Cyclic Dinucleotide–Adjuvanted Dengue Virus Nonstructural Protein 1 Induces Protective Antibody and T Cell Responses

Diego A. Espinosa,* P. Robert Beatty,* Gabrielle L. Reiner,† Kelsey E. Sivick,† Laura Hix Glickman,‡ Thomas W. Dubensky, Jr.,§ and Eva Harris* 

Endothelial dysfunction and vascular leak, pathogenic hallmarks of severe dengue disease, are directly triggered by dengue virus (DENV) nonstructural protein 1 (NS1). Previous studies have shown that immunization with NS1, as well as passive transfer of NS1-immune serum or anti-NS1 mAb, prevent NS1-mediated lethality in vivo. In this study, we evaluated the immunogenicity and protective capacity of recombinant DENV NS1 administered with cyclic dinucleotides (CDNs), potent activators of innate immune pathways and highly immunogenic adjuvants. Using both wild-type C57BL/6 mice and IFN-α/β receptor–deficient mice, we show that NS1-CDN immunizations elicit serotype-specific and cross-reactive Ab and T cell responses. Furthermore, NS1-CDN vaccinations conferred significant homotypic and heterotypic protection from DENV2-induced morbidity and mortality. In addition, we demonstrate that high anti-NS1 Ab titers are associated with protection, supporting the role of humoral responses against DENV NS1 as correlates of protection. These findings highlight the potential of CDN-based adjuvants for inducing Ab and T cell responses and validate NS1 as an important candidate for dengue vaccine development. The Journal of Immunology, 2019, 202: 000–000.

As an immunogen, formulations based on recombinant NS1 induce strong immune responses and confer protection in rodent models of dengue disease (5–8). Furthermore, passive transfer of polyclonal sera from NS1-immunized mice or anti-NS1 mAb into naive mice conferred protection against vascular leak disease, likely by promoting lysis of infected cells and/or by blocking the pathogenic effects of secreted NS1 (5, 9, 10). Importantly, NS1 is ~64–79% conserved across the four DENV serotypes (11, 12), and immunodominant regions of this protein have been identified in both NS1-immunized and DENV-infected mice, as well as in naturally infected humans (10, 13–16). The role of NS1-specific CD4+ T cells against DENV infection is a topic of active investigation, and studies have provided evidence of the likely protective effect of these cells (17–20). Taken together, these findings support further research of NS1 as an important Ag for dengue vaccine development.

One of the main challenges of nonreplicating vaccines is the use of adjuvants capable of eliciting strong memory T cells and protective Abs (21, 22). Cyclic dinucleotides (CDNs) are ubiquitous second messengers synthesized by bacteria, which are capable of activating the cytosolic receptor stimulator of IFN genes (STING), resulting in the activation of different immune pathways (23–27). Due to their immunostimulatory properties, CDNs were initially used as vaccine adjuvants to elicit protective Ab and T cell responses against pathogenic extracellular bacteria (28–30). However, more recent studies have shown that CDN compounds also have a significant capacity to induce potent antitumor responses (31–33) and to elicit protective Th1 and Th17 cellular immune responses against Mycobacterium tuberculosis infection (34).

In this study, we evaluated the immunogenicity of DENV NS1 proteins together with CDN compounds in comparison with monophosphoryl lipid A (MPLA) adjuvant, a TLR4 agonist capable of eliciting strong Th1 responses and high Ab titers (35–38) that has been approved for human use in vaccines for hepatitis B virus and human papillomavirus infection (39, 40). Using both wild-type C57BL/6 (WTB6) and IFN-α/β receptor–deficient C57BL/6 (Ifnar−/−) mice, we measured IgG titers and T cell responses after immunizations with each NS1 from all four DENV
serotypes. We found that NS1-CDN vaccinations induced balanced Ab responses against all DENV NS1 serotypes, which were comparable or higher in magnitude to those elicited by NS1-MPLA immunizations. Furthermore, NS1-CDN–immunized mice developed serotype-specific and cross-reactive T cell responses, greater than MPLA-adjuvanted NS1, underscoring the ability of CDN compounds to induce cellular immunity. Finally, using a mouse model of lethal DENV infection, we show that NS1 combined with CDNs confers significant protection against DENV-induced morbidity and mortality.

Materials and Methods

Ethics statement

All experimental procedures involving the use of animals were preapproved and performed according to the guidelines of the Institutional Animal Care and Use Committee of the University of California, Berkeley.

Mice

WTB6 and Ifnar−/− mice were bred and coted in specific pathogen-free conditions at the University of California, Berkeley, Animal Facility. Five- to eight-week-old male and female mice were used for all experimental procedures.

Recombinant NS1 proteins

Recombinant NS1 proteins from DENV serotypes 1 (Nauru/Western Pacific/1974), 2 (Thailand/16681/84), 3 (Sri Lanka D3/H/IMTSSA-SRI/2000/1266), and 4 (Dominica/814669/1981) were purchased from The Native Antigen Company (Oxford, U.K.).

Viruses

DENV2 D220 was generated in our laboratory from the parental strain DENV2 PLO46 (41). The virus was propagated in A. albopictus C6/36 cell line (American Type Culture Collection) and titered by plaque assay on baby hamster kidney cells (BHK21, clone 15). The parental strain DENV2 PLO46 was obtained originally from H.-Y. Lei (National Cheng Kang University, Taiwan).

NS1 immunizations

Mice were immunized s.c. two times (study days 0 and 21) with 20 μg of NS1 or OVA in combination with 5–15 μg CDN compounds or 1 μg MPLA, each formulated in AddaVax (0.5% sorbitan trioleate, 5% squalene, 0.5% Tween-80 in 10 mM sodium citrate buffer). CDN compounds dithio-(Rp,Rp)–cyclic[G2(5′)pA(3′,5′)p] (mixed-linkage dithio cGMP-AMP (ML-RR-cGAMP)) and a derivative of this compound consisting of cyclic [G(2′,5′)pA(3′,5′)p] comprising Rp,Rp-bisphosphorothioate linkages (ML-RR-cGAMP-D) were produced by Aduro Biotech as previously reported (31, 34, 42). MPLA from Salmonella minnesota R595 and AddaVax were acquired from InvivoGen (San Diego, CA).

Anti-NS1 ELISA

Ab responses induced by NS1 were evaluated 1 wk following the second immunization. Blood samples were collected via submandibular bleed to evaluate polyclonal sera against recombinant NS1 by ELISA. Briefly, MaxiSorp ELISA plates (Thermo Scientific Nunc) were coated with 50 μl of NS1 (0.5 μg/ml) and incubated overnight at room temperature. After blocking with 1% BSA in PBS (1% BSA-PBS), wells were incubated for 1 h at room temperature with 100 μl of serial dilutions of polyclonal sera. Plates were then washed and incubated for 1 h at room temperature with peroxidase-labeled goat anti-mouse secondary Abs (Jackson ImmunoResearch) at 0.5 μg/ml in 1% BSA-PBS. After a washing step, the assay was developed using an ABTS-HRP substrate kit (KPL), according to the manufacturer’s specifications. Ab titters are reported as area under the curve, calculated using GraphPad Prism 6 software.

T cell assays

T cell responses in mouse spleen were evaluated 1 wk after the second immunization. For ELISPOT assays, plates were coated with anti-mouse IFN-γ Ab (BD Biosciences) and incubated overnight at 4°C. Plates were then blocked with tissue culture media (RPMI 1640, 10% FCS, and 5% penicillin-streptomycin), and single-cell suspensions (2 × 106 cells/well) were incubated with 1 μg/ml NS1 protein, 1 μM OVA CD4 peptide (263-277 SIINFEKL), or 1 μM OVA CD8 peptide (266-TETWSSNVMEERKIVK,269) for a minimum of 18 h at 37°C. Biotinylated anti-IFN-γ Ab (BD Biosciences) was then added, followed by a washing step and addition of streptavidin-conjugated alkaline phosphatase. The assay was developed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate substrate (Thermo Fisher Scientific). For intracellular cytokine staining (ICS), 2 × 106 cells were incubated at 37°C for 1 h with 1 μg/ml DENV NS1 protein, followed by 4 h in the presence of 1 μg/ml brefeldin A (GolgiPlug; BD Biosciences) and 2 μM monensin (GolgiStop; BD Biosciences). Cells were stained with the viability dye Zombie Green (BioLegend), CD8α-BUV395, CD4-BUV737, Ly6G-FITC, CD90.2 V500 (BD Biosciences), and MHCII-FITC (BioLegend). Cells were then treated with Cytotox/Cytoperm Fixation/Permeabilization Kit (BD Biosciences) and stained with IL-2 PE, T NF-α-PE/Cy7, IFN-γ APC/647 (BioLegend).

In vitro assessment of ADE

Serial dilutions of polyclonal sera were mixed with DENV2 D220 for 1 h at 37°C and then mixed with human erythrocytemic K562 cells at a multiplicity of infection of 1 for 24 h in a 96-well plate. Cells were then washed with FACS buffer and fixed in 2% paraformaldehyde for 10 min at room temperature. Cells were stained using 2.5 μg/ml 4G2-Alexa 488 (Invitrogen) upon permeabilization with FACS buffer containing 0.1% saponin (Sigma-Aldrich). K562 cells were then washed, and the percentage of infection was determined using Guava flow cytometer (EMD Millipore) by gating Alexa 488–positive cells.

DENV challenge experiments

To evaluate the protective capacity of DENV NS1-CDN vaccinations, immunized Ifnar−/− mice were challenged with 5 μg of 4G2 (anti-envelope mAb) 20–24 h prior to infection with 3 × 105 PFU of DENV2 D220. Virus challenge was administered by i.v. injection 4 wk after the second immunization, and mice were monitored every 12 h using a morbidity scoring system on a scale of 1–5 (41). Mice were immediately euthanized when they became moribund (score of 5).

Statistical analysis

Data were analyzed and plotted using GraphPad Prism 6 software. Differences in Ab and T cell responses between two treatment groups were evaluated using a two-sided unpaired Student t test. Comparison of survival rates was conducted using a log-rank (Mantel–Cox) test and graphed as Kaplan–Meier survival curves.

Results

CDN compounds induce robust anti-NS1 Ab and T cell responses

To characterize immune responses elicited by NS1 vaccination, WTB6 and Ifnar−/− mice were immunized with DENV2 NS1 in combination with the CDN compound ML-RR-cGAMP, a stable, lipophilic derivative of the endogenous mammalian CDN ML-cGAMP, or MPLA, a TLR4 agonist, each formulated in a squalene-in-water emulsion (AddaVax). As determined by ELISA, WTB6 animals immunized with NS1 in combination with CDN compounds developed higher total IgG responses compared with those vaccinated with MPLA. However, total IgG titers in Ifnar−/− mice were comparable among all NS1-immunized groups, irrespective of the adjuvant used. Characterization of IgG subclass titers revealed higher Th1-associated IgG2b and IgG2c isotype responses in WTB6 animals immunized with NS1 and ML-RR-cGAMP or a ML-RR-cGAMP derivative (ML-RR-cGAMP-D), although MPLA induced higher IgG2b titers in Ifnar−/− mice. Furthermore, the two CDN compounds and MPLA induced comparable anti-DENV2 NS1 IgG1 and IgG3 titers in WTB6 and in Ifnar−/− mice (Fig. 1A, 1D). ELISPOT analysis of T cell responses in spleen showed higher IFN-γ production in mice immunized with NS1 in combination with ML-RR-cGAMP or ML-RR-cGAMP-D as compared with MPLA-NS1–vaccinated animals (Fig. 1B, 1E). A similar trend was observed by ICS analysis, which also revealed higher TNF-α and IL-2 production as a result of CDN-NS1 immunizations, indicating a polyfunctional T cell activation status (Fig. 1C, 1F). In addition, immunizations...
were immunized twice with 20 μg of DENV2 NS1 in combination with ML-RR-cGAMP, ML-RR-cGAMP-D, or MPLA, each formulated in AddaVax, and immune responses were evaluated 1 wk following the second immunization. Anti-NS1 IgG titers were measured by direct ELISA using sera from WTB6 and Ifnar−/− (A) and Ifnar−/− (D) mice. Calculations of area under the curve (AUC) based on serially diluted serum samples are shown. Ag-specific IFN-γ responses induced by recombinant DENV2 NS1 were measured by ELISpot with splenocytes from WTB6 (B) and Ifnar−/− (E) mice. Frequencies of DENV2 NS1-specific IFN-γ−, TNF-α−, and IL-2-producing CD4+ T cells in the spleen of WTB6 (C) and Ifnar−/− (F) mice were measured by ICS. Data are representative of two independent experiments (mean ± SEM; n = 3–4). *p < 0.05, **p < 0.01.

ML-RR-cGAMP-D elicits balanced anti-NS1 humoral responses against all DENV NS1 serotypes and induces a Th1-associated Ab profile in WTB6 mice

To evaluate the effect of CDNs on the induction of serotype-specific and cross-reactive DENV NS1 Ab responses, mice were immunized with NS1 from DENV serotypes 1–4 in combination with ML-RR-cGAMP-D or MPLA. Ab responses in polyclonal sera were measured by ELISA using recombinant NS1 from all four DENV serotypes. In general, immunization with DENV NS1 in combination with ML-RR-cGAMP-D or MPLA resulted in comparable anti-NS1 responses in both WTB6 and Ifnar−/− mice. However, WTB6 mice immunized with DENV3 NS1 in combination with ML-RR-cGAMP-D developed higher anti-DENV1 NS1 and anti-DENV3 NS1 IgG titers than when MPLA was used as an adjuvant. Similarly, DENV2 NS1-ML-RR-cGAMP-D-immunized WTB6 animals generated higher anti-DENV2 NS1 titers than DENV2 NS1-MPLA-immunized mice (Supplemental Fig. 1). In Ifnar−/− mice, vaccination with DENV1 NS1 or DENV4 NS1 adjuvanted with ML-RR-cGAMP-D resulted in higher Ab titers against DENV1 NS1 than those elicited with MPLA formulations. Furthermore, Ifnar−/− mice immunized with DENV4 NS1-ML-RR-cGAMP-D had higher anti-DENV3 NS1 IgG titers than DENV4 NS1-MPLA-immunized mice. In contrast, Ifnar−/− mice vaccinated with DENV3 NS1-MPLA developed higher anti-DENV1 NS1 titers than those immunized with DENV3 NS1-ML-RR-cGAMP-D (Fig. 2B).

To further characterize humoral responses induced by NS1-ML-RR-cGAMP-D immunization, we quantified anti-NS1 IgG subclass responses. Notably, IgG2c responses, typically associated with skewing of immune responses toward a Th1 phenotype, were significantly higher in WTB6 mice immunized with ML-RR-cGAMP-D and NS1, regardless of the DENV NS1 serotype used for immunization (Fig. 2C). This trend, however, was not observed in Ifnar−/− mice (Fig. 2D). Compared with MPLA-adjuvanted NS1 immunization, IgG1 titers were significantly higher in WTB6 mice vaccinated with DENV2 NS1 or DENV3 NS1 in combination with ML-RR-cGAMP-D, although in Ifnar−/− mice, higher IgG1 titers were achieved upon DENV1 NS1- or DENV4 NS1-ML-RR-cGAMP-D vaccination (Supplemental Fig. 2A, 2B). In contrast, MPLA-adjuvanted DENV2 NS1 and DENV3 NS1 induced stronger IgG2b responses than those elicited by ML-RR-cGAMP-D-adjuvanted vaccinations in Ifnar−/− mice, although no significant differences were found in WTB6 animals (Supplemental Fig. 2B, 2E). Finally, IgG3 titers elicited by ML-RR-cGAMP-D or MPLA in combination with NS1 were comparable among all DENV NS1 serotypes, in both WTB6 and Ifnar−/− mice (Supplemental Fig. 2C, 2F).

ML-RR-cGAMP-D induces serotype-specific and cross-reactive DENV NS1 T cell responses

Several studies have established that CDN compounds are effective inducers of T cell immune responses (reviewed in Ref. 27). To assess the ability of ML-RR-cGAMP-D to elicit cellular immunity against the different DENV serotype NS1 proteins, we measured responses in the spleen of immunized mice using ELISpot assays. In WTB6 mice, immunization with DENV2 NS1 or DENV3 NS1 in combination with ML-RR-cGAMP-D elicited significantly higher IFN-γ serotype-specific production than MPLA-NS1 vaccination (Fig. 3A). A similar trend was observed in Ifnar−/− mice, where DENV2 NS1 and DENV4 NS1 adjuvanted with ML-RR-cGAMP-D induced significantly stronger responses than MPLA-adjuvanted
immunization (Fig. 3C). Cross-reactive responses were assessed by stimulating splenocytes with each of the NS1 proteins from the DENV serotypes that were not used for immunization (e.g., splenocytes from mice immunized with DENV1 NS1 were stimulated with NS1 proteins from DENV serotypes 2, 3, and 4). Notably, in both WTB6 and Ifnar−/− mice, immunization with DENV3 NS1 in combination with ML-RR-cGAMP-D resulted in strong IFN-γ responses against DENV1 NS1. However, immunization with NS1 from DENV serotypes 1, 2, and 4 failed to induce considerable cross-reactive responses, and none of the MPLA-adjuvanted immunizations elicited significant IFN-γ production (Fig. 3B, 3D). Finally, ICS analysis of cross-reactive CD4+ T cell responses

**FIGURE 2.** ML-RR-cGAMP-D induces Ab responses against all four DENV NS1 serotypes and skews immunity toward the Th1 phenotype in WTB6 mice. Mice were immunized twice with 20 μg of NS1 from each DENV serotype in combination with ML-RR-cGAMP-D or MPLA formulated in AddaVax. Total IgG titers against NS1 from DENV1–4 were assessed by direct ELISA 1 wk after the second immunization in WTB6 (A) and Ifnar−/− (B) mice. Anti-NS1 IgG2c responses in WTB6 (C) and Ifnar−/− (D) mice were used as surrogate markers of Th1 responses. Titers are shown as area under the curve (AUC) based on serially diluted serum samples. Data are representative of two independent experiments (mean ± SEM; n = 4). *p < 0.05, **p < 0.01.
responses from immunized WTB6 mice showed similar results to those from ELISpot assays. Vaccination with DENV2 NS1 or DENV3 NS1 adjuvanted with ML-RR-cGAMP-D elicited significant serotype-specific IFN-γ, TNF-α, and IL-2 production, whereas only DENV3 NS1 immunization resulted in strong cross-reactive responses against DENV1 NS1 (Supplemental Fig. 3).

**ML-RR-cGAMP-NS1 vaccination confers significant protection against DENV-induced pathogenesis**

Given the ability of CDN compounds to induce substantial Ab and T cell responses, we tested whether immunization with NS1 in combination with ML-RR-cGAMP could prevent lethal DENV disease in Ifnar−/− mice. ML-RR-cGAMP is a potent activator of T cell immunity, capable of binding all common STING alleles (data not shown).

### **ML-RR-cGAMP-NS1 vaccination confers significant protection against DENV-induced pathogenesis**

Given the ability of CDN compounds to induce substantial Ab and T cell responses, we tested whether immunization with NS1 in combination with ML-RR-cGAMP could prevent lethal DENV disease in Ifnar−/− mice. ML-RR-cGAMP is a potent activator of T cell immunity, capable of binding all common STING alleles (data not shown). Ifnar−/− mice were immunized two times with 20 μg of DENV1-4 NS1 and ML-RR-cGAMP over a 3-wk period (days 0 and 21) and challenged with DENV2 D220 4 wk after the last immunization (day 49) using ADE conditions. Vaccination with DENV1 NS1, DENV2 NS1, or DENV3 NS1 in combination with ML-RR-cGAMP resulted in significant protection compared with OVA-immunized animals and naive controls (Fig. 4A). Vaccination with DENV1 NS1, DENV2 NS1, or DENV3 NS1 in combination with ML-RR-cGAMP resulted in significant protection compared with OVA-immunized mice and naive controls (Fig. 4A). Vaccination with DENV1 NS1, DENV2 NS1, or DENV3 NS1 in combination with ML-RR-cGAMP resulted in significant protection compared with OVA-immunized mice and naive controls (Fig. 4A).

**Anti-DENV2 NS1 Ab responses induced by ML-RR-cGAMP-NS1 vaccination are associated with protection against lethal dengue challenge**

Previously, we showed that passive transfer of NS1-immune serum and anti-NS1 mAb prevents NS1-induced lethality in vivo (5). To determine if Ab responses in mice immunized with ML-RR-cGAMP-NS1 were associated with protection, serum samples were tested by ELISA against NS1 proteins from the four DENV serotypes. Notably, mice that were protected against lethal DENV challenge developed significantly higher anti-DENV2 NS1 IgG titers than those that succumbed to the infection. Ab titers against DENV1 NS1 and DENV3 NS1 showed a similar trend, albeit differences between protected and nonprotected groups were not statistically significant (Fig. 6).

**Sera from mice immunized with ML-RR-cGAMP-NS1 do not enhance DENV infection in vitro**

ADE is the phenomenon by which weakly neutralizing and/or insufficient amounts of pre-existing anti-DENV Abs facilitate DENV infection, mortality was significantly delayed (p < 0.05) compared with OVA-immunized animals. As a positive control, a low-dose (10^5 PFU) primary homologous infection with the parental DENV2 strain PL046 conferred 100% protection against lethal challenge (Fig. 4B). Finally, in two independent experiments, immunizations with ML-RR-cGAMP in combination with DENV2 NS1 conferred similar or greater protection than MPLA-DENV2 NS1 vaccinations against lethal DENV2 D220 infection (Fig. 5).
infection of FcR-bearing cells. This results in increased viral replication and can potentially lead to more severe disease. Because NS1 is not a structural component of the DENV virion, anti-NS1 Abs should not enable ADE. To examine whether sera from mice immunized with ML-RR-cGAMP-NS1 could enhance DENV infection in vitro, pooled serum samples were incubated with DENV2 D220 virus and used to infect K562 cells, a human erythroleukemic cell line that expresses FcγRIIA (CD32A) and is nonpermissive in the absence of enhancing Abs. Importantly, sera from mice vaccinated with DENV NS1 in combination with ML-RR-cGAMP did not allow infection of K562 cells. In contrast, sera from mice immunized with PL046 enabled DENV2 D220 infection, with peak enhancement between 1:135 and 1:405 serum dilutions (Supplemental Fig. 4).

![Image of Figure 4](https://example.com/image4.png)

**FIGURE 4.** Immunization with DENV NS1 and ML-RR-cGAMP significantly decreases DENV-induced morbidity and mortality in Ifnar−/− mice. Mice were immunized s.c. with 20 μg of each DENV NS1 serotype (n = 10) or OVA (n = 9) in combination with ML-RR-cGAMP/AddaVax on days 0 and 21, or were infected with a sublethal dose (1 × 10^5 PFU) of DENV2 PL046 (n = 6) on day 0. On day 49, mice were challenged i.v. with lethal Ab-enhanced DENV2 D220 infection. (A) Signs of DENV-induced morbidity were assessed daily for 10 d. The percentage of each group of mice displaying the indicated signs is shown. (B) A Kaplan–Meier survival curve is shown. Mice immunized with NS1 were significantly protected compared with OVA controls. Data are pooled from two similar experiments (naive group, n = 10). *p < 0.05, **p < 0.01, ***p < 0.001.
Discussion

Earlier research had indicated that NS1 antigenemia in DENV-infected patients is associated with disease severity (43–45); however, it was not shown until recently that NS1 is directly involved in triggering endothelial permeability and vascular leak, and that immune responses against NS1 can prevent lethal DENV infection (5, 46). On the basis of these findings, we sought to characterize immune responses and assess the protective efficacy of immunization with NS1 in combination with the CDN compound ML-RR-cGAMP or ML-RR-cGAMP-D, which are phosphodiesterase resistant, highly lipophilic activators of all common human STING alleles (31). Because immunocompetent WTB6 mice are not susceptible to DENV infection, immune responses to NS1-CDN vaccinations were assessed in parallel in Ifnar\(^{-/-}\) mice, an in vivo model for mimicking key features of severe human dengue (41).

Compared with DENV2-MPLA vaccination, both WTB6 and Ifnar\(^{-/-}\) mice developed similar or higher IgG responses upon immunization with DENV2 NS1 in combination with ML-RR-cGAMP or ML-RR-cGAMP-D. With the exception of IgG2b titers in Ifnar\(^{-/-}\) mice, IgG1, IgG2c, and IgG3 subclass titers were also superior or equivalent in mice immunized with NS1-CDNs compared with NS1-MPLA–immunized animals. The ability of MPLA to induce robust Ab responses against clinically relevant Ags is well documented (47); however, few studies have assessed humoral responses elicited by viral proteins in combination with CDN compounds (48–51). Therefore, these results provide additional evidence demonstrating the ability of mammalian cGAMP homologs to induce robust and protective Ab responses.

In both WTB6 and Ifnar\(^{-/-}\) mice, immunization with NS1 in combination with CDNs induced overall more robust T cell responses than NS1-MPLA vaccination. Similar results were obtained when using OVA as an immunogen, which also allowed us to assess CD8\(^+\) T cell responses by circumventing the lack of cytotoxic T cell epitopes in DENV NS1 for the C57BL/6 (H-2K\(^{b}\)) background. As expected, immunization of NS1 adjuvanted with ML-RR-cGAMP and ML-RR-cGAMP-D resulted in higher frequencies of cytokine-producing CD4\(^+\) and CD8\(^+\) T cells than immunization with MPLA. Nonetheless, previous research indicates that MPLA is capable of inducing considerable T cell responses at higher immunization doses (52, 53). There is a paucity of studies addressing the possible protective role of NS1-specific T cells against DENV infection. However, investigations in DENV-infected patients indicate that CD4\(^+\) T cells preferentially target NS1, the envelope, and capsid, the same proteins thought to be recognized by B cells (18). In clinical trials, the NIH TV-003 DENV vaccine candidate induced NS1-specific CD4\(^+\) and CD8\(^+\) T cells against DENV infection (17). Thus, these data suggest that the
induction of NS1-specific CD4+ T cell responses by dengue vaccine candidates may significantly contribute to protective efficacy.

Immunization with each DENV NS1 serotype in combination with ML-RR-cGAMP/AddaVax were tested by direct ELISA against DENV1 NS1 (panel A), DENV2 NS1 (panel B), DENV3 NS1 (panel C), and DENV4 NS1 (panel D). These are the same mice evaluated for morbidity and mortality in Fig. 4. Mice that did not succumb to DENV2 D220 infection developed significantly higher anti-DENV2 NS1 IgG titers than nonprotected mice. Data are representative of two independent experiments (protected, n = 23; nonprotected, n = 17). *p < 0.05.

**FIGURE 6.** Anti-DENV2 NS1 Ab titers are associated with protection from DENV2-induced mortality. Sera from mice immunized with each DENV NS1 serotype in combination with ML-RR-cGAMP/AddaVax were tested by direct ELISA against DENV1 NS1 (A), DENV2 NS1 (B), DENV3 NS1 (C), and DENV4 NS1 (D). These are the same mice evaluated for morbidity and mortality in Fig. 4. Mice that did not succumb to DENV2 D220 infection developed significantly higher anti-DENV2 NS1 IgG titers than nonprotected mice. Data are representative of two independent experiments (protected, n = 23; nonprotected, n = 17). *p < 0.05.
such as MPLA, may be suboptimal for T cell priming against infectious disease Ags.

The induction of potent T cell responses by soluble protein-in-adjuvant formulations has remained a difficult task. CDNs have shown promising results as vaccine adjuvants for intracellular pathogens in animal models, and their immunostimulatory properties can be readily optimized through chemical synthesis (27). Importantly, phase I clinical trials for a synthetic human STING-activating CDN (ADU-S100) as a chemotherapeutic agent alone and in combination with checkpoint inhibition are currently in progress (ClinicalTrials.gov NCT02675439 and NCT03172936). However, to date there are no human studies assessing CDNs as adjuvants against infectious agents.

In conclusion, we demonstrate that NS1-CDN immunizations induce potent Ab and T cell responses in both WT6 and Iffd−/− mice and confer significant protection from lethal DENV infection. We also show that anti-NS1 Ab titers in NS1-immunized mice are associated with protection, adding to previous research indicating that humoral responses against DENV NS1 are potential correlates of protection. Taken together, our results support the inclusion of NS1 in dengue vaccine candidates.

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Disclosures
G.L.R. and K.E.S. are both employees of Aduro Biotech, Inc. and own stock in the company. L.H.G. owns Aduro Biotech, Inc. stock. G.L.R., K.E.S., L.H.G., A.E. Gould, and T.W.D. may be inventors on patent applications that apply to the CDN technology. However, to date there are no human studies assessing CDNs as adjuvants against infectious agents.

References


SUPPLEMENTARY FIGURE 1. ML-RR-cGAMP and ML-RR-cGAMP-D induce higher frequency of antigen-specific T-cell responses than MPLA in WTB6 and Ifnar\(^{-/-}\) mice. Mice were immunized twice with 20 \(\mu\)g of OVA in combination with ML-RR-cGAMP, ML-RR-cGAMP-D or MPLA, each formulated in AddaVax; immune responses were evaluated 1 week following the second immunization. Splenic antigen-specific IFN-\(\gamma\) responses induced by OVA were measured by ELISpot upon stimulation with OVA CD4 peptide (265TEWTSSNVMEERKIKV\(_{280}\)) or OVA CD8 peptide (257SIINFEKL\(_{264}\)) in WTB6 (A) and Ifnar\(^{-/-}\) (D) mice. The frequency of OVA-specific IFN-\(\gamma\)-, TNF-\(\alpha\)- and IL-2-producing CD4\(^+\) and CD8\(^+\) T cells in spleen were measured by ICS upon stimulation with OVA CD4 peptide and OVA CD8 peptide, respectively; responses were measured in WTB6 (B-C) and Ifnar\(^{-/-}\) (E-F) mice. Data are representative of 2 independent experiments (mean \(\pm\) SEM; \(n=3\); * \(p<0.05\)).
SUPPLEMENTARY FIGURE 2. ML-RR-cGAMP-D and MPLA induce comparable IgG subclass responses in WTB6 and Ifnar−/− mice. Mice were immunized two times with 20 μg of each DENV NS1 serotype in combination with ML-RR-cGAMP-D or MPLA, each formulated in AddaVax. IgG1, IgG2b and IgG3 titers against homotypic NS1 proteins (i.e., against the same NS1 protein serotype used for immunization) were assessed by direct ELISA 1 week after the second immunization in WTB6 (A-C) and Ifnar−/− (D-F) mice. Titers are shown as area under the curve (AUC) based on serially diluted serum samples. Data are representative from 2 independent experiments (mean ± SEM; n=4; * p<0.05, ** p<0.01, *** p<0.001).
SUPPLEMENTARY FIGURE 3. ML-RR-cGAMP-D elicits serotype-specific and cross-reactive anti-NS1 CD4+ T-cell responses in WTB6 mice. T-cell responses in spleens from mice immunized twice with 20 μg of each DENV NS1 serotype in combination with ML-RR-cGAMP-D (white symbols) or MPLA (gray symbols) formulated in AddaVax were assessed by ICS 1 week after the second immunization. Serotype-specific (A-C) and cross-reactive (D-F) IFN-γ, TNF-α and IL-2 production by CD4+ T cells was measured upon stimulation with NS1 proteins from DENV1 (square), DENV2 (circle), DENV3 (triangle) or DENV4 (diamond). Mean ± SEM; n=4; * p<0.05, ** p<0.01, *** p<0.001.
SUPPLEMENTARY FIGURE 4. Immune sera induced by DENV NS1-ML-RR-cGAMP/AddaVax immunizations do not enhance *in vitro* DENV infection. DENV2 D220 was incubated with serially diluted pooled serum samples from *I*fnar*Δ/Δ* mice immunized with DENV NS1 or OVA in combination with ML-RR-cGAMP and used to infect K562 cells. Sera from mice immunized with DENV2 PLO46 were used as positive control. Data are plotted as the average of duplicate values, where the absolute percent infection of K562 cells is shown on the y-axis. Data are representative from 2 independent experiments.