The STING of inflammatory adjuvants: Is the Toll too high?

Ross Darling

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The STING of inflammatory adjuvants: Is the Toll too high?

by

Ross Jeffrey Darling

A dissertation submitted to the graduate faculty in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Immunobiology

Program of Study Committee:

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The student author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this dissertation. The Graduate College will ensure this dissertation is globally accessible and will not permit alterations after a degree is conferred.

Iowa State University
Ames, Iowa
2019

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DEDICATION

This dissertation is dedicated to my family for their constant unwavering support and encouragement through the ups and downs of pursuing this research. Without them this would not have been possible.
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<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>AT</td>
<td>Anthrax toxins</td>
</tr>
<tr>
<td>BAFF</td>
<td>B cell activating factor</td>
</tr>
<tr>
<td>BMDC</td>
<td>Bone marrow-derived dendritic cell</td>
</tr>
<tr>
<td>BMM</td>
<td>Bone marrow macrophage</td>
</tr>
<tr>
<td>cDC</td>
<td>Classical dendritic cell</td>
</tr>
<tr>
<td>cdG</td>
<td>cyclic di-GMP</td>
</tr>
<tr>
<td>CDN</td>
<td>Cyclic dinucleotide</td>
</tr>
<tr>
<td>cGAS</td>
<td>Cyclic GMP-AMP synthase</td>
</tr>
<tr>
<td>CMI</td>
<td>Cell mediated immunity</td>
</tr>
<tr>
<td>CPH</td>
<td>1,6-bis(p-carboxyphenoxy) hexane</td>
</tr>
<tr>
<td>CPTEG</td>
<td>1,8-bis(p-carboxyphenoxy)-3,6-dioxaoctante</td>
</tr>
<tr>
<td>DAMP</td>
<td>Damage associated molecular pattern</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>ECAR</td>
<td>Extracellular acidification rate</td>
</tr>
<tr>
<td>EF</td>
<td>Edema Factor</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>LCMV</td>
<td>Lymphocytic choriomeningitis virus</td>
</tr>
<tr>
<td>LF</td>
<td>Lethal Factor</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MAMP</td>
<td>Microbial associated molecular pattern</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>moDC</td>
<td>Monocyte-derived dendritic cell</td>
</tr>
<tr>
<td>MPLA</td>
<td>Monophosphoryl lipid a</td>
</tr>
<tr>
<td>mROS</td>
<td>Mitochondrial reactive oxygen species</td>
</tr>
<tr>
<td>MST</td>
<td>Mitochondrial stress test</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NP</td>
<td>Nanoparticle</td>
</tr>
<tr>
<td>OCR</td>
<td>Oxygen consumption rate</td>
</tr>
<tr>
<td>ODN</td>
<td>Oligodeoxynucleotide</td>
</tr>
<tr>
<td>Ova</td>
<td>Ovalbumin</td>
</tr>
<tr>
<td>PA</td>
<td>Protective antigen</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen associated molecular pattern</td>
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<tr>
<td>pDC</td>
<td>Plasmacytoid dendritic cell</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptor</td>
</tr>
<tr>
<td>RIG</td>
<td>Retinoic acid-inducible gene</td>
</tr>
<tr>
<td>SA</td>
<td>Sebacic Acid</td>
</tr>
<tr>
<td>STING</td>
<td>Stimulator of interferon genes</td>
</tr>
<tr>
<td>SLE</td>
<td>Systemic lupus erythematosus</td>
</tr>
<tr>
<td>TBK1</td>
<td>Tank binding kinase 1</td>
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<tr>
<td>TLR</td>
<td>Toll like receptor</td>
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ABSTRACT

The worldwide impact of vaccines on human health through the prevention of death, disease, and suffering caused by infectious disease cannot be overstated. The use of inactivated or live-attenuated microbes, as well as alum or oil-in-water adjuvanted vaccines has been successful in limiting or eradicating disease across the world. However, each of these existing vaccine formulations carries its own shortcomings and limitations associated with their use. These deficiencies will need to be addressed and overcome through the identification and development of new vaccine adjuvants and/or delivery platforms. It would also be beneficial to those populations of individuals who exhibit increased susceptibility to infectious disease or who respond poorly to vaccination to identify adjuvants capable of enhancing efficacy in these immunologically deficient groups. While providing for antigen delivery, the studies described herein demonstrate that polyanhydride nanoparticles are capable activation of innate immune cells, particularly dendritic cell populations, in a manner that avoids induction of an overtly proinflammatory environment. These effects are associated with improved induction of cytotoxic CD8+ T cell memory, as compared to the inflammatory TLR ligand CpG, and resultant decreased tumor progression. The stimulation of the STING pathway using cyclic dinucleotides also demonstrated differential activation of the innate immune system as compared to TLR ligands. STING mediated stimulation of innate immune cells results in a differential activation phenotype that avoids production of innate, antimicrobial effector molecules. This led to increased BAFF production which was associated with higher vaccine-induced antibody titers in healthy adult mice, as well as in aged animals. This is potentially a unique outcome given the fact that aged individuals often respond poorly to TLR ligand adjuvanted vaccine formulations. This suggests that by
leveraging pathways and/or materials that avoid undesirable aspects associated with innate inflammatory response against microbial infection leads to a more hospitable inflammatory environment that results in a better adaptive immune response following vaccination.
CHAPTER 1. GENERAL INTRODUCTION AND LITERATURE REVIEW

Vaccination

Since its inception, prophylactic vaccination has proven to be one of the most effective and impactful medical strategies to improve human and animal health. Vaccination is unique as an intervention in the control of infectious disease as it has the ability to confer indirect protection to individuals who did not receive the vaccine through herd immunity [1]. Natural infection has long been known to provide long-lived immunity to reinfection (assuming you survive). Knowing this, most strategies of vaccination have centered around mimicking a state of natural infection, while avoiding onset of disease or illness.

Early effective vaccine formulations contained attenuated live strains of bacteria or virus. These formulations have proven successful in the overall prevention of disease dating back to the original Jenner smallpox vaccine of the 18th century [2,3]. This strategy of vaccination though successful carries some risk, albeit very low, that the attenuated strain may genetically revert to a highly virulent state and cause the very disease it was meant to prevent [4]. Inactivated vaccines removed this potential complication but can exhibit lower efficacy relatively decreased duration of protection, particularly in populations that tend to respond poorly such as the young or aged [5,6]. Highly purified recombinant protein subunit-based vaccines have also become common as they avoid undesirable reactogenicity caused by presence of various microbial components though this further reduces the immunogenicity and efficacy [7].

In order to improve these responses to inactivated vaccines or recombinant subunit vaccines, the inclusion of immune stimulatory adjuvants has become common [8]. The
mostly commonly used adjuvants in approved vaccine formulations consist of aluminum salts, oil-in-water emulsions, and more recently TLR ligands like MPLA [9,10]. While exact mechanisms for some of these is largely unknown the overall effect is to induce activation and inflammatory responses of the innate immune system via pathogen-mimicking or danger signals, comparable to the recognition of bacteria or viruses [11]. In large portions of the population these strategies have been successful in inducing efficacious antibody titers, though vaccines for disease that require generation of memory cytotoxic CD8+ T cells has lagged behind [12]. The best method to induce CMI, aside from naturally occurring infection (if you survive), has been to utilize a multiple dose prime-boost strategy which can lead to poor patient compliance and longer time required for efficacious immunity to develop [13]. New strategies involving the incorporation of various aspects of pathogen mimicking, such as various PRRs, have proven to be potent adjuvants in the area of vaccine development [14,15]. However, few have led to commercially available FDA licensed products [16].

**Dendritic Cells**

Dendritic cells were discovered to be potent stimulators of T cells capable of processing and presenting antigen in in the context of MHCI and MHCII [17–19]. Dendritic cells are divided into three major populations consisting of classical DCs (cDC), monocyte-derived DCs (moDC), and plasmacytoid DCs (pDC).

In mice the, cDC populations consist of CD8α+, CD103+, and CD11b+ subpopulations. The cDC family are generally found in barrier tissues and organ points of entry. Upon encounter with foreign antigen and/or MAMPs, activation is initiated resulting in increased antigen uptake, processing, and presentation on MHCI/MHCII, proinflammatory cytokine production, costimulatory molecule upregulation (B7 family molecules), and
eventual trafficking to draining, secondary lymphoid tissues [20]. The CD8α+ DCs found in lymphoid tissues, and CD103+ DCs found in the periphery have been described to be specialized in the cross-presentation of antigens, not normally found in the cytosol, in the context of MHCI [21–24]. This activity is critical in the cross-priming induction of CD8+ T cell populations [25,26]. The activity of CD11b+ DCs remains somewhat undefined as this is a heterogeneous population of cells [27].

Plasmacytoid DCs, are a distinct subset of nonclassical dendritic cell with unique functional characteristics [28]. Plasmacytoid DCs are found circulating in the blood as well as in organ tissues in the periphery [29]. The primary function of pDCs upon activation is their ability to secrete large amounts of type 1 IFNs (IFNa, IFNb) upon activation, primarily via TLR7 and TLR9 [29,30]. Despite the expression of MHCI and MHCII and associated antigen presentation pDCs, as compared to other DC populations, are relatively poor at presenting antigen to CD4+ and CD8+ T cells [31]. Despite poor performance as antigen presentation to T cells, pDCs play an important role in response to viral infection by the production of type 1 interferons.

Another nonclassical population of DCs are the monocyte derived DCs. This population commonly arises as a result of infection or inflammation [32]. Monocytes are recruited to sites of active inflammation where recognition of MAMPs, and subsequent proinflammatory cytokine environment, initiates activation and differentiation of the infiltrating monocytes to DCs or macrophages [33,34]. The moDC population shares functional similarities to the CD11b+ cDC populations relating to antigen presentation and costimulation of T cells [35]. In vitro GM-CSF bone marrow derived DCs (BMDCs) are phenotypically the most similar to moDCs allowing them to be modeled in vitro, though the
Toll-Like Receptors

Toll-like receptors (TLRs) are a family of receptors that are broadly expressed on cells of the innate immune system, B cells, as well as mucosal facing epithelial and endothelial cells. These receptors recognize a wide variety of microbial or pathogen-associated molecular patterns (MAMPs/PAMPs). TLRs 1, 2, 4, 5, and 6 are found on the surface of innate immune cells and detect extracellular MAMPs consisting of, lipopeptides recognized by TLRs 1/2/6/10, endotoxin or lipopolysaccharides (LPS) are recognized by TLR4, and flagellins bind to TLR5 [39–42]. Intracellularly expressed TLRs, TLR 3, 7, 8, and 9 recognize nucleic acid products such as double-stranded RNA which is recognized by TLR3, single-stranded RNA binds to TRL7/8, CpG DNA motifs are ligands for TLR9, ribosomal RNA is recognized by TLR13, and profilin binds to TLR 11/12 [40, 43–46].

Upon recognition of their ligands, these TLRs largely signal through the adapter molecule myeloid differentiation primary response gene 88 (MyD88), or through TIR domain containing adapter inducing interferon-β (TRIF) [47]. These signaling events ultimately lead to the activation of innate immune cells and the production and secretion of proinflammatory cytokines enhanced by transcription factors including NF-κβ, IRF5, and AP-1 [48–50]. This induction of an activated pro-inflammatory state via TLR stimulation also results in the production of antimicrobial innate immune effector molecules. This includes production of nitric oxide via nitric oxide synthase 2 (NOS2/iNOS), as well as reactive oxygen species by DCs and MΦs [51–53]. This recognition of MAMPs is critical in
the activation and mobilization of the innate immune system to quickly respond to and control an infection while adaptive immunity develops should it be necessary.

Figure 1-1. Toll-Like Receptor Signaling. Representative overview of the TLR family of receptors, their ligands, and downstream signaling events [54].

Nitric Oxide

Nitric oxide (NO) can be produced by three different isoforms of nitric oxide synthase (NOS) enzymes. Neuronal NOS (nNOS/NOS1) expressed in neurons to regulate blood pressure and smooth muscles, inducible NOS (iNOS/NOS2 expressed in activated cells of innate immune system, and endothelial NOS (eNOS/NOS3) is expressed in endothelial cells to control vasodilation and blood pressure [55]. The immunologically associated NOS2 is a component of the rapid innate immune response to microbial encounter and it is not constitutively expressed in resting state innate immune cells, but is transcriptionally upregulated upon recognition of MAMPs via activation of NF-κβ [51,56]. Interferon-γ (IFNγ) can also induce the expression of NOS2 via activation of JAK/STAT pathway and subsequent induction of interferon response factor 1 (IRF-1) [57]. Synergistic effects can also be observed on the transcription of NOS2 when IFNγ is combined with TLR stimulation [58]. Multiple other transcription factors, including HIF-1, have also been implicated in the induction of NOS2 [59].

The NOS2 production of NO in innate immune cells in response to infection plays an important role in the clearance of pathogens, as demonstrated by increased susceptibility to various infections in NOS2 deficient animals [51,60–63]. This is further supported by the fact that some microbes have evolved mechanisms to inhibit and evade NOS activity [64,65]. The antimicrobial mechanism ascribed to NO is largely associated with the inhibition of DNA synthesis, and breakage of DNA strands as well through formation of peroxynitrite, another reactive downstream product of NO, which can nitrosylate and oxidize nucleotides [66,67].

Despite its role as a potent, rapidly induced antimicrobial mediator, NO production by the innate immune system is very much a double-edged sword. The induction of NO has
been implicated in numerous deleterious or inhibitory effects on the adaptive immune system. T cell activation, proliferation, and effector function is inhibited by NO produced by innate immune cells [68–72]. B cells are also vulnerable bystanders to the cytotoxic effects of monocyte derived NO leading to depressed antibody responses [73]. A baseline level of intrinsic NO does seem to be required for normal function of B and T lymphocytes and plasmas cell populations suggesting a source, amount, and context dependent role for NO and the outcome of an adaptive immune response to infection or vaccination [74,75].

**Reactive Oxygen Species**

Reactive oxygen species are a family of innate immune anti-microbial effector molecules that also possess second messenger functions in various signaling pathways [76,77]. This family consists mainly of anionic superoxide, hydroxyl radicals, hydrogen peroxide, and peroxynitrite that can oxidize DNA, lipid, and proteins to interfere with microbial biochemical processes [78,79]. The production of ROS occurs in activated innate immune cells upon recognition of PAMPs and subsequent production IFN\(\gamma\) and its induction and enhancement of ROS production [80–83].

Reactive oxygen intermediates can be produced by two major potential sources. Phagocytic cells express the membrane bound multiprotein electron transferase, NADPH oxidase (NOX2), which is a major source ROS in the innate immune system [78]. This NOX2 derived ROS in particular has been deemed essential in the innate immune clearance of pathogens as illustrated by deficiencies in NOX2 which results chronic granulomatous disease (CGD) and the associated susceptibility to infections [84,85]. The mitochondria of a cell can also be a key source of ROS production [86]. Recognition of MAMPs via TLR ligands, as well as the proinflammatory cytokine IFN\(\gamma\), lead to the induction of mitochondrial
ROS generation through repurposing of mitochondria via Krebs cycle breakage, thought the full mechanism is not fully described. [87–89].

Despite the critical nature of ROS in clearing pathogens, the production of high concentrations is not without drawbacks. Induction of ROS has been linked to inhibition of IFNβ production via the STING pathway and decreased functionality of APCs and T cells [90–93]. B cell phenotypic differentiation (plasma cell, GC B cell) is also directly affected by transcriptional control exerted by ROS [94]. In addition to playing a proinflammatory role through the regulation of transcription factors, ROS can cause DNA damage to neighboring cells in a paracrine fashion [95]. While high ROS concentrations can exert negative effects on immunological function there is also dysfunction associated with abnormally low levels of ROS as well [96]. These results, while somewhat conflicting, indicate that the role for ROS in immunological function are not fully understood and are likely to be concentration and context dependent.

**BAFF**

B cell activation factor (BAFF, also known as TNFSF 13b, BLyS, TALL-1, THANK, zTNF4) and its receptor, BAFF-R, are described to display an essential role in the survival of B cell populations [97–100]. In particular the B2 B cells, marginal zone and follicular B cells, critically rely on BAFF/BAFF-R signaling for maintenance and survival while the peripheral innate-like B1 B cells do not [101]. The BAFF-R is expressed on the surface of all mature B cells, including memory B cells, but is not present on differentiated short- or long-lived plasma cells [102,103]. BAFF itself is primarily produced, either secreted or in membrane-bound form, by cells of the innate immune system such as MΦs, DCs, monocytes, and neutrophils though it can also be produced by T cells and activated B cells [104].
In addition to providing static survival signals to various B cell populations, BAFF also plays a major role in supporting the proliferation of B cells and subsequent overall outcome of adaptive humoral responses [105]. Despite the fact that serum IgG levels are lower in BAFF knock-out mice, relative to that observed for wild-type mice, upon immunization, there is normal germinal center formation in these mice. Normal germinal center formation is also observed in wild type mice when BAFF is inhibited [106]. However, these germinal centers are not maintained and dissipate much more rapidly than in BAFF sufficient mice which is, at least in part, due to the lack of an effective follicular DC network [107]. T independent antibody responses are also impaired when BAFF is not present at normal levels though this is directly related to the decreased numbers of B cells and associated proliferation rather than germinal center maintenance [101].

While BAFF signaling is critical for the normal development of humoral responses, conversely, overexpression of BAFF is associated with the generation of autoimmune conditions with the clinical manifestations similar to those of systemic lupus erythematosus (SLE) [104]. This partially appears to be caused by the constitutively high concentrations of BAFF supporting the survival of autoreactive low-affinity B cells. Some studies have suggested that patients with SLE exhibit higher concentrations of BAFF, similar to the overexpressing transgenic mice, however, this is controversial [108]. This suggests that temporarily high concentrations of BAFF upon immunization may be correlated with improved antibody titers, while avoiding the long-term negative effects of autoimmunity.
**Bacillus anthracis History and Pathogenesis**

*Bacillus anthracis* is spore-forming, gram-positive bacterium that induces a disease whose historical description date to pre-biblical eras. It was not discovered that *B. anthracis* was the causal agent of anthrax, also known as wool sorters disease, until the 1870s [109,110]. Cases in textile, and other animal processing industries, dropped greatly after the introduction of the Sterne vaccine for livestock [111,112]. The only major outbreaks of anthrax in humans, after the introduction of the Sterne vaccine, occurred in 1979 by an accidental spore release from a Soviet Union facility. In 2001, the first use of *B. anthracis* spores as a biological weapon, occurred in the United States when spores were dispersed through the federal mail system [113,114]. The events in 2001 led to renewed public health concern about anthrax as potential weapon of biological warfare that was no longer just a weapon of nation states but could be carried out by small groups or individuals [115,116].

The major virulence factors of *B. anthracis* infection are the anthrax toxins (AT). The ATs consist of lethal factor (LF), edema factor (EF), and protective antigen (PA). The ATs are considering amongst the family of AB toxins, where the A subunit, LF and EF in this case, is catalytic component within a target cell, and a B subunit, PA in this case, that binds to the cell and facilitates entry of the catalytic A subunit into the cytosol [112]. PA functions by binding to its ubiquitously expressed receptors, tumor endothelial marker 8 (TEM8/ANTXR1), and capillary morphogenesis protein (CMG/ANTXR2), and their controversially associated coreceptor lipoprotein receptor-related protein 6 (LRP6) on the surface of cells [117–119]. After binding to the cellular surface occurs, the whole PA_{83} is cleaved by a member of the furin family of proteases leaving a free PA_{20} N terminal subunit and a receptor-bound PA_{63} subunit [120]. This receptor associated PA_{63} subunit self assembles into a heptameric complex known as the prepore. The oligomeric prepore is able
to bind three subunits of LF or EF before being endocytosed by the target cell [121]. As the pH drops in the endosome, it leads to a conformational change resulting in the heptameric pore inserting itself into the endosomal membrane where it can facilitate the entry of LF and EF into the cytosol [122,123].

Upon translocation into the cytosol, LF cleaves and inactivates the family of mitogen-activated protein kinase kinases (MEKs). This causes the inactivation of MAPK family members JNK, p38, and ERK [124]. This damage leads to apoptotic cell death and suppression of the activity of the innate immune system [125]. The second catalytic toxin secreted by *B. anthracis*, EF, is an adenylyl cyclase that converts ATP to cyclic AMP (cAMP). This conversion rapidly increases the cellular levels of cAMP, and decreases levels of ATP [126]. This increase in cAMP leads to massive fluid secretion and edema of tissues and associated organ damage [119]. Together these toxins lead to disarming of the early immune response to infection with *B. anthracis* assisting with widespread bacterial dissemination. Lethal factor causes vascular collapse as its primary effects are on cardiomyocytes and smooth muscle, while EF results in multi-organ damage and failure [112,127,128].

The reliance of EF and LF on the pore forming activity of PA to function in the pathogenesis of *B. anthracis* makes it an ideal vaccine antigen candidate as effective PA neutralization would block the function both catalytic toxins.
Figure 1-2. Protective antigen facilitates Lethal Toxin and Edema Toxin entry into cells. An overview of *B. anthracis* toxin entry into cells [122].


**Anthrax Treatments and Practical Considerations**

With *B. anthracis* as a major concern for its potential use as an agent of biological warfare, current preventative and therapeutic treatment options are far from ideal. Use of antibiotics to treat anthrax infection has had success in improving survival after an exposure [129,130]. However, due to the resilience of the *B. anthracis* spore, disease can potentially
occur long after the initial exposure, in some cases up to 100 days [131]. This necessitates a rigorous long-term regimen of prophylactic antibiotics that can have poor patient adherence [132,133]. Such an extensive antibiotic schedule would require large and readily available stockpiles of the appropriate drugs ready to distribute immediately upon an exposure event [134]. This limits the overall practical effectiveness of antibiotics against *B. anthracis*.

Monoclonal antibodies (mAbs) have also been developed to target neutralizing epitopes of the *B. anthracis* PA toxin component. Many of these mAbs have been successful in improving survival subsequent to challenge in laboratory animal species [135–137]. However, the widespread usage of mAbs would be burdened by similar challenges as antibiotics in regards to the production, stockpiling, multiple doses due to antibody half-life, and distribution in the event of a large-scale anthrax spore release.

This leaves vaccination as a more cost-effective and practical option. That said, current anthrax vaccination strategies are not without their own shortcomings. The current alum adsorbed AVA BioThrax vaccine requires three doses over the course of six months along with regular boosters to maintain efficacy [138]. This makes mass vaccination of a large population of potentially exposed people largely impractical as it would take a great number of doses, and considering the boosters, far too lengthy a time period until protection would be possible. The alum component of the AVA BioThrax vaccine has also been implicated in blocking or biasing the humoral response towards non-neutralizing epitopes, likely limiting its potential efficacy [139–142]. Long-term shelf stability is also an area of concern for the AVA BioThrax vaccine [143,144]. Improvements in storage stability, and moving towards single dose efficacy would make vaccination a much practical to prepare for, and easier to distribute material for during the event of a large-scale anthrax release.
**Polyanhydride Particle Platform**

The primary rationale behind the development and use of polyanhydride nano- or microparticle formulations, consisting of the copolymers of the anhydrides 1,6-bis(p-carboxyphenoxy)hexane (CPH), 1,8-bis(p-carboxyphenoxy)-3,6-dioxaoctane (CPTEG), and sebacic acid (SA) as vaccine adjuvants and recombinant antigen delivery vehicles is to provide specific aspects of pathogen-mimicking that other adjuvant formulations cannot. The use of pattern recognition receptor (PRR) signaling molecules, such as TLRs, or sustained inflammatory effects, as with alum or oil-water emulsions, can provide potent innate immune recruitment and activation to improve adaptive immune responses to vaccination \[9,10,145\].

Polyanhydride particle formulations are capable of providing some similar effects to the traditional adjuvant formulations, such as inducing activation of DCs and MΦs as measured by co-stimulatory molecule upregulation, cytokine production, and antigen presentation \[146,147\]. As opposed to traditional adjuvants, particles also provide other aspects of pathogen-mimicking that can improve vaccine responses. These include extended payload release kinetics that can mimic the antigen duration of a natural infection, a small particulate context for antigen that is comparable to bacteria and viruses, and hydrophobic regions that can mimic similar aspects of various pathogens \[147–149\].

In addition to innate immune activating, pathogen-mimicking properties, polyanhydride formulations have also demonstrated the ability to provide stability to encapsulated antigen \[150–153\]. This provides a dual benefit for polyanhydride particle-based formulations, that in addition the inherent adjuvant benefits, that the encapsulated antigen can prolong storage times at a variety of temperatures. This would particularly be advantageous for recombinant antigens that are particularly prone to degradation at room temperature conditions, or where extended storage, in case of an outbreak, would improve
the distribution of doses.

These characteristics have been harnessed to provide efficacious, single-dose vaccine induced immunity to a number of infectious diseases \[150,154,155\]. Polyanhydride particles have also demonstrated the ability to induce CD8 cytotoxic T cell responses in addition to antibody \[156–158\]. Polyanhydride formulations are also unique in the sense they are providing innate immune activation and protective vaccine responses without overt injection site inflammation, reactogenicity, or side-effects as is seen with a traditional adjuvant such as alum \[159,160\]. While polyanhydrides exhibit effectiveness as a vaccine adjuvant, the exact biological mechanisms underpinning their mode of action remain undefined. Further experimentation is necessary to elucidate their mechanism of action and how their strengths may be best harnessed to rationally design the vaccine delivery and adjuvant formulations of the future.

**STING Pathway**

The stimulator of interferon genes (STING/MPYS/ERIS/TMEM173/MITA) is a relatively recently discovered endoplasmic reticulum (ER) anchored protein that was discovered to be a key pathway mediator in the TLR independent innate immune recognition of double-stranded DNA (dsDNA) \[161,162\]. Stimulator of interferon genes is predominately expressed in macrophages (MΦ), dendritic cells (DC), T cells, as wells epithelial and endothelial cells \[162,163\]. The STING pathway does not directly recognize dsDNA, instead it recognizes cyclic dinucleotides (CDNs) either directly secreted from bacteria, or generated through the enzymatic activity of cyclic GMP-AMP synthase (cGAS) which converts dsDNA into a cyclic dinucleotide \[164,165\]. STING exists as an inactive homodimer on the ER that upon binding of CDNs undergoes conformational changes \[166\].
This allows STING to bind tank binding kinase 1 (TBK1) and subsequently translocate to perinuclear endosomes via the Golgi [167]. There it activates proinflammatory transcription factors including IRF3 and NF-κβ [168,169]. In addition to its sensing of cytosolic DNA, significant crosstalk between the cGAS-STING and RIG-I-MAVS has been reported suggesting that STING is playing a role in the recognition of RNA as well [170].

Upon recognition of CDNs, STING signaling in innate immune cells induces the production of proinflammatory cytokines, particularly the type 1 interferon, IFNβ, via the transcription factors IRF3, IRF7, NF-κβ, STAT6, as well as playing a role in inflammasome activation [171–173]. This combination of transcription factors leads to a balanced pattern of Th1 (IFNγ, IFNβ, TNFα) vs Th2 responses (IL-4) via IRF3/NF-κβ and STAT6, respectively [174]. This balanced cytokine milieu is also accompanied by an activated, yet not fully characterized, “mixed” polarization phenotype of antigen presenting cells (APCs) [175,176]. The STING pathway is also not an exclusive detector of MAMPs as STING will also recognize self dsDNA from damaged cells in the absence of a pathogen, where persistent activation can induce auto-inflammation [177–179].

This potentially places STING recognition of DNA in a category more akin to “danger-sensing” rather than exclusively pathogen sensing. This leaves much of the effects of CDNs on the immune system as yet undescribed particularly as multiple contexts of STING activation come to light [176]. Despite a number of unknowns relating to the regulation and function of the STING pathway, CDNs have been described to possess potent adjuvant properties [180–183]. As a relatively recently discovered DNA sensing pathway further characterization will be necessary in order to fully appreciate the exact effects of
STING on the innate immune system and the resultant adaptive responses to damage, infection, and vaccination.

Figure 1-3. STING pathway activation. Diagram of ligands of STING and downstream transcription factors as a result of STING activation [184].


**Immunometabolism of Macrophages and Dendritic Cells**

Many decades ago it was originally observed that activated macrophages, as well as other immune cells, require certain metabolites upon activation [185–187]. However, the metabolism of immune cells is an area of research that, until recent years, was largely left
unexplored. The metabolic pathways involved are the glycolytic pathway, the TCA (Krebs/citric acid) cycle, the pentose phosphate pathway, fatty acid synthesis, fatty acid oxidation, and amino acid pathways. Each of these plays a key role in the providing the necessary ATP and molecular building blocks for immune cell function from rapidly proliferating T cells to M2 macrophages and everything in between (Reviewed in depth [188]).

Aerobic glycolysis as a result of innate immune cell activation and its importance to functional proinflammatory responses has been studied extensively. The induction of aerobic glycolysis as a result of activation, despite its poor efficiency at generating ATP, provides critical precursor building blocks for the biosynthesis of cellular products such as cytokines [189,190]. In addition to contributing to biosynthesis, a state of aerobic glycolysis can improve survival at sites of inflammation, such as a tumor or bacterial infection, where immune cells may be forced to compete for nutrients in an environment that is often hypoxic [191]. This is contrasted to the wound healing, tolerogenic, or regulatory phenotypes where mitochondrial respiration, fed by varying sources such as fatty acids, is the main source of ATP to meet energetic demands [188].

Macrophages, depending upon their general activation state and phenotype exhibit distinct metabolic demands [192–194]. Upon recognition of TLR ligands and IFNγ, resting macrophages assume a proinflammatory or “classically” activated M1 phenotype; in contrast exposure to IL-4 and IL-13 can promote an “alternatively” activated or M2 macrophage phenotype [195,196]. Activated M1 macrophages exhibit a distinct metabolic phenotype that is characterized by high rates of aerobic glycolysis and little to no mitochondrial oxidative phosphorylation [197]. This inhibited rate of oxidative phosphorylation also frees up the
mitochondrial machinery for repurposing itself to the production of ROS which in addition to antimicrobial effects promotes proinflammatory cytokine production [87,198,199]. Another key metabolic trait of M1 macrophages is their metabolism of arginine. Activated proinflammatory M1 macrophages utilize arginine as a substrate for NOS2 production of nitric oxide [200].

**Figure 1-4. Arginine metabolism.** Overview of the potential fates of arginine [201].


Conversely, activated M2 macrophages utilize arginine to fuel arginase activity that promotes down-regulation of NOS2, induction of tissue regeneration, and wound healing
Upon activation M2 macrophages also differ in their utilization of metabolic pathways to meet energetic demands. M2 macrophages exhibit lower utilization of glycolysis and increased mitochondrial oxygen consumption rates as compared to resting or M1 macrophages [203]. In particular, M2 macrophages critically rely on fatty acid oxidation to fuel mitochondrial respiration, as inhibition of these pathways blunts the M2 phenotype development and function [203].

Dendritic cells exhibit comparable distinct patterns of activation in the context of differential metabolic changes. Upon recognition of a MAMP, such as a TLR ligand, inflammatory DCs exhibit an immediate upregulation of glycolysis [204,205]. In addition to this early glycolytic burst, DCs also exhibit long-term commitment to aerobic glycolysis driven by NOS2 activity. Consequently, NOS2 derived NO causes nitrosylation of proteins in the electron transport chain, specifically complex I, complex II, and complex IV, blocking consumption of oxygen and ATP production via mitochondrial respiration [206–209]. Conversely tolerogenic DCs have a metabolic phenotype that has limited commitment to glycolysis and high capacity for mitochondrial respiration [210,211].

In nearly all cell types of the immune system, metabolism has been inextricably linked to functional phenotype. Inhibition of metabolic pathways can directly alter phenotype and skew immunological outcomes, a noteworthy example of this is the use or rapamycin to blunt glycolysis and CD8+ T cell effector expansion leading to increased memory generation [213–219]. While great strides in the understanding of immunometabolism and its role in immune function have occurred in recent years, the question of which comes first, the metabolism or the functional phenotype remains largely unanswered. What is clear though, is
that metabolism is an incredibly sensitive and useful technique to observe activation of
immune cells in real time, and insight into the functional phenotype of the cells as well.

Figure 1-5. Dendritic cell metabolism and nitric oxide. Representation of how NO plays a
role in the induction of aerobic glycolysis in dendritic cells upon TLR stimulated activation
[212].

Thwe PM, Amiel E. The role of nitric oxide in metabolic regulation of Dendritic cell immune
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Aging and Immunity

Advancements in the biological sciences and medical fields have led to the developments and improvement in the prevention and management of countless types of illnesses and medical conditions. This has led to overall improved quality of life and longevity [220,221]. This increase in lifespan is not entirely mirrored by equivalent increases in healthspan, or years without major health issues affecting quality of life. The underlying effects of aging and age-related disease means that a greater number of individuals are at risk for complications related to the aging process itself [222]. One of the more concerning dysfunctions that arises with advanced age is immunosenescence and the associated chronic low-level inflammation of the innate immune system known as “inflammaging” [223].

In advanced age, the innate immune system is profoundly altered. Neutrophils have blunted chemotactic ability, which is thought to contribute to sustained local inflammation, as well as decreased ability to phagocytose opsonized particulates, though interestingly not un-opsonized particles [224–226]. Neutrophil cytokine signaling, as well as the production of innate effector molecules such as NO and ROS is also reduced, leading to diminished ability to clear pathogens [227]. Macrophages and DCs exhibit diminished phagocytosis, decreased antigen processing/presentation, lower costimulatory molecule expression, as well as diminished cytokine secretion and responsiveness upon recognition of MAMPs, particularly the TLR PRRs [228–231]. This hyporesponsiveness of APCs has been linked to the poor activation of CD4+ and CD8+ T cells [232–234]. Myeloid derived suppressor cells have also been shown to increase in relative number in aged population potentially contributing to poor development of adaptive immunity [235]. Whether these deficiencies are due to decreased receptor expression or signaling defects is contested, leaving the opportunity to identify pathways that may be less affected by aging or create an inflammatory environment that is
more conducive to the generation of adaptive immune responses.

The adaptive immune system is also impacted by advanced age. Aged individuals have decreased numbers of circulating B cells as well as a narrowed repertoire of available naïve B cells in lymphoid tissues [236,237]. B cells from the aged also display decreased activation-induced cytidine deaminase (AID) activity, the key enzyme of somatic hypermutation and class switching of the B cell receptor, which in turn leads to decreased generation of high affinity antibodies [238,239]. Similar to B cells, naïve T cells are also present in decreased number in lymphoid tissues [240]. These naïve T cells also represent a limited repertoire of TCR specificities [241]. These numerous age-induced immunological disadvantages lead to worse health outcomes from infections and poor efficacy of vaccines [242,243]. This highlights a need to develop more effective vaccination strategies that are capable of navigating the complications of immunosenescence and inflammaging to provide protection from the inherent age-related susceptibility to infectious disease.

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CHAPTER 2. POLYANHYDRIDE NANOPARTICLES PROVIDE FOR LOW INFLAMMATORY DENDRITIC CELL ACTIVATION AND ANTIGEN DELIVERY RESULTING IN IMPROVED CD8+ T CELL MEMORY AND DELAYED TUMOR PROGRESSION

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**Author Contributions**

RD, SS, BN, ART, and MW contributed to the conception and design of the studies. RD and SS contributed to the execution of *in vitro* DC related studies. RD, JC, and LL contributed to the execution of *in vivo* murine vaccination and tumor challenge experiments. RD, SS, JC, LL, ART, BN, and MW contributed to drafting, revising, and approval of the final version of the manuscript.
Abstract

A need exists for new and effective immunotherapies capable of activating cell-mediated adaptive immunity and eliminating malignancies when administered in conjunction with chemotherapeutic, surgical, and checkpoint blockade interventions. Such approaches require immunization regimens that appropriately activate antigen-presenting cells, most notably dendritic cells, to induce efficacious cell-mediated immunity directed toward tumor-specific or pathogen associated antigens. In this regard, micro- and nanoparticles formulated from polyanhydride copolymers represent a candidate platform for immunotherapy, as they can be effectively internalized by and activate dendritic cells in vitro. In this study, polyanhydride nanoparticles induced activation and upregulation of costimulatory molecules on various dendritic cell populations in vitro, most notably CD8α+ DCs. However, nanoparticle-activated DCs did not induce/secrete large amounts of pro-inflammatory cytokines (e.g., TNF), chemokines (e.g., CXCL9, CXCL10), drive characteristic metabolic changes of activation or induce production of innate antimicrobial effector molecules, such as nitric oxide. Using an in vivo tumor model system where protection is restricted to CD8+ T cells, a single-dose, prophylactic polyanhydride-based nanovaccine encapsulating a model tumor antigen (e.g., ovalbumin) induced a protective CD8+ T cell memory response, decreased tumor progression, and increased time on study for mice that received it. Together, these results suggest that the use of a polyanhydride-based nanovaccine platform can be an effective approach to inducing antigen-specific CD8+ T cell memory and preventing tumor progression by providing antigen delivery and DC activation while avoiding the overtly inflammatory and immunologically inhibitory responses commonly associated with adjuvants.
1. Introduction

Cytotoxic CD8+ T cells are critical mediators of immunological protection against tumors and intracellular pathogens [1]. Although naturally-occurring infections are often effective at inducing long-lived CD8+ T cell memory, commonly used adjuvants such as alum, oil-in-water emulsions or innate immune stimulating Toll-like receptor (TLR) ligands have not proven as successful for the induction of cell-mediated immunity (CMI) [2]. Many efficacious immunization strategies that induce rapid and robust/durable memory CD8+ T cell responses have either employed a multiple dose regimen (i.e., prime-boost) or treated animals with adoptively transferred DCs pulsed in vitro with antigen [3–5]. Research indicates that DC vaccines capable of generating a large number of memory CD8+ T cell populations in a single administration possess the relatively unique property of enhancing both antigen presentation and co-stimulation without inducing the overt inflammatory response that often results when using more standard antigen-adjuvant combinations [4]. Many adjuvants are incorporated into vaccine formulations to induce inflammation and innate immune activation to facilitate induction of long-lived antibody titers to vaccine antigens [2]. Indeed, adjuvant-associated inflammation has been demonstrated to interfere not only with the ability of DC vaccination to induce CD8+ T cell memory, but also impair effector CD8+ T cell trafficking and function [4,6]. These blunted responses lead to decreased immunotherapeutic efficacy when administered to tumor-bearing mice [4,6]. Another common class of adjuvants, TLR agonists, not only induce overt inflammation, but also cause production of innate immune effector molecules such as nitric oxide (NO) [7]. Nitric oxide in particular has been described to cause deleterious effects on DC activation via altered metabolism, decreased survival and impaired co-stimulatory upregulation [8–10]. Although effective, the use of low inflammatory DC vaccines also comes with increased
costs and the challenges associated with the personalized nature of generating *ex vivo* DC populations to be used therapeutically [11]. Consequently, identification and development of novel low inflammatory vaccine adjuvants and antigen delivery systems would be beneficial to provide DC vaccine-like outcomes in a more accessible format.

To improve a vaccine formulation’s ability to induce optimal cytotoxic CD8$^+$ T cell memory, novel immune stimulatory strategies and antigen delivery systems that mimic the beneficial effects of DC vaccination could be used. Polyanhydride particle-based vaccines in particular represent a unique alternative to existing vaccines that employ more traditional adjuvants. Nanoparticles consisting of combinations of sebacic acid (SA), 1,6-bis($p$-carboxyphenoxy) hexane (CPH), and 1,8-bis($p$-carboxyphenoxy)-3,6-dioxaoctante (CPTEG) are readily phagocytosed by DCs and provide activation as measured by cytokine and costimulatory molecule upregulation [12]. Following administration in vivo, these polyanhydride formulations are also known to persist in the local tissues, thus, facilitating the controlled release of their encapsulated payload [13,14]. When delivered subcutaneously, polyanhydride NPs induce a mild inflammatory response with no evidence of adverse histopathological reactions [13,15]. Previous studies have also illustrated the ability of polyanhydride formulations to prophylactically enhance CD8$^+$ T cell memory responses and elicit protection in a tumor challenge model. However, the addition of a TLR ligand such as CpG oligodeoxynucleotides (ODN) to the polyanhydride platform decreased vaccine effectiveness indicating that traditional adjuvants may be deleterious to generating CD8$^+$ T cell memory responses [16,17]. Considering the unique innate immune stimulatory properties of polyanhydride NPs, the ability of this platform to activate DCs and induce an effective memory CD8$^+$ T cells was tested and compared to the inflammatory TLR agonist CpG ODN,
by using an antigen-specific tumor challenge model where protection is restricted to MHCI/CD8\(^+\) T cell-mediated immune responses.

### 2. Materials and Methods

#### 2.1 Synthesis Materials

Chemicals used for the polymer and nanoparticles synthesis: 4-p-hydroxybenzoic acid, triethylene-glycol, 1,6-dibromohexane were obtained from Sigma Aldrich (St. Louis, MO); dimethyl formamide, acetic acid, acetonitrile, acetic anhydride, toluene, methylene chloride, pentane were purchased from Fisher Scientific (Fairlawn, NJ) and 4-p-fluorobenzonitrile was purchased from Apollo Scientific (Cheshire, UK).

#### 2.2 Polyanhydride Copolymer Synthesis

Monomers of CPTEG and CPH were used to synthesize a 20:80 CPTEG:CPH copolymer via melt polycondensation reaction as previously described [18]. The purity and molecular weight of the copolymer was characterized using \(^1\)H nuclear magnetic resonance spectroscopy in deuterated chloroform. The molecular weight of the copolymer was about 5.3 kDa, consistent with previous work [19].

#### 2.3 Nanoparticle Synthesis

20:80 CPTEG:CPH nanoparticles encapsulating 5 wt.% ovalbumin (Ova) were synthesized using flash nanoprecipitation as previously described [20]. Briefly, a solution of 20:80 CPTEG:CPH and ovalbumin in methylene chloride at a concentration of 20 mg/mL was poured into pentane at a solvent to anti-solvent ratio of 1:250. The nanoparticles were then collected using vacuum filtration. The particle morphology and size were examined
using scanning electron microscopy. The average particle size of nanoparticles used for these studies was about 200 nm.

2.4 Animals

Female BALB/c mice, aged six to eight weeks, were obtained from Charles River Laboratories (Wilmington, MA) for \textit{in vitro} studies. Female C57BL/6 mice, aged six to eight weeks, were obtained from Charles River and Envigo (Somerset, NJ). Studies involving the use of mice were conducted in accordance with Iowa State University guidelines for the care and use of animals and upon approval of the Institutional Animal Care and Use Committee.

2.5 Cell Culture

The E.G7-OVA (ATCC, Manassas, VA) cell line was cultured and maintained in RPMI 1640 (Cat #10-040-CM, Corning) medium containing 2 mM L-glutamine 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, 1 mM sodium pyruvate, 0.05 mM 2-mercaptoethanol, 0.4 mg/mL G418 (Cat #30-234-CI, Cellgro), 100 U/mL penicillin, 100 µg/mL streptomycin, and 10 % fetal bovine serum. The EL4 (ATCC) cell line was cultured and maintained in DMEM (Cat #15-013-CV, Corning) supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin and 10 % horse serum.

2.6 Bone Marrow DC Generation

Naïve female BALB/c mice were used to generate BMDC cultures. Bone marrow was collected from femurs and tibias. Cells were washed and plated in RPMI 1640 medium supplemented with 100 U/mL penicillin and 100 µg/mL streptomycin, 2 mM glutamine, and 10 % FBS in 100 mm petri plates, at a density of $4 \times 10^6$ cells per plate with 10 mL of
medium supplemented with GM-CSF (Peprotech, Rocky Hill, NJ) at 10 ng/ml. On day three of culture, 10 mL of GM-CSF containing medium was added. On days six and eight of culture, 10 mL of culture medium was exchanged for fresh GM-CSF containing medium. DCs were harvested on day 10 of culture by gently rinsing and collecting non-adherent cells.

2.7 Splenic DC Isolation

Naïve female BALB/c mice were sacrificed to collect spleens. Spleens from eight to ten mice were harvested, pooled, and homogenized into a population of single cells. Using these splenocytes, a highly enriched populations of DCs were recovered using a pan-DC isolation kit (13-100-875) (Miltenyi Biotec, Auburn, CA) on an autoMACS Pro separator (Miltenyi Biotec) according to manufacturer’s protocol.

2.8 BMDC and sDC stimulation

DCs were plated at 5 x 10^5 cells/well in a 96-well round bottom tissue culture plate in 200 µL of RPMI 1640 medium supplemented with 100 U/mL penicillin and 100 µg/mL streptomycin, 2 mM glutamine, and 10 % FBS. Stimulations consisted of 100 µg/well of 20:80 CPTEG:CPH NPs encapsulating 5 µg of Ova, 5 µg/mL of CpG ODN 1668 (Cat #16E17-MM InvivoGen) with 5 µg Ova, or non-stimulated control wells. Stimulations were carried out for 48 hours after which supernatants and cells were harvested for cytokine analysis and cell surface maker expression by flow cytometry.
2.9 Nitric Oxide Quantification

Supernatants from stimulated DCs were analyzed for nitric oxide indirectly via nitrite concentration by Griess assay. A standard curve was created using two-fold dilutions of sodium nitrite ranging from 100 µM to 0 µM. 100 µL of supernatant was incubated with 100 µL of Griess reagents (Cat. No. 03553, Sigma-Aldrich) in a 96 well microtiter plate. Samples were incubated for 15 minutes at room temperature and read at 540 nm on a SpectraMAX 190 (Molecular Devices, Sunnyvale, CA).

2.10 Extracellular Flux Analysis

BMDCs were stimulated in RPMI 1640 medium supplemented with 100 U/mL penicillin and 100 µg/mL streptomycin, 2 mM glutamine, and 10 % FBS for 18 hours with 100 µg of 20:80 CPTEG:CPH encapsulating 5 µg of Ova, 5 µg/mL of CpG ODN with 5 µg Ova, or no stimulation control in 5 mL polypropylene tubes. Treated BMDCs were washed with Seahorse assay media consisting of Agilent Seahorse XF Base medium (Cat #102353, Agilent, Santa Clara, CA) supplemented with 1mM sodium pyruvate, 2 mM L-glutamine, and 10 mM glucose with a pH adjusted to 7.4, were seeded into 24 well Seahorse plates coated with Cell-Tak (Corning, Corning NY) at a density of 2.5 x 10^5 cells per well. Metabolic phenotyping was conducted on a Seahorse XFe24 (Agilent). Mitochondrial function was analyzed via mitochondrial stress test according to manufacturer specifications [21]. Final concentrations of 1 µM oligomycin, 2 µM FCCP, and 0.5 µM rotenone and antimycin were used (Agilent) and prepared in Seahorse assay medium.
2.11 Murine Vaccination

For the high antigen dose study (Figure 2-1) (2.0 mg Ova), female C57BL/6 mice were vaccinated with formulations consisting of 250 µg Ova encapsulated in 5 mg of 20:80 CPTEG:CPH (5 % loaded) plus 1.75 mg Ova soluble (NP), 2 mg soluble Ova (sOva), or unvaccinated control. In the prime-boost experiment (Figure 2-2) the mice were vaccinated with formulations consisting of 1.75 mg soluble Ova along with 5 mg of 20:80 CPTEG:CPH polyanhydride nanoparticles encapsulating 250 µg Ova with and without a boost (NPx1, NPx2), 2 mg soluble Ova with 5 mg of blank 20:80 CPTEG:CPH polyanhydride nanoparticles (Blank NP x2), or PBS control. Mice that received a booster (indicated by x2) were given the same formulations at half the original dose of antigen and particle mass. In the low antigen dose study (Figure 2-5), mice were vaccinated with formulations consisting of 75 µg soluble Ova plus 500 µg of 20:80 CPTEG:CPH polyanhydride nanoparticles encapsulating 25 µg Ova (NP), 100 µg soluble Ova (sOva), or PBS control. In the multi-adjuvant experiment (Figure 2-6) mice received formulations consisting of 75 µg soluble Ova plus 500 µg of 20:80 CPTEG:CPH polyanhydride nanoparticles encapsulating 25 µg Ova (NP), 100 µg soluble Ova adjuvanted with 20 µg CpG ODN 1668 (CpG), a combination of 75 µg soluble Ova plus 500 µg of 20:80 CPTEG:CPH polyanhydride nanoparticles encapsulating 25 µg Ova adjuvanted with 20 µg CpG ODN (NP+CpG), or PBS control. All formulations were delivered subcutaneously at the nape of the neck.

2.12 Tumor Challenge

C57BL/6 mice were challenged subcutaneously on the flank with 2-5 x 10^6 E.G7 Ova expressing lymphoma cells or 2.5 x 10^6 EL4 lymphoma cells and washed and suspended in PBS respectively. Tumor growth was monitored three times a week and volumes were
calculated by the volume of an ellipsoid. Mice were removed from study when tumor volume surpassed 1000 mm$^3$.

Tumor Volume = \((4/3)\pi r_1 r_2 r_3\)

2.13 Cytokine and Chemokine Analysis

Cytokine quantification was performed using supernatants from the previously described DC stimulations. A Millipore Milliplex cytokine/chemokine panel (MCYTOMAG-70K-32, Burlington, MA) was used to detect cytokines and analyzed on a Bio-Plex 200 System (Bio-Rad, Hercules, CA) according to manufacturer’s specifications.

2.14 Flow Cytometry

Splenic DCs and BMDCs were analyzed for costimulatory marker expression using flow cytometry. 5 x 10$^5$ DCs were aspirated from a 96 well plate and transferred to FACS tubes. Prior to labeling with specific monoclonal antibodies, Fc receptors on DCs were blocked to prevent non-specific antibody binding by incubating the cells with 100 µg/mL of rat IgG (Sigma Aldrich) and 10 µg/mL of anti-CD16/32 (eBioscience, San Diego, CA). Subsequently, DCs were stained with fluorescently conjugated antibodies for CD80 (Biolegend, San Diego, CA, PerCP-Cy5.5, clone 16-10A1), CD86 (eBioscience, FITC, clone GL1), CD40 (eBioscience, APC, clone 1C10), CD11c (Biolegend, APC-Cy7, clone N418), MHCII (eBioscience, AF700, clone M5/114.15.2), and CD8α (Biolegend, BV421, clone 53-67) diluted in FACS buffer. Samples were fixed using BD stabilizing fixative (BD Bioscience, Franklin Lakes, NJ). Mitochondrial superoxide production was evaluated using live cells stained with MitoSOX Red (Cat # M36008, Invitrogen, Carlsbad, CA) according to
manufacturer’s specifications. All data was collected on a FACSCanto II (BD Bioscience, Franklin Lakes, NJ). Data was analyzed using FlowJo (FlowJo LLC, Ashland, OR).

### 2.15 Serum IgG detection

Where applicable, immunized mice were bled via saphenous vein 5 weeks post-immunization. Anti-Ova serum IgG titers were measured via indirect ELISA. Costar 3590 96-well EIA/RIA high binding plates (Corning) were coated with 100 µL of Ova (5 µg/mL PBS) or PA (0.5 µg/mL PBS) and incubated overnight at 4°C. Plates were blocked using 2.5% (w/v) powdered skim milk PBS containing 0.05% Tween-20 (PBS-T), that has been heat inactivated at 56°C for hours to inactivate any phosphatase activity, for two hours at room temperature. After three washes using PBS-T, serum samples were titrated across the plate using two-fold serial dilutions, starting at 1:100, in PBS-T and 1% (v/v) normal goat serum. Samples were incubated overnight at 4°C. After three washes in PBS-T, an alkaline phosphatase conjugated goat anti-mouse IgG (H+L) secondary detection antibody (Cat# 115-005-003, Jackson ImmunoResearch, West Grove, PA) was diluted 1:1000 in PBS-T and added to the wells and allowed to incubate at room temperature for two hours. Plates were washed three times with PBS-T and alkaline phosphatase substrate was added at 1 mg/mL in buffer containing 50 mM sodium carbonate, 2 mM magnesium chloride, and sodium bicarbonate was titrated into the solution in order to achieve a pH of 9.3. Plates were allowed to develop for 30 minutes and analyzed using the SpectraMAX 190 at a wavelength of 405 nm.
2.16 Statistical Analysis

Data generated during flow cytometry assays, metabolic assays, Griess assays, and tumor volume experiments were analyzed via one-way ANOVA with a Tukey, Dunnett, or Sidak post-test for multiple comparisons. Survival data were analyzed using a log-rank (Mantel-Cox) test with a Bonferroni correction for multiple comparisons. All analyses were performed using GraphPad Prism 8.0 (GraphPad Software, La Jolla, CA).

3. Results

3.1 High antigen dose polyanhydride nanoparticle vaccination reduced tumor burden and increased time on study

Previous studies from our lab have shown that immunization with high dose (i.e., 2 mg) of Ova antigen in a polyanhydride particle-based vaccine regimen induced transgenic OTI CD8$^+$ T cell memory and the ability to expand these CD8$^+$ T cells upon reencounter with antigen [18]. Here, the ability of polyanhydride nanoparticles to induce efficacious endogenous antigen-specific CD8$^+$ T cell memory and subsequent effector expansion upon challenge was evaluated. C57BL/6 mice were vaccinated prophylactically with high antigen dose (2.0 mg Ova) formulations consisting of polyanhydride nanoparticles (NP), sOva alone, or a PBS control. Six weeks later, mice were challenged with the subcutaneous implantation of Ova-expressing E.G7 tumor cells and tumor progression was tracked. Vaccination with the NP formulation significantly reduced tumor progression as measured on day 19 post-challenge in comparison to naïve control mice, while the unadjuvanted sOva formulation did not inhibit tumor progression (Figure 2-1a-b). NP vaccination also significantly improved time on study (i.e., % of mice with tumors < 1000 mm$^3$) compared to control animals (Figure 2-1c). No significant improvements in survival were observed for mice receiving sOva alone.
compared to controls. To determine whether antigen-specific immune activation was responsible for the observed anti-tumor responses, another set of mice was challenged with the EL4 parent tumor that does not express Ova. Tumor volume was evaluated 12 days post-challenge; neither the mice receiving the NP formulation or sOva experienced a significant decrease in tumor burden compared to naive control mice (Supplemental Figure 2-8a-b). Additionally, neither immunization regimen provided a significant increase in time on study (Supplemental Figure 2-8c). Together, these data demonstrate that the NP formulation is able to induce efficacious antigen-specific CD8+ T cell memory capable of responding to re-exposure to antigen (i.e., tumor implantation).

**Figure 2-1.** Polyanhydride nanoparticles enhance CD8+ T cell memory generation with high dose antigen. C57BL/6 female mice (n=12) were immunized subcutaneously with formulations consisting of 1.75 mg soluble Ova plus 5 mg of 20:80 CPTEG:CPH
3.2 Encapsulation of antigen in polyanhydride NPs was critical for inducing optimal CD8+ T cell memory

Even though our high antigen dose (2.0 mg Ova) vaccine formulation contained a large mass of unencapsulated soluble antigen (1.75 mg), we hypothesized that the portion of antigen encapsulated within the polyanhydride NPs would be more important to the induction of CD8+ T cell memory than the soluble bolus. This hypothesis was tested by vaccinating C57BL/6 mice with a single-dose NP formulation, a prime-boost of the same NP formulation, a prime-boost of blank NPs with no encapsulated antigen (i.e., soluble Ova only), or injecting PBS alone (i.e., control). Six weeks after their last immunization, all mice were challenged subcutaneously with the Ova-expressing E.G7 tumor, and the tumor progression and time on study for each treatment compared (Figure 2-2a-b). The only treatments that significantly inhibited tumor progression compared to PBS injections were the single-dose NP and the prime-boost NP regimens. Mice immunized twice with blank NPs
plus sOva tended to have an improved median survival time compared to naïve control mice; however, this difference was not significant.

Figure 2-2. Encapsulated antigen is crucial to induce antigen-specific CD8+ T cell memory. C57BL/6 female mice were immunized subcutaneously at the base of the neck with formulations consisting of 1.75 mg soluble Ova with 5 mg of 20:80 CPTEG:CPH
polyanhydride nanoparticles encapsulating 250 µg Ova with and without a boost (NPx1, NPx2) (n=16); 2 mg soluble Ova with 5 mg of blank 20:80 CPTEG:CPH polyanhydride nanoparticles (Blank NP x2) (n=8); or PBS control (n=16). Mice that received a booster (indicated by x2) were given the same formulations at half the original dose subcutaneously at the base of the neck 28 days after the primary immunization. Mice were challenged subcutaneously in the flank with E.G7 Ova expressing tumor cells 42 days after the primary or boost immunization, respectively and (a) tumor volume of individual mice was tracked. (b) Survival was evaluated 31 days post-challenge. Significance was determined using a Log-rank (Mantel-Cox) test with a Bonferroni correction for multiple (6) comparisons. Significance from control is indicated in the legend as *p ≤ 0.0083. Median survival of each group is also reported.

3.3 Polyanhydride NPs upregulated costimulatory molecule expression on DCs but only induced low levels of inflammatory cytokine and chemokine production.

Previous work on polyanhydride particle formulations illustrated that bone marrow-derived dendritic cells (BMDCs) readily take up and respond to these particles by upregulating co-stimulatory molecules and cytokine secretion similar to other pathogen mimicking moieties [12,14,22]. The low inflammatory response induced by polyanhydride NPs is similar to that induced by DC vaccination as it relates to the induction of CD8+ T cell memory [15]. Herein, the ability of the 20:80 CPTEG:CPH polyanhydride NP formulation to activate BMDC was compared to the effects of CpG ODN. After 48 hours of in vitro stimulation with NPs or CpG ODN, BMDCs respond by upregulating the co-stimulatory molecules CD86 and CD40 as compared to non-stimulated control BMDCs (Figure 2-3a)
indicating that both stimulants activated the BMDCs and increased co-stimulatory molecule expression. Supernatants from stimulated BMDCs were also evaluated for induction of inflammatory cytokine and chemokine production. Other than MIP2, KC and IL-6, the NP formulation induced markedly less inflammatory cytokine secretion when compared to that induced by CpG ODN (Supplemental Figure 2-9a-c).

Dendritic cell populations isolated from the spleens of naïve mice were also evaluated for their response to stimulation. Splenic DCs (sDC) were stimulated with either the NP formulation or CpG ODN for 48 hours; control sDC were not stimulated. The CD11c+ population of the sDC was evaluated for upregulation of the costimulatory molecules CD86 and CD40. The NP formulation upregulated CD86 and CD40 expression while CpG ODN only upregulated CD40 as compared to non-stimulated control sDC (Figure 2-3b). The sDC CD8α+ subpopulation has been previously reported to cross prime CD8+ T cells [4,23–25]. To determine if NP stimulation had any effect on the activation of this population of sDC, we measured co-stimulatory marker expression on CD8α+ sDC. CpG ODN induced only a moderate upregulation of co-stimulatory molecules on CD8α+ sDC; however, NP stimulation induced a marked increase in the expression of CD86, CD80, and CD40 (Fig. 2-3c). These findings suggest that NPs are capable of activating a critically important DC subtype involved in the presentation of antigen to and activation of CD8+ T cells. Supernatants from total population of stimulated sDC were also evaluated for cytokine production via a multiplex assay. As observed for the BMDC, a lower magnitude of inflammatory cytokine and chemokine responses were observed for NP stimulated sDC compared to those induced by CpG ODN (Supplemental Figure 2-10.)
Figure 2-3. Polyanhydride nanoparticles activated BMDCs and splenic DC populations.

Dendritic cells isolated or generated from naïve BALB/c mice were stimulated for 48 hours with 20:80 CPTEG:CPH polyanhydride nanoparticles encapsulating Ova (5% w/w) (NP); CpG ODN plus Ova (CpG); or unstimulated control as described in materials and methods section. (a) BMDCs gated on MHCII+ and CD11c+, (b) the total MHCII+ and CD11c+ population of DCs isolated from the spleens, and (c) the CD8α+ subpopulation of splenic
DCs was analyzed for costimulatory expression via flow cytometry. Significance was determined via one-way ANOVA with a Tukey multiple comparison test. P value is indicated as follows *p ≤ 0.05. All graphs represent the treatment average ± SEM.

3.4 Polyanhydride nanoparticles activated BMDCs without inducing innate effector molecules or altering cellular metabolism

Upon natural infection or after stimulation with TLR ligands such as CpG ODN, activated APCs produce innate effector molecules such as nitric oxide (NO) or reactive oxygen species (ROS). Although these molecules provide critical microbicidal activity during acute infections [26,27], NO and ROS can also have inhibitory effects on innate [8,10,28] and adaptive immune responses [10,29–32]. After 48 hours of in vitro stimulation, NO production by BMDC was measured indirectly by assessing nitrite content in supernatants. Unlike CpG ODN, which induced high concentrations of NO, stimulation of BMDCs with polyanhydride NPs did not result in significant amounts of NO (Fig. 2-4a). Lower concentrations of mitochondrial superoxide (mROS) were also observed following NP stimulation compared to CpG ODN treatment (Supplemental Figure 2-11).

After encountering TLR ligands, murine BMDCs exhibit a profound metabolic shift away from mitochondrial oxidative phosphorylation and towards sustained aerobic glycolysis [33]. This extended commitment to aerobic glycolysis is a consequence of the concentration of the innate effector molecule NO; yet, NO plays an important antimicrobial role during acute infections and in response to TLR-stimulation [8,9,34]. Because polyanhydride nanoparticles were observed to activate BMDC without inducing NO, we hypothesized that NP stimulation would result in an BMDC activation phenotype that maintained functional mitochondria. After an 18-hour stimulation with NP or CpG, a mitochondrial stress test
(MST) was performed on BMDCs and revealed marked differences in metabolic states. CpG ODN stimulation resulted in the expected inhibition of ATP production, loss of spare respiratory capacity, and increased proton leak (Figure 2-4a-b). In contrast, NP stimulation of BMDCs resulted in an overall maintenance of mitochondrial functionality resembling that of non-stimulated BMDCs (Figure 2-4a-b). During the MST, the extracellular acidification rate (ECAR) was also measured as an indicator of glycolysis. As expected, the CpG ODN stimulated BMDCs exhibited an elevated glycolytic rate while NP stimulated cells did not (Supplemental Figure 2-12). Together, these results demonstrate that polyanhydride nanoparticles, while inducing effective dendritic cell activation, are resulting in a distinctly less overt activation phenotype that avoid production of innate effector molecules and consequently avoids reliance on aerobic glycolysis.

Figure 2-4. Polyanhydride nanoparticles induce a differential metabolic profile of activation and no induction of innate effector molecules.
BMDCs were stimulated with either NPs, CpG ODN, or control as described in materials and methods. (a) Nitric oxide production was measured indirectly via Griess assay as nitrite concentration in the cell supernatant 48 hours after stimulation. BMDCs generated from BALB/c mice were stimulated for 18 hours with, CpG ODN, or non-stimulated control. Stimulated BMDCs were seeded into 24 well seahorse plates coated with Cell-Tak at a density of 2.5 x 10^5 cells per well and a mitochondrial stress test (MST) was conducted. (b) Kinetic results of the MST oxygen consumption rate (OCR) are shown along with (c) ATP production, proton leak, maximal respiratory capacity, and spare respiratory capacity calculated from the MST. Significance between treatments was determined via one-way ANOVA with a Tukey posttest. P value is indicated as *p ≤ 0.05. All bar graphs and symbols represent the treatment average ± SEM.

3.5 Low antigen dose polyanhydride nanoparticle vaccination reduced tumor burden and increased time on study

Although high doses of antigen and polyanhydride nanoparticles were effective at inducing CD8\(^+\) T cell memory upon vaccination, experiments were designed to determine
whether lower doses of both Ova and polymer particles could also induce an efficacious immune response. A lower dose of Ova (100 µg) and nanoparticle amount (500 µg) were administered to mice and the induction of antigen-specific CD8+ T memory was evaluated. C57BL/6 mice were vaccinated prophylactically with formulations consisting of the polyanhydride nanoparticles (NP-Ova), sOva alone, or a PBS control. Six weeks later, mice were challenged subcutaneously with Ova-expressing E.G7 tumor cells and the progression of tumor volume monitored (Figure 2-5a). Tumor volumes were compared among treatments 12 days post-tumor challenge, and time on study was evaluated at 30 days post-challenge. On day 12 post-challenge, tumor volumes in mice immunized with either the NP formulation or sOva alone were significantly lower compared to those of non-vaccinated control mice (Figure 2-5b). However, only the mice immunized with the NP formulation experienced a significant increase in survival at day 30 compared to the non-vaccinated controls (Figure 2-5c). Together, these results demonstrate that a lower dose NP vaccine regimen was as effective as the high dose regimen with respect to the induction of an efficacious immune response.

Figure 2-5. Polyanhydride nanoparticles induce CD8+ T cell memory with a low antigen dose.
Figure 2-5 Continued

C57BL/6 female mice were immunized subcutaneously with formulations consisting of 75 µg soluble Ova plus 500 µg of 20:80 CPTEG:CPH polyanhydride nanoparticles encapsulating 25 µg Ova (NP) (n=12); 100 µg soluble Ova alone (sOva) (n=11); or PBS control (n=12). Mice were challenged subcutaneously in the flank with E.G7 Ova expressing tumor cells 42 days post-immunization and (a) tumor volume of individual mice was tracked. (b) Tumor volume was compared when the first mouse was removed from study on day 12. Treatment groups were compared to PBS control using an ordinary one-way ANOVA with a Dunnett’s multiple comparison test. Significance is indicated as follows *p ≤ 0.05. Bar graphs indicate the group mean ± SEM. (c) Survival was evaluated 30 days post-challenge. Significance from PBS control was determined using a Log-rank (Mantel-Cox) test with a Bonferroni correction for multiple (2) comparisons. Significance from control is indicated in the legend as follows *p ≤ 0.025. Median survival of each group is also indicated parenthetically.
3.6 Polyanhydride NPs provided a more efficacious induction of CD8+ T cell memory upon vaccination compared to CpG ODN

The observed low inflammatory effects of polyanhydride NPs on DCs, as compared to the TLR agonist CpG ODN, lead to the hypothesis that the magnitude and/or consequences of differentially inflammatory adjuvants would alter the efficacy of the ensuing immune response. This hypothesis was tested by subcutaneously immunizing C57BL/6 mice with vaccine formulations containing a low dose of Ova (100 µg) as described above: NP, CpG ODN, NP + CpG ODN, or a naïve PBS only control. After implantation of the EG.7 cells, tumor volumes were tracked (Figure 2-6a) and compared among groups at 14 days post-tumor challenge. As expected, the tumor volumes of mice receiving the overtly inflammatory regimen, CpG ODN + Ova, were not significantly different than those of the non-vaccinated control mice. However, tumor progression in mice immunized with either of the two NP formulations containing encapsulated Ova was significantly limited (Figure 2-6b). At 30 days post-tumor challenge, time on study was significantly improved for mice immunized with either the NP + Ova formulation or the combination NP + CpG ODN + Ova formulation compared to unvaccinated control (Figure 2-6c). Vaccination with CpG ODN + Ova lead to no improvement in survival time compared to unvaccinated controls. These results suggest that polyanhydride NPs containing encapsulated antigen are able to initiate effective T cell memory on their own as well as when co-administered with the inflammatory TLR agonist CpG ODN.
Figure 2-6. Polyanhydride nanoparticles induce effective CD8+ T cell memory as compared to CpG ODN.
C57BL/6 female mice (n=12) were immunized subcutaneously with formulations consisting of 75 µg soluble Ova plus 500 µg of 20:80 CPTEG:CPH polyanhydride nanoparticles encapsulating 25 µg Ova (NP); 100 µg soluble Ova adjuvanted with 20 µg CpG ODN (CpG); a combination of 75 µg soluble Ova plus 500 µg of 20:80 CPTEG:CPH polyanhydride nanoparticles encapsulating 25 µg Ova plus 20 µg CpG ODN (NP+CpG); or PBS control. Mice were challenged subcutaneously in the flank with E.G7 Ova expressing tumor cells 42 days post-immunization and (a) tumor volume of individual mice was tracked. (b) Tumor volume was compared when the first mouse was removed from study on day 14. Treatment groups were compared to PBS control using an ordinary one-way ANOVA with a Tukey posttest. Significance between treatments is indicated as follows *p ≤ 0.05. Bar graphs indicate the group mean ± SEM. (c) Survival was evaluated 30 days post-challenge. Significance from PBS control was determined using a Log-rank (Mantel-Cox) test with a Bonferroni correction for multiple (3) comparisons. Significance from naïve control mice is indicated in the legend as follows *p ≤ 0.016. Median survival of each group is also reported.
4. Discussion

In this work, the ability of a polyanhydride nanoparticle-based vaccine to induce CD8+ T cell memory was investigated. Polyanhydride nanoparticles, encapsulating tumor antigen, enhanced the development of CD8+ T cell memory upon vaccination. This effect occurs without inducing the overt inflammatory responses commonly associated with innate immune stimulating microbial associated molecular patterns [7]. Direct in vivo comparison of the TLR agonist CpG ODN and the polyanhydride nanoparticle formulation illustrates the benefits of a low inflammatory vaccine adjuvant formulation on the resulting immune response.

Previously, inclusion of a TLR agonist in the nanoparticle formulation negatively affected the ability 20:80 CPTEG:CPH polyanhydride NPs to inhibit tumor progression [12]. These observations suggest that the inclusion of a TLR agonist in the vaccine formulation inhibited or hindered the efficacy of the polyanhydride particle vaccine to induce either CD8+ T effector or memory cells. Inflammatory adjuvants, while effective at enhancing antibody production and expansion of terminally differentiated effector CD8+ T cells, often fail to induce optimal T cell memory populations among other adverse reactions [1,6,32,35,36]. Vaccination strategies that have been described to rapidly induce high numbers of CD8+ T cell memory, such as DC vaccination or antigen coated particle formulations, share an important quality [4,35,37]. The common feature of these strategies is that they both induce CD8+ T cell memory more effectively when the antigen is delivered in the absence of overt inflammation.

One component of the overt inflammatory responses induced by pathogens or TLR-based adjuvants is the production of microbicidal innate immune effector molecules such as NO [38,39]. Although NO is important for the innate immune system in overcoming acute
pathogenic infections [40], it has potential negative autocrine effects on APCs as well as paracrine effects on neighboring cells. When NO is produced by BMDCs, it inhibits the electron transport chain (i.e., oxidative phosphorylation), leading to a dependence on aerobic glycolysis for ATP production and cellular survival [9,33]. This sustained dependence on aerobic glycolysis, while not necessary for activation, has been described to provide the necessary energy demands for BMDC survival when stimulated via a TLR agonist [41]. Previous studies have also indicated that mitochondrial deficiencies are associated with suboptimal antigen processing and presentation [42]. In the present studies, the ability of the NP formulations to avoid adverse impacts on mitochondrial function in APCs and the resultant reliance on sustained glycolysis (Figure 2-4) may have contributed to a DC phenotype that more effectively promoted the induction of antigen-specific CD8+ T cell memory responses (Figures 2-1,2-5). Furthermore, production of high amounts of NO by DCs following TLR ligand stimulation can lead to decreased co-stimulatory capacity and survival [9,43]. This NO production by DCs and other innate immune cells has also been implicated in the direct inhibition of B cell (i.e., antibody production) and T cell function (i.e., effector and memory phenotypes) [29,31,44,45]. Pharmacological inhibition of NO production can prevent some of these deleterious effects on the adaptive immune response [29,43]. Compared to the robust stimulation of DCs by TLR ligands, the low inflammatory activation phenotype observed in DCs following polyanhydride particle stimulation likely contributes to the enhanced anti-tumor immune response demonstrated in our studies (Figures 2-3,2-4).

Previous intervention strategies targeting the inflammatory response to enhance CD8+ T cell memory have focused on interfering with chemokine actions and chemokine effects on
lymphocytes [46–49]. Other strategies have inhibited the expansion of effector cells at strategic timepoints after immunization, such as dosing with the mTOR inhibitor rapamycin to interfere with the metabolic requirements of expanding effector cells to shift differentiation toward memory development [43,50,51]. Although these approaches have shown positive effects on DCs and memory CD8+ T cell induction following vaccination or after infection, the systemic effects of using these inhibitors may have unintended consequences. Reducing the magnitude of the inflammatory response associated with vaccine adjuvants may be able to provide many of these same benefits without the necessary post-vaccination intervention with inhibitors such as rapamycin. Together, these studies along with the current observations, suggest that obtaining effective adaptive memory following vaccination can be induced using adjuvant formulations that provide optimal innate activation of APCs with “just right” adjuvant-induced inflammatory effects [52].

Polyanhydride nanoparticle adjuvants have been shown to be endocytosed by APCs, provide innate immune activation, and induce co-stimulatory molecule upregulation. These activities of polyanhydride nanoparticles have contributed to the successful generation of long-lived protective antibody responses to various encapsulated protein antigens [12,14,53–55]. In the present work, it was shown that a 20:80 CPTEG:CPH polyanhydride nanoparticle formulation induced antigen-specific CD8+ T cell memory capable of providing protection against a tumor challenge distant from the vaccination site at both high (Figure 2-1) and low dosages of antigen (Figure 2-5). This immune-enhancing effect was associated with the ability to activate DCs, particularly the CD8α+ subpopulation of DCs associated with cross-priming CD8+ T cells (Figure 2-3). Such activation was accomplished in the absence of deleterious vaccination site inflammation, which can lead to dysfunctional CD8+ T cell
trafficking and effector functions. Further, it was demonstrated that the delivery of empty nanoparticles with soluble antigen did not provide the same benefits as particulate formulations including encapsulated antigen. This finding suggests that there is a critical role for antigen encapsulation in the capability of polyanhydride NP formulations to induce CD8+ T cell memory (Figure 2-2). The benefits of antigen encapsulation with a single dose regimen that provide comparable results of prime-boost formulations was also described (Figure 2-2). Direct in vivo comparison of polyanhydride nanoparticles with the TLR agonist CpG ODN, illustrates the effectiveness of the low inflammatory nature at work to provide significantly greater survival and decreased tumor burden while the CD8+ T cell memory induced by CpG ODN vaccination failed to do so (Figure 2-6). Related to its phlogistic potential, the addition of CpG ODN to vaccine formulations has been shown to limit the effectiveness of the polyanhydride nanovaccine [4,17,56]. This suggests that the nature of the innate inflammatory phenotype induced by a vaccine plays a key role in the outcome of CD8+ T cell response. It is also of note that in this study the addition of CpG ODN to the polyanhydride nanoparticle formulation did not have the same decreased survival. This observation suggests that the encapsulation of antigen and persistence of the polyanhydride particles allows the host to overcome the apparent negative impact of the acute inflammatory response induced by CpG ODN at the time of immunization (Figure 2-6).

In conclusion, these results suggest that the beneficial effects of polyanhydride nanoparticles arise, in part, from their ability to activate DCs without excessive induction of inflammatory cytokines, chemokines, and effector molecules (e.g., NO) associated with a pathogenic infection or TLR ligand administration. The use of adjuvants that closely mimic the immunological response to infection has been successful in generating protective
antibody mediated immunity, but many of these adjuvants are associated with poor CD8+ T cell responses. By virtue of their low inflammatory activation properties (i.e., selectively pathogen mimicking), polyanhydride nanoparticles are emerging as a vaccine option to more effectively generate CD8+ T cell memory without the induction of deleterious innate inflammatory effects.

5. Supplemental Data

Figure 2-7. 20:80 CPTEG:CPH polyanhydride nanoparticles provide highest degree of tumor protection. C57BL/6 female mice (n=12) were immunized subcutaneously with formulations consisting of 1.75 mg soluble Ova plus 5 mg of either 20:80 CPTEG:CPH, 50:50 CPTEG:CPH, or 20:80 CPH:SA encapsulating 250 µg Ova, or PBS (control). Mice were challenged subcutaneously in the flank with E.G7 Ova expressing tumor cells 47 days post-immunization and tumor volume of individual mice was tracked. Graph indicates the group mean ± SEM.
Figure 2-8. Polyanhydride nanoparticles provide no protection against the parent EL4 non-Ova expressing tumor. C57BL/6 female mice (n=4) were immunized subcutaneously with formulations consisting of 1.75 mg soluble Ova plus 5mg of 20:80 CPTEG:CPH polyanhydride nanoparticles encapsulating 250 µg Ova (NP); 2 mg soluble Ova (sOva); or PBS (control). Mice were challenged subcutaneously in the flank with EL4 tumor cells 40 days post-immunization and (a) tumor volume of individual mice was tracked. (b) Tumor volume was compared when the first mouse was removed from study on day 12. Treatment groups were compared to PBS control using an ordinary one-way ANOVA with a Dunnett’s multiple comparison test. Significance is indicated as follows *=p<0.05. Bar graphs indicate the group mean ± SEM. (c) Survival was evaluated 30 days post-challenge. Significance from PBS control was determined using a Log-rank (Mantel-Cox) test with a Bonferroni correction for multiple (2) comparisons. Significance from control is indicated in the legend as follows *p ≤ 0.025. Median survival of each group is also reported parenthetically.
Figure 2-9. BMDCs stimulated with polyanhydride nanoparticles secrete lower levels of proinflammatory cytokine and chemokines than those stimulated with CpG ODN.

BMDCs generated from naïve BALB/c mice were stimulated for 48 hours with (a) CpG
ODN plus Ova; (b) 20:80 CPTEG:CPH polyanhydride nanoparticles encapsulating Ova (5% w/w); (c) or unstimulated control as described in Materials and Methods section. Cytokine concentration in the supernatants were quantified using a 42-plex assay. Numbers denoting concentric circles represent the cytokine concentration (pg/mL). Each cytokine measured in the supernatant is denoted by a solid red line.

Figure 2-10. Splenic DCs stimulated with polyanhydride nanoparticles secrete lower levels of proinflammatory cytokine and chemokines than those stimulated with CpG ODN.
Figure 2-10 Continued

Splenic DCs isolated from naïve BALB/c mice were stimulated for 48 hours with (a) CpG ODN plus Ova; (b) 20:80 CPTEG:CPH polyanhydride nanoparticles encapsulating Ova (5% w/w); (c) or unstimulated control as described in Materials and Methods section. Cytokine concentration in the supernatants were quantified using a 42-plex assay. Numbers denoting concentric circles represent the cytokine concentration (pg/mL). Each cytokine measured in the supernatant is denoted by a solid red line.

Figure 2-11. Polyanhydride nanoparticle stimulation of BMDCs does not result in the induction of mitochondrial superoxide. 20:80 CPTEG:CPH polyanhydride nanoparticles
encapsulating Ova (5% w/w) (NP), CpG ODN (CpG) Ova, or unstimulated control (Cont.) were used to stimulate BMDCs as described in Materials and Methods section were assayed for mitochondrial superoxide production via MitoSOX staining and resulting MFI collected via flow cytometry. Significance was determined via one-way ANOVA with a Sidak’s multiple comparison test. Significance is indicated as *=p<0.05. All bars and symbols represent the treatment average ± SEM. Data include two stimulation replicates

Figure 2-12. Polyanhydride nanoparticles do not upregulate BMDC glycolytic metabolism in response to stimulation. BMDCs generated from BALB/c mice were stimulated for 18 h with 20:80 CPTEG:CPH polyanhydride nanoparticles (NP), CpG ODN (CpG), or non-stimulated control (Cont.). Stimulated BMDCs were seeded into 24 well seahorse plates coated with Cell-Tak at a density of 2.5 x 10⁵ cells per well and a mitochondrial stress test (MST) was conducted. (a) Kinetic results of a portion of the MST extracellular acidification rate (ECAR) are shown along with (b) basal ECAR. Significance between treatments was determined via one-way ANOVA with a Tukey posttest. P value is indicated as *p ≤ 0.05. All bar graphs and symbols represent the treatment average ± SEM.
Figure 2-13. Vaccinated mice generated anti-Ova IgG antibody titers. C57BL/6 female mice (from Fig. 6) (n=12) were immunized subcutaneously with formulations consisting of 75 µg soluble Ova plus 500 µg of 20:80 CPTEG:CPH polyanhydride nanoparticles encapsulating 25 µg Ova (NP), 100 µg soluble Ova adjuvanted with 20 µg CpG ODN (CpG), a combination of 75 µg soluble Ova plus 500 µg of 20:80 CPTEG:CPH polyanhydride nanoparticles encapsulating 25 µg Ova adjuvanted with 20 µg CpG ODN (NP+CpG), or PBS control. Serum samples were collected 5 weeks post-immunization. Total IgG antibody titers were measured using indirect ELISA. Limit of detection indicated by the dashed line. Significance between each treatment and control was determined via one-way ANOVA with a Dunnett’s posttest. P value is indicated as *p ≤ 0.05. All bar graphs and symbols represent the treatment average ± SEM.
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7. References


CHAPTER 3. STING PATHWAY STIMULATION RESULTS IN A DIFFERENTIALLY ACTIVATED INNATE IMMUNE PHENOTYPE ASSOCIATED WITH LOW NITRIC OXIDE AND ENHANCED ANTIBODY TITERS IN YOUNG AND AGED MICE

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RD, MW, and MK contributed to conception and design of the studies. RD, SS, SK contributed to the execution of animal vaccination studies. RD and SS carried out laboratory *in vitro* assays. RD, SS, SK, MK, BN, and MW were involved in drafting, revising, and approval of the final version of the manuscript.

**Abstract**

*Background:* One of the most concerning public health issues, related to vaccination and disease prevention, is the inability to induce durable immune responses following a
single-dose immunization. In this regard, the nature of the inflammatory environment induced by vaccine adjuvants can negatively impact the resulting immune response. To address these concerns, new strategies to vaccine design are needed in order to improve the outcomes of immune responses, particularly in immunologically disadvantaged populations.

Methods: Comparisons of the scope of innate immune activation induced by TLR agonists versus cyclic dinucleotides (CDNs) was performed. Their effects on the activation characteristics (e.g., metabolism, cytokine secretion) of bone marrow derived dendritic cells (BMDCs) were studied. In addition, the differential effects on in vivo induction of antibody responses were measured.

Results: As compared to TLR ligands, the stimulation of BMDCs with CDNs induced distinctly different metabolic outcomes. Marked differences were observed in the production of nitric oxide (NO) and the cytokine BAFF. These distinct differences were correlated with improved (i.e., more rapid and persistent) vaccine antibody responses in both aged and young mice.

Conclusions: Our results illustrate that the innate immune pathway targeted by adjuvants can critically impact the outcome of the immune response post-vaccination. Specifically, CDN stimulation of APCs induced an activation phenotype that was characterized by decreased innate effector molecule production (e.g., NO) and increased BAFF. This was attributed to the induction of an innate inflammatory environment that enabled the host to make the most of the existing B lymphocyte potential. The use of adjuvants that differentially engage mechanisms of innate immune activation would be particularly advantageous for the generation of robust, single dose vaccines. The results of this study demonstrated that CDNs
induced differential innate activation and enhanced vaccine induced antibody responses in both young and aged mice.

1. Introduction

As a result of their relatively low immunogenicity, recombinant subunit-based vaccine formulations generally require the addition of adjuvants to induce protective immunological responses [1,2]. One of the often-selected families of adjuvants are Toll-like receptor (TLR) ligands [3]. These are chosen for their ability to provide activation (i.e., induce inflammation) of the innate and adaptive immune system through ligation of pattern recognition receptors (PRRs) to effectively mimic the presence of an active infection. While effective at activating innate immune responses, TLR engagement leads to the production of reactive nitrogen and reactive oxygen species by innate immune cells [4].

While production of nitric oxide (NO) and mitochondrial reactive oxygen species (mROS) are often central in the clearance of pathogenic infections prior to development of adaptive immunity, each are known to have deleterious effects on the magnitude of activation as well as the phenotype and survival of innate and adaptive immune cells [5–10]. For example, mice infected with lymphocytic choriomeningitis virus (LCMV) which develop severe lymphopenia have a poor adaptive immune response (e.g., no neutralizing antibody) that has been attributed to the large amounts of NO produced by inflammatory monocytes in the lymph nodes [11]. The elevated levels of NO leads to B cell death, decreased production of BAFF (i.e., cytokine promoting B cell survival), and subsequent loss of antibody production [12–15]. NOS2 derived NO from innate immune cells has also been described to interfere with lymphatic function and flow that can impair the adaptive immune response [16]. ROS have also been described to influence the phenotypic differentiation of B cells to plasma cells or immunoglobulin class switching in germinal center B cells. Varying levels of
ROS will react with and inhibit the ability of heme to regulate critical B cell transcription factors determining B cell fate [17]. While beneficial for acute innate immune responses, excessive levels of NO and/or ROS may have a negative impact on the adaptive immune response. In this regard, proper “management” of the inflammatory response induced by vaccine adjuvants needs to be carefully considered especially for older adults [18].

Those with immunological deficiencies, such as older adults developing an age-related narrowing of the naïve lymphocyte repertoire leading to less than adequate antibody responses, must make the most of the limited number of naïve cells remaining [19–21]. This will require the identification of more optimal activation pathways for adjuvants to target or avoid targeting, and will be critical in order to achieve the broadest protective response to recombinant subunit vaccines. Besides TLRs, another, independent receptor family interacting with microbial associated molecular patterns (MAMPs) or damage associated molecular patterns (DAMPs) are the cytosolic nucleic acid sensors, such as Stimulator of Interferon Genes (STING) [22–25]. Herein, the ability of STING targeting cyclic dinucleotides (CDNs), specifically a synthetic analog of cyclic di-GMP (cdG), to provide effective innate immune activation and adjuvanticity was evaluated. While STING stimulation induces inflammation, we show that CDNs did not induce deleterious amounts of innate effector molecules (e.g., NO and ROS) that are associated with TLR agonist-based adjuvants [26,27]. We also illustrate that the activation of the STING pathway results in a distinct metabolic profile of DCs which is indicative of a unique activation phenotype as compared to TLR agonist. The results of this study furthers our understanding of adjuvant properties that contribute to the induction of durable protective immunity while avoiding the adverse reactions associated with adjuvants such as Alum or TLR agonists. Specifically,
these studies demonstrate that the use of CDNs induce a more favorable early innate immune activation phenotype and associated inflammatory environment which is linked to a rapid and greater magnitude of the vaccine-induced antibody responses in both aged and young mice.

2.0 Materials and Methods

2.1 Animals

Female BALB/c or C57BL/6 mice of six to eight weeks of age were obtained from Charles River (Wilmington, MA) for young mouse vaccination studies as well as in vitro APC studies. Aged female BALB/c mice of ≥ 20 months of age were obtained from Jackson Laboratory (Bar Harbor, ME). All studies involving the use of animals was carried out in accordance with current institutional guidelines for the care and use of animals.

2.2 Vaccination Studies

Young (6-8 weeks) and aged (18+ months) female BALB/c mice were immunized subcutaneously at the nape of the neck with formulations consisting of 50 µg ovalbumin (Ova), and 20 µg of the indicated TLR ligand or CDN (dithio-RP,RP-cyclic di-guanosine monophosphate) (Aduro Biotech, Berkley, CA) adjuvant, or where indicated Ova alone. Where applicable N-[[3-(aminomethyl)phenyl]methyl]-ethanimidine, dihydrochloride (1400W) (Cayman Chemical, Ann Arbor, MI) was administered intraperitoneally at 5 mg/kg body weight, every eight hours for seven days following vaccination. Female C56BL/6 mice (6-8 weeks of age) were immunized with 20 µg of recombinant protective antigen (PA) from B. anthracis (BEI, NR-3780) along with 20 µg of CDNs, or with 50 µL of the commercial BioThrax Anthrax Vaccine Adsorbed (Emergent Biosolutions, Rockville, MD) subcutaneously at the base of the neck.
2.3 Extracellular Flux Analysis

For mitochondrial stress tests (MST), BMDCs were stimulated overnight with 0.5 µg/mL CDNs, 1 µg/mL LPS or MPLA, 5 µg/mL imiquimod, CpG ODN, or no stimulation control in 5 mL polypropylene tubes (to avoid cell adherence). Treated BMDCs were seeded into 24 well seahorse plates coated with Cell-Tak (Corning, Corning NY) at a density of 2.5 x 10^5 cells per well. Mitochondrial stress test was carried out according to manufacturer’s MST protocol (Agilent, Santa Clara, CA). Concentrations of 1 µM oligomycin, 2 µM FCCP, and 0.5 µM rotenone and antimycin were used (Agilent, Santa Clara, CA). Kinetic stimulation assays were conducted with non-stimulated BMDCs seeded at 2.5 x 10^5 cells per well in 24 well seahorse plates coated with Cell-Tak (Corning, Corning NY). Stimulants were injected at the concentrations outlined in the APC stimulation section, after the third baseline measurement interval. Metabolic phenotyping was conducted on a Seahorse XFe24 (Agilent, Santa Clara, CA).

2.4 Bone marrow dendritic cell and macrophage generation

Bone marrow was collected from femurs and tibias of BALB/c mice. Cells (4 x 10^6 cells per 100 mm plate) were washed and plated in 10 mL of complete RPMI 1640 medium (100 U/mL penicillin and 100 µg/mL streptomycin, 2 mM glutamine, and 10 % FBS) was supplemented with 10 ng/mL of GM-CSF (Peprotech, Rocky Hill, NJ). On day three of culture, 10 mL of GM-CSF containing complete medium was added. On days six and eight of culture, 10 mL of culture medium was exchanged for 10 mL of fresh GM-CSF containing complete medium. On day 10 of culture, DCs were harvested by gently rinsing and collecting non-adherent cells. The same protocol was used to generate BMMs but substituting M-CSF in place of GM-CSF.
2.5 In vitro APC stimulation

BMDCs or BMMs were plated at $5 \times 10^5$ cells/well in a 96-well round bottom tissue culture plate in 200 µL of the previously described complete RPMI 1640 medium. Stimulants used included 5 µg/mL CpG ODN 1668, 5 µg/mL imiquimod, 1 µg/mL LPS, 1 µg/mL MPLA, 0.5 µg/mL cyclic di-GMP (cdg) CDN, or non-stimulated control wells (i.e., medium alone). Stimulations were carried out for 48 hours and supernatants and cells were harvested for cytokine analysis and cell surface marker expression by flow cytometry. In \textit{in vitro} experiments, the NOS2 inhibitor 1400W was used at a concentration of 50 µM.

2.6 Flow Cytometry

Following stimulation APCs were analyzed for costimulatory marker expression using flow cytometry. $5 \times 10^5$ DCs were aspirated from a 96 well plate and transferred to polystyrene tubes. Prior to labeling with specific monoclonal antibodies, Fc receptors on DCs were blocked to prevent non-specific antibody binding by incubating the cells with 100 µg/mL of rat IgG (Sigma Aldrich, St. Louis, MO) and 10 µg/mL of anti-CD16/32 (eBioscience). Subsequently, DCs were stained with fluorescently conjugated antibodies for CD80 (Biolegend, PerCP-Cy5.5, clone 16-10A1), CD86 (eBioscience, FITC, clone GL1), CD11c (Biolegend, APC-Cy7, clone N418), MHCII (eBioscience, AF700, clone M5/114.15.2) diluted in FACS buffer. Mitochondrial superoxide production was evaluated using live cells stained with MitoSOX Red according to manufacturer’s specifications (ThermoFisher Scientific). Samples were fixed using BD stabilizing fixative where applicable (BD Bioscience, Franklin Lakes, NJ). Data was collected on a FACSCanto II (BD Bioscience, Franklin Lakes, NJ), and analyzed using FlowJo (Flowjo LLC).
2.7 Nitric oxide quantification

Nitric oxide (NO) was quantified in BMDC supernatants via Griess assay. A sodium nitrite standard curve was created using two-fold serial dilutions with concentrations ranging from 100 µM to 0 µM. 100 µL of standard or supernatant was added to 100 µL of Griess reagent (Cat. No. 03553, Sigma-Aldrich) in a 96 well microtiter plate. Samples were allowed to react for 15 minutes at room temperature and the optical density at 540 nm was recorded using a SpectraMAX 190 (Molecular Devices, Sunnyvale, CA). Concentrations of nitrite were calculated using a linear regression method.

2.8 BAFF quantification

Quantification of serum BAFF, collected at seven days post-vaccination, and DC supernatant BAFF was performed via ELISA. A murine BAFF/BLYS/TNFSF13B Quantikine ELISA kit (R&D Systems, Cat. No. MBLYS0, Minneapolis, MN) was used according to manufacturer’s instructions and the optical density at 540 nm was recorded using the SpectraMAX 190. Concentrations of serum and supernatant BAFF were calculated using a linear regression method.

2.9 Serum antibody detection

Vaccinated mice were bled via saphenous vein at the indicated timepoints post-vaccination. Anti-Ova serum antibody titers were measured via indirect ELISA. Costar 3590 96-well EIA/RIA high binding plates (Corning, Corning NY) were coated with 100 µL of Ova (5 µg/mL PBS) or PA (0.5 µg/mL PBS) and incubated overnight at 4C. Plates were blocked using 2 % (w/v) Difco gelatin in PBS (0.05 M, PH 7.2) containing 0.05% Tween-20 (PBS-T) for two hours at room temperature. After three washes using PBS-T, serum samples
were titrated across the plate using two-fold serial dilutions, starting at 1:200, in PBS-T and 1% (v/v) normal goat serum. Samples were incubated overnight at 4°C. After three washes in PBS-T, an alkaline phosphatase conjugated goat anti-mouse IgG (H+L) secondary detection antibody (Cat# 115-005-003, Jackson ImmunoResearch) was diluted 1:1000, added to the wells and allowed to incubate at room temperature for two hours. Plates were washed three times with PBS-T and alkaline phosphatase substrate was added at 1 mg/mL in buffer containing 50 mM sodium carbonate, 2 mM magnesium chloride, and sodium bicarbonate added to achieve a pH of 9.3. Plates were allowed to develop for 30 minutes and analyzed using the SpectraMAX 190 at a wavelength of 405 nm. For isotype specific a similar ELISA protocol was used, but serum samples were diluted 1:200 and IgG1 (Cat# 115-055-205, Jackson ImmunoResearch and IgG2a (Cat# 115-055-206, Jackson ImmunoResearch) specific secondary antibodies were used.

3. Results

3.1 CDNs result in higher antibody titers when compared to TLR agonists

Studies incorporating the use of CDNs in recombinant subunit vaccine studies have illustrated the ability of CDNs to enhance the induction of durable, high titer antibody after vaccination [28]. Herein, the antibody titers induced in mice that received TLR agonist or CDNs were compared (Figure 3-1). Mice were vaccinated with 50 µg of Ova alone, or adjuvanted with either 20 µg of monophosphoryl lipid A (MPLA) or Imiquimod as representative TLR ligands, or 20 µg of CDN. Antibody titers were measured at 14- and 28-days post-vaccination. Animals that had received vaccination with CDNs as the adjuvant had higher serum anti-Ova IgG titers compared to mice that had been vaccinated using TLR agonists as the adjuvant at both 14 and 28 days after vaccination (Figure 3-1a-b). CDNs were
also compared to a vaccine containing the commonly used adjuvant alum. To demonstrate that the difference in the magnitude of the immune response induced with CDNs was not specific to Ova, it was also shown that higher serum antibody titers were achieved after vaccination with the protective antigen (PA) of *B. anthracis* admixed with CDNs as compared to the commercially available alum-based vaccine, Biothrax (Supplemental Figure 3-7).

**Figure 3-1.** Assessment of the murine serum antibody response to ovalbumin (Ova) when adjuvanted with cyclic dinucleotides (CDNs) or TLR-ligands. Young (6-8-week-old) female BALB/c mice were immunized subcutaneously with formulations consisting of 50 µg Ova alone or with the addition of 20 µg of the indicated TLR ligand (Imiquimod (Imiq) or monophosphoryl lipid A (MPLA)) or CDN as an adjuvant (n=8). Serum antibody titer to Ova was quantified via ELISA at (a) 2-weeks and (b) 4-weeks post-immunization. Titer values were Log₂ transformed and compared for statistical significance via an ordinary one-way ANOVA with a Dunnett’s multiple comparison test with each group being compared back to
CDN. P value is indicated as follows (*=p<0.05). Individual animals are shown with bars indicating mean ± SEM.

3.2 CDN stimulation of BMDCs results in early dual metabolic burst and sustained mitochondrial respiration

Stimulation and activation of BMDCs has been described to cause distinct acute, and long term, metabolic alterations in response to encounter and activation upon exposure to TLR agonists [29–31]. Immediately upon stimulation with TLR ligands, a hallmark glycolytic burst is observed. Stimulation with CDNs also results in a comparable uptick in glycolytic rate upon activation, albeit a somewhat slower response (Figure 3-2a-b). While TLR ligand stimulation results in no immediate increase in mitochondrial respiration after stimulation, CDN stimulation results in an upregulation of oxygen consumption rate in addition to the observed glycolytic burst (Figure 3-2c-d).

Mitochondrial function was analyzed after an 18-h stimulation with CDNs and TLR agonists. The TLR ligand results are consistent with previous observations that BMDCs are driven to a persistent state of aerobic glycolysis and inhibited mitochondrial function [29,32]. CDN mediated activation results in a sustained basal oxygen consumption rate, ATP production, decreased proton leak, and maintenance of spare capacity (Figure 3-2e-f) while TLR stimulation results in depressed functionality of mitochondria (Figure 3-2e-f). Similar decreases in mitochondrial respiration are also observed in the monocyte like J774 cell line after 18 h of stimulation with TLR agonists, but not with CDNs (Supplemental Figure 3-8). The relationship between metabolism and phenotypic outcome of cells is described to be closely linked [33–35]. These results illustrate a differential metabolic phenotype arose, as
well as a corresponding distinct activation phenotype, depending on the PRR activation pathway engaged by these adjuvants.

**Figure 3-2. Acute metabolic responses and long-term mitochondrial function.** Acute and chronic metabolic responses of bone marrow derived dendritic cells (BMDCs) stimulated with cyclic dinucleotides (CDNs) or TLR ligands. For acute assays non-stimulated BMDCs...
were seeded at 2.5 x 10^5 cells per well in a Seahorse plate coated with Cell-Tak. Extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) were measured as indicators of glycolysis and mitochondrial respiration respectively. (a-d) After three baseline readings either CDNs, Imiquimod, MPLA, or medium control were injected into each well at the indicated time point (n=3). (b) ECAR % and (d) OCR % change relative to baseline readings at 100 minutes. For longer term mitochondrial stress test (MST), (e) BMDCs were stimulated for 18 h with CDNs, Imiquimod (Imiq), lipopolysaccharide (LPS), CpG, or non-stimulated control in 5 mL polypropylene tubes. Stimulated BMDCs were seeded into seahorse plates coated with Cell-Tak at a density of 2.5 x 10^5 cells per well and oxygen consumption rate (OCR) was measured. (f) Basal respiration, ATP production, maximal respiratory capacity, and spare capacity are calculated from the MST. Significance (p<0.05) was determined via one-way ANOVA with a Dunnett’s (b) or Sidak’s (d-f) multiple comparison test. Significance is indicated as compared to control with (*) and as compared to CDN with (#), respectively. All bars and symbols represent the group average ± SEM. Data shown is a single experimental replicate that is representative of at least one additional experimental repeat.

3.3 CDN stimulation results in decreased innate immune effector molecule production and increased BAFF

The sustained aerobic glycolysis observed in murine BMDCs after TLR stimulation is the direct result of the production of high concentration of nitric oxide (NO) leading to nitrosylation of electron transport chain molecules [31,36]. This results in mitochondrial deficiency and dependence on glycolysis for survival and the metabolic demands of activation [32]. After observing the acute characteristic metabolic effects of activation, yet
maintenance of mitochondrial function of CDN stimulated BMDCs at later time points, it was hypothesized that there would be low to no production of the innate effector molecule NO. After 48 hours, CDN stimulation results in control levels of NO accumulation in the supernatant, while TLR ligands induce a dramatic upregulation of NO concentrations (Figure 3-3a). Bone marrow macrophages (BMM) as well as monocyte like J774 cells also show similar patterns of low NO production after stimulation with CDN (Supplemental Figure 3-9). As glycolytic reprogramming upon activation in macrophages has been linked to production of reactive oxygen species, we also assayed stimulated BMDCs for mitochondrial superoxide (mROS) [37]. Similar patterns are again observed in the production of mROS after stimulation. CDNs result in control levels of mROS generation while TLR ligand stimulation overall resulted in an increase (Supplemental Figure 3-10).

BAFF is a critical cytokine in promoting B cell and plasma cell survival, as well as maintenance of germinal centers and B cell follicles [12,13,15,38]. As NO is a known inhibitor of the production of BAFF expression, it was hypothesized that the extremely low production of NO from CDN stimulated BMDC, would allow for the generation of higher levels of BAFF expression. Supernatant concentrations of BAFF were measured after 48 hours of stimulation with CDNs or TLR ligand via ELISA. As hypothesized CDN stimulation lead to higher concentrations of supernatant BAFF (Figure 3-3b).
Figure 3-3. Comparison of nitric oxide (NO) and BAFF produced by bone marrow derived dendritic cells (BMDCs) stimulated with cyclic dinucleotides (CDNs) or TLR-ligands. Supernatants were collected from BMDCs after 48 hrs. of stimulation with CDNs, CpG, Imiquimod (Imiq), lipopolysaccharide (LPS), monophosphoryl lipid A (MPLA), or Non-stimulated control as described in Materials and Methods section 3.6. The supernatants were assayed for (a) NO production was measured via Griess assay detection of supernatant nitrite, and (b) supernatant BAFF concentrations were measured via ELISA. All histograms represent the group average ± SEM. Significance was determined via one-way ANOVA with a Sidak’s multiple comparison test. Significance is indicated as compared to control with (*) and as compared to CDN with (#), respectively. P value is indicated as follows (* or #=p<0.05). Data shown is a single experimental stimulation with (a) n=4 Griess replicates, or (b) n=2 BAFF ELISA replicates.
3.4 *In vitro* NOS2 inhibition improves TLR induced BAFF production and costimulatory expression, but not CDNs.

It has been illustrated via the use of NOS2 knockout mice and *in vitro* NOS2 inhibitors that NO suppresses TLR agonist-mediated costimulatory molecule upregulation on BMDCs [14,36]. Therefore, BMDCs were stimulated with CDNs or TLR ligands in the presence or absence of the NOS2 inhibitor 1400W. After 48 hours, supernatants were harvested and assayed for NO production. Results showed that the NOS2 inhibitor was effective at significantly decreasing NO concentrations in TLR agonist stimulated cells (Figure 3-4a). BMDC culture supernatants were also analyzed for BAFF production after stimulation with or without 1400W. Increases in BAFF production in TLR stimulation groups that have NOS2 activity inhibited are observed, but not in CDN stimulated cells (Figure 3-4b). Like BAFF production, upregulation of the costimulatory molecules CD86 and CD80 after stimulation with CDNs is unaffected by inhibition of NOS2 while TLR ligand stimulated groups have enhanced upregulation of these molecules (Figure 3-4c-d). This suggests that CDN stimulation of the STING pathway in BMDCs leads to an enhanced upregulation of the costimulatory molecules and increased BAFF production, compared to TLR ligands, by avoiding production of the innate effector molecule NO.
Figure 3-4. Effect of NOS2 inhibition on the costimulatory expression and BAFF secretion of innate immune cells. BMDCs were stimulated for 48 hrs. with CDNs, CpG, Imiquimod, LPS, MPLA, or non-stimulated control as described in Materials and Methods section 3.6. Each stimulant was given/used alone (-) or with the NOS2 inhibitor 1400w (+) and supernatants collected and were assayed for (a) NO production via Griess assay detection of supernatant nitrite, (b) supernatant BAFF concentrations via ELISA, and costimulatory expression of (c) CD86 and (d) CD80 expression via flow cytometry. All histograms represent the group average ± SEM. Statistical significance was determined between each stimulant alone (-), and the stimulant with 1400W present (+) via multiple Unpaired t-tests.
(one-tailed). P value is indicated as follows (*=p<0.05). Data shown includes three independent experiments.

3.5 Short term In vivo inhibition of NO results in improved antibody titers

As improved costimulatory expression and BAFF secretion was observed in vitro with TLR agonist/1400W treated BMDCs, it was hypothesized that inhibiting in vivo NOS2 activity during the acute inflammatory period after vaccination would result in increased antibody titers. This was tested by vaccinating mice with Ova alone, Ova adjuvanted with MPLA, or Ova adjuvanted with CDNs. In addition, mice also received an injection of 1400W or a sham injection three times daily for 7 days. Serum antibody titers were measured at 2 weeks, 6 weeks, and 32 weeks post-vaccination. Anti-Ova IgG titers at 2 weeks post-vaccination reveal improved titers in MPLA mice receiving inhibitor as compared to MPLA only mice, but treatment with the NOS2 inhibitor did not improve the antibody response in the CDN treated mice (Figure 3-5a). 6 weeks after vaccination the inhibitor did not result in a statistically significant increase in titer when inhibitor was added relative to adjuvant alone (Figure 3-5b). At 32 weeks post-vaccination time point, beneficial effects were observed for mice treated with MPLA and the NOS2 inhibitor. Interestingly, the CDN treated mice receiving the NOS2 inhibitor also exhibited an increase in titer as compared to those receiving CDNs alone (Figure 3-5c). No notable difference in IgG1 vs IgG2 isotypes were observed (Supplemental Figure 3-11).
Figure 3-5. Assessment of the murine serum antibody response to ovalbumin (Ova) when adjuvanted with cyclic dinucleotides (CDNs) or TLR-ligand in the presence or absence of a NOS2 inhibitor. Young (6-8 weeks) female BALB/c mice were immunized subcutaneously with formulations consisting of 50 µg Ova alone (sOva) or with 20 µg of MPLA or CDN as an adjuvant. Mice were treated with MPLA or CDNs alone (-) or were treated intraperitoneally with the NOS2 inhibitor 1400W (+) for 7 days post-immunization (n=6). Serum antibody titer was quantified via ELISA at (a) 2-weeks and (b) 6-weeks (c) 32-weeks post-immunization. Titer values were Log₂ transformed and each treatment group was compared to itself with and without 1400W. Individual values for each mouse in a given treatment group are depicted by the symbols and the group average ± SEM is also indicated. Statistical significance was determined via multiple Unpaired t-tests (one-tailed). P value is indicated as follows (*=p<0.05), ns = not significant.

3.6 CDN vaccination results in increased BAFF and antibody titer in aged recipients

NO production following in vitro stimulation, or vaccination with a TLR ligand as the adjuvant has deleterious effects on the generation of critical cytokines (e.g. BAFF), and of
antibody titers (Figure 3-5) [10,11]. It was hypothesized that immunologically compromised populations that often respond poorly to vaccination, such as older adults, would benefit (i.e., higher Ag-specific titers) from a vaccine formulation containing CDNs as opposed to a TLR ligand. Aged mice (≥ 20 months old) were vaccinated with Ova adjuvanted with either CDNs or the TLR7 ligand, imiquimod. Serum anti-Ova antibody titers were measured at 28- and 75-days post-vaccination. Mice receiving the CDN adjuvanted vaccine regimen had higher antibody titers as compared to mice receiving Imiquimod at both time points (Figure 3-6a). We hypothesized that mice receiving the CDN formulation would have increased BAFF concentration after vaccination as compared to Imiquimod as CDNs do not result in NO production. Serum concentrations of BAFF were measured 7 days post-vaccination, and aged mice receiving CDNs had significantly increased serum BAFF concentrations as compared to those treated with imiquimod (Figure 3-6b).

Figure 3-6. Assessment of serum BAFF and antigen-specific antibody responses in aged mice following vaccination. Aged (20+ months) female BALB/c mice were immunized
subcutaneously with formulations consisting of 50 µg Ova plus 20 µg of imiquimod (Imiq) or cyclic dinucleotides (CDN) (n=7). (a) Serum antibody titer of anti-ova antibodies was quantified via ELISA at 28- and 75-days post-immunization. Titer values were Log2 transformed and CDNs were compared to Imiquimod for statistical significance at each timepoint via Unpaired t-tests (one-tailed). (b) Serum was collected from immunized mice 7 days post-immunization and BAFF concentrations were determined via ELISA. Statistical significance was determined via Unpaired t-test (one-tailed). P value is indicated as follows (*=p<0.05). All bars and symbols indicate mean ± SEM.

4. Discussion

As the trend to use recombinant proteins in the development and design of new vaccine formulations expands, there is need to develop immunization strategies that induce durable, protective immunity in the absence of adverse reactions [39]. Because of the low immunogenicity of many recombinant proteins, there is a need to include adjuvants in the vaccine formulation to induce an inflammatory response in order to drive higher antibody titers [40,41]. While safety of any adjuvant deployed for use in humans or animals is a major concern, patients’ perceptions of the discomfort (i.e., pain) associated with vaccines that employ inflammatory adjuvants (e.g., Alum, ASO4) often impacts patient willingness to receive a booster immunization or be immunized at all [39]. That said, these studies were designed to evaluate host and cellular responses induced by CDNs in comparison to TLR ligands in order to gain insights into functional inflammatory characteristics that would avoid some adverse negative aspects of inflammation and improve the outcome of an adaptive immune response [18].
In these studies, the differential effects of TLR agonists and CDNs on the outcome of antigen-specific antibody responses subsequent to vaccination were evaluated. The inclusion of CDNs as an adjuvant in a vaccine regimen, as compared to MPLA or Imiquimod, resulted in higher antibody titers at all time points after vaccination (Figure 3-1) suggesting that the phenotype of the inflammatory environment induced by the TLR ligands may have muted the magnitude of the antibody response. Therefore, studies were performed to elucidate the differing effects on the innate immune responses that could be responsible for affecting the magnitude of the observed humoral response.

It has been shown that elevated induction of NO and ROS can inhibit B cell responses [10,11]. Innate immune cell derived NO in particular, has been linked to B cell death in the lymph node, and a suppressive effect on antibody titers [10,11]. Furthermore, levels of ROS have been linked to determining B cell fate (i.e., plasma cell vs class switching recombination in B cells) by reacting with heme and affecting its regulation of transcription factors (e.g., BACH2, BLIMP-1) that are critical in determining B cell phenotypes [17,42].

In the current study, distinct differences in the metabolic phenotype of DCs and the resultant levels of NO and ROS following stimulation indicated that the activation phenotype induced by CDN stimulation was markedly different than that induced by TLR agonists (Figure 3-2). Studies assessing metabolism of immune cells has inextricably linked the metabolic demands/profile of various immune cells to their phenotype (e.g. M1 vs M2, effector vs memory vs regulatory, etc.) [33,43,44]. In this regard, CDN stimulation of DCs resulted in the immediate upregulation of glycolysis, as well as mitochondrial respiration, resulting in a metabolic “double-dipping” that is indicative of a distinct phenotype in contrast to that induced by TLR agonist that solely upregulate glycolysis. The CDN-induced activation
phenotype of DCs diverged further from that induced by TLR-ligands based on the production of lower levels of innate immune effector molecules (i.e. NO/ROS) and the differential effects on mitochondrial function at a later timepoint (e.g. 18 hours). Observed differences in adaptive immune responses may be the result of the contextual activation of APCs via innate signals linked to recognition of “infectious-nonself” activators (e.g., TLR signaling pathway) compared to danger/damage signals, which are not exclusively a result of microbial encounter (e.g., CDNs and the STING pathway) [41].

In addition to having direct effects on B lymphocytes, NO from inflammatory monocytes and monocyte derived DCs has been shown to inhibit the production of BAFF, as well as expression of DC costimulatory molecules [45]. To demonstrate the negative impact of NO on DC functions, increased expression of costimulatory molecules on BMDCs stimulated with TLR agonists was observed in conjunction with the use of the NOS2 inhibitor, 1400W. As expected, the increased level of CD80 and CD86 expression on DCs stimulated with CDN was not affected by presence or absence of 1400W (Figure 3-4c-d).

BAFF is a key cytokine responsible for B cell survival, maintenance of germinal centers, as well as for plasma cell survival, directly affecting the maintenance of antibody titers after vaccination. As an adjuvant that enhances B cell responses, the very low levels of NO produced by BMDCs stimulated with CDNs (Figure 3-3a) correlated with higher BAFF production as compared to the significantly lower (p ≤ 0.05) levels of BAFF induced by TLR-ligands in the presence of higher amounts of NO production (Figure 3-3a). In vivo, the heightened amounts of BAFF induced by CDNs correlated with elevated titers of anti-Ova antibody compared to mice immunized with Ova plus MPLA (Figure 3-5a). This effect is also observed in the serum BAFF concentrations of aged mice immunized with Ova plus
CDN when compared to aged mice immunized with Ova plus imiquimod (Figure 3-6a) and the significantly higher ($p \leq 0.05$) antibody titers to Ova in the aged mice immunized with the CDN formulation (Figure 3-6b).

Results of this study also indicated that *in vivo* inhibition of NOS2 derived NO with 1400W after vaccination lead to improved antibody titers at 2 weeks post-vaccination in animals vaccinated with MPLA. Not unexpectedly, the administration of 1400W had no effect on the antibody response of mice receiving Ova plus CDNs. This suggests that even mice immunized with MPLA, a detoxified TLR ligand, are at a disadvantage relative to those receiving CDNs as a consequence of the induction of NO that contributed to lower antibody titers. However, at late time points (e.g., 32 weeks), mice immunized with vaccines incorporating either MPLA or CDNs presented with higher antigen-specific antibody titers when NOS2 was inhibited during the first week post-vaccination. In this situation, the observed benefits to mice immunized with Ova adjuvanted with CDNs are likely explained by the fact that CDNs, while inducing extremely low levels of NO relative to TLR agonists, do produce detectable levels of NO that likely have a minor effect on diminishing the resultant antibody response. This may be explained in part by the propensity of CDNs to induce interferon beta, a type 1 interferon, which have been described to inhibit the production of NO. This is at best a partial explanation as it has been shown that TLR 7/9 agonists also stimulate the production of interferon alpha, another type 1 interferon, yet there was demonstrable production of NO *in vitro* (Figure 3-3 and 3-4) [10,22,23]. To achieve higher antibody titers post-immunization, use of an adjuvant (e.g., CDNs) that induces very low amounts of NO would avoid the negative impacts on the antibody response without the need to include a NOS2 inhibitor as part of the vaccination strategy [14,46].
Recombinant subunit-based vaccine formulations will continue to require the addition of effective adjuvants in order to induce elevated and durable antibody responses at a given immunogen dose and in as few injections as possible. In this regard, it has been shown that multiple administrations of the DTaP vaccine to humans in the presence of inflammatory adjuvants induced isotype class switching such that the ratio of IgG1 to IgG4 decreased [47]. While IgG4 effectively neutralized toxins, this isotype does not fix complement nor does it effectively bind to FcRgIIIB or FcRgIIa (i.e., a poor opsonin) [48]. Traditionally, the general strategy in selection of adjuvants often involved mimicking various characteristics of naturally-occurring infections consistent with infectious-nonsself (i.e. pathogen or microbial associated) activation pathways [40,41]. In many situations, currently approved oil-in-water, TLR agonist, or aluminum salt-based adjuvants will induce efficacious immune responses and be effective components of vaccine formulations. However, they are not without their shortcomings, such as, induction of adverse, local inflammation (i.e., reactogenicity) is associated with oil-in-water- or alum-based adjuvants and the negative impact these responses have on patient compliance (i.e., avoiding booster immunization) [39]. In addition, these adjuvants often fail to induce optimal CD8+ T cell responses (i.e., cell-mediated immunity), and can be implicated in undesirable effects after subsequent re-encounter with antigen, such as dysfunction of T cell populations [49,50]. This is further corroborated by work in our laboratory that CDNs have also exhibit improved induction of vaccine-associated memory CD8+ T cell generation as compared to the TLR agonist CpG (data not shown).

In conclusion, as new and more optimized vaccine formulations are identified, developed, and tested, the optimal immunological outcome (i.e., high titer, durable antibody responses, improved memory) may include/encompass a paradigm shift away from the need
to induce an innate immune response similar to that which occurs during infection [41]. The results of this study demonstrate that the choice of an adjuvant that induces a disparate inflammatory response from that induced by traditional MAMP-based adjuvants, that often induce antimicrobial effector molecules (e.g., NO, ROS), and that can result in the induction of effective humoral immune responses in both young and aged mice. CDN as a vaccine adjuvant are an ideal candidate to induce rapid generation of high titer antibody responses that were durable through at least 32 weeks. In the broadest sense, individuals that may benefit the most from the use of CDNs in vaccine regimen would be older adults or others presenting as poor vaccine responders. Unlike more traditional adjuvants (e.g., alum, TLR ligands), these studies demonstrated that the distinct phenotype of APCs stimulated by CDNs contribute to enhanced antibody responses while avoiding the overt production of NO and mROS induced by more traditional vaccine adjuvants (e.g., TLR ligands) that may attenuate the resultant immune response.

5. Supplemental Data

**Figure 3-7.** Comparison of the serum antibody response in mice given a vaccine containing cyclic dinucleotides versus alum. Young (6-8 weeks) female C57BL/6 mice
were immunized subcutaneously with formulations consisting of 20 µg of the protective antigen (PA) from *Bacillus anthracis* with 20 µg CDNs, or 50 µL of BioThrax. Serum antibody titer of anti-PA antibodies was quantified via ELISA at (a) 4-weeks and (b) 13-weeks post-immunization. Titer values were Log2 transformed. Individual values are symbols along with the group average ± SEM. n = 16 for each treatment group. Statistical significance was determined between treatments at each timepoint via Unpaired t-tests (two-tailed). P value is indicated as follows (*=p<0.05).

**Figure 3-8. Metabolic effects of CDN stimulation on J774 cells.** After stimulation, metabolic responses of J774s to cyclic dinucleotides (CDNs) or TLR ligands was measured. Mitochondrial stress test (MST), (a) J774s were stimulated overnight with CDNs, Imiquimod (Imiq), lipopolysaccharide (LPS), CpG, or non-stimulated control in 5 mL polypropylene tubes. Treated J774s were seeded into seahorse plates coated with Cell-Tak at a density of 1.7 x 10^5 cells per well and oxygen consumption rate (OCR) was measured. (d) Basal respiration, ATP production, maximal respiratory capacity, and spare capacity are calculated from the MST. Significance was determined via one-way ANOVA with a Sidak’s multiple
comparison test. Significance ($p \leq 0.05$) is indicated as a comparison to control (*) or in comparison to CDN stimulation (#), respectively. All histograms and line graphs represent the group average ± SEM. Data shown is a single experimental replicate that is representative of two independent experiments.

**Figure 3-9.** Induction of nitric oxide by bone marrow derived macrophages (BMMs) and J774 cells stimulated with cyclic dinucleotides (CDNs) or TLR ligands. Culture supernatants were collected at 48 hrs post-stimulation from (a) BMMs or (b) J774 cells treated with CDNs, CpG, Imiquimod, LPS, or medium (control) and assayed for NO production via Griess assay as described in Materials and Methods. All histograms represent the group average ± SEM. Significance was determined via one-way ANOVA with a Sidak’s multiple comparison test. Significance ($p \leq 0.05$) is indicated as compared to control with (*) and as compared to CDN with (#), respectively. Data shown depict the results from a single experiment with n=3 for each treatment.
Figure 3-10. Bone marrow derived dendritic cells (BMDC) production of mitochondrial superoxide after stimulation. BMDCs after 48 h of stimulation with CDNs, CpG, Imiquimod, LPS, MPLA, or unstimulated control were assayed for mitochondrial superoxide production via MitoSOX staining and MFI collected via flow cytometry. All bars and symbols represent the group average ± SEM. Significance was determined via one-way ANOVA with a Sidak’s multiple comparison test. Significance (p ≤ 0.05) is indicated as compared to control with (*) and as compared to CDN with (#), respectively. Data shown is a single experimental stimulation with n=2 replicates.
Figure 3-11. Assessment of the murine serum antibody isotype specific response to ovalbumin (Ova) when adjuvanted with cyclic dinucleotides (CDNs) or TLR-ligand in the presence or absence of a NOS2 inhibitor. Young (6-8 weeks) female BALB/c mice were immunized subcutaneously with formulations consisting of 50 µg Ova alone (sOva) or with 20 µg of MPLA or CDN as an adjuvant. Mice were treated with MPLA or CDNs alone (-) or were treated intraperitoneally with the NOS2 inhibitor 1400W (+) for 7 days post-immunization (n=6). Serum antibody titer was quantified via ELISA at (a) 2-weeks and (b) 4-weeks post-immunization. Isotype ratios (IgG2a/IgG1) values were calculated. Individual values for each mouse in a given treatment group are depicted by the symbols and the group average ± SEM is also indicated.

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7. References


CHAPTER 4. COMBINATION ADJUVANT FORMULATION AS A HIGH TOXIN NEUTRALIZING TITER B. ANTHRACIS VACCINE

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Abstract

Bacillus anthracis, the causative agent of anthrax, continues to be one of the most prominent biological warfare and bioterrorism treats. Vaccination is likely to remain the most cost effective and patient friendly protective regimen for the foreseeable future. The currently available AVA BioThrax, while currently the best option available, has a number of shortcomings where improvement would lead to a more practical and effective vaccine for use in the case of an exposure event. Identification of more effective adjuvants and novel delivery platforms is necessary in order to improve not only the effectiveness of the anthrax vaccine, but also enhance shelf stability and ease-of-use to improve patient compliance. In
previous studies, cyclic dinucleotides have proven to be uniquely effective at inducing a beneficial inflammatory environment that leads to high titer antibodies post-vaccination capable of providing rapid protection against *Yersinia pestis*. This makes them an ideal adjuvant candidate for an antitoxin vaccine where rapid generation of neutralizing titers is critical. Polyanhydride particles have proven to be effective at adjuvancing the vaccine associated adaptive immune response, as well as enhancing stability of encapsulated antigen. Here, we evaluate the individual contributions of cyclic dinucleotides, polyanhydride nanoparticles, and a combination thereof to elicit high neutralizing antibody titers against the recombinant protective antigen from *B. anthracis*.

1. Introduction

*Bacillus anthracis* is a gram-positive, spore-forming, aerobic bacterium that is the causative agent of anthrax disease, observations of which date back to biblical times [1]. Anthrax spores are found in soil and cause disease largely amongst herbivores such as cattle, sheep, and goats, but can infect a wide variety of livestock and wildlife species. Humans have also become infected, largely by being exposed to infected animals or by working in facilities that handle products from infected animals (e.g. wool sorters disease). *B. anthracis* has three major routes of infection, cutaneous, gastrointestinal, and inhalation, leading to varied severity of disease with inhalation having the highest lethality [2]. The long-lasting, highly durable spores of *B. anthracis* are resistant to varied environmental conditions as well as the host immune system. The stability and dispersibility of anthrax spores contribute to them being a leading concern among biological warfare weapons [3–6].

The major virulence factors of *B. anthracis* infection are the anthrax toxins (AT). The ATs consist of lethal factor (LF), edema factor (EF), and protective antigen (PA) [7]. The
ATs are placed within the family of AB toxins, where the A subunit, LF and EF in this case, is the catalytic component within a target cell, and the PA is the B subunit that binds to the target cell and facilitates entry of the catalytic A subunit. The PA functions by binding to two potential receptors, namely, tumor endothelial marker 8 (TEM8/ANTXR1) or capillary morphogenesis gene-2 (CMG2/ANTXR2) on the cellular surface. At the cell surface, PA undergoes proteolytic cleavage by furin or furin-like proteases [8,9] which allows PA to form a heptamer that is capable of binding and then, via a ratchet-like mechanism, translocating LF and EF into the cytosol where their toxic effects occur [10,11].

This dependence on the activity of PA for the lethality of the ATs makes PA a logical choice as a target for development of a prophylactic vaccine. Neutralizing this single bacterial component would effectively inhibit the major disease-causing virulence attribute of B. anthracis infection. This has proven to be the case with the currently approved and widely used AVA BioThrax (AVA) vaccine. However, this vaccine formulation is not without its shortcomings. AVA consists of undefined components derived from culture supernatants, is variable in its ability to induce protective immunity across species, and requires a 6-month long schedule of vaccinations and regular boosters in order to be effective [12]. Studies have shown that the result immune response to the AVA formulation can block responses to functionally active regions of PA; however, much of the AVA-induced antibodies are skewed towards non-neutralizing epitopes [13–16]. More recently, vaccines targeting PA have moved toward using recombinant PA (rPA) to provide a more consistent immunogen as well as reduce the adverse reactogenicity associated with the use of the bacterial culture supernatant in AVA. However, poor shelf stability of rPA formulation has led to loss of rPA’s ability to induce neutralizing antibody titers [17,18]. In order to improve on AVA
BioThrax, and protect at-risk military personnel, workers with occupational risks of exposure, and to be prepared for the possibility of malicious wide-spread dispersion of *B. anthracis* spores, novel vaccine formulations are required to provide shelf and thermal stability to rPA, as well maintains its ability to induce high titer neutralizing antibodies up on vaccination.

Studies have evaluated the effects of polyanhydride nanoparticles to provide chemistry-dependent shelf stability to encapsulated antigen. The polyanhydride nanoparticles provided enhanced stability, as compared to alum adsorbed rPA or rPA alone, when stored at multiple storage temperatures. Polyanhydride nanoparticles formulated with the CPTEG:CPH chemistries, in particular, helped to retain the antigenicity of rPA during storage and the stored polyanhydride nanoparticles encapsulating rPA effectively induced neutralizing antibody responses in vivo [19,20]. Evaluations of the adjuvant activity of cyclic dinucleotides have illustrated their unique ability to induce high titer antibody responses upon vaccination [21–24]. Herein, we evaluate the combination of polyanhydride nanoparticles in conjunction with CDNs to induce high titer neutralizing antibody against the rPA from *B. anthracis* using a single dose immunization regimen.

### 2. Materials and Methods

#### 2.1 Synthesis Materials

Chemicals used for the polymer and nanoparticles synthesis: 4-p-hydroxybenzoic acid, tri-ethylene-glycol, 1,6- dibromohexane were obtained from Sigma Aldrich (St. Louis, MO); dimethyl formamide, acetic acid, acetonitrile, acetic anhydride, toluene, methylene chloride, pentane were purchased from Fisher Scientific (Fairlawn, NJ) and 4-p-flourobenzonitrile was purchased from Apollo Scientific (Cheshire, UK).
2.2 Polymer Synthesis

Monomers of 1,8-bis(p-carboxyphenoxy)-3,6-dioxaoctante (CPTEG) and 1,6-bis(p-carboxyphenoxy) hexane (CPH) was used to synthesize the copolymer 20:80 CPTEG:CPH via melt polycondensation reaction as previously described [25,26]. The purity and molecular weight of the copolymer was characterized using $^1$H nuclear magnetic resonance spectroscopy in deuterated chloroform. The molecular weight of the copolymer was near 5.3 kDa.

2.3 Particle Synthesis

Polyanhydride nanoparticles encapsulating 6.8% recombinant protective antigen (PA) (BEI resources, Cat# NR-3780, Manassas, VA), from Bacillus anthracis, by weight were synthesized using 20:80 CPTEG:CPH, via flash nanoprecipitation as essentially as previously described [27]. Briefly, a solution of 20:80 CPTEG:CPH and PA in methylene chloride at a concentration of 20 mg/mL. This solution was poured into pentane at a solvent to anti-solvent ratio of 1:250. The nanoparticles were then collected using vacuum filtration.

2.4 Animals

Female BALB/cAnNHsd, A/J, and C57BL/6Crl or C57BL/6NHsd mice of six to eight weeks of age were obtained from Jackson Laboratory (Bar Harbor, ME) or Envigo (Somerset, NJ), respectively. All studies involving the use of animals was carried out in accordance with and approval from the Iowa State University institutional animal care and use committee.
2.5 Vaccinations

Separate groups of 6-8 week old female BALB/c, C57BL/6, or AJ mice were immunized subcutaneously (sc) with one of the following vaccine formulations: 1) 5 µg rPA encapsulated in 73.5 µg of 20:80 CPTEG:CPH (6.8% wt/wt) nanoparticles (NP), 2) 5 µg soluble rPA adjuvanted with 25 µg cyclic dinucleotide cdG (dithio-RP,RP-cyclic di-guanosine monophosphate, CDN) (Aduro Biotech, Berkley, CA), 3) a combination of 5 µg rPA encapsulated in 73.5 µg of 20:80 CPTEG:CPH (6.8% wt/wt) nanoparticles adjuvanted with 25 µg CDN (NP+CDN), or 4) 5 µg soluble rPA alone (rPA).

AVA BioThrax comparison study separate groups of mice were immunized sc with one of the following formulations 1) 5 µg rPA encapsulated in 73.5 µg 20:80 CPTEG:CPH (6.8% wt/wt) nanoparticles plus 15 µg soluble rPA (NP), 20 µg rPA adjuvanted with 20 µg CDN, 3) 5 µg rPA encapsulated in 73.5 µg of 20:80 CPTEG:CPH (6.8% wt/wt) nanoparticles plus 15 µg soluble rPA adjuvanted with 20 µg CDN, 4) 50 µL of AVA BioThrax (Emergent BioSolutions, Rockville, MD), or 5) PBS control.

2.6 Serum antibody detection

Vaccinated mice were bled via saphenous vein at the indicated time points post vaccination and serum was collected and stored at – 20 °C until used for analysis. Anti-rPA serum antibody titers were measured via indirect ELISA. Costar 3590 96-well EIA/RIA high binding plates (Corning, Corning NY) were coated with 100 µL of rPA (0.5 µg/mL in PBS) and incubated overnight at 4 °C. Plates were blocked using 2 % (w/v) Difco gelatin in PBS (0.05 M, PH 7.2) containing 0.05% Tween-20 (PBS-T) for two hours at room temperature. After three washes using PBS-T, serum samples were titrated across the plate using two-fold serial dilutions, starting at 1:200, in PBS-T containing 1% (v/v) normal goat serum. Samples
were incubated overnight at 4 °C. After three washes in PBS-T, an alkaline phosphatase conjugated goat anti-mouse IgG (H+L) secondary detection antibody (Cat# 115-005-003, Jackson ImmunoResearch, West Grove, PA) was diluted 1:1000, and 100 µL was added to the wells and allowed to incubate at room temperature for two hours. Plates were washed three times with PBS-T and alkaline phosphatase substrate (Cat# BP2534, Fischer Scientific, Hampton, NH) was added at 1 mg/mL in buffer containing 50 mM sodium carbonate, 2 mM magnesium chloride, and sodium bicarbonate added to achieve a pH of 9.3. Color was allowed to develop for 2 hours and analyzed using the SpectraMAX 190 (Molecular Devices, San Jose, CA) at a wavelength of 405 nm.

2.7 Peptide Array

102 peptides (13- to 20-mers) with 10-11 overlapping amino acids (BEI resources, Cat# NR-527), spanning the entireity of *B. anthracis* PA were dissolved into DMSO at 10 mg/mL to ensure dissolution, then diluted 10-fold in water to 1 mg/mL. Each peptide solution was diluted to bring the final concentration to 0.5 mg/mL in 1x print buffer (5% (v/v) DMSO, 137 mM NaCl, 9 mM KOH, 11.3 mM NaH₂PO₄). Slides were printed with 16 arrays per slide, each containing duplicate spots for each peptide, onto Nexterion AL slides (Schott, Louisville, KY) using a BioRobotics MicroGRID II microarray printer (Genomic Solutions, Inc. Ann Arbor, MI). Following printing, slides were vacuum sealed and stored at -20 °C until further use.

Microarray slides were allowed to warm to room temperature and then unsealed and placed on a slide warmer at 37 °C for 30 min to ensure minimal moisture presence. The slides were then placed immediately in a blocking solution consisting of 1 % BSA (w/v) in PBS-T for one hour. Slides were subsequently blocked a second time for one hour in 1 %
(v/v) goat serum PBS-T, then thrice washed with PBS-T and placed into a 16-well incubation chamber (Nexterion IC-16) to separate individual arrays. 120 µL of each serum sample was added at a 1:20 dilution and allowed to incubate for one hour at room temperature with gentle agitation. Slides were washed three times with PBS-T and biotinylated goat anti-mouse IgG (H+L) secondary antibody (Jackson ImmunoResearch CAT# 115-065-003) was added to wells at 1:1000 dilution for one hour at room temperature with gentle agitation. Slides were again washed thrice with PBS-T. Streptavidin Alexa Fluor 555 conjugate (Life Technologies, Cat# S21381, Carlsbad, CA) was added to wells at a 1:1000 dilution for 30 min at room temperature with gentle agitation. Slides were removed from incubation chambers, washed thrice with PBS-T, followed by another three washes with PBS. Slides were spun dry by centrifugation and read on the Scanarray 5000 laser scanner (GSI Lumonics, Bedford, MA).

The scanned images of the microarray slides were analyzed using SoftWorRx Tracker v2.8 software (Applied Precision, Inc., Issaquah, WA) to detect and quantify the fluorescent intensity of each spot. The background-corrected fluorescence of each spot was calculated as the mean fluorescence intensity (MFI) of each spot minus the neighboring median background surrounding the spot. The mean fluorescence intensity for each peptide was calculated as the average of the corresponding duplicate background-corrected fluorescence spot values.

2.8 Cell Culture

J774 cells were cultured and maintained in DMEM (Cat #15-013-CV, Corning) medium supplemented with 100 U/mL penicillin and 100 µg/mL streptomycin, 2 mM glutamine, and 10 % FBS.
2.9 Toxin neutralization assay

In order to assess neutralizing activity of serum from vaccinated mice, a J774 cell-based killing assay was used essentially as previously described [28,29]. Briefly, J774s were seeded into 96-well flat bottom plates (Costar, Cat #3595) at a concentration of 4 x 10^4 cells/well and allowed to adhere for 18 hours. Serum samples were prepared in separate 96-well plates by adding 100 µL of serum at a 1:200 dilution and serially diluting them across the plate with 2-fold dilutions. The serum samples were then incubated for 30 minutes with PA and lethal factor (LF) (BEI resources, Cat# NR-28544), at constant concentrations of 50 ng/mL and 40 ng/mL respectively, then added to the prepared J774s. The cell-serum-toxin mixture was allowed to incubate for 4 hours after which 25 µL of a 5 mg/mL of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) reagent (Invitrogen, Cat# M6494, Carlsbad, CA) was added. After 2 hours of incubation, supernatants were removed and 100 µL of dimethyl sulfoxide (Fisher Scientific, Cat# D128-500, Hampton, NH) was added to lyse and solubilize formazan crystals. The OD at 555 nm was measured on a SpectraMAX 190 and recorded for each sample.

2.10 Statistical Analysis

Data generated during anti-PA serum IgG ELISAs were log2 transformed and analyzed within each timepoint via one-way ANOVA with a Tukey post-test for multiple comparisons. Cell based toxin neutralization experiments were analyzed by calculating an area under the curve for each treatment group with the baseline set at the negative control. Difference between treatments were determined via one-way ANOVA with a Tukey post-test for multiple comparison. All analyses were performed using GraphPad Prism 8.0 (GraphPad Software, La Jolla, CA).
3.1 A co-adjuvant is required to drive high titer anti-PA antibody responses.

In order to achieve a more complete picture of the murine immune response induced by the various vaccine formulations, three strains of mice were evaluated. The C57BL/6, BALB/c, and A/J mice, as the standard Sterne strain *B. anthracis* model, were chosen to further our understanding in how the optimal adjuvant formulation may translate across strains [30,31]. The Th1 biased, poor antibody producers in the C57BL/6, Th2 biased high antibody producers in the BALB/c, and the A/J strain that is generates high titers in response to PA and is easily protected from disease [32–37]. Separate groups of mice from each of these three strains of mice were vaccinated with one of the following vaccine formulations: i) PA encapsulated in 20:80 CPTEG:CPH nanoparticles (NP), ii) PA adjuvanted with CDNs (CDN), iii) a combination of PA encapsulated in 20:80 CPTEG:CPH nanoparticles co-adjuvanted with CDNs (NP+CDN), or iv) PA alone (sPA). Serum samples were collected at 2-, 4-, and 8-weeks post-vaccination, along with an additional serum sample collected at 15-weeks post-immunization from the AJ mice. Anti-rPA total IgG antibody titers were evaluated at each timepoint.

The C57BL/6 mice exhibited lower magnitude anti-rPA titers across all timepoints as well as a waning titer in all treatment groups by 56 DPI. The only vaccine formulation capable of improving titers over sPA alone was the CDN formulation, which was observed at all timepoints. CDN adjuvanted titers were also significantly higher as compared to that induced by the NP formulation at 2-weeks, and significantly higher than that induced by NP+CDNs at 4-weeks post-vaccination (Figure 4-1a).

The BALB/c mice exhibited overall higher and steadily increasing antibody responses over the course of the experiment as compared to C57BL/6 mice. The CDN, and NP+CDN
formulations were both capable of significantly improving antibody titers in the BALB/c mice when compared to rPA alone, and the NP formulation at all timepoints evaluated. CDN
s also provided significantly higher anti-rpA titers two weeks post-vaccination as compared to the combination NP+CDN formulation (Figure 4-1b).

The A/J mice quickly attained high anti-rPA titers and maintained them over time. The pattern of responsiveness to adjuvant formulation is comparable to the responses observed in BALB/c mice. The only formulations able to significantly improve the A/J antibody titers over that induced by sPA alone were CDNs and the combination of NP+CDNs. The 15-week timepoint does indicate the combination formulation of NP+CDNs had the greatest titers and maintained this elevated titer compared to rPA alone (Figure 4-1c).

Taken together, the results using these three strains of mice with varied genetic propensity to produce antibody suggest that NP formulations alone are not able to provide any adjuvant benefit to anti-PA titers compared to that induced by rPA alone and will require the inclusion of a more inflammatory co-adjuvant to produce high titer antibody responses. The combination of NP+CDN overall provided better responses than NP alone, however, for the C57BL/6NHsd mice, rPA + CDNs performed the best. This suggests that the use of a formulation with 100 % of the immunogen encapsulated in NPs may be less than optimal to promote the generation of high titer antibodies, particularly at early timepoints. This could potentially be due to the lack of a significant bolus of antigen immediately available upon vaccination.
Figure 4-1. Multi-strain combination nanovaccine induced antibody titers against protective antigen.
(a) C57BL/6NHsd (n=6), (b) BALB/cAnNHsd (n=6), and (c) A/J (n=4) mice were vaccinated subcutaneously against the PA protein from *B. anthracis*. The formulations consisted of 5 µg PA encapsulated in 20:80 CPTEG:CPH nanoparticles (NP), 5 µg soluble PA adjuvanted with 25 µg CDNs (CDN), a combination of 5 µg PA encapsulated in 20:80 CPTEG:CPH nanoparticles adjuvanted with 25 µg CDN (NP+CDN), or 5 µg soluble PA alone (sPA). Total serum IgG antibody titer to PA was quantified via ELISA at 2-weeks, 4-weeks, and 8-weeks post-immunization. Titer values were Log2 transformed and compared at each timepoint for statistical significance between treatment groups via an ordinary one-way ANOVA with a Tukey’s multiple comparison test. P value is indicated as
follows for each comparison indicated (*#@&=p<0.05) (#=NP, &=CDN, @=Combo, *=sPA). Individual animals are shown with bars indicating mean ± SEM.

3.2 CDNs induce higher titer anti-PA antibody responses as compared to AVA BioThrax in a single dose.

In an effort to compare the antibody responses of our CDN and nanoparticle containing formulations to the commercially available AVA BioThrax formulation, a soluble antigen component was added to the NP groups and the overall total antigen increased to match that administered with CDN (i.e., group ii below). Separate groups of C57BL/6 mice were immunized subcutaneously with one of the following formulation: i) rPA encapsulated in 20:80 CPTEG:CPH NPs plus soluble rPA (NP), ii) soluble rPA adjuvanted with CDNs (CDN), iii) a combination of rPA encapsulated in 20:80 CPTEG:CPH nanoparticles plus soluble rPA adjuvanted with 20 µg of CDNs (NP+CDN), iv) the commercially available AVA BioThrax, or v) sham vaccinated controls (PBS). Vaccine induced total anti-rPA IgG antibody titers were compared between adjuvant groups. All groups that were vaccinated induced detectible titers as compared to sham vaccinated control mice. The CDN and CDN+NP groups induced significantly higher antibody titers and compared to NP alone at all time points evaluated. The anti-PA IgG responses induced by rPA + CDNs were also significantly higher at 4-weeks and beyond as compared to the AVA formulation, as well as at 13-weeks compared to the NP+CDN combo (Figure 4-2).
Figure 4-2. Combination nanovaccine comparison with commercial BioThrax formulation. C57BL/6Crl (n=16) mice were vaccinated subcutaneously against the PA protein from *B. anthracis*. The formulations consisted of i) 5 µg PA encapsulated in 20:80 CPTEG:CPH nanoparticles plus 15 µg soluble PA (NP), ii) 20 µg PA adjuvanted with 20 µg of CDN (CDN), iii) a combination of 5 µg PA encapsulated in 20:80 CPTEG:CPH nanoparticles plus 15 µg soluble PA adjuvanted with 20 µg of CDN (NP+CDN), iv) 50 µL of AVA, or v) PBS control. Serum IgG antibody titer to PA was quantified via ELISA at 2-weeks, 4-weeks, 6-weeks, 8-weeks, and 13-weeks post-immunization. Titer values were Log2 transformed and compared at each timepoint for statistical significance between treatment groups via an ordinary one-way ANOVA with a Tukey’s multiple comparison test. P value is indicated as follows for each comparison indicated on the higher magnitude group (*#@&=p<0.05) (#=NP, &=CDN, @=Combo, $=AVA *=PBS). Individual animals are shown with bars indicating mean ± SEM.
3.3 Nanoparticle formulations enhance antibody responses to a linear neutralizing epitope of PA.

In order to evaluate generation of antibodies to known linear epitopes of the *B. anthracis* PA, an array of 102 overlapping peptides was printed onto microarray slides. C57BL/6 (from Figure 4-2) mice were immunized subcutaneously with a formulation consisting of rPA encapsulated in 20:80 CPTEG:CPH NPs plus soluble rPA (NP), soluble rPA adjuvanted with CDNs (CDN), a combination of rPA encapsulated in 20:80 CPTEG:CPH nanoparticles plus soluble PA adjuvanted with 20 µg of CDNs (NP+CDN), the commercially available AVA BioThrax, or unvaccinated PBS control. Serum samples were collected at 4- and 13-weeks post-immunization and assayed for reactivity to a microarray of PA peptides (Figure 4-3a-b). While there is large variability in responses within each individual treatment group, vaccine formulations that contained rPA encapsulated into polyanhydride nanoparticles induced antibodies that recognized an epitope spanning peptides 31 and 32 near the furin binding region. The CDN formulation, despite the overall higher antibody titers (Figure 4-2), did not result in any detectible antibody to this region. This indicates that while formulations containing NP encapsulated rPA had lower titers (Figure 4-2), this vaccine regimen induced antibody responses that recognized differential linear epitopes than formulations without polyanhydride encapsulated antigens.
Figure 4-3. Microarray peptide walk of vaccinated mice to evaluate anti-PA reactive linear epitopes.
C57BL/6 (n=16) mice were vaccinated subcutaneously against the PA protein from *B. anthracis*. The formulations consisted of 5 μg PA encapsulated in 20:80 CPTEG:CPH nanoparticles plus 15 μg soluble PA (NP), 20 μg PA adjuvanted with 20 μg of CDNs (CDN), a combination of 5 μg PA encapsulated in 20:80 CPTEG:CPH nanoparticles plus 15 μg
soluble PA adjuvanted with 20 µg of CDNs (NP+CDN), 50 µL of AVA, or PBS control. Serum samples from vaccinated mice were collected, diluted 1:20, and analyzed at (a) 4- and (b) 13-weeks post immunization for reactivity against 102 peptides (13- to 20-mer) with 10-11 overlapping amino acids spanning the entire length of *B. anthracis* PA.

**3.4 CDNs and the combination of CDNs and NPs induce high magnitude PA neutralizing antibody titers in a single dose.**

While evaluating linear neutralizing epitopes can provide a partial picture of vaccine induced antibody responses to PA, it is necessary to evaluate the overall induction of neutralizing antibodies. Many of these neutralizing epitopes may be conformational in nature and would not be detected by a series of linear peptides or an ELISA. In order to compare the overall neutralizing anti-PA titer, C57BL/6 (from Figure 4-2) mice were vaccinated with a NP formulation containing soluble and encapsulated rPA (NP), rPA adjuvanted with CDNs (CDN), a combination of the NP formulation containing soluble and encapsulated rPA adjuvanted with CDNs (NP+CDN), the AVA BioThrax vaccine, and PBS controls. Serum samples were collected eight weeks post-vaccination and assayed for PA neutralizing antibody titers in an *in vitro* cell based killing assay.

All vaccinated groups were able to induce detectible levels of neutralizing antibodies; however, differences were observed in the magnitudes of these responses that largely mirrored total IgG titer. The CDN, NP+CDN, and AVA BioThrax treated groups were statistically improved over the control mice. The highest neutralizing titer was observed in mice that received the CDN containing formulations as both CDNs and NP+CDNs had significantly increased neutralizing titers as compared to NP alone and the alum containing AVA BioThrax vaccines (Figure 4-4).
Figure 4-4. Evaluation of protective antigen neutralizing titer in B6 mice 8 weeks after vaccination. C57BL/6 (n=16) mice were vaccinated subcutaneously with the PA protein from *B. anthracis*. The individual vaccine formulations consisted of i) 5 µg PA encapsulated in 20:80 CPTEG:CPH nanoparticles plus 15 µg soluble PA (NP), ii) 20 µg PA adjuvanted with 20 µg of CDNs (CDN), iii) a combination of 5 µg PA encapsulated in 20:80 CPTEG:CPH nanoparticles plus 15 µg soluble PA adjuvanted with 20 µg of CDNs (NP+CDN), iv) 50 µL of AVA, or v) PBS control. Neutralizing antibody titers were determined in the 8-week post-vaccination serum via an MTT assay. Dilution values were Log2 transformed and the mean area under the curve (AUC) was calculated for each treatment group. The AUC was compared between treatment groups via one-way ANOVA with a Tukey’s multiple comparison test. P value is indicated as follows (*#@&=p<0.05) (#=NP, &=CDN, @=Combo, $=AVA *=Control) as compared to all other groups in the figure legend. The upper dashed line indicates the no toxin control average OD (3.6), and the
lower dotted line indicates the no sample control average OD (0.7). Group averages are shown with error bars indicating mean ± SEM.

4. Discussion

In this work, the ability of a combination polyanhydride nanoparticle vaccine platform co-adjuvanted with CDNs to induce neutralizing antibodies against the protective antigen of *B. anthracis* was evaluated. The results of this work illustrate the induction of high titer neutralizing antibodies with a single-dose formulation of rPA adjuvanted with CDNs, as well as the induction of antibodies to a linear neutralizing epitope (peptides 31-32) following immunization with polyanhydride nanoparticle vaccine regimen that encapsulated rPA. The two vaccine regimens (CDNs and NP+CDNs) that included CDNs in the formulation outperformed the commercially available AVA BioThrax vaccine with respect to overall anti-PA titer and the induction of toxin neutralizing antibody.

While natural inhalation infection of humans with *B. anthracis* spores is rare, its durable spores and high lethality capable of infecting hundreds of thousands of individuals with a single aerosol dispersion, places *B. anthracis* in a position of prominence as a major biological warfare agent [6,38,39]. The difficulty in diagnosing anthrax, with its early onset of cold-like symptoms, is one of the major hurdles in managing the response to a widespread release event of *B. anthracis* spores [2,38]. If anthrax infection is identified early after exposure, intervention or prophylaxis with antibiotics is critical to prevent serious illness and death. This antibiotic regimen must be adhered to for an extended period of time as spores can persist in a dormant state and cause disease well after the initial exposure [39–41]. In order to respond to a large-scale release, a therapeutic intervention would require a large-scale stockpile of antibiotics to be readily available at all times and would require that health
care workers follow up with individuals to improve upon historically poor patient compliance [42]. Another class of therapeutic interventions in development are toxin neutralizing monoclonal antibodies (mAb). Neutralizing mAbs would provide benefits over antibiotic usage as the two to four-week half-life of the mAbs after infusion could prove effective at preventing severe disease with limited treatments [43,44].

While these effective treatment options are available, deploying them on any large scale remains a public health obstacle. Treatments including vaccination for higher risk populations or those who have been exposed or were potentially exposed remains the most cost effective and most patient friendly option available [45,46]. Even as one of the ideal approaches to dealing with B. anthracis as a biological agent, current vaccines targeting the PA have room for improvement. The major issues that need solving in next generation anti-PA vaccines are i) reducing the number of doses required to effectively induce high neutralizing titer, ii) identification of adjuvants that skew responses to neutralizing epitopes rather than towards non-neutralizing epitopes, and iii) improving the shelf-life stability of the rPA protein and formulated vaccines [13–18].

Encapsulation of protein payloads, including rPA, into polyanhydride particle formulations has illustrated the stability enhancing properties that encapsulation can provide during storage and upon protein release [19,47]. This enhanced stability provided by polyanhydride polymers would be particularly advantageous in the stockpiling and storage of vaccine doses to be readily distributed should the need arise. In immunization studies evaluating the immune response of multiple strains of mice, the NP formulation with 100 % of the rPA encapsulated did not result in a measurable increase in anti-PA antibody titers compare to that induced by rPA alone (Figure 4-1). The inclusion of CDNs as a co-adjuvant,
either alone or combined with the NP formulation, led to increases in anti-PA titers across all three strains of mice. The lone exception to this observation was injection of the NP+CDN combination in C57BL/6NHsd mice (Figure 4-1). At 108 days after the single dose immunization of A/J mice, the total anti-PA IgG response induced by the NP + CDN vaccine exhibited the greatest titer suggesting there is a potential immunological benefit when antigen is encapsulated within and released from nanoparticles over time (Figure 4-1). While undefined, the data indicated that there was a decided influence of mouse genetics on the magnitude of the anti-PA antibody response regardless of vaccine regimen.

In order to improve the overall poor anti-PA IgG response in C57BL/6 mice, the particle containing formulations was modified to also include soluble rPA in addition to the encapsulated rPA. While this modified vaccine regimen did improve anti-PA titers as compared to the previous study (Figure 4-1a), the only group of C57BL/6 mice that developed significantly higher antibody titers in comparison to that induced by the Alum adjuvanted AVA BioThrax formulation was the CDN formulation (Figure 4-2). While total IgG responses are an important indicator of the effectiveness of an PA-containing vaccine formulation, not all PA binding antibodies are neutralizing [48]. While there are no notable differences in the linear epitopes recognized by antisera from mice immunized with the CDN alone or AVA BioThrax vaccine regimen, the formulations containing polyanhydride nanoparticles induced IgG to a neutralizing epitope (peptides 31,32) near the furin binding domain of PA (Figure 4-3). This particular stretch of amino acids is described as a linear neutralizing epitope near the furin binding domain and is a binding location of the monoclonal antibodies (mAb) from the 19D9, a neutralizing IgG, and 20G7 a non-neutralizing IgM, hybridomas [49]. This suggests that some aspect of how the PA is being
presented to B Cells, or through interaction with the polyanhydride particles, degraded polyanhydride strands, or the formulation and delivery process influenced the induction of antibodies to this particular domain. The pH environment can alter the propensity of PA to bind to its receptors and is critical in the formation of a functional pore capable of facilitating LF and EF entry into the cytosol [9–11,50]. The local environment associated with degrading polyanhydrides is likely more acidic than the tissue environment. This may be playing a role in altering rPA interactions with receptors, promoting aggregation, or altering its structure in some way as to make this epitope more available to cross-linking the BCR on B cells.

Conformational epitopes also play a role in effective neutralization of functional PA activity and would not be detected [48]. In cell-based toxin neutralization assays, all tested vaccine formulations induced detectible neutralization of the holotoxin as evidenced by the inhibition of cell death. The only vaccine formulations to significantly improve neutralizing antibody titer, over that induced by the commercially available AVA BioThrax, were the rPA + CDN, and rPA encapsulated in polyanhydride nanoparticles coadjuvanted with CDNs (Figure 4-4). The early high titer responses observed at 2-weeks post immunization in the mice receiving CDNs illustrate a potential strength for vaccination after a large-scale dispersal event. This propensity of CDNs to induce rapid high titers could lead to rapid immunity for individuals in an effected area and limit the danger of the resulting exclusion zone created by B. anthracis spores. Rapid generation of neutralizing antibodies would also have the potential of reducing the extended (60+ days) of prophylactic antibiotic treatments used when potential exposure has occurred.

In conclusion, these studies demonstrate the potential of CDNs to effectively adjuvant rPA to generate high titer total IgG as well as high titer neutralizing antibody responses.
Inclusion of polyanhydride nanoparticles with encapsulated rPA in the vaccine formulation, in an effort to provide potential shelf stability to the rPA led to moderate decreases in overall and neutralizing titer as compared to CDNs alone, however still outperformed the AVA BioThrax formulation. Interestingly, generally lower titer as compared to CDNs alone, NP containing formulations did have a positive effect on the antibody response to a linear neutralizing epitope suggesting a potential skewing towards a higher percentage of neutralizing antibodies. This may suggest that there is some advantageous protein polymer interaction, or other aspect of the polyanhydride particle formulation that facilitate the antibody response toward this particular epitope. In order to take advantage of the superior shelf-life stability offered by polyanhydride copolymer, further studies will be needed to fully elucidate the protein-polymer interactions to enhance the induction of neutralizing antibody titers when using a vaccine formulation with 100% encapsulated antigen with a CDN co-adjuvant.

5. Supplemental Data

Table 4-1. Amino acid sequences of peptides analyzed in the PA peptide microarray.

This table indicates the sequences used for each of the 102 overlapping peptides spanning the length of protective antigen from *Bacillus anthracis*.

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Figure 4-5. Example PA peptide microarrays.
Figure 4-5 Continued

Representative array images of vaccinated mouse serum 13 weeks post immunization of each of the 102 overlapping peptides spanning the length of protective antigen from *Bacillus anthracis*. The whole intact PA protein is printed near the bottom right of each array duplicate and indicated by a white arrow in each array.

6. Acknowledgements

The authors thank Dr. Chris Minion and Andrew Petersen for assisting with the development of the peptide microarray and heatmap generation. The authors also thank Drs. David Kanne, Chudi Ndubaku, and Thomas Dubensky, Jr at Aduro Biotech for providing the cdG used in the *in vitro* and *in vivo* experiments. Funding was provided by support from NIH-NIAID [R01 AI111466]; B.N. also acknowledges the support of the Vlasta Klima Balloun Faculty Chair.
7. References


CHAPTER 5. GENERAL CONCLUSIONS

Conclusions

This work highlights the unique effects polyanhydride nanoparticles and cyclic dinucleotides have on the activation of the innate immune system. In contrast to the overtly inflammatory TLR ligand CpG, the relatively low grade proinflammatory effects polyanhydride nanoparticles activate populations of dendritic cells and upregulate costimulatory function to effectively induce antigen-specific CD8$^+$ T cell memory to improve survival of immunized mice upon tumor challenge. STING pathway stimulation results in activated APCs that exhibit a distinct metabolic profile and little to no induction of NO or ROS. In contrast to TLR ligands, each of these adjuvant-induced phenotypes leads to a beneficial inflammatory environment that allows for improved adaptive immunity upon vaccination.

Polyanhydride particle formulations have previously been described to possess a number of pathogen-mimicking properties [1,2]. For instance, particles are readily taken up by phagocytic cells where they can deliver encapsulated payloads to either the endosomal or cytosolic compartments [3,4]. This interaction induces the activation and subsequent upregulation of costimulatory molecules and secretion of proinflammatory cytokines [5,6]. In addition to antigen delivery and APC activation, the slow degrading properties of polyanhydride particles allows them to persist at the site of injection. As they release their payload over an extended time it can mimic the persistence of antigen over the course of an infection [7,8]. Evaluation of safety and injection site reactogenicity reveals that the in vivo inflammatory response induced by polyanhydride nanoparticles is much lower than traditional adjuvants such as MPLA, alum, or incomplete Freund’s adjuvant [9,10].
Herein, we directly compared the TLR ligand CpG to 20:80 CPTEG:CPH nanoparticles for their ability to activate DC populations and subsequently, generation of memory CD8\(^+\) T cells. Dendritic cells generated from bone-marrow, as well as unfractionated splenic CD11c\(^+\) DCs and the subpopulation of CD8\(\alpha\)^+ DCs were evaluated for their responses. Despite the limited classical signs of activation, such as the induction of proinflammatory cytokines, nitric oxide, and induction of aerobic glycolysis, polyanhydride nanoparticles potently upregulated the expression of costimulatory molecules of all DC populations tested. In contrast, CpG was limited in its ability to upregulate costimulatory expression on CD8\(\alpha\)^+ DCs (Figures 2-3, 2-4, 2-9, 2-10). An analogous low inflammatory vaccine strategy is the use of adoptively transferred DCs, which are capable of rapidly and preferentially inducing high number of CD8\(^+\) memory T cells. In cases where these DCs are exposed to an inflammatory stimulant, such as a TLR ligand, prior to adoptive transfer, leads to increased CD8\(^+\) T cell effector expansion and limits the generation of memory [11]. Indeed, direct comparison of prophylactic vaccine formulations consisting of 20:80 CPTEG:CPH polyanhydride nanoparticles, as compared to CpG, led to relatively greater induction of CD8\(^+\) T cell memory as measured by an antigen-specific tumor challenge (Figure 2-6). This suggests that polyanhydride nanoparticle formulations may be ideal candidates to induce efficacious cell-mediated immunity following the administration of a single dose; an outcome other adjuvant formulations have largely been unable to achieve without employing prime-boost strategies [12].

The STING pathway is a recently discovered PRR pathway that senses nucleic acids in the form of cyclic dinucleotides [13]. CDNs can be released from bacteria or generated from double-stranded DNA, including host-DNA, via the enzyme cGAS [14,15]. The potent
immune stimulatory effects of CDNs are well documented as well as its effectiveness at inducing high titer antibodies when included in vaccine formulations [16–18]. The current literature suggests that CDN stimulation of the immune system results in a balanced or “mixed” response, suggesting STING stimulation results in a unique non-polarized inflammatory environment. In order to elucidate the mechanism mediating the ability of CDN-adjuvanted vaccines to induce such remarkably rapid and high titer antibody responses as compared to TLR ligands (Figure 3-1), various stimulatory effects on innate immune cells were compared to cells stimulated by the TLR family of receptors.

BMDCs stimulated with CDNs exhibited distinctly divergent acute metabolic responses as compared to those stimulated with TLR ligands, particular in the immediate upregulation of mitochondrial respiration (Figure 3-2). Due to the close relationship between metabolic requirements and immunological phenotype, this is indicative of a functionally distinct phenotype [19]. CDN-induced activation results in the lack of production of innate effectors NO and mROS (Figures 3-3, 3-9, 3-10). In BMDCs and the monocyte-like J774 cell line, CDN-induced activation results in cellular activation without the metabolic commitment/shift to aerobic glycolysis (Figure 3-3, 3,8) for survival in the presence of antimicrobial NO. NO has also been implicated in the inhibition of B cells and resultant antibody responses in addition to the inhibition of pro-survival cytokines, particularly BAFF [20,21]. We hypothesized that this low production of NO would improve the production of BAFF via CDN stimulation. Indeed, inhibition of NOS2 led to reduction in NO and increased BAFF secretion from BMDCs stimulated with TLR ligands, comparable to the uninhibited CDN group (Figure 3-4). We also tested the effects of NO in vivo and found that short-term inhibition of NOS2 in mice receiving an MPLA adjuvanted vaccine, led to improved
antibody titers at 2-weeks and 32-weeks post vaccination (Figure 3-5). This leads us to conclude that the inflammatory environment created by CDN administration results in an improvement of B cell survival and subsequently, greater magnitude antibody production. While an antimicrobial innate inflammatory environment, as a result of vaccination, may not lead to major shortcomings of vaccine efficacy in young and healthy individuals, immunologically disadvantaged individuals, such as the aged, may be less capable of efficacious adaptive immunity in such an environment [22]. We hypothesized that the use of CDNs as a vaccine adjuvant would lead to improved vaccine-induced humoral responses in aged animals as compared to a TLR-targeting adjuvant. Indeed, the CDN adjuvanted formulation led to relatively greater concentrations of serum BAFF and higher antigen specific antibody titers at both 28- and 75-days post-vaccination (Figure 3-6). These results suggest that the potently activating, yet less pathogen-mimicking nature of CDNs and the STING pathway, avoid negative effects associated with innate immune responses involved in the clearance of microbes. These properties led to an overall more rapid induction and higher magnitude of adaptive immune responses to vaccine antigens.

Polyanhydride nanoparticles, in addition to their adjuvant capacity, are also capable of stabilizing encapsulated antigen [8,23,24]. This would be particularly advantageous for vaccine formulations containing antigens that exhibit poor shelf stability, such as PA from Bacillus anthracis. Previous work evaluating combination CDN and NP vaccines have proven successful in generating rapid immunity to Yersinia pestis [18]. In order to determine the ideal formulation targeting PA from B. anthracis, a PA containing combination NP and CDN formulation was evaluated, along with each adjuvant individually. Overall the NP formulation did not significantly improve antibody responses over soluble PA alone, while
CDNs and the combination of NPs and CDN lead to significantly higher antibody titers (Figure 4-1, 4-2). The single-dose CDN and combination formulations also lead to significantly increased titers of PA-specific neutralizing antibody as compared to the currently approved AVA BioThrax vaccine (Figure 4-4). This suggest that a combination CDN and NP vaccine would deliver both shelf stability as well single-dose high magnitude neutralizing antibody titers able to rapidly protect against infectious disease and avoid the need for long-term antibiotic treatments.

Overall, the results of these studies suggest that the innate immune environment created as a consequence of vaccine adjuvants has a major impact on the magnitude and nature of the resultant humoral and T cell responses. The generation of antimicrobial NO and ROS, while critical in the innate immune-mediated clearance and control of many pathogens, imposes a cost on the adaptive immune response. In the formulation of recombinant subunit-based vaccines, where no MAMPs are present and innate immune-mediated clearance is superfluous, identification of adjuvants and pathways that can avoid paying this adaptive cost of innate immune activation (i.e., a Toll too high) can result in improvements in vaccine-induced immunity and may represent a paradigm shift in vaccine design.

The properties of CDNs and polyanhydride nanoparticles make them ideal candidates as vaccine adjuvants. This would be particularly advantageous for individuals who have an underlying immunological deficiency or in situations where the rapid induction of high titer neutralizing antibody is needed, such as an act of biological warfare where induction of rapid immunity is required. Lastly, as vaccine formulations are designed and tested, we need to consider the effects of artificially signaling through a single pathway in a relative vacuum. Whether it be TLR ligands generating STING inhibiting ROS or STING
generation of NO inhibiting type 1 interferons, natural infection results in activation of multiple pathways that exhibit cross-regulating properties. Care needs to be taken to ensure that vaccine adjuvants are not resulting in a more extreme phenotype of activation than occurs with natural infection.

**Bibliography**


