Published in final edited form as:

Vaccine. 2019 May 06; 37(20): 2721–2730. doi:10.1016/j.vaccine.2019.04.004.

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

Ross J Darling¹, Sujata Senapati², Sean M Kelly², Marian L Kohut^{3,4}, Balaji Narasimhan^{2,4}, and Michael J Wannemuehler^{1,4}

¹Iowa State University, Department of Veterinary Microbiology and Preventative Medicine

²Iowa State University, Department of Chemical and Biological Engineering

³Iowa State University, Department of Kinesiology

⁴Nanovaccine Institute, Iowa State University

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.

Corresponding Author: Michael J Wannemuehler, mjwannem@iastate.edu.

Author contributions

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.

Conflict of interest statement

The authors have no potential conflicts of interest to disclose.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

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.

2.0 Introduction

Because of their relative 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, the TLR family signaling through MYD88 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 (i.e., activated antigen-specific cells and serum BAFF) which is linked to a rapid and greater magnitude of the vaccine-induced antibody responses in both aged and young mice.

3.0 Materials and Methods

3.1 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.

3.2 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]-ethanimidamine, 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.

3.3 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 2.5×10^5

cells per well. Mitochondrial stress test was carried out according to manufacturer's MST protocol (Agilent, Santa Clara, CA). Concentrations of 1 μ M oligomycin, 2 μ M FCCP, and 0.5 μ M rotenone and antimycin were used (Agilent, Santa Clara, CA). Kinetic stimulation assays were conducted with non-stimulated BMDCs seeded at 2.5×10^5 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).

3.4 Bone marrow dendritic cell and macrophage generation

Bone marrow was collected from femurs and tibias of BALB/c mice. Cells (4×10^6 cells per 100 mm plate) were washed and plated in 10 mL of complete RPMI 1640 medium (100 U/mL penicillin and 100 μ g/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.

3.5 *In vitro* APC stimulation

BMDCs or BMMs were plated at 5×10^5 cells/well in a 96-well round bottom tissue culture plate in 200 μ L of the previously described complete RPMI 1640 medium. Stimulants used included 5 μ g/mL CpG ODN 1668, 5 μ g/mL imiquimod, 1 μ g/mL LPS, 1 μ g/mL MPLA, 0.5 μ g/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 *in vitro* experiments, the NOS2 inhibitor 1400W was used at a concentration of 50 μ M.

3.6 Flow Cytometry

Following stimulation APCs were analyzed for costimulatory marker expression using flow cytometry. 5×10^5 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 μ g/mL of rat IgG (Sigma Aldrich, St. Louis, MO) and 10 μ g/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), CD40 (eBioscience, APC, clone 1C10), CD11c (Biolegend, APC-Cy7, clone N418), MHCII (eBioscience, AF700, clone M5/114.15.2), PDCA-1 (eBioscience, PE-eFluor610, clone eBio927), CD103 (Biolegend, Brilliant Violet 610, clone 2e7), and CD8 α (Biolegend, BV421, clone 53-67) 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).

3.7 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 μ M to 0 μ M. 100 μ L of standard or supernatant was added to 100 μ L 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.

3.8 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.

3.9 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 μ L of Ova (5 μ g/mL PBS) or PA (0.5 μ g/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 4C. 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.

4.0 Results

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

Studies incorporating the use of CDNs in recombinant subunit vaccine studies have illustrated an 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 1). Mice were vaccinated with 50 μ g of Ova alone, or adjuvanted with either 20 μ g of monophosphoryl lipid A (MPLA) or Imiquimod as representative TLR ligands, or 20 μ g 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 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 1).

4.2 CDN stimulation of BMDCs results in early glycolytic 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 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 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 2e–f) while TLR stimulation results in depressed functionality of mitochondrial activity (Figure 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 2). 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.

4.3 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 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). 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 4).

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 3b).

4.4 *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 of 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 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 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 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 innate effector molecule NO.

4.5 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 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 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 5c).

4.6 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 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 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 6b).

5.0 Discussion

As the trend to use recombinant proteins in 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 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 very different than that induced by TLR agonists (Figure 2). Studies assessing immunometabolism 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 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 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 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 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 6a) and the significantly higher ($p < 0.05$) antibody titers to Ova in the aged mice immunized with the CDN formulation (Figure 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 because 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 and 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 FcRgIIIa (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-nonsel (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. CDNs 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

The authors 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

Funding was provided by support from NIH-NIAID [R01 AI111466]; the Iowa State University Nanovaccine Institute; B.N. acknowledges the support of the Vlasta Klima Balloun Faculty Chair.

Abbreviations

APC	Antigen presenting cell
BAFF	B cell activating factor
BDC	Bone marrow-derived dendritic cells
BMM	Bone marrow macrophage
cdG	cyclic di-GMP
CDN	Cyclic dinucleotide
DAMP	Damage associated molecular pattern
DC	Dendritic cell
LCMV	Lymphocytic choriomeningitis virus
MAMP	Microbial associated molecular pattern
MPLA	Monophosphoryl lipid a
mROS	mitochondrial reactive oxygen species
MST	Mitochondrial stress test

NO	Nitric oxide
Ova	Ovalbumin
PA	Protective antigen
PRR	Pattern recognition receptor
STING	Stimulator of interferon genes
TLR	Toll like receptor

Bibliography

- [1]. Bonam SR, Partidos CD, Halmuthur SKM, Muller S. An Overview of Novel Adjuvants Designed for Improving Vaccine Efficacy. *Trends Pharmacol Sci* 2017;38:771–93. doi:10.1016/j.tips.2017.06.002. [PubMed: 28668223]
- [2]. Coffman RL, Sher A, Seder RA. Vaccine adjuvants: Putting innate immunity to work. *Immunity* 2010;33:492–503. doi:10.1016/j.immuni.2010.10.002. [PubMed: 21029960]
- [3]. O'Hagan DT, Fox CB. New generation adjuvants - From empiricism to rational design. *Vaccine* 2015;33:B14–20. doi:10.1016/j.vaccine.2015.01.088. [PubMed: 26022561]
- [4]. Abdul-Cader MS, Amarasinghe A, Abdul-Careem MF. Activation of toll-like receptor signaling pathways leading to nitric oxide-mediated antiviral responses. *Arch Virol* 2016;161:2075–86. doi:10.1007/s00705-016-2904-x. [PubMed: 27233799]
- [5]. Serbina N V, Salazar-Mather TP, Biron CA, Kuziel WA, Pamer EG. TNF/iNOS-producing dendritic cells mediate innate immune defense against bacterial infection. *Immunity* 2003;19:59–70. doi:10.1016/S1074-7613(03)00171-7. [PubMed: 12871639]
- [6]. García-Ortiz A, Serrador JM. Nitric Oxide Signaling in T Cell-Mediated Immunity. *Trends Mol Med* 2018;24:412–27. doi:10.1016/j.molmed.2018.02.002. [PubMed: 29519621]
- [7]. Lee M, Rey K, Besler K, Wang C, Choy J. Immunobiology of Nitric Oxide and Regulation of Inducible Nitric Oxide Synthase. *Results Probl Cell Differ* 2017;62:181–207. doi:10.1007/978-3-319-54090-0_8. [PubMed: 28455710]
- [8]. Blaser H, Dostert C, Mak TW, Brenner D. TNF and ROS Crosstalk in Inflammation. *Trends Cell Biol* 2016;26:249–61. doi:10.1016/j.tcb.2015.12.002. [PubMed: 26791157]
- [9]. Olekhnovitch R, Ryffel B, Müller AJ, Bousso P. Collective nitric oxide production provides tissue-wide immunity during *Leishmania* infection. *J Clin Invest* 2014;124:1711–22. doi:10.1172/JCI72058. [PubMed: 24614106]
- [10]. Deguchi M, Inaba K, Muramatsu S. Counteracting effect of interferon- α and - β on interferon- γ -induced production of nitric oxide which is suppressive for antibody response. *Immunol Lett* 1995;45:157–62. doi:10.1016/0165-2478(94)00246-N. [PubMed: 7558167]
- [11]. Sammiceli S, Kuka M, Di Lucia P, de Oya NJ, De Giovanni M, Fioravanti J, et al. Inflammatory monocytes hinder antiviral B cell responses. *Sci Immunol* 2016;1:1–11. doi:10.1126/sciimmunol.aah6789.
- [12]. Schneider P, Tschopp J. BAFF and the regulation of B cell survival. *Immunol Lett* 2003;88:57–62. doi:10.1016/S0165-2478(03)00050-6. [PubMed: 12853163]
- [13]. Shah HB, Joshi SK, Rampuria P, Devera TS, Lang GA, Stohl W, et al. BAFF- and APRIL-Dependent Maintenance of Antibody Titers after Immunization with T-Dependent Antigen and CD1d-Binding Ligand. *J Immunol* 2013;191:1154–63. doi:10.4049/jimmunol.1300263. [PubMed: 23797666]
- [14]. Giordano D, Draves KE, Li C, Hohl TM, Clark EA. Nitric oxide regulates BAFF expression and T cell-independent antibody responses. *J Immunol* 2014;193:1110–20. doi:10.4049/jimmunol.1303158. [PubMed: 24951820]
- [15]. Vora KA, Wang LC, Rao SP, Liu Z-Y, Majeau GR, Cutler AH, et al. Cutting Edge: Germinal Centers Formed in the Absence of B Cell-Activating Factor Belonging to the TNF Family

- Exhibit Impaired Maturation and Function. *J Immunol* 2003;171:547–51. doi:10.4049/jimmunol.171.2.547. [PubMed: 12847217]
- [16]. Schmid-Schonbein GW. Nitric oxide (NO) side of lymphatic flow and immune surveillance. *Proc Natl Acad Sci* 2012;109:3–4. doi:10.1073/pnas.1117710109. [PubMed: 22178757]
- [17]. Jang KJ, Mano H, Aoki K, Hayashi T, Muto A, Nambu Y, et al. Mitochondrial function provides instructive signals for activation-induced B-cell fates. *Nat Commun* 2015;6:6750. doi:10.1038/ncomms7750. [PubMed: 25857523]
- [18]. Okin D, Medzhitov R. Evolution of inflammatory diseases. *Curr Biol* 2012;22:R733–40. doi:10.1016/j.cub.2012.07.029. [PubMed: 22975004]
- [19]. Min H, Montecino-Rodriguez E, Dorshkind K. Effects of aging on early B- and T-cell development. *Immunol Rev* 2005;205:7–17. doi:10.1111/j.0105-2896.2005.00263.x. [PubMed: 15882341]
- [20]. Simon AK, Hollander GA, McMichael A. Evolution of the immune system in humans from infancy to old age. *Proc R Soc B Biol Sci* 2015;282. doi:10.1098/rspb.2014.3085.
- [21]. Tabibian-Keissar H, Hazanov L, Schiby G, Rosenthal N, Rakovsky A, Michaeli M, et al. Aging affects B-cell antigen receptor repertoire diversity in primary and secondary lymphoid tissues. *Eur J Immunol* 2016;46:480–92. doi:10.1002/eji.201545586. [PubMed: 26614343]
- [22]. Banete A, Seaver K, Bakshi D, Gee K, Basta S. On taking the STING out of immune activation. *J Leukoc Biol* 2018;1–7. doi:10.1002/JLB.2MIR0917-383R.
- [23]. Jenal U, Reinders A, Lori C. Cyclic di-GMP: Second messenger extraordinaire. *Nat Rev Microbiol* 2017;15:271–84. doi:10.1038/nrmicro.2016.190. [PubMed: 28163311]
- [24]. Ebensen T, Schulze K, Riese P, Link C, Morr M, Guzmán CA. The bacterial second messenger cyclic diGMP exhibits potent adjuvant properties. *Vaccine* 2007;25:1464–9. doi:10.1016/j.vaccine.2006.10.033. [PubMed: 17187906]
- [25]. Danilchanka O, Mekalanos JJ. Cyclic Dinucleotides and the Innate Immune Response. *Cell* 2013;154:962–70. doi:10.1016/j.cell.2013.08.014. [PubMed: 23993090]
- [26]. Cai X, Chiu Y-HH, Chen ZJJ. The cGAS-cGAMP-STING pathway of cytosolic DNA sensing and signaling. vol. 54 Cell Press; 2014. doi:10.1016/j.molcel.2014.03.040.
- [27]. Qiao JT, Cui C, Qing L, Wang LS, He TY, Yan F, et al. Activation of the STING-IRF3 pathway promotes hepatocyte inflammation, apoptosis and induces metabolic disorders in nonalcoholic fatty liver disease. *Metabolism* 2018;81:13–24. doi:10.1016/j.metabol.2017.09.010. [PubMed: 29106945]
- [28]. Wagner-Muñiz DA, Kelly SM, Peroutka-Bigus N, Darling RJ, Petersen AC, Bellaire BH, et al. Single-Dose Combination Nanovaccine Induces Both Rapid and Long-Lived Protection Against Pneumonic Plague. To Be Submitt NPJ Vaccines n.d.
- [29]. Krawczyk CM, Holowka T, Sun J, Blagih J, Amiel E, DeBerardinis RJ, et al. Toll-like receptor-induced changes in glycolytic metabolism regulate dendritic cell activation. *Blood* 2010;115:4742–9. doi:10.1182/blood-2009-10-249540. [PubMed: 20351312]
- [30]. Everts B, Pearce EJ. Metabolic control of dendritic cell activation and function: Recent advances and clinical implications. *Front Immunol* 2014;5:203. doi:10.3389/fimmu.2014.00203. [PubMed: 24847328]
- [31]. Thwe PM, Amiel E. The role of nitric oxide in metabolic regulation of Dendritic cell immune function. *Cancer Lett* 2018;412:236–42. doi:10.1016/j.canlet.2017.10.032. [PubMed: 29107106]
- [32]. Everts B, Amiel E, Huang SCC, Smith AM, Chang CH, Lam WY, et al. TLR-driven early glycolytic reprogramming via the kinases TBK1-IKKe supports the anabolic demands of dendritic cell activation. *Nat Immunol* 2014;15:323–32. doi:10.1038/ni.2833. [PubMed: 24562310]
- [33]. Simioni PU, Fernandes LGGR, Tamashiro WMSCM. Downregulation of L-arginine metabolism in dendritic cells induces tolerance to exogenous antigen. *Int J Immunopathol Pharmacol* 2017;30:44–57. doi:10.1177/0394632016678873. [PubMed: 27903843]
- [34]. O'Neill LAJ, Pearce EJ. Immunometabolism governs dendritic cell and macrophage function. *J Exp Med* 2016;213:15–23. doi:10.1084/jem.20151570. [PubMed: 26694970]
- [35]. Araki K, Youngblood B, Ahmed R. The role of mTOR in memory CD8+ T-cell differentiation. *Immunol Rev* 2010;235:234–43. doi:10.1111/j.0105-2896.2010.00898.x. [PubMed: 20536567]

- [36]. Everts B, Amiel E, Van Der Windt GJW, Freitas TC, Chott R, Yarasheski KE, et al. Commitment to glycolysis sustains survival of NO-producing inflammatory dendritic cells. *Blood* 2012;120:1422–31. doi:10.1182/blood-2012-03-419747. [PubMed: 22786879]
- [37]. Mills EL, Kelly B, Logan A, Costa ASH, Varma M, Bryant CE, et al. Succinate Dehydrogenase Supports Metabolic Repurposing of Mitochondria to Drive Inflammatory Macrophages. *Cell* 2016;167:457–470.e13. doi:10.1016/j.cell.2016.08.064. [PubMed: 27667687]
- [38]. Schweighoffer E, Vanes L, Nys J, Cantrell D, McCleary S, Smithers N, et al. The BAFF Receptor Transduces Survival Signals by Co-opting the B Cell Receptor Signaling Pathway. *Immunity* 2013;38:475–88. doi:10.1016/J.IMMUNI.2012.11.015. [PubMed: 23453634]
- [39]. Mitchell TC, Casella CR. No pain no gain? Adjuvant effects of alum and monophosphoryl lipid A in pertussis and HPV vaccines. *Curr Opin Immunol* 2017;47:17–25. doi:10.1016/j.coi.2017.06.009. [PubMed: 28728074]
- [40]. Medzhitov R, Janeway CA. Decoding the patterns of self and nonself by the innate immune system. *Science* 2002;296:298–300. doi:10.1126/science.1068883. [PubMed: 11951031]
- [41]. Matzinger P The danger model: a renewed sense of self. *Science* 2002;296:301–5. doi:10.1126/science.1071059. [PubMed: 11951032]
- [42]. Igarashi K, Kurosaki T, Roychoudhuri R. BACH transcription factors in innate and adaptive immunity. *Nat Rev Immunol* 2017;17:437–50. doi:10.1038/nri.2017.26. [PubMed: 28461702]
- [43]. Van den Bossche J, O'Neill LA, Menon D. Macrophage Immunometabolism: Where Are We (Going)? *Trends Immunol* 2017;38:395–406. doi:10.1016/j.it.2017.03.001. [PubMed: 28396078]
- [44]. Zhang L, Romero P. Metabolic Control of CD8 + T Cell Fate Decisions and Antitumor Immunity. *Trends Mol Med* 2017;24. doi:10.1016/j.molmed.2017.11.005.
- [45]. Si C, Zhang R, Wu T, Lu G, Hu Y, Zhang H, et al. Dendritic cell-derived nitric oxide inhibits the differentiation of effector dendritic cells. *Oncotarget* 2016;7:74834–45. doi:10.18632/oncotarget.11361 [doi]. [PubMed: 27556858]
- [46]. Yamamoto MD, Fazle Akbar MF, Masumoto, Onji. Increased nitric oxide (NO) production by antigen-presenting dendritic cells is responsible for low allogeneic mixed leucocyte reaction (MLR) in primary biliary cirrhosis (PBC). *Clin Exp Immunol* 1998;114:94–101. doi:10.1046/j.1365-2249.1998.00696.x. [PubMed: 9764609]
- [47]. Diavatopoulos DA, Edwards KM. What is wrong with pertussis vaccine immunity?: Why immunological memory to pertussis is failing. *Cold Spring Harb Perspect Biol* 2017;9:a029553. doi:10.1101/cshperspect.a029553. [PubMed: 28289059]
- [48]. Vidarsson G, Dekkers G, Rispens T. IgG subclasses and allotypes: From structure to effector functions. *Front Immunol* 2014;5:1–17. doi:10.3389/fimmu.2014.00520. [PubMed: 24474949]
- [49]. Hailemichael Y, Dai Z, Jaffarizad N, Ye Y, Medina MA, Huang X-F, et al. Persistent antigen at vaccination sites induces tumor-specific CD8+ T cell sequestration, dysfunction and deletion. *Nat Med* 2013;19:465–72. doi:10.1038/nm.3105. [PubMed: 23455713]
- [50]. Bingisser RM, Tilbrook PA, Holt PG, Kees UR. Macrophage-derived nitric oxide regulates T cell activation via reversible disruption of the Jak3/STAT5 signaling pathway. *J Immunol* 1998;160:5729–34. doi:10.1017/CBO9781107415324.004. [PubMed: 9637481]

Highlights

- CDNs induce high titer vaccine antibody responses.
- STING pathway stimulation leads to innate immune activation with low NO and ROS production.
- Low NO production is associated with higher DC secretion of and concentration of serum BAFF.
- *In vivo* inhibition of NO production by a TLR based adjuvant leads to improved antibody responses.
- Relative to TLR agonists, CDNs result in high titer antibody responses in aged animals.

Figure 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.

Figure 2. Acute metabolic effects 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 2.5×10^5 cells per well in a Seahorse plate coated with Cell-Tak. Extracellular acidification rate (ECAR) was measured as an indicator of glycolysis. **(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 2.5×10^5 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 $\pm$ SEM. Data shown is a single experimental replicate that is representative of at least one additional experimental repeat.

Figure 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 $\pm$ 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.

Figure 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 $\pm$ 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.

Figure 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 μ g Ova alone (sOva) or with 20 μ g 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 $\pm$ 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.

Figure 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 μ g Ova plus 20 μ g 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 $\pm$ SEM.