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CpG Oligodeoxynucleotides Protect Newborn Mice from a Lethal Challenge with the Neurotropic Tacaribe Arenavirus

João A. Pedras-Vasconcelos,* David Goucher,* Montserrat Puig,† Leonardo H. Tonelli,‡ Vivian Wang,* Shuichi Ito,‡ and Daniela Verthelyi1*§

The innate immune system is key to limiting the early spread of most pathogens and directing the development of Ag-specific immunity. Recently, a number of synthetic molecules that activate the innate immune system by stimulating TLRs have been identified. Among them, synthetic oligodeoxynucleotides (ODNs) containing unmethylated CpG motifs (CpG ODNs) were shown to activate TLR9-bearing B cells, macrophages, and dendritic cells to induce a strong proinflammatory milieu and a type 1-biased immune response that protects mice from a variety of parasitic, bacterial, and viral infections. Although the protective effect of CpG ODN in adult mice was well established, its effectiveness in neonates, which have lower numbers of dendritic, B, and T cells and tend to favor Th2 responses, was unclear. This study uses the New World arenavirus Tacaribe, a neurotropic pathogen that is lethal in newborn mice, to explore the effectiveness of TLR-mediated innate immune responses. Neonatal BALB/c mice treated with CpG ODN at the time of infection had reduced viral load (p < 0.01) and increased survival (52%, p < 0.001 i.p.; 36%, p < 0.05 intranasally). Protection was achieved in mice treated no later than 3 days postchallenge and appears to be mediated by an increase in Ag-specific Abs (IgG and IgM) and to require inducible NO synthase expression and NO production. To our knowledge, this is the first study assessing the mechanisms by which CpG ODN can protect mice from a neurotropic viral infection. The Journal of Immunology, 2006, 176: 4940–4949.

The neonatal period is characterized by increased susceptibility to infections, particularly by upper respiratory tract pathogens (1, 2). Several factors are thought to contribute to this susceptibility, including lack of pre-existing memory to common pathogens, immature secondary lymphoid organs, impaired neuron activation (3), and weaker B cell responses, as neonates tend to have lower IgG2a in mice and IgG2 in humans (4). In addition, newborn mice tend to have fewer CD4+ T cells and a Th2 bias as compared with adults (1). Recent reports suggest that the innate immune cell activation via TLR may be impaired in newborns as well (4–6).

Over the past decade, TLRs and their ligands (natural and synthetic) have been considered as potential targets for immunoprotective therapies through direct stimulation of the immune system. In particular, the activation of TLR7 and TLR8 by guanosine- and uridine-rich ssRNA or synthetic imidazoquinoline-like molecules such as resiquimod (R848) or the activation of TLR9 by bacterial DNA or synthetic oligodeoxynucleotides (ODNs) containing unmethylated CpG motifs triggers an immune cascade that results in the proliferation, differentiation, and maturation of multiple immune cells, including B and T lymphocytes, NK cells, monocytes, macrophages, and dendritic cells (7–9). Together, these cells secrete cytokines and chemokines that create a proinflammatory immune milieu.

In murine models, treatment with CpG ODN reduces the severity and time course of infection and facilitates the clearance of bacteria, parasites, and viruses such as herpes simplex virus type 2, Friend retrovirus, and influenza (10–12). In turn, topical application of imidazolines has been effective against infections such as human papillomavirus and herpes simplex virus (13).

Recent studies demonstrating that resident CNS cells, including microglia, astrocytes, and neurons, express Toll receptors and are capable of generating an innate immune response suggested that TLR ligands might be used to control neurotropic infection (14–16). However, direct administration of CpG ODN intracerebrally induced acute local inflammation and death, and it was uncertain whether systemic administration of a Toll agonist would activate the immune cells in the CNS in vivo to control an infection.

To assess whether TLR agonists can act as immunoprotective agents against a neurotropic pathogen in neonatal mice, we used a murine model of the Arenaviridae family. The New World arenavirus (Tacaribe serocomplex) is a growing family of enveloped, segmented RNA viruses of increasing medical importance that currently has 23 identified members (17). Four of those viruses (the Junin, Machupo, Guanarito, and Sabia viruses) are causative agents of South American hemorrhagic fevers. Infections occur usually via the respiratory route as a consequence of inadvertent human contact with viruses in rodent feces and urine. Because of their high pathogenic potential, ease of growth in culture, and plastic genomic structure, several arenaviruses have been included in the list of potential biowarfare agents (class 1A) by the Center for Disease Control and Prevention (Atlanta, GA) (18). The Tacaribe virus (TCRV) is a biochemically and serologically close relative of...
the Junin virus but has a low pathogenic potential for humans and is more easily amenable to laboratory study. Experimentally, it causes lethal meningoencephalitis in mice younger than 1 wk old with death occurring 1–3 wk later, depending on the mouse strain, dose, and route of inoculation (19). In this study we demonstrate that CpG ODN treatment can activate the newborn’s innate immune system to protect against an otherwise lethal neurotropic infection with the New World arenavirus Tacaribe.

Materials and Methods

Reagents and Media
Phosphorothioate CpG ODN (5′-GCTAGACGTTAGCGT-3′; underlined portion represents the active CpG motif) and control ODN (5′-GCTA-GAGCTTAGCCTG-3′) were synthesized at the Center for Biologies Evaluation and Research (CBER; Rockville, MD) core facility. All ODNs had <0.1 endotoxin units of endotoxin per milligram of ODN as assessed by a Limulus amebocyte lysate assay (QCL-1000; BioWhittaker). R848 (re-siquimod) was resuspended as per the manufacturer’s instructions (InvivoGen).

mAbs to TCRV were provided by Dr. M. J. Buchmeier of the Scripps Research Foundation (La Jolla, CA) and have been previously described (20). Convalescent sera were generated from 21-day-old mice that had been challenged with TCRV on day 7 of life. Abs to CD45, IL-12 (C15.6 and C17.8), and IL-6 (MP-20F3 and MP-35C21) were obtained from BD Biosciences. Abs to glial fibrillary acidic protein (GFAP) and to neuron-specific enolase (NSE) were purchased from Chemicon International. Abs to TCRV strain TRVL 11573 were obtained from American Type Culture Collection (Rockville, MD), MT/B cell KO mice were provided by Dr. D. Jankovic (National Institute of Allergy and Infectious Diseases, Bethesda, MD). Mice were housed in sterile microisolator cages in the CBER specific pathogen-free animal facility and bred at 6–12 wk of age. All experiments were approved by the Food and Drug Administration Animal Care and Use Committee.

Neonatal mice were infected with 2000 TCID50 of TCRV i.p., intranasally (i.n.) (10–20 μl), or intracranially (i.c.) (10 μl) 1–3 days after birth, alone or with CpG ODN, control ODN (50 μg/mouse), or R848 (25 μg/mouse) using syringes (Hamilton) and 30-gauge needles (BD Biosciences). Mice that died within 24 h after inoculation were excluded from the study. Uninfected mice that received CpG ODN or saline were used as controls. Administration was performed i.n. by placing a 10-μl drop of TCRV and/or CpG ODN or control ODN on the nostrils. The mice were kept in prone position and allowed to inhale the solution (2–4 min). Delivery and absorption (i.n.) was confirmed by confocal microscopy using fluorescently labeled ODN.

The immunomodulatory treatment was administered at the time of infection (day 0), 3 days prior to infection (day −3), or 3 or 6 days postinfection (day 3 or day 6) as described in Results. In some experiments neonatal mice were treated with the NO synthase inhibitor aminoguanidine (AMG) (40 μg/mouse in 20 μl; Sigma-Aldrich) daily, starting on the day of infection and for a period of 10–12 days. Some mice received convalescent serum transfers (30 μl i.p.) 3 and 8 days postinfection obtained from mice infected on day 7 and bled on day 21 postinfection. The convalescent sera derived from infected mice or from infected mice treated with CpG ODN had similar levels of IgG and IgM Abs to TCRV. For each condition was assessed in 2–5 independent experiments. As previously reported (22), neonatal mice treated with CpG ODN i.p. or i.n. at therapeutic doses experienced no obvious delay in development or weight loss. CpG ODN administered i.c. induced acute encephalitis and death within 24 h. The mice were monitored daily, but, unless stated, infections were allowed to proceed to their natural outcome. Sera were prepared in Microtainer serum separator tubes (BD Biosciences) and kept frozen until used. Brains and spleens were collected under sterile conditions.

Immunohistochemistry
Brain pathology was assessed at 5 and 10 days postchallenge. Briefly, mice were euthanized and perfused intracardially with cold PBS followed by 4% paraformaldehyde. Brains were then dissected, cryopreserved, and mounted in OCT (Triangle Biochemical Sciences) freezing medium. Serial sagittal sections (15-μm thick) were then collected at −400 μm intervals spanning an entire hemisphere, allowing for systematic analysis of the cerebellum, upper spinal cord, and brain stem. After blocking in 4% normal goat serum plus 0.01% Triton X-100 (Sigma-Aldrich) in PBS for 1 h at room temperature, the slides were labeled with Abs to TCRV (1/500), GFAP, NSE, CD45, and/or Diablo (all at 1/250) for 1 h at room temperature followed by anti-donkey-Cy5, anti-mouse-Cy3, or anti-rabbit-Cy5 (at 1/400 for 1 h at room temperature; Chemicon International). Lastly, samples were rinsed with PBS and mounted with a fluorophore stabilizer (Invitrogen Life Technologies). Controls (not shown) included slides unstained and stained with isotype and species-matchedAbs or with secondary Abs alone. Slides were coded and analyzed by a “blinded” reader. Tissues were examined with a Zeiss LSM Pascal laser confocal microscope. Multipass emissions were collected through Cy3 (bandpass 560–615) and Cy-5 (longpass 650) filters. High-resolution images using the same settings were collected for three separate cerebellar folia, the cerebellar nucleus, and the upper spinal chord of each brain. All acquired images were exported from Zeiss LSM version 2.8 as full-resolution merged TIFF images for analysis with IMARIN (www.fda.gov/cber/research/imaging/imageanalysis.html). All images were then batch analyzed and assessed for individual pixel counts. Viral Ag coclonization analysis was confirmed by acquiring Z-stacks of selected areas with the x63 objective.

Quantitative real-time RT-PCR
Total RNA was prepared from individual brains of neonatal mice using TRIzol (Invitrogen Life Technologies) and then purified with RNaseasy (Qiagen). For mRNA cytokine detection, total RNA (500 ng/sample) was reverse transcribed into cDNA using an iScript cDNA synthesis Kit (BioRad) as per the manufacturer’s instructions. cDNA samples were treated with RNase H (Invitrogen Life Technologies) 30 min at 37°C and stored at −20°C until used for quantitative RT-PCR.

Quantitative RT-PCR was conducted using the IQ SYBR Green Supermix kit (Bio-Rad) as described (23). Primers used are given in Table I. Values for each target gene were normalized using rat 18S rRNA. Expression values were calculated using the

Table 1. Primers used for RT-PCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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<tr>
<td>TNF-α</td>
<td>5′-TGAGCAGCAGAAGGAGCTAGC-3′</td>
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<tr>
<td>3′-GCAGGAATGAGAAGGCTAGAC-3′</td>
<td></td>
</tr>
<tr>
<td>IFN-γ</td>
<td>5′-GGATGGCAGCATGAAATGTCG-3′</td>
</tr>
<tr>
<td>3′-GCCGTAGATGTTGACTAGG-3′</td>
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2−ΔΔCt method (24). To assess the expression of type-1 IFN-inducible genes, total RNA (1 μg/sample) was reverse transcribed into cDNA using the First-Strand cDNA Synthesis Kit (Amersham Biosciences) as per the manufacturer’s instructions. Relative mRNA levels for the Prkr, Irf7, Isg54, Mx2, and Oas1γ were assessed using a mouse multigene-12 RT-PCR profiling kit (SuperArray Bioscience) as per the manufacturer’s instructions. Relative amounts of mRNA for the individual IFN-inducible genes were calculated by first normalizing with the endogenous gene (GADPH) and subsequently calculating the fold increase in respect to the background (control animals) by using Image Gage 4.1 (Fuji Photo Film) software on gel-scanned images.

**Cytokine and Ab assays**

Cytokine levels were assessed in supernatants (72 h) of splenocytes (5 × 10⁷ cell/well) cultured in R10 medium at 37°C in the presence or absence of heat-killed 10⁵ TCID₅₀ viruses/well. Levels of IFN-α, IFN-γ, IL-12 q70, and IL-6 were assessed by ELISA as described (25). Briefly, 96-well Immunomullon 2 HB plates (Thermo Electron) were coated with cytokine-specific Ab and then blocked with PBS plus 1% BSA (Sigma). After washing, the plates were overlaid with the supernatant for 3 h and then washed and treated with the appropriate biotinylated secondary Ab followed by alkaline phosphatase-conjugated avidin (BD Biosciences). Standard curves using recombinant cytokines were generated to quantify the responses. Virus-specific Ab levels were tested in sera using Immunomullon 1 HB plates coated with UV-irradiated TCRV (at 1/500 in PBS). After blocking, pooled mouse sera (1/50) was added to the wells (2 h) followed by washing and alkaline phosphatase-conjugated Abs. (Southern Biotech Associates). Absorbance was read at 405 nm after 30 min. Pooled sera from surviving adult mice boosted with virus injection i.p. (10⁶ TCID₅₀/mouse) were used as positive control reference standard. The number of cells secreting cytokines and Abs were determined by ELISPOT as described (26).

**Statistical analysis**

Differences in survival curves were evaluated using the Kaplan-Meier method to determine survival fractions and the Mantel-Haenszel log rank test to determine p values. Changes in Ab or cytokine expression were analyzed by parametric or nonparametric ANOVA as appropriate. p values of <0.05 were considered significant.

**Results**

**Effect of CpG ODN administration on survival of neonatal mice infected with the TCRV**

Activation of the innate immune system by administration of CpG ODN or R848 results in the protection from infection in several animal models, leading to their consideration as immunoprotective agents (9, 27, 28). To assess whether R848 and/or CpG ODN can protect neonatal mice from infection, BALB/c mice were infected (i.p.) with TCRV on days 1–3 of life. Untreated mice infected with TCRV developed limb paralysis and died within 18 days of challenge. Treatment with R848 (25 μg/mouse i.p.) did not prevent death; however, 52% of mice treated with CpG ODN (50 μg) i.p. on the day of infection survived into adulthood (Fig. 1A; p < 0.001). The survival rates were similar in mice treated with 25, 50, or 100 μg/mouse (data not shown). The effect was TLR9-dependent, as no protection was evident when mice were treated with ODN lacking an active CpG motif (control ODN; Fig. 1A) or in mice lacking TLR9 expression (Fig. 1B).

Because New World arenaviruses are often transmitted to people via the respiratory route, we next assessed whether CpG ODN could also improve the survival of mice infected by this route. Mice infected with the TCRV i.n. died within 15 days of challenge. Intranasal administration of CpG ODN (50 μg) immediately following infection resulted in 36% survival (Fig. 1C; p < 0.01). Although i.p. administration of CpG protected mice from an i.n. challenge (50% survival), i.n. administration of CpG did not protect the mice from an i.p. TCRV infection (not shown).

**CpG ODN protects mice from an ongoing Tacaribe infection**

Several studies suggest that the optimal time for the administration of CpG ODN varies depending on the pathogen. Empirical data indicate that delivery of CpG ODN following infection resulted in 36% survival (Fig. 1C; p < 0.01).

**Statistical analysis**

Differences in survival curves were evaluated using the Kaplan-Meier method to determine survival fractions and the Mantel-Haenszel log rank test to determine p values. Changes in Ab or cytokine expression were analyzed by parametric or nonparametric ANOVA as appropriate. p values of <0.05 were considered significant.

**FIGURE 1.** CpG ODNs (concurrent or 3 days postchallenge) protect neonatal mice from lethal TCRV infection in a TLR9-dependent manner. Neonatal BALB/c (A, C, and D) or TLR9 KO (B) mice (1–3 days old) were infected with TCRV (2000 TCID₅₀ of strain TR11573 in 10 μl) i.p. (A, B, and D) or i.n. (C). A, Infected mice were treated i.p. with CpG ODN, control ODN lacking the CpG motif (50 μg/mouse), or R848 (resiquimod; 25 μg/mouse i.p.) at the time of infection. B, TLR9 KO mice treated with CpG ODN i.p. on the day of the challenge. C, Mice were challenged and treated (i.n.) on the day of the infection. D, Mice treated (i.p.) 3 days after the virus challenge (i.p.). The mice were monitored daily, but TCRV infection was allowed to proceed to its natural outcome. Note the improved survival in mice treated with CpG ODN (i.p. concurrent: ***, p < 0.001(A); i.p. postchallenge: **, p < 0.01 (D); i.n. concurrent: ***, p < 0.001(C)) compared with untreated mice. CpG ODN alone did not modify survival of the neonates. Results show data from 3 to 5 experiments. For statistical analysis, the Kaplan-Meier method was used to determine survival fractions, and the Mantel-Haenszel log rank test was used to determine p values, post-inf, postinfection;
suggest that, in infections with rapidly dividing pathogens, optimal protection is evident when the CpG ODNs are administered 3–6 days before infection (29). In contrast, for infections that have slower kinetics such as leishmaniasis, CpG ODN can induce a protective response even when administered weeks after infection (30). We next assessed the optimal time for CpG ODN treatment of mice infected with the TCRV. BALB/c mice were challenged with the TCRV i.p. on day 4 of life and treated with CpG ODN i.p. on days 1, 4, 7, or 10 of life. Untreated mice served as controls. The protective effect of CpG ODN was best when the treatment was administered at the time of challenge (Fig. 1A). Mice treated 3 days after the challenge (Fig. 1D) were protected to a lesser, but still significant, degree (30% survival; p < 0.01). However, no protection was evident in mice that were treated with CpG ODN either 3 days before or 6 days postchallenge (not shown).

**Effect of CpG ODN on intracranial Tacaribe infections**

The TCRV is a neurotropic virus. In untreated mice it rapidly migrates to the brain where it replicates. We reasoned that the lack of protection observed when the CpG ODNs were administered >3 days after infection might reflect an inability of the CpG ODN to control the infection once the virus reached the CNS. To test whether the protective effect of CpG ODN was restricted to acting in the periphery, reducing the viral load or even preventing the virus from reaching the brain, neonates were infected i.c. (10 μl/mouse) with TCRV and treated with CpG ODN i.p. Mice infected i.c. died sooner than animals infected i.p. or i.n., as 100% of the mice were dead by day 12 (Fig. 2A). Of note, treatment with CpG ODN i.p. resulted in a 20% survival rate (p < 0.01). The protective effect of CpG ODN was still evident in mice treated 3 days after i.c. infection (23% survival; p < 0.01) (Fig. 2B).

**FIGURE 2.** CpG ODNs protect neonatal mice from lethal intracranial (i.c.) TCRV infection. BALB/c neonates (1–3 days old) were infected i.c. with TCRV and treated with CpG ODN i.p., either concurrently (A; **, p < 0.01) or 3 days postchallenge (B; ***, p < 0.01), as described in the Fig. 1 legend. Control mice received CpG ODN and control ODN lacking the CpG motif either i.p. or i.c. Results show data from 3 to 5 independent experiments. Statistical analysis of survival rates was determined by the Kaplan-Meier/Mantel-Haenszel log rank test. post-inf, postinfection.

**Effect of CpG ODN on viral load**

To examine the mechanism by which CpG ODN increased survival, neonatal mice were infected with TCRV i.p. and then sacrificed at specific time points after infection. Viral loads in spleen and brain were assessed by TCID₅₀ on days 1 (3 h after challenge), 3, 7, and 10 after infection. No live viruses were recovered from spleen or sera. However live viruses were cultured from brain tissue 7 days after infection, regardless of the route of infection. As shown in Fig. 3A, on day 7 untreated mice had viral titers of 10⁶ TCID₅₀. The increased survival observed in the CpG-treated mice was associated with a decreased, yet detectable, viral load (10⁴ TCID₅₀) in the brains of those mice. Importantly, no live virus could be isolated from the brains of CpG-treated mice past weaning age (not shown), indicating that the surviving mice cleared the virus.

To better understand the progression of the infection, direct visualization of TCRV Ags in brain sections was performed by immunohistochemistry using an Ab specific for the viral surface glycoprotein. Viral Ag was detectable by day 5 (Fig. 4, C and D) in the upper spinal chord and cerebellum and was most evident in the subarachnoid space (Fig. 4C). Viral Ag was quantitated using 25–35 images per mouse brain. Similar levels of virus Ags and distribution were evident at day 5 in treated and untreated animals.

**FIGURE 3.** CpG ODNs decrease the viral load in the brains of TCRV-infected neonatal mice. A, Viral loads in the brains of BALB/c neonates (three per group) were determined by the TCID₅₀ method 3 h and 4, 7, and 10 days post i.p. infection (post-inf). Results are shown as means of viral loads ± SD. B, Enumeration of viral Ag levels (pixel counts) in brain sections from i.p. infected TCRV, TCRV plus CpG ODNs (TCRV + CpG), and uninfected control BALB/c neonates (3–4 mice/group) 5 or 10 days postinfection. Pixel counts (mean ± SD of >25–35 images/mouse brain) were performed using IMAJIN software. Statistical analysis was done using Student’s t test comparing TCRV to TCRV plus CpG at each specific time point (A) and one-way ANOVA (B) (**, p < 0.01; ***, p < 0.001).
FIGURE 4. CpG treatment decreases levels of TCRV Ag detectable in infected neonatal mouse brains. Confocal microscopy images of the cerebellum of mice 5 or 10 days postinfection. TCRV Ag is shown in red, and GFAP is shown in green. Yellow denotes colocalization of TCRV with GFAP, indicating that the virus infects astrocytes. A, H&E-stained image of a neonatal (5 days old) mouse brain; boxes indicate the five sites where the virus was quantified. B, Cerebellar folia of age-matched uninfected mouse at day 6. C and D, Analogous sections from day 5 post-TCRV infection in untreated (TCRV) (C) or treated (TCRV plus CpG) mice (D). E and F, Day 10 postinfection from TCRV (E) and TCRV plus CpG (F) mice. G–I, Colocalization of TCRV Ag (red) with GFAP (green) (G), CD45 (blue) (H), or NSE (green) (I) 5 days postinfection using confocal microscopy and IMAJIN software. Colocalization was confirmed by Z-stack analysis. J and K, TCRV Ag (red), GFAP (green) and Diablo (second mitochondria-derived activator of caspase (SMAC); blue) in the brains of untreated (J) and CpG ODN-treated (K) mice 10 days postinfection. Note that Tacaribe-infected astrocytes undergoing apoptosis appear white, whereas other infected apoptotic cell types appear magenta.

(Figs. 3B and 4, C and D). By day 10, however, untreated animals showed greater levels of virus Ag, whereas those treated with CpG ODN did not (Figs. 3B and 4, E and F). In terms of cell tropism, on day 5 TCRV localized primarily in GFAP+ cells but was also evident in NSE+ neurons and CD45high microglia (Fig. 4, G–I). CpG ODN treatment did not change the cellular distribution of the TCRV (not shown). Ten days after infection the brains of untreated mice showed clear signs of disorganization of the parenchyma,
astrocytosis and apoptosis (Diablo+ cells), whereas those from treated animals showed only mild viral infiltration, and their astrocytes and neurons were Diablo− (Fig. 4, E, F, J, and K).

**Role of TNF-α and IFN-γ in CpG ODN-mediated immunoprotection from Tacaribe meningoencephalitis**

CpG ODN-mediated immunoprotection against other intracellular pathogens is mediated by the proinflammatory and type 1 adaptive immune response it fosters (7). Because IFNs are known to inhibit replication of other arenaviruses (31), we next investigated whether CpG ODN treatment enhanced the IFN-γ response to the virus. Spleen cells from treated and untreated mice were collected 4 and 7 days postinfection and restimulated in vitro with heat-inactivated TCRV. As shown in Fig. 5A, 4 days after challenge splenocytes from TCRV-infected mice produced high levels of IFN-γ in response to TCRV Ags regardless of treatment. By day 7 after infection, however, higher IFN-γ levels were produced by splenocytes from untreated mice than from mice treated with CpG ODN (p < 0.05; Fig. 5A).

Because the in vitro response of splenocytes to a neurotropic virus may not reflect the immune milieu at the site of infection, the expressions of IFN-γ, TNF-α, and type 1 IFN-inducible genes were assessed in the brain 5 and/or 13 days after challenge. Results indicate that infected mice had significantly increased levels of IFN-γ and TNF-α mRNA compared with uninfected controls (Fig. 5, B and C). Further, although IFNα was not readily detectable, mRNA for several type 1 IFN-inducible genes (Prkr, Irf7, Isgf3g, Mx2, and Oas1g) were increased 5 days after infection, suggesting that the virus does induce type 1 IFN expression (Fig 5D). Of note, the mRNA levels for mice treated with CpG ODN tended to be lower than those for the untreated ones, particularly at day 13, likely reflecting the comparatively lower viral load. This suggested that the increased survival and reduced viral load of CpG ODN-treated mice were unlikely to be mediated by enhanced type1 cytokine production.

**Increased virus-specific Ab levels are seen in infected animals that are CpG treated**

Previous studies had indicated that Abs play an important role in protection from arenaviruses (18, 32). Because B cells express TLR9 and are activated directly by CpG ODN, we hypothesized that the improved survival rate could result from increased Ag-specific Ab levels. As shown in Fig. 6A, Ag-specific Abs, mainly IgM, were evident 10 days after infection in untreated TCRV-infected mice. In contrast, mice treated with CpG ODN developed high levels of IgM and IgG Ag-specific Abs by day 5 (Fig. 6A and B). The increased expression of IgG Abs included both IgG1 and IgG2a subisotypes (not shown). Of note, no shift in the ratio of IgG1: IgG2a was evident in treated mice, further supporting the notion that CpG ODN does not induce a Th1 shift in this disease model.

To establish whether early expression of Abs to TCRV was protective, infected newborn mice were treated with sera from convalescent or naive mice on days 3 and 8 postchallenge. As shown in Fig. 6C, the early administration of sera from older mice improved survival. Survival was optimal in mice that received sera from convalescent mice that had been treated with CpG ODN at the time of infection (89% survival; p < 0.05), compared with 56% survival in mice that received convalescent sera from mice not treated with CpG ODN and 29% in those that received sera from age-matched mice never exposed to the virus. Although the mice that received sera from CpG ODN-treated convalescent mice had better survival rates than those that received sera from untreated convalescent mice, the difference did not reach statistical significance.

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**FIGURE 5.** CpG-mediated protection is not associated with increased type 1 cytokine production. A, IFN-γ levels were measured by ELISA in supernatants of splenocytes from infected BALB/c mice collected 4 or 7 days postinfection and restimulated in vitro with 10^5 TCID_50 heat-inactivated TCRV per well for 72 h. Results are means ± SD of two representative experiments. B and C, mRNA levels in brain of infected (i.n.) C57BL/6J mice (2–3 mice/group) 5 and 13 days postinfection (post-Inf.), mRNA levels for IFN-γ and TNF-α were assessed by real-time quantitative PCR using the iQ SYBR Green/Cycler system. D, mRNA for type1 IFN-inducible genes were assessed using a profiling kit from SuperArray Bioscience (one of five mice tested in two independent experiments). Statistical analysis was done by Student’s t test (*, p < 0.05; **, p < 0.01).
NO production by iNOS is required for the immunoprotective effect of CpG ODN

CpG ODNs are known to induce the expression of iNOS in vitro in microglial cells (36), and NO production has been shown to be critical for CpG ODN-mediated protection against Listeria monocytogenes (37). Because previous studies showed that iNOS also plays a major role in the survival of mice infected with the arenavirus Junin (38), we next determined whether iNOS activation plays a role in CpG ODN-mediated protection against the TCRV.

BALB/c mice were treated daily with AMG (40 μg/mouse/day), an iNOS blocker. By itself, AMG had no deleterious impact on neonatal health, nor did it modify TCRV viral titers (Fig. 7, A and B). When administered to CpG ODN-treated mice challenged with Tacaribe, however, AMG impeded the rescue of BALB/c mice (Fig. 7A). Unlike μMT KO mice, no reduction in brain viral load was evident in CpG ODN-treated BALB/c mice that received AMG (Fig. 7B). Infection of mice lacking a functional iNOS (iNOS KO) confirmed that iNOS is needed for CpG ODN-mediated protection (Fig. 7C). Further, as with AMG-treated BALB/c mice, CpG ODN treatment did not reduce the viral load in iNOS KO mice. We next assessed whether the lack of protection in iNOS KO mice could be due to a defect in their Ab response to TCRV. As shown in Fig. 7E, 10 days post infection the level of IgM Abs to TCRV in iNOS KO mice was similar to that found in C57BL/6 mice; however, the CpG ODN-induced increase in IgG anti-TCRV Abs was not present, suggesting that iNOS treated mice may have a defect in their CpG ODN-mediated Ab response that was responsible for their lack of clinical effect. We reasoned that, if the lack of survival was secondary to a defect in IgG Abs to TCRV, passive transfer of convalescent sera would rescue these animals. However, unlike BALB/c and μMT KO mice, transfer of convalescent sera did not rescue iNOS KO mice (Fig. 7F), indicating that a functional iNOS, in addition to an enhanced Ab response, is required for survival.
Vaccine development remains the most effective and efficient manner to reduce susceptibility to infection by pathogens with high prevalence in the population. However, newly developed immunomodulators provide an alternative approach to limit the spread and pathogenicity of emerging or sporadic pathogens against which vaccine development is not a practical alternative. Accumulating evidence suggests that immunomodulators might be effective even in populations where vaccines have frequently failed, such as the newborn, the elderly or the immunocompromised (39, 40). In this study we report that treatment with CpG ODN protects 20–50% of neonatal mice from a lethal challenge with the neurotropic New World arenavirus Tacaribe. Protection was attained when the mice were treated (i.p. or i.n.) at the time or up to 3 days postchallenge. The protective effect was TLR9 dependent and associated with reduced type-1 IFN-inducible genes, IFN-β and TNF-α expression in the brain, and increased virus-specific IgM and IgG Abs in sera. Protection also required the expression of iNOS. To our knowledge, this is the first study assessing the mechanisms by which CpG ODN can protect neonatal mice from an otherwise lethal neurotropic virus infection.

Numerous studies in adult mice had shown that the activation of the innate immune system and the fostering of a strong Th1–type response by CpG ODN leads to reduced susceptibility to pathogens as diverse as Listeria, Francisella, Klebsiella, Anthrax, Mycobacterium, Plasmodium, or Leishmania (30, 41–43). In addition, recent studies show that systemic administration of CpG ODN improved recovery of mice infected with the Friend leukemia virus and senescent mice infected with the influenza virus (12, 44). Protection in all cases was linked to induction of a Th1 response.

In newborn mice infected with the TCRV, the lethal meningoencephalitis they develop appears to be T cell-mediated as demonstrated by the survival of thymectomized and nude nu/nu mice (45). Further, preliminary studies from our laboratory have shown that mice lacking IFN-β (IFN-β KO) or treated with anti-TNF-α Abs have improved survival (J. Pedras-Vasconcelos and D. Vertheleyi, unpublished observations). These findings are in agreement with studies from Riviere et al. (46) showing that the severity of the choriomeningitis in suckling mice correlated with the IFN production. In this context, it was a concern that CpG ODN treatment might be detrimental to the disease progression. However, as shown in Fig. 5, CpG ODN treatment was associated with relatively lower in situ and systemic levels of TNF-α, IFN-β, and type 1 IFN-inducible genes compared with untreated infected animals.

Although most of the early studies that assessed the immunoprotective effects of CpG ODN used the i.p. route, recent studies have favored compartmentalized delivery of CpG ODN in the prevention of a localized infection (47). In our studies i.p. administration of CpG ODN achieved better protection than the i.n. route. Moreover, CpG ODN administered i.p. protected mice against i.p., i.n., and even i.c. challenges, whereas the i.n. route did not improve the survival of mice challenged i.p.
Regardless of the route of infection, TCRV circulates at very low concentrations in the periphery and only begins to replicate in earnest when it reaches the spinal chord and the brain between days 4 and 5 (19). Confocal images clearly show that on day 5 postinfection viral Ags are present in astrocytes and neurons and, to a lesser extent, in CD45<sup>inh</sup> microglia, and appear to be located in the areas adjacent to the meninges. Treated and untreated mice have similar viral loads in the brain with comparable cellular distribution. By day 10, however, the viral infection in the untreated mice has progressed as shown by higher viral load and a change in the distribution of the viral Ag, which can be seen penetrating a disorganized parenchyma. Furthermore, at this time infected and surrounding astrocytes appear engorged, and staining for the cytoplasmic Diablo protein, an early marker for apoptosis, is evident in local astrocytes and neurons (Fig. 6, and data not shown) (48). The similar viral progression in treated and untreated mice early in infection suggests that the antiviral effect does not take place in the periphery by reducing the virus titer that reaches the brain, but rather is the result of the immune response that takes place in the brain.

Previous studies had shown that CpG ODN-activated I-κB kinase and JNK in astrocytes induce cytokine and chemokine production. Microglial cells treated with CpG ODN up-regulate B7-1, B7-2, and CD40 and secrete increased levels of TNF-α, IL-12, and NO (15, 16, 49). It is unclear at this time whether in vivo CpG ODN crosses the blood brain barrier to act directly on local glial cells, induces the activation of immune cells that migrate to the brain (although CD45<sup>bright</sup> cell are not evident until late in the infection), or causes a systemic change in the immune milieu (cytokine, chemokine, NO, etc.) that modifies the local environment or response to the virus.

Our studies suggest that CpG ODN mediates protection by accelerating the development of virus-specific Abs. This hypothesis is in line with the previously reported treatment of hemorrhagic fever by transfer of convalescent sera (45). The mechanism of action of the Abs is not entirely clear. One possibility is that they act in the periphery by reducing the viral load that reaches the brain; however, our studies show similar viral loads in brain on day 4 or 5 after infection in treated and untreated animals. More likely, the Abs are required for viral clearance as described for Sindbis virus (reviewed in Ref. 50) and the mouse hepatitis virus (34), two virus models where the Abs help control virus replication in CNS. Of note, sera is known to exert immunoregulatory effects independent from the presence of Ag-specific Abs, such as the Fc receptor-dependent induction of anti-inflammatory cytokines (51, 52). The increased survival of infected wild-type and μMT KO neonatal mice following treatment with sera from naive mice would support this notion (Fig. 6, C and F, respectively).

Although Abs clearly can protect mice from infection, their absence does not accelerate disease. Indeed, μMT KO mice, which cannot mount an Ab response to the virus, do not succumb more rapidly to Tacaribe infections. This finding suggests that in the natural history of Tacaribe infections in wild-type (BALB/c or C57BL/6) neonate mice, the development of Abs occurs too late to modify disease outcome. Therefore, it is only when the development of Abs is accelerated (by CpG ODN treatment or by transfer of immune sera) that the Abs can affect disease outcome.

In addition to accelerating the development of Abs, CpG ODN may induce a qualitative change in the Abs generated. Mice that received convalescent sera from animals treated with CpG ODN at the time of infection consistently tended to have higher survival rates relative to those that received sera from infected but untreated mice. The observation that the IgG and IgM levels of anti-TCRV Abs transferred were similar suggests a "qualitative" difference in the Abs generated in the CpG ODN-treated mice. Although theoretically possible, it is unlikely that the difference in survival is due to the presence of CpG ODN in the sera, because the convalescent sera were obtained 15 days after the administration of CpG ODN to the serum donor. Further studies will be necessary to determine whether mice treated with CpG ODN generate Abs that have higher affinity than untreated ones. Assessment of IgG1 and IgG2a Abs to TCRV did not suggest a shift in the IgG subtype generated (data not shown).

iNOS KO mice could not be rescued by CpG ODN treatment, and the inhibition of iNOS using AMG in infected BALB/c mice negated the protective effect of CpG-ODN. This finding is in accordance with previous studies showing that iNOS plays an important role in the clearance of several pathogens, including Leishmania and Klebsiella (53), and is required for CpG ODN-mediated protection from Listeria (54). The assessment of Ab levels in iNOS KO mice and AMG-treated mice (not shown) suggested that these mice make lower levels of virus-specific IgG Abs. However, the serum transfer studies show that iNOS KO mice cannot be rescued by exogenous Abs, underscoring the role of iNOS in survival. In differentiating the contribution of iNOS and Ab to the CpG ODN-mediated protection, it is important to underscore that μMT mice have normal levels of iNOS mRNA expression and function (data not shown). Studies by Creon (55) and Karupiah et al. (56) suggested that iNOS inhibits viral replication by S-nitrosylation of cysteine residues in essential viral proteins. An alternative mechanism is suggested by studies in Junin arenavirus and Sindbis alphavirus (57). In both models, inhibition of iNOS did not impact viral load but increased virus-induced pathology (38). In the arenavirus study, decreased mortality was associated with increased astrocytosis, whereas in mice infected with encephalomyelitic Sindbis alphavirus, specific inhibition of iNOS with N<sup>ω</sup>-nitro-L-arginine methyl ester decreased the survival of infected neurons (57). Lastly, NO could act by decreasing the IFN-γ production and indirectly promoting anti-inflammatory responses, as suggested by studies of herpes simplex virus type 1 (58) and the influenza virus (59). Of note, although iNOS expression in infected areas was confirmed by immunohistochemistry, its local expression was only evident 10 days after infection on CD45<sup>bright</sup> infiltrating monocytes (data not shown). The role of local vs systemic iNOS expression is yet unclear and will be the focus of future studies.

In summary, the studies presented show that activation of the innate immune system via TLR9 in neonates accelerates the host’s Ab response to a neurotropic virus, improving the survival of the challenged animals. Importantly, neonatal exposure to CpG ODN appears to be safe, because no changes in growth rate or adverse events were observed in this or other studies (60). These findings support the development of CpG ODNs as immunoprotective agents in humans.

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Disclosures

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