB) as well as proinflammatory cytokines such as type 1 interferons (IFNs).

Figure 1: Schematic diagram of TLRs, adapter molecules and downstream products.
1.2 Inflammatory Endogenous Nucleic Acids and TLRs

Endosomal TLRs 3, 7, 8 and 9 primarily serve to moderate and control viral and bacterial infections by sensing non-self nucleic acids. TLR7 recognizes specific sequences in guanosine and uridine-rich ssRNA, whereas TLR3 and TLR9 sense dsRNA and unmethylated CpG motifs in dsDNA, respectively. TLR8 identifies viral ssRNA and regulates IFN production, in humans. Endosomal and cell surface TLRs serve to keep the host safe from harmful stimuli.

Problems arise, however, when circulating immune complexes that contain self-nucleic acids reach the endosomal compartment they can cause inappropriate activation of TLRs. Although mechanisms do exist to prevent this inappropriate activation of TLRs by self nucleic acids, initiation of aberrant immune responses can occur if there is inadequate repression. For example, if self-nucleic acids released by dying cells form a complex with a stabilizing protein such as the high mobility group box (HMGB1), this complex can activate endosomal TLRs. This response, if left unchecked, can stimulate the inflammatory cytokine signaling cascade, thus potentially leading to an autoimmune disease state (figure 2).
Figure 2: Working model of nucleic acid mediated inflammation. Nucleic acids released from dead and dying cells can induce inflammatory responses and inflammatory diseases.

1.3 Inflammatory Self Nucleic Acids and Autoimmunity

Many complex autoimmune disorders are thought to be initiated by inappropriate activation of immune cells via self nucleic acids and nucleic-acid immune complexes including rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), inflammatory bowel disease (IBD), multiple sclerosis (MS), and psoriasis.\textsuperscript{21-23} Nucleic acid-containing microparticles expelled from apoptotic and necrotic cells have been identified in the blood of patients with inflammatory disorders\textsuperscript{14,24,25}. Furthermore, immune cells including plasmacytoid dendritic cells (pDCs) and B cells
have been shown to play a significant part in systemic lupus erythematosus (SLE) autoimmune disease onset and development due to their ability to induce pro-inflammatory cytokines and auto-reactive antibodies\textsuperscript{26}. Upon stimulation, pDCs produce a large quantity of type I interferons (IFNs) which in turn stimulates conventional dendritic cell (cDC) maturation and additional pro-inflammatory cytokine production\textsuperscript{26,27}. Self-nucleic acid TLR ligands can also serve to activate B cells during autoimmune disease progression \textsuperscript{28-30}. This stimulation results in production of aberrant antibodies. Similarly, autoimmune disorders such as multiple sclerosis and rheumatoid arthritis have been shown to be caused by such immune cell activation as well TLR stimulation \textsuperscript{31,32}. TLR agonists have been used to induce inflammation and autoimmune symptoms. Conversely, blocking the negative regulators of TLRs can also result in autoimmune symptom development through the initiation of the proinflammatory cytokine cascade\textsuperscript{23,33}.

\textbf{1.3.1 Blocking TLR-Activated Inflammation in Autoimmunity}

Blocking the activation of endosomal TLRs directly via self-ligands is effective in treating autoimmune disorders\textsuperscript{34,35}. Current therapies that have been shown to slow down SLE progression focus on direct inhibition of TLR7 and TLR9 via immunoregulatory DNA sequence (IRS) \textsuperscript{954}\textsuperscript{35}. For example, IRS954 treatment of lupus prone NZBWF1 mice results in lessened disease state. Mice have lower levels of circulating autoantibodies production and reduced glomerulonephritis \textsuperscript{35}. Furthermore,
mice lacking both TLR7 and 9 genes in a lupus background have a reduced disease onset and development. Both of these studies illustrate the power of targeting TLRs to treat SLE and autoimmunity altogether.

Despite the success of blocking TLRs 7 and 9 ameliorating SLE symptoms and progression, it is not an ideal solution. TLRs are essential for a functioning immune system, and blocking their function would leave the host vulnerable to viral and bacterial infections. Long-term therapeutics that directly block TLR7 and 9 are likely to inhibit the host’s ability to defend against such infections. Therefore, an alternative and potentially safer therapeutic approach would be to target and neutralize pro-inflammatory self-nucleic acids prior to TLR binding. This would allow TLRs to recognize and respond normal to pathogenic stimuli, thus leaving the immune system relatively in tact.

1.3.2 Nucleic Acid Scavenging

Nucleic Acids are inherently negatively charged due to their polyphosphate backbones. Studies done in the field of aptamer development, nucleic acid based therapeutics, demonstrated the use of cationic polymers to bind nucleic acids regardless of sequence or chemistry through simple charge based interactions. Nucleic acid scavenging polymers (NASPs) form stable polyplexes with RNA and DNA molecules (figure 3). Initially designed as aptamer antidotes, these cationic polymers lend
themselves to the development of novel therapeutics for nucleic acid-mediated disorders. 

Figure 3: Cationic NASPs bind the negatively charged backbone of nucleic acids and form stable polyplexes.

1.3.3 Nucleic Acid Scavenging Polymers

Three cationic polymers were identified with the capability of neutralizing nucleic acid activity. This was first tested in an aptamer system due to the fact that the activity of such nucleic acids could be directly examined. The three polymers chosen for their efficacy and safety profiles were: cationic cyclodextrin polysaccharide (CDP), a cationic cyclodextrin, polysaccharide; HexaDimethrine Bromide (HDMBr), a linear quarternary amine; and Polyamidoamine G3.0 (PAMAM-G3), a globular, branched dendrimer. All three were originally designed for the purpose of siRNA delivery, and were found to be capable of reversing the effects of eight distinct aptamers (figure 4).
These polymers were capable of rapidly reversing aptamer therapeutics in both *in vitro* and *in vivo* systems (Figure 5).\textsuperscript{6,40-42}

**Figure 5:** Aptamer and universal antidote (CDP) in swine. (a) Factor IXa RNA aptamer anticoagulation of swine (blue). The ability of protamine (a) and CDP (b) to reverse Ch-9.3t activity in vivo was assessed using ACT assays.\textsuperscript{40}

### 1.3.4 Scavenging TLR Agonists

Since NASPs act as molecular scavengers, and neutralize simply based on charge, such were effective in neutralizing the activity of pro-inflammatory nucleic acids, specifically known agonists of TLRs (Figure 4). NASPs were able to block...
inflammatory cytokine production from murine macrophages stimulated with nucleic acid-based agonists of TLRs 3 and 9. 6-8,10,43-45.

Figure 6: NASPs are able to inhibit TLR 7 and 9 agonists in immune cells. RAW264.7 cells were co-incubated with synthetic agonists of TLR3 (poly I:C) and TLR9 (CpG) along with PBS or nucleic acid binding polymers (CDP, HDMBr, PAMAM-G3, PPADPA, poly L-lysine and protamine sulfate). Culture supernatants were collected and analyzed for TNFα (L) & IL-6(R).

1.3.5 Nucleic Acid Scavenging in Primary Cells

The ability of NASPs to neutralize nucleic acids in both a synthetic system and in cell lines lends to their candidacy as a promising autoimmune therapeutic.21,40

Experiments performed in primary murine immune cells serve to further bolster their potential. Conventional dendritic cells were harvested and cultured from wild type C57BL6/J and lupus prone NZBWFl mice. CDCs were then stimulated with a TLR 4 agonist (LPS) or a TLR 9 agonist (CPG). NASPs were able to block inflammatory cytokine output from the TLR9 agonist, and endosomal TLR, but not from the TLR4
agonist, a cell surface TLR. Therefore, NASPs are in fact specific for nucleic acids, and are capable of binding agonists of TLRs (figure 7).\cite{46}

Figure 7: NASs block TLR activation by nucleic acid agonists but not LPS in DCs isolated from NZBW F1 animals similarly to wild-type mice. (A and B) NASs block IL-6 and TNFα cytokine production during CpG but not LPS stimulation in both wild type and lupus-prone (NZBW F1) mice. Bone marrow-derived dendritic cells (DCs) were cultured as previously described. DCs were then cultured in the presence of LPS and CpG as well as 20 µg/mL of each NAS (HDMBr, CDP, and PAMAM-G3). IL-6 and TNFα cytokine production was assessed 18 h later using ELISA. Data are representative of three independent experiments. n = 9 mice per group, **P < 0.01.\cite{46}

Additionally NASPs have demonstrated the ability to block the maturation of dendritic and B cells, as well as the up-regulation of MHC class II molecules. This stimulation is a hallmark of the innate immune system and disease progression\cite{47} (Figure 8).
Figure 8: Effect of Cationic Polymers on B Cell Proliferation Cationic polymers (20mg/ml) selectively inhibit CpG induced B-cell proliferation in vitro from C57BL/6 (B6) and lupus mice (NZBWF1), but do not interfere with non-nucleic acid induced proliferation of B-cells (LPS-induced). Cell proliferation was monitored by CFSE staining for 3 days following treatment with CpG/LPS and polymer. Mock = untreated cells without stimulation (red) or stimulated in the absence of polymer (blue). Percentages indicate the percent of cells that proliferated in a particular B-cell sample.⁴⁷

2. Nucleic Acid Scavengers and Viral Infection In Vitro

The text and figures of Chapter 2 were previously published in the July 2013 issue of PLOS ONE. All portions used are from K L Shumansky’s contribution to the paper. The paper was written in conjunction with first author Eda K. Holl. The full citation is:


PLoS One does not require permission for authors to reuse their own articles.
2.1 Introduction

The overarching goal is to develop novel autoimmune therapeutics that do not suppress normal immune function. Most current therapies act nonspecifically by acting upon immune receptors themselves, or targeting their downstream signaling cascade. Thus, leaving the host susceptible to infection.34 If a therapeutic could neutralize the source of the stimulus, prior to TLR interaction; both the deleterious inflammation could be quelled without compromising the immune system as a whole. Cationic polymers have shown great potential to be used as scavengers for inflammatory self nucleic acids released from dead and dying cells (figure 2). Studies have demonstrated their specificity for nucleic acids, and ability to inhibit cytokine production from said agonists.21,46 To further evaluate the efficacy of NASPs as novel therapeutics, it must be determined they have unwanted immunosuppressive effects. Thus NASPs were evaluated during infection to determine if they produced any non-specific or selective suppression on immune cell functions. Previous studies have demonstrated that nucleic acids from microbial and viral sources acids exist in a shielded state and therefore, differ from endogenous pro-inflammatory nucleic acids48 (figure 9). Therefore, our polymers will bind exclusively to endogenous nucleic acids based on location and accessibility.
2.2 Viruses

The in vitro efficacy of the NASPs was evaluated in a viral system of infection to ensure they did not have negative immunosuppressive side effects. Scavengers were evaluated against both RNA and DNA viruses.

2.2.1 Vesicular Stomatitis Virus

Vesicular stomatitis virus (VSV), part of the family Rhabdoviridae virus is a prototypical negative stranded RNA virus. To date VSV is one of the best-characterized animal viruses. The VSV genome is 11kb and is comprised of five genes encoding nucleocapsid (N) protein, phosphoprotein (P), matrix (M) protein, G protein and large polymerase (L). VSV’s G protein that enables the virus to infect almost all mammalian cells. Furthermore, the host’s main antiviral response to VSV infection is through the
production of type 1 interferons\textsuperscript{49}. Thus, making VSV an ideal RNA virus for a NASP challenge infection.

2.2.1.2 VSV G Protein

Though VSV is an RNA virus and predominately sensed through endosomal TLR \textsuperscript{7}\textsuperscript{50,51}, evidence has demonstrated that the G protein of VSV is recognized by TLR4\textsuperscript{52}. Thus, it is imperative to evaluate the efficacy of NASPs during VSV in the presence of a TLR4 inhibitor such as CLI-095. This would ensure that this virus is only recognized by nucleic acid-sensing TLRs.

2.2.2 Vaccinia Virus

Vaccinia virus (VV) is a large prototypical DNA virus belonging to the family Poxviridae. VV encodes 250 genes and replicates in the cytoplasm\textsuperscript{53}. Poxviruses are particularly complex viruses an employ many strategies to evade the immune system\textsuperscript{54}. Vaccinia virus is an extraordinary regulator of the immune system, notably cytokine production. Studies have shown its ability to significantly reduce TNF\textalpha\textsuperscript{54,55}. Thus, it may be difficult to discern the effects of viral infection in conjunction with scavengers with respect to TNF\textalpha production. IL-6 and IFN \textalpha/\textbeta may be better markers of immune response in relation to Vaccinia viral production.

2.3 NASP: PAMAM-G3

For the studies in chapters 2 and 3 only NASP, PAMAM-G3 was utilized. As compared to CDP and HDMBr, it had the best batch-to-batch consistency and least
overall toxicity. Its 32 surface amines (figure 4) that allowed for high-affinity binding to nucleic acids, whereas lower-generation PAMAM dendrimers were not as effective at inhibiting nucleic acid-mediated TLR activation in our previous in vitro studies with model TLR ligands\textsuperscript{21}.

\section*{2.3 Materials and Methods}

\subsection*{2.3.1 Ethics Statement}

All studies were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Duke University Institutional Animal Care and Use Committee (protocol number A011-11-01).

\subsection*{2.3.2 Mice}

Female C57BL/6 were obtained from the Jackson Laboratory (Bar Harbor, ME). Mice were housed in a pathogen-free barrier facility at Duke University.

\subsection*{2.3.3 Nucleic Acid Scavenging Polymers}

PAMAM-G3 (Cat# 412422) was obtained from Sigma-Aldrich. All polymers were resuspended in pharmaceutical grade saline prior to use.

\subsection*{2.3.4 Cell Culture}

\subsubsection*{2.3.4.1 Bone Marrow Dendritic Cells}

Murine bone marrow DCs were isolated C57BL/6 mice and were cultured in the presence of GM-CSF and IL-4 as previously described\textsuperscript{26}. 
2.3.4.2 Plasmacytoid Dendritic Cells

Murine bone marrow was isolated from C57BL/6 mice and was cultured in the presence of Flt3 ligand for 10 days. Cells were then isolated and purified using a mouse pDC isolation kit (Mylteni) with purity assessed at .90%.

2.3.5 Viruses

Vesicular stomatitis virus (Indiana strain) was originally obtained from Dr. John Rose (Yale University). Virus was propagated on BHK cells and titered using a standard plaque assay on BHK cells. Vaccinia virus Western Reserve strain was obtained from BEI Resources. Virus was propagated and titered on HeLa cells using a standard plaque assay as described57.

2.3.6 Virus Assays

Bone marrow derived dendritic cells and plasmacytoid dendritic cells were plated on 24well tissue culture plates at a density of 16106 cells/well in 500 mLs complete RPMI media. Cells were infected with vesicular stomatitis virus at an MOI of 5 or with vaccinia virus at an MOI of 10 in the presence or absence of 20 mg/mL PAMAM-G3. CpG 1668 and 1585 were used as controls for cell stimulation respectively. Cells were incubated at 37uC for 24 hours and supernatants were collected.

Bone marrow derived dendritic cells were stimulated with 100 ng/mL LPS, 1 mM CpG or VSV and treated with 0.5 mg/mL CLI-095 (TLR-4 inhibitor), 2 mM IRS954 and/or 20 mg/mL PAMAM-G3.
2.3.7 Cytokine ELISA

Murine IL-6, TNFa and IFNa in cell culture media were quantified using Mouse IL-6 ELISA Ready-SET-Go (eBioscience), Mouse IFN alpha Platinum ELISA (eBioscience), or VeriKine™ Mouse Interferon-Alpha ELISA Kit (PBL) according to the manufacturers protocol. Each cultured sample was run as undiluted and then followed by 5 serial 2 fold dilutions and assayed in duplicate.

2.3.8 Statistical Analysis

Statistical significance was determined with two-tailed Student’s t test or analysis of variance (ANOVA). All p values less than 0.05 were considered significant.

2.4 Results

2.4.1 Effects of PAMAM-G3 Dendrimer on the Immune Response to Viral Infection

We have demonstrated that PAMAM-G3 is capable of neutralizing the inflammatory effects induced by the TLR9 agonist CpG. Since, pro-inflammatory self nucleic acids are recognized by endosomal TLRs that are thought to also respond to viral RNA and DNA, we evaluated whether PAMAM-G3 would inhibit immune cell activation by nucleic acid containing viruses\textsuperscript{12,37,38}. We infected BMDCs and plasmacytoid DCs with vesicular stomatitis virus (VSV) and vaccinia virus (a prototype RNA virus, and DNA virus respectively) in the presence of PAMAM-G3 dendrimer. BMDCs produce pro-inflammatory cytokines including TNFa and IL6 upon viral infection. Both VSV and vaccinia virus have been shown to activate TLRs, including
nucleic acid sensing TLRs, and trigger an inflammatory response. PDCs play an important role in viral immunity by producing large amounts of the signature cytokine IFNα upon viral infection\textsuperscript{58}. In our study, cell supernatants were analyzed by ELISA for the presence of inflammatory cytokines IL-6 (BMDCs) and IFNα (pDCs). Neither IL-6 nor IFNα levels were decreased in virally infected cells treated with PAMAM-G3 as compared to infected BMDCs or pDCs without PAMAM-G3 treatment (Figure 10). These data demonstrate that PAMAM-G3 does not interfere with the cell’s ability to detect and respond to two prototypical RNA and DNA encapsulated viral pathogens. Together these observations suggest that NASPs can be used as potential therapeutic agents to bind accessible, extracellular self- nucleic acids without interfering with normal immune responses to viral pathogens.

![Figure 10](image1.jpg)

**Figure 10:** (A) Polymers do not affect TNFα production post VSV stimulation. DCs were generated from bone marrow cells of WT animals cultured in the presence of IL4 and GMCSF for 7 days. Cells were then stimulated with live VSV at a multiplicity of infection (MOI) of 1 in the presence of 20 mg/mL PAMAM-G3. Culture supernatants
were analyzed for secretion of IL6 by ELISA, 24 h later. (B) Polymers do not affect TNFα production post vaccinia stimulation. DCs were generated from bone marrow cells of WT animals cultured in the presence of IL4 and GMCSF for 7 days. Cells were then stimulated with live vaccinia at a multiplicity of infection (MOI) of 1 in the presence of 20 mg/mL PAMAM-G3. Culture supernatants were analyzed for secretion of IL6 by ELISA, 24 h later. (C) Polymers do not affect IFNα production post VSV stimulation. pDCs were generated from bone marrow cells of WT animals in the presence of Flt-3L for 10 days. Cells were then stimulated with live VSV at an MOI of 1 in the presence of 20 mg/mL PAMAM-G3. Supernatants were analyzed for the presence of IFNα by ELISA 24 h later. Data are presented as mean+/-2 SD and are representative of at least 3 independent experiments. n = 9 mice per group.

2.4.2 Mechanism of PAMAM-G3 Differs From Direct TLR Inhibitors

To determine if this mechanism of inhibition, targeting the agonist, is fundamentally different from direct inhibition of TLR receptors themself, we next compared PAMAM-G3 to a direct TLR7/9 inhibitor and a TLR4 inhibitor. As previously reported\(^{59,60}\), we observed that agents which inhibit directly target and inhibit TLR7/9 and TLR4 resulted in reduced cytokine production by DCs following viral challenge. This result clearly demonstrates that TLR7/9 are important receptors for mounting an anti-VSV response. By striking contrast, the NASP PAMAM-G3 had no effect on cytokine production post viral challenge (figure 11) even though this NASP inhibits CpG DNA activation of TLR9. These data demonstrate that NASPs act through a different mechanism than traditional therapeutic agents under development that directly target the TLRs. This difference may represent significant advantage for the treatment of autoimmune disorders because NASPs only inhibit activation of TLRs by accessible nucleic acid and do not prevent immune cell recognition of shielded nucleic acids, such as those present in viruses.
Figure 11: Polymers do not affect IL6 production post VSV stimulation as compared to TLR inhibitors. DCs were generated from bone marrow cells of WT animals cultured in the presence of IL4 and GMCSF for 7 days. Cells were then stimulated with live VSV at an MOI of 1 in the presence of 20 mg/mL PAMAM-G3, CLI-095 and IRS954. Supernatants were analyzed for the presence of IL6 by ELISA 24 h later. Data are presented as mean+/2 SD and are representative of at least 3 independent experiments. n = 9 mice per group. *p<0.05.

2.5 Discussion

Previous studies have illustrated the ability of NASPs to bind a variety of inflammatory nucleic acids, and in turn prevent the activation of nucleic acid-sensing endosomal TLRs\textsuperscript{21}. This study demonstrates that despite the ability of NASPs to bind and neutralize extracellular nucleic acids, they do not interact with those that protected or encapsulated such as within a viral capsid. Thus, NASPs do not interfere with, or impede virus-triggered immune responses. This presents a novel and exciting new therapeutic agent due to their unique mechanism of action. NASPs potentially could be
used to treat inflammatory and autoimmune disorders without causing detrimental suppression to the host’s immune system.

Endosomal TLRs 3, 7 and 9 can be stimulated by endogenous nucleic acids released from dead or dying cells (figure 2)\textsuperscript{19,61,62} This stimulation and subsequent inflammation, can lead to a positive feedback loop resulting in an autoimmune disorder.\textsuperscript{36,63,64} Experiments in mice lacking one or more of the TLR genes have demonstrated that these receptors help regulate the progression of inflammatory disorders\textsuperscript{65}. TLR3, activated by dsRNA, plays a key role in the induction of downstream IFN genes\textsuperscript{66}. Lupus prone mice, MRL/lpr, lacking TLR7 present improved signs of disease progression\textsuperscript{67}. TLR9 is essential in controlling production of autoantibodies during disease development\textsuperscript{30}. These data demonstrate the role endosomal TLRs play in autoimmune disease progression, and highlight the significance of inflammatory nucleic acids in autoimmune disease development.

Existing autoimmune therapeutics, such as systemic administration of IRS954, serve to prevent the downstream inflammatory cascade by blocking the TLR7 and 9 receptors directly\textsuperscript{35}. Although this has proved to be somewhat efficacious, this is far from an ideal solution. IRS954 is highly specific to a set of nucleic acid receptors known to be involved in autoimmunity, thus still allowing other receptors to contribute to disease development. Furthermore, as we have demonstrated (Figure 11) and as has been previously reported, the direct blocking of TLR7 and 9 receptors impairs the ability
of the cell expressing these receptors to respond to exogenous stimuli. This immune suppression could be extremely harmful to the patient. Conversely, our study clearly illustrates that NASPs do inhibit cellular cytokine by viruses such as VSV and VV, while direct TLR7/9 protein inhibition via IRS954 does (Figure 11). Thus NASPs have a property that may prove to be valuable in the development of safer anti-inflammatory agents that block aberrant cell activation while leaving normal immune responses intact.

3. Nucleic Acid Scavengers and Influenza Infection In Vivo

The text and figures of Chapter 3 were previously published in the August 2016 issue of PNAS. All portions used are from K. L. Shumansky’s contribution to the paper. The paper was co-authored Eda K. Holl. The full citation is:


PNAS does not require permission for authors to reuse their own articles.

3.1 Background

Chapter 2 demonstrates the unique scavenging properties of NASPs. They are able to bind and neutralize inflammatory nucleic acids and TLR agonists without
directly inhibiting TLR function and signaling directly. Furthermore, infected primary immune cells (figure 10) treated with NASPs are capable of responding to viral stimuli. However, cells are far less complex than the people such therapeutics would eventually be designed for. Therefore, the next step would be to explore the potential use of NASPs in vivo, specifically their ability to neutralize inflammatory agonists upstream of TLR binding, thus allowing normal responses to exogenous stimuli. This would highlight their novel properties as non-immunosuppressive agents for therapeutic use in a number of inflammatory and autoimmune diseases.

3.1.1 Influenza Virus

3.1.1.2 Influenza Virus Overview

In vitro studies using NASPs were performed using VSV and vaccinia due to their efficacy in cell culture studies. However, when moving on to in vivo studies, influenza was chosen due to its clinical relevance in human populations. The three subclasses of influenza viruses, A, B, and C, are among the most common human respiratory infections worldwide. It is associated with strikingly high morbidity and mortality, with upwards of 250,000 hospitalizations and 40,000 deaths in the United States during a single flu season. During pandemic flu outbreaks, up to 50% of the population may be afflicted with the virus, cause hospitalization and death rates to skyrocket. A total of five pandemics have occurred since 1889, the worst being the 1918 flu which killed approximately 50 million people worldwide.
3.1.1.2 Influenza Biology

Influenza viruses, members of the family Orthomyxoviridae, are enveloped negative-strand RNA viruses with segmented genomes comprising of seven to eight gene segments. Influenza A is the most common of the three subclasses and are responsible for most seasonal infection as well as pandemics. Influenza A viruses are sorted by antigenic characterization of the hemagglutinin (HA) and neuraminidase NA surface glycoproteins. A total of sixteen HA and 9 NA subtypes have been described.

3.1.1.3 Mouse Adapted Influenza

Influenza virus does not normally infect mice. Thus, a mouse adapted strain, PR8 was created to allow influenza to infection be modeled in mice. It is important to note that there are inherent difference between the course of human influenza infection and that of a mouse. Generally, human influenza viruses bind to sialic acid residues with an α2,6 linkage to galactose which are found largely in the upper airways. However, in the mouse respiratory tract, α2,6 linked sialic acid residues are not widely found. Rather, sialic acids with an α2,3 linkages are the predominate type found in the airways of mice. Thus, upon infection, mice tend to experience a lower respiratory infection rather than an upper respiratory tract infection seen in human influenza cases. Despite these differences in infection course, the potential efficacy of NASPs should still be able to be elucidated.
3.2 Materials and Methods

3.2.1 Mice

C57BL/6 and NZBW F1 mice were obtained from The Jackson Laboratory. Mice were housed in a pathogen-free barrier facility at Duke University. Only female mice were used in all of our studies. All studies were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Duke University Institutional Animal Care and Use Committee (Protocol A011-11-01).

3.2.2 Influenza Infection Studies

Mouse-adapted virus strain, influenza A/Puerto Rico/8/34 (H1N1; PR8), was obtained from Charles River. Ten-week-old mice were anesthetized with vaporized isoflurane. Virus was administered intranasally in a total volume of 40 µL of sterile pharmaceutical-grade saline. Control mice were mock treated with pharmaceutical grade saline only. PAMAM-G3 was injected subcutaneously or intraperitoneally at 20 mg/kg in a total volume to 200 µL of pharmaceutical-grade saline. Weight loss and survival of infected mice were followed over a period of 14 d. Mice that lost 15% or more of their body weight were euthanized and recorded as dead per Duke University Institutional Animal Care and Use Committee guidelines.
### 3.2.3 Virus Microneutralization Assay

Virus microneutralization assay was performed as described previously with modifications. Briefly, virus and serum dilutions were performed as described and then mixed with 100 µL of freshly trypsinized MDCK-London cells containing $1.5 \times 10^4$ cells in 96-well cell culture- treated plates. Negative controls consisted of cells alone, whereas positive controls contained virally infected cells. Plates were incubated for 20 h before fixation with acetone. Endogenous biotin in wells was blocked with PBS containing 0.1% avidin (Life Technologies) for 15 min followed by washes, and any bound avidin was blocked with PBS containing 0.01% biotin (Sigma Aldrich) for 15 min. Plates were analyzed for positive infection via ELISA. Mouse monoclonal biotinylated anti-NP antibodies MAB8257B and MAB8258B (Millipore) (dilution, 1:6,000) and HRP–streptavidin conjugate (BD Biosciences) (dilution, 1:4,000) were used in the ELISA. Color was developed using OPD substrate (Sigma-Aldrich) in citrate buffer (Sigma-Aldrich), and optical density was measured at 490 nm in plate reader (Molecular Devices). The highest serum dilution that generated >50% specific signal was determined to be the neutralization titer: $50\%$ specific signal = $(\text{OD}_{490} \ \text{virus control} - \text{OD}_{490} \ \text{cell control})/2 + \text{OD}_{490} \ \text{cell control}$.

### 3.2.4 Antibodies and FACS

Monoclonal Abs included the following: B220, CD4, CD8, and GL7 (eBioscience). Single-cell suspensions of C57BL6/J and NZBW/1 spleens cells were counted; $1 \times 10^6$ cells
were suspended in FACS buffer [PBS plus 2% (vol/vol) FBS] and stained with various Ab combinations. Flow cytometry was performed on a Gallios flow cytometer and FACSCanto. All data were analyzed with FlowJo software.

3.2.5 Statistical Analysis

Statistical significance was determined with two-tailed Student’s t test or ANOVA. Log-rank Mantel–Cox test was performed on all survival curve graphs. All P values less than 0.05 were considered significant.

3.3 Results

3.3.1 NASPs Do Not Increase Morbidity Or Mortality In Lupus Prone Mice During PR8 Influenza Infection

Currently marketed autoimmune disease-combatting drugs result in severe immune suppression and an array of side effects\(^8\). To determine whether NASP treatment results in immune suppression as well, we evaluated the effects of PAMAM-G3 treatment in a viral infection model \textit{in vivo} using the same dosing strategy that proved effective in the lupus-prone mice (PAMAM-G3, 20 mg/kg, twice per week)\(^8\). Lupus-prone animals were challenged intranasally with the mouse-adapted influenza A H1N1 strain, PR8 at a mouse lethal dose of 10% (mLD10) to determine whether NASP treatment could result in increased morbidity and mortality. Mice were monitored daily and sacrificed if they lost >15% of their body weight. NASP treatment did not affect animal mortality following influenza infection at an mLD10 (figure 12a). Moreover this treatment did not increase morbidity, as monitored by weight loss (figure 12b). At this
relatively low dose, however, we observed that influenza challenge resulted in 16% fatality in lupus-prone animals not treated with the NASP, whereas none of the NASP-treated animals died.

Figure 12: PAMAM-G3 treatment does not suppress the immune system of NZBW F1 animals during PR8 influenza infection. NZBWF1 mice were intranasally infected with PR8 influenza and injected subcutaneously twice per week with PBS or PAMAM-G3 (20 mg/kg). Mice were monitored for survival (A) and weight loss (B).

3.3.2 NASPs Do Not Suppress The Immune System In Lupus Prone Mice

To explore the ability of these animals to mount an immune response to influenza, we further analyzed the percentages of germinal center cells in the spleen. The analysis of splenic cells indicates that germinal center maturation is also not affected
by NASP treatment (figure 13). These data demonstrate that PAMAM-G3 treatment does not suppress the antiviral immune response in lupus-prone animals and suggest that, if adequately developed, NASPs may become inherently safer, anti-inflammatory agents.

Figure 13: PAMAM-G3 treatment does not affect the ability of NZBWF1 mice to mount a germinal center response after a sublethal dose of PR8 influenza treatment. Spleens from PR8 influenza-infected NZBWF1 mice were isolated 14 d post treatment with PBS or PAMAM-G3. Percentages of GL7+B220+ B cells were determined by flow cytometry. Data are representative of two independent experiments. N = 4.

3.3.3 NASPs Protect Wild Type Mice From Lethal PR8 Influenza Infection

Our observation that NASP treatment may improve survival of lupus-prone mice when the animals are challenged at low mLDs, led us to investigate whether PAMAM-G3 might have beneficial effects on normal mice challenged with higher doses of influenza. Therefore, C57BL6/J mice were infected with an mLD50 of influenza A virus PR8 (H1N1) and treated with PAMAM-G3 at the time of viral challenge (20 mg/kg, twice per week). Remarkably, as shown in figure 14a, NASP treatment significantly

29
improved survival following flu infection, reducing mortality from 75% to only 14% (P = 0.0126). Similarly, morbidity was dramatically improved as seen by a significant reduction in weight loss following viral challenge (figure 14b).

Figure 14: PAMAM-G3 treatment protects C57BL6/J mice from lethal PR8 influenza infection. (A) C57BL6/J mice were intransally infected with PR8 influenza (PR8) or treated with saline (mock) and injected intraperitoneally twice per week with PBS or PAMAM-G3 (20 mg/kg). Mice were monitored for survival. n = 7 per group. Graphs are representative of at least three independent experiments. **P < 0.01. (B) Treated mice were monitored for weight loss throughout the study.
3.3.2 Immune Responses Remain Normal in Wild Type Mice Treated with NASPs during Lethal PR8 Infection

To explore the ability of these animals to mount an immune response to influenza, we measured the level of neutralizing Abs to PR8 in the serum (figure 15).

Figure 15: PAMAM-G3 treatment protects C57BL6/J mice from lethal PR8 influenza infection. C57BL6/J mice were intranasally infected with PR8 influenza (PR8) or treated with saline (mock) and injected intraperitoneally twice per week with PBS or PAMAM-G3 (20 mg/kg). Mice were monitored for survival. n = 7 per group. Anti-influenza neutralizing Ab titers from infected mice were analyzed by microneutralization assay.

These studies revealed that anti-influenza Ab titers are not impacted by NAS treatment. Furthermore, the analysis of splenic cells indicates that germinal center maturation is also not affected by NASP treatment (figure 16). These results suggest that, by binding
extracellular nucleic acid debris released from virally infected, dying cells, NASPs can limit viral-induced, acute pathological inflammation.

Figure 16: PAMAM-G3 treatment does not affect the ability of wild-type mice to mount a germinal center response after lethal dose of PR8 influenza treatment. Spleens from PR8 influenza-infected C57BL/6 mice were isolated 14 d post treatment with PBS or PAMAM-G3. Percentages of GL7+B220+ B cells were determined by flow cytometry. Data are representative of two independent experiments. N = 5.

3.4 Discussion

Inflammation is a complex biological process that is necessary for clearance of pathogens. However, when acute inflammation turns chronic, it can lead to inflammatory disorders that are hard to control. Dead or dying cells release RNA and DNA into circulation. If these self-nucleic acids are not properly cleared, they can trigger activation of endosomal TLRs such as TLR7, 8, and 9\textsuperscript{59,82,83}. This in turn results in further downstream activation of signaling pathways and production of proinflammatory cytokines\textsuperscript{26,84}. In fact, multiple autoimmune disorders are characterized by elevated
levels of circulating proinflammatory cytokines and autoantibodies\textsuperscript{85-87}. To date, numerous studies and clinical trials have focused on addressing circulating self-RNA and DNA, TLR activation, proinflammatory cytokines and circulating autoantibodies\textsuperscript{80}. Many of these drugs act on a single component or cell type of the inflammatory response, have short-term effects, and do not break the TLR activation cycle\textsuperscript{88}.

Additionally, a number of these compounds are associated with increased susceptibility to infection and decreased pathogen clearance\textsuperscript{3,4}. The goal of this study was to break the cycle of deleterious inflammation by targeting self-nucleic acids prior to their binding of endosomal TLRs. To address our goals, we used a newly identified class of compounds, NASPs, which bind circulating nucleic acids/ nucleic acid- protein complexes, and block TLR activation. Treatment of immune cells from both C57BL6/J wild-type animals and NZBWF1, lupus-prone animals with nucleic acid agonists in the presence of NASPs resulted in diminished proinflammatory cytokine production (IL-6 and TNF-\(\alpha\)) \textit{in vitro} (figure 7)\textsuperscript{81}. Cell activation through non-endosomal TLRs remained intact, thus further demonstrating the specificity of our compounds for nucleic acids (figure 7). Moreover, these compounds inhibited nucleic acid- driven TLR activation in cultures of DCs derived from SLE-prone animals, suggesting that these compounds can potentially be effective in an autoimmune disease setting (figure 7)\textsuperscript{81}.

Our study also aimed to addresses immunosuppression, a very important aspect of all therapeutic agents attempting to target deleterious inflammation. Sustained
treatment with anti-inflammatory agents can lead to overall immune suppression and increased susceptibility to infections\textsuperscript{3,4,89,90}. To determine whether our therapeutic approach impacts immune responses to pathogens, we infected NASP-treated animals with PR8 influenza to mimic human flu infection. We did not observe immune suppression in treated animals, as NASP-treated lupus-prone mice were able to recover similarly to untreated controls. To our surprise, C57BL6 animals that received lethal doses of PR8 in the presence of NASPs did not succumb to infection at the same rate as the control-treated mice. These findings suggest that NASPs may have broader applications in not only controlling aberrant inflammation but in also improving the immune response to pathogens.

Thus, NASPs represent a class of drugs to potentially treat SLE and autoimmune disorders, as well as a wide variety of infectious diseases, particularly those caused by highly pathogenic viruses such as pandemic influenza and Ebola. Their potential as therapeutics should now be explored.

4. Conclusions

4.1 Thesis Summary

The overall goal of this work was to explore a novel class of nucleic acid binding agents, and their potential use as therapeutics. There is an immense need for drugs to treat autoimmune and inflammatory diseases that do not have immunosuppressive side effects. First, NASPs were shown to inhibit inflammation from TLR agonists without
directly impeding the TLR proteins. This essential finding was expounded upon when it was shown that NASPs did not inhibit immune cells from recognizing or responding to pathogenic viruses. Data from both wild type and lupus prone animals illustrates the potential power of such polymers for inflammatory nucleic acid scavenging.

Next NASPs were introduced into an *in vivo* model of murine influenza infection where two major conclusions were drawn. The first set of findings tied in nicely with the *in vitro* experiments done previously. Influenza infection lupus prone mice did not fare worse when treated with NASP, PAMAM-G3. In fact, mice treated with PAMAM-G3 had slightly slower morbidity and mortality rates. Germinal center responses were similar between both infected groups regardless of NASP treatment, further suggesting that PAMAM-G3 did not inhibit the immune response to influenza.

The second conclusion from the influenza study was perhaps the most intriguing. Wild type mice challenged with influenza and treated with PAMAM-G3 survived the infection far better than mock treated infected mice. Overall morbidity was significantly decreased as well. As with the lupus prone mice, infected wild type mice showed similar germinal center development as well as neutralizing antibody titers. This protection against influenza infection presented an entirely new area of therapeutic development for NASPs: antivirals.
4.2 Future Studies

4.2.1 Mechanism

The studies in chapter three illustrate PAMAM-G3 as a potential antiviral. However, it is key to understand the mechanism of action before pursuing it as a potential therapeutic. Based on the in vitro studies in chapter 2, it is unlikely that PAMAM-G3 is interacting directly with the virus or cytokine output would have been directly affected. Thus, it is likely to be an immune regulator. Even though the work described in chapter 3 was done in influenza, it may be better to work with a blood born virus as influenza infection has many drawbacks. It is hard to get good consistency between animals with intranasal infections, and there is a huge variation in susceptibility depending on mouse strain chosen. Of course there is always the possibility of loss of phenotype if the pathogen is changed.

If studies are continued with influenza, DBA2/J mice might be a better mouse strain to work with due to their susceptibility to influenza. They present with a more robust disease state and higher cytokine production upon infection. Both serum and bronchoalveolar lavage (BAL) should be examined for cytokines throughout the acute phase of infection. Pathology should be performed on the lungs through the course of infection for signs/amelioration of damage and/or infiltrating immune cells. Flow cytometry of immune cell populations in the lungs or BAL would provide further data. This could allow insight into the mechanism of action of NASP’s.
Another tool that would help elucidate the mechanism of NASPs would be a biotinylated or fluorescently tagged polymer. In the influenza studies, mice were injected intraperitoneally or subcutaneously, yet influenza in mice primarily targets the lungs. A fluorescently tagged polymer would allow it to be tracked within the animal in real time, while a biotinylated NASP would allow insight into what exactly the polymer is binding to. Both would be key to understanding the biology of how NASPs ameliorate influenza infection.
References


12. Schulz, O. *et al.* Toll-like receptor 3 promotes cross-priming to virus-infected


24. Pisetsky, D. S., Gauley, J. & Ullal, A. J. Microparticles as a source of


34. A, S. et al. Protection against autoimmune nephritis in MyD88-deficient


69. Dienz, O. *et al.* Essential role of IL-6 in protection against H1N1 influenza virus by promoting neutrophil survival in the lung. **5**, 258–266 (2012).


89. Davies, R. J. et al. Rituximab in the treatment of resistant lupus nephritis:

