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Prophylaxis with cationic liposome–DNA complexes protects hamsters from phleboviral disease: Importance of liposomal delivery and CpG motifs☆

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ABSTRACT

Cationic liposome–DNA complexes (CLDC) are cationic/neutral lipid carriers complexed with plasmid DNA that when administered systemically results in a robust T_H1 cytokine response. CLDC have been shown to be effective in prophylaxis and therapeutic treatment of animal models of viral disease. To determine the contribution of liposomal delivery and CpG content of the plasmid DNA to the efficacy of CLDC; plasmid, CpG-free plasmid DNA, or CpG-containing oligodeoxynucleotides (ODN) with and without liposomes, as well as poly(I:C_{12}U), were evaluated for their ability to elicit protection against lethal Punta Toro virus (PTV, *Bunyaviridae*, *phlebovirus*) challenge in hamsters. CLDC-containing plasmid significantly improved survival, decreased systemic and liver viral loads, and reduced liver damage due to progression of viral infection. Mouse-reactive ODNs complexed with liposomes failed to protect hamsters, whereas ODNs known to cross-react with human and mouse (CpG 2006) or non-liposomal poly(I:C_{12}U) showed survival benefit but did not limit liver injury. Liposomes complexed with a non-CpG motif-containing plasmid reduced liver viral load and tissue damage, but did not protect hamsters from death. To evaluate the mechanisms of the enhanced activity of CLDC, microarray experiments examined differences in the gene expression profile. The results suggest a broad T_H1 response elicited by liposomal delivery of a diverse sequence containing CpG and non-CpG elements may be a more effective antiviral treatment than other nucleic acid based immunotherapeutics.

1. Introduction

Infection of humans with Rift Valley fever virus (RVFV, *Bunyaviridae*, *phlebovirus*) can lead to severe disease in the form of viral hemorrhagic fever, encephalitis and retinitis (Peters and Meegan, 1981). The virus has been recognized as a cause of human illness for more than 75 years (Flick and Bouloy, 2005). RVFV is also a significant pathogen of agricultural importance, with young sheep and cattle being highly susceptible to lethal disease and infection of pregnant females often resulting in abortion (Gerdes, 2002). Of the viruses known to cause viral hemorrhagic fever, the ability to also cause severe disease in livestock is unique to RVFV. As a result, RVFV is classified as an overlap select agent by the Department of Health and Human Services and the US Department of Agriculture. In addition, the potential of RVFV to be used as a bioterror agent has led to its inclusion in a group of the highest priority pathogens that pose the greatest threat to national biosecurity (NIAID, 2002).

Closely related to RVFV, Punta Toro virus (PTV) is a phlebovirus that generally only causes mild self-limiting disease in humans (Peters and LeDuc, 1984), but produces lethal disease in hamsters and mice with some of the characteristic features of human cases of RVF (Anderson et al., 1990; Fisher et al., 2003; Gowen and Holbrook, 2008; Pifat and Smith, 1987). This attribute has led to the use of these small animal PTV infection models for early pre-clinical studies evaluating candidate experimental therapies targeted at identifying treatments for RVF (Gowen et al., 2006a,c, 2007a; Sidwell et al., 1988, 1992, 1994). Although the pathological findings seen in hamsters more closely resemble RVF disease in humans (Anderson et al., 1990), the availability of mouse reagents and genetically modified mice has facilitated characterization of

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phleboviral disease and drug mode of action studies in vivo (Gowen et al., 2006b,c, 2007b).

We have previously demonstrated that a single treatment with cationic liposome–DNA complexes (CLDC) can protect mice against PTV challenge (Gowen et al., 2006a). For immunotherapeutics that rely on the stimulation of the host through cellular mechanisms, it is critical to show that antiviral activity can be elicited in multiple animal models since this would suggest activity in other species including humans. Here, we investigated the activity of CLDC in the hamster PTV infection model and examined the contribution of liposomal delivery and DNA content in the stimulation of protective immunity.

2. Materials and methods

2.1. Animals

Female 7–8-week-old Syrian hamsters were obtained from Charles River Laboratories (Wilmington, MA). Hamsters were acclimated to the Laboratory Animal Research Facility at Utah State University for 5 days prior to use and weighed approximately 100–115 g at the time drug treatments were started. Hamster procedures complied with USDA guidelines and were approved by the Utah State University Institutional Animal Care and Use Committee.

Mouse studies were performed at Colorado State University. Female 6-week-old BALB/cJ mice were obtained from the Jackson Laboratory (Bar Harbor, ME) and housed under specific pathogen-free conditions for 2 weeks prior to use. Mouse studies were conducted under Colorado State University Animal Care and Use Committee approved protocols.

2.2. Virus

The Adames strain of PTV was received from Dr. Dominique Pifat of the U.S. Army Medical Research Institute for Infectious Diseases, Ft. Detrick (Frederick, MD). The virus used was from a stock prepared following four passages of the original virus through LLC-MK_2 monkey kidney cells (American Type Culture Collection, Manassas, VA) and one passage in hamsters. Viral stocks made from pooled hamster liver homogenates containing high titers of infectious PTV were diluted in minimal essential medium just prior to infectious challenge.

2.3. Test materials

Cationic liposomes (DOTIM lipid + cholesterol) in 10% sucrose solution and plasmid DNA (pMB75.6; 3 mg/ml) were provided by Juvaris BioTherapeutics (Burlingame, CA). A detailed description of the 4242 base pair empty vector plasmid pMB75.6 and the formulation of CLDC have been previously described (Gowen et al., 2006a). Briefly, the CLDC were prepared by the sequential addition of nuclease-free water (NFW), DOTIM, and plasmid DNA. A 0.05 ml volume of this preparation contained 5 μg of liposome–complexed DNA. Experimental doses are indicated in μg amounts, referring to the total DNA content complexed with the cationic lipid. Further dilutions of CLDC were made in NFW.

In addition to the standard CLDC preparation, the second study also included DNA–liposome complexes formed with the mouse restrictive CpG-containing 1826 ODN (5′-TCC ATG ACG TTC CTG ACG TT-3′) or the non-CpG 1982 control ODN (5′-TCC AGG ACT TCT CTC AGG TT-3′) from Proligo (Boulder, CO). A third experiment included standard CLDC, as well as CLDC formulated with the human and mouse-reactive CpG-containing 2006 ODN (5′-TCG TCG TTT TGT CGT TTT GTC GTT-3′) or a CpG-free version of the 2006 ODN, both synthesized by Sigma Genosys (The Woodlands, TX). In addition CLDC were also prepared with plasmid pCpG Giant (Invivogen, San Diego, CA). The pCpG Giant is a high molecular weight plasmid entirely devoid of CpG dinucleotide. This plasmid was purified in the same manner as pMB75.6. A description of the various treatments is included in Table 1.

Table 1
Descriptions of plasmid and oligodeoxynucleotide (ODN) treatments.

Treatment	Type	CpGs	Complexed with liposomes
CLDC (pMB75.6)	Plasmid	Yes	Yes
nCLDC (pCpG Giant)	Plasmid	No	Yes
Plasmid DNA (pMB75.6)	Plasmid	Yes	No
Non-CpG plasmid (pCpG Giant)	Plasmid	No	No
1826 ODN (mouse-reactive CpG)	ODN	Yes	No
1826 CLDC (mouse-reactive CpG)	ODN	Yes	Yes
1982 ODN (non-CpG control for 1826)	ODN	No	No
1982 CLDC (non-CpG control for 1826)	ODN	No	Yes
2006 CLDC (human- and mouse-reactive CpG)	ODN	Yes	Yes
n2006 CLDC (human- and mouse-reactive CpG)	ODN	No	Yes

Ribavirin was supplied by ICN Pharmaceuticals, Inc. (Costa Mesa, CA) and served as the positive control for all challenge studies. Poly(I:C_{12}U), a mismatched double-stranded RNA, trade name Ampligen®, was included in the initial experiment as an additional positive control. It was provided by HEMISPHERx Biopharma (Philadelphia, PA) at a concentration of 2.4 mg/ml. Ribavirin was dissolved in sterile saline and poly(I:C_{12}U) was prepared for injection in NFW. All treatments were given by intraperitoneal (i.p.) injection.

2.4. In vivo challenge studies

Hamsters were weighed prior to the start of experiments and sorted into groups of five hamsters per cage so that mean cage weights across the entire experiment varied by less than 5 g. Groups of hamsters received treatments as specified in the figure legends. Following treatment, animals were inoculated by the subcutaneous route with approximately 50 plaque-forming units of PTV. Five animals from each treatment group were sacrificed on day 4 of infection to measure viral loads (assessed by viremia and liver virus) and serum alanine aminotransferase (ALT) activity. Serum was collected for assaying systemic viral burden and ALT activity and livers were harvested and homogenized for viral titer determination as described below. The remaining animals in each group were held 21 days for observation. Several sham-infected animals were included in each experiment as normal controls in order to establish reference values for all tested parameters.

2.5. Determination of liver and serum virus titers

Virus titers were assayed using an infectious cell culture assay as previously described (Sidwell et al., 1988). In brief, dilutions of liver homogenate or serum were serially diluted and added to triplicate wells of LLC-MK_2 cell monolayers in 96-well microplates. Viral cytopathic effect (CPE) was determined 6–7 days after exposure to the samples and the 50% endpoints were calculated as described (Reed and Muench, 1938). The assay detection range was 2.75–9.5 $\log_{10}$ cell culture 50% infectious doses ($CCID_{50}$)/g of liver or 1.75–8.5 $CCID_{50}$/ml of serum. For statistical analysis, in samples presenting with undetectable liver or serum virus, a value of 2.75 or

1.75 $\log_{10}$ was assigned, respectively. In cases where virus exceeded the detection range, a value of 9.5 or 8.5 $\log_{10}$ was assigned. Thus, where samples are shown to be at the outer limits of detection, group mean viral loads may be over or underestimates of the actual burden.

2.6. Measurement of serum ALT activity

Serum ALT levels serve as a good marker for liver disease (Amacher, 1998). ALT activity in hamster serum samples was measured using the ALT (SGPT) Reagent Set (Pointe Scientific, Lincoln Park, MI) per the manufacturer's recommendations. The reagent volumes were adjusted for large sample size analysis on 96-well microplates.

2.7. Quantitative RT-PCR analysis of hamster cytokine induction

Hamsters treated with 30 μg CLDC were sacrificed at the indicated times after treatment. Spleen tissue was harvested and immediately stored in RNA*later*® (Ambion, Austin, TX) until time of RNA purification. Spleens were homogenized using a Tissue Tearor™ (Biospec Products, Bartlesville, OK) and total RNA was isolated using RNeasy reagents from Qiagen (Valencia, CA) as indicated by the manufacturer. All RNA preparations were treated with DNase (Turbo DNA-*free*™, Ambion) for removal of contaminating genomic DNA prior to quantitative (q)RT-PCR analysis. Superscript III Platinum® One-Step Quantitative RT-PCR reagents from Invitrogen (Carlesbad, CA) were used to measure relative IFN-γ, TNF-α, IL-12p40 and IL-21 transcript levels. Primers and probe sequences for these genes and the housekeeping normalization gene, γ-actin, have been previously described (Gowen et al., 2008).

2.8. Statistical analysis

Kaplan–Meier survival plots were generated using the Prism software package (GraphPad Software, San Diego, CA). Survival curves were compared using the log-rank test. The Mann–Whitney test (two-tailed) was performed to analyze the differences in mean virus titers and serum ALT levels.

2.9. Microarray analysis

Groups of 5 BALB/c mice were injected i.p. with 500 μl sterile PBS alone, sterile PBS with 50 μg CpG 1826 ODN or CLDC containing 20 μg DNA. At 6 h post-treatment mice were sacrificed and spleens were removed and homogenized using a Polytron PT10-35 rotor-stator homogenizer (Kinematica, Lucerne, Switzerland). Total spleen RNA was extracted with TRIzol reagent (Invitrogen) according to the manufacturer's instructions, treated with 4 U DNase I Amplification Grade (Invitrogen) in a volume of 100 μl and further purified using the RNeasy Mini Kit (Qiagen) RNA cleanup protocol. Labeled cDNAs were produced by reverse transcribing 50 μg total RNA using 5 μl 5× first-strand reaction buffer, 2.5 μl DTT (100 mM), 1.5 μl random primers (3 mg/ml), 2.5 μl of a mixture of 5 mM dATP, dGTP, dCTP and 0.5 mM dTTP, 1 μl (200 U) Superscript II reverse transcriptase (Invitrogen), and 1 μl Cy3-dUTP or Cy5-dUTP (GE Healthcare, Little Chalfont Buckinghamshire, UK) in a final volume of 25 μl at 42 °C for 90 min. Labeled cDNAs were purified using Microcon YM30 columns (Millipore, Billerica, MA).

Murine whole genome oligonucleotide arrays were produced by the Rocky Mountain Regional Center for Excellence Genomics Core at Colorado State University. Hybridization was performed in humidified chambers at 42 °C for 15 h. Post-hybridization, the arrays were washed twice with 1× SSC/0.05% SDS and twice with 0.06× SSC, centrifuged, and dried. Slides were scanned using a GenePix 4000B Scanner (Molecular Devices, Sunnyvale, CA). The fluorescence for each channel of the array (Cy3 and Cy5) was normalized to the mean channel intensity and analyzed using GeneSifter analysis software (VizX Labs, Seattle, WA). Statistical significance was determined by application of Student's *t* test and Benjamini and Hochberg correction to analyses of all mean normalized data. Any transcript that showed at least two-fold change in expression level between experimental sample and control sample with a *P* value cutoff of <0.05 was considered significant.

2.10. Analysis of mouse IFN gene expression by qRT-PCR

Spleen total RNA from PBS-, CpG 1826 ODN-, and CLDC-treated mice were prepared as described above in Section 2.9. Reverse transcription of 4 μg total RNA was carried out with the SuperScript III First-Strand Synthesis Kit for RT-PCR (Invitrogen) according to the manufacturer's instructions using an oligo dT primer. Levels of relative cytokine gene expression were determined using specific primers and FAM dye-labeled TaqMan probes purchased from Applied Biosystems Inc., Foster City, CA. Probe and primer sequences for IFN-α4 and IFN-β were published previously (Karaghiosoff et al., 2003). IFN-γ probe and primers were designed by Applied Biosystems. Reactions were prepared with Taqman Universal PCR Master Mix (Applied Biosytems) and run in 96-well format on an iCycler (BioRad, Hercules, CA). Cytokine gene expression was normalized to abundance of the hypoxanthine guanine phosphoribosyl transferase 1 (HPRT1) housekeeping gene. The $\Delta\Delta$Ct method of relative quantification was used to calculate fold change in cytokine gene expression of treated (CpG ODN and CLDC) samples compared to untreated (PBS) samples.

3. Results

3.1. Treatment of PTV infection in hamsters with CLDC

CLDC have been shown to be effective in eliciting protective immunity against PTV infection in mice (Gowen et al., 2006a). Here we investigated its ability to induce protection in hamsters, which are more susceptible to the virus. The highest dose of CLDC tested, 30 μg, was found to be moderately effective, protecting 50% of the animals from a highly lethal challenge dose (Fig. 1A). Moreover, hamsters receiving the high dose of CLDC that succumbed to the infection survived considerably longer (9.4 ± 1.8 days) than those from the NFW placebo group (4.8 ± 1.3 days; $P < 0.01$). The lower dose of 15 μg was slightly protective as it was able to significantly improve disease outcome by delaying the time of death. An additional control group consisting of hamsters treated with only the liposome component of the CLDC was also included and produced a survival curve similar to that of the placebo group (Fig. 1A). The positive control drug, ribavirin was highly effective to the extent that 90% of the hamsters were protected and the only fatality occurred on day 11. Poly(I:C_{12}U) did not protect to the same degree as seen previously (Gowen et al., 2006c), which may have been due to the more lethal challenge dose used in the current study. The tested doses of CLDC have been previously shown to be well-tolerated in hamsters (data not shown).

Various disease parameters were also measured on day 4 of the infection. Due to the death of certain animals prior to the time of sacrifice in some of the groups, including 5 of the 10 from the NFW placebo group, the data are likely an underestimate of the true disease severity in those groups. No detectable virus and normal ALT levels were observed for the four hamsters in the 30 μg CLDC group (Fig. 1B–D). The death of one hamster in this group prior to the time of sacrifice was surprising considering that in the parallel

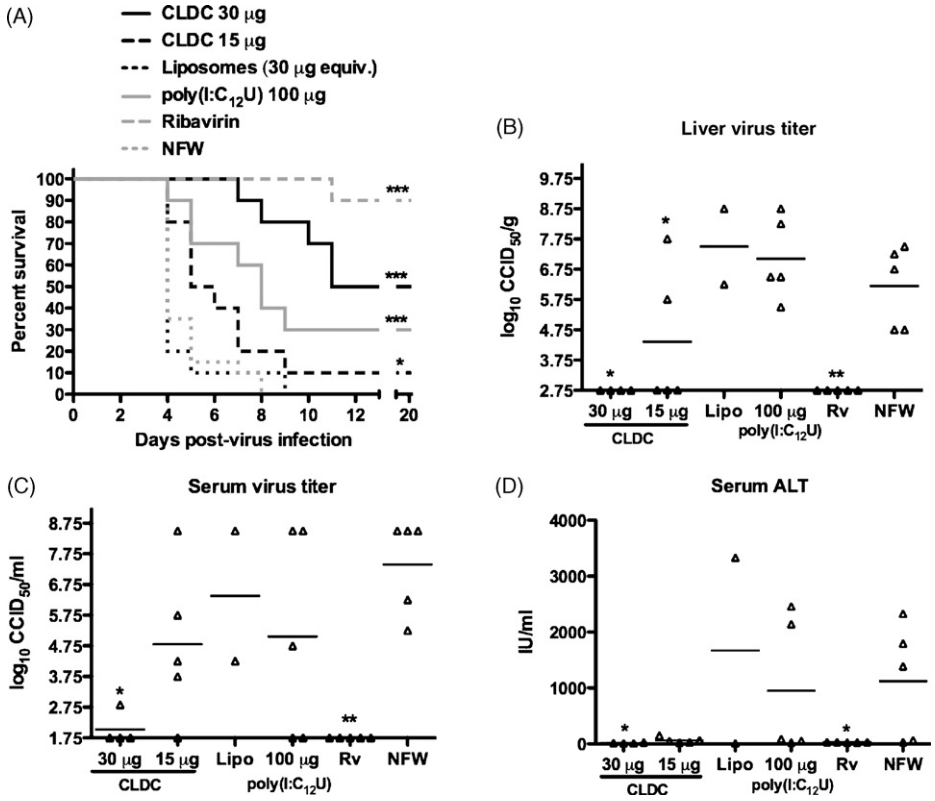

Fig. 1. Prophylactic intervention of PTV infection in hamsters with CLDC. Groups of 15 hamsters each (30 for the placebo group) were treated with a single i.p. injection of 30 or 15 μg of CLDC, liposomes only, 100 μg of poly(I:C_{12}U) or NFW placebo, 4 h prior to PTV challenge. Ribavirin (40 mg/kg/day) was administered twice per day for 6 days starting 4 h before challenge. Ten hamsters per group (20 for the placebo) were observed daily and (A) mortality was plotted over a 21-day period. The remaining five hamsters in each group (10 for the placebo) were sacrificed on day 4 of the infection for determination of (B) liver and (C) serum virus titers, and (D) serum ALT levels. Due to death prior to time of sacrifice, disease parameters could not be determined for 1, 3 and 5 hamsters from the CLDC 30 μg, liposomes only and NFW placebo groups, respectively. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ compared to NFW-treated animals. Mean virus titers and ALT levels for each group in graphs B–D are represented by horizontal lines. Equivalent, equiv.; nuclease-free water, NFW; liposomes, Lipo; ribavirin, Rv.

treated and challenged cohort of 10 animals held for survival (Fig. 1A), all survived a minimum of 6 days. The lower dose of CLDC significantly reduced liver viral burden, and decreased serum virus and ALT to levels that approached statistical significance compared to the decimated placebo NFW group. Although remarkable, this activity was not sufficient to improve overall number of survivors. Consistent with the survival data, only two of the five animals that received liposomes alone survived through day 4 and no appreciable decrease in viral load or ALT levels was observed (Fig. 1B–D). Poly(I:C_{12}U) was less effective than the 15-μg CLDC group in reducing liver virus titers ($P < 0.05$). Like the hamsters that were treated with 15 μg of CLDC, all poly(I:C_{12}U)-treated animals survived until the time of sacrifice, so caution must be used when comparisons with the placebo group are made since only the animals with the least severe disease are included in the latter. Hamsters in the ribavirin group had undetectable viral loads and normal ALT values.

3.2. Treatment of PTV infection in hamsters with CLDC formulated with mouse-reactive CpG-containing ODNs

In the first experiment, CLDC were found to be moderately effective in protecting hamsters from a highly lethal PTV challenge dose. To examine whether the effect of CLDC in the hamster system results from exposure to unmethylated DNA (CpG) islands contained in the bacterial plasmid DNA used in its formulation, CpG-containing ODN and a control non-CpG ODN were used to generate the DNA liposome complexes. As shown in Fig. 2A, only the standard preparation of CLDC protected animals from death. Neither plasmid nor ODN alone elicited protective immunity against PTV, although the time to death was delayed for many of the animals, suggesting that there may be a slight hint of some beneficial activity (Fig. 2B).

Similar to the first experiment, 50% of the NFW placebo died, as well as several animals in some of the other treatment groups, the night before the day of sacrifice. As before, not being able to include the hamsters that died prior to sacrifice complicates the analysis of the data and limits the statistical power. Nevertheless, the data that were collected suggest that the standard CLDC formulation was more effective at reducing viral burden and ALT compared to the uncomplexed plasmid and ODNs, as well as the liposome–complexed ODNs (Fig. 2C–E). There was no evidence that plasmid DNA and ODNs alone, or ODNs in conjunction with liposomes, were effective at reducing viral titers. As expected, ribavirin significantly abrogated liver disease (viral burden and ALT) compared to the five placebo-treated hamsters included in the analysis.

3.3. Treatment of PTV infection in hamsters with CLDC formulated with mouse- and human-reactive CpG-containing ODNs and plasmid DNA lacking CpG motifs

Since it does not appear that known mouse-reactive CpG ODNs evoke a protective antiviral response to PTV in hamsters we next

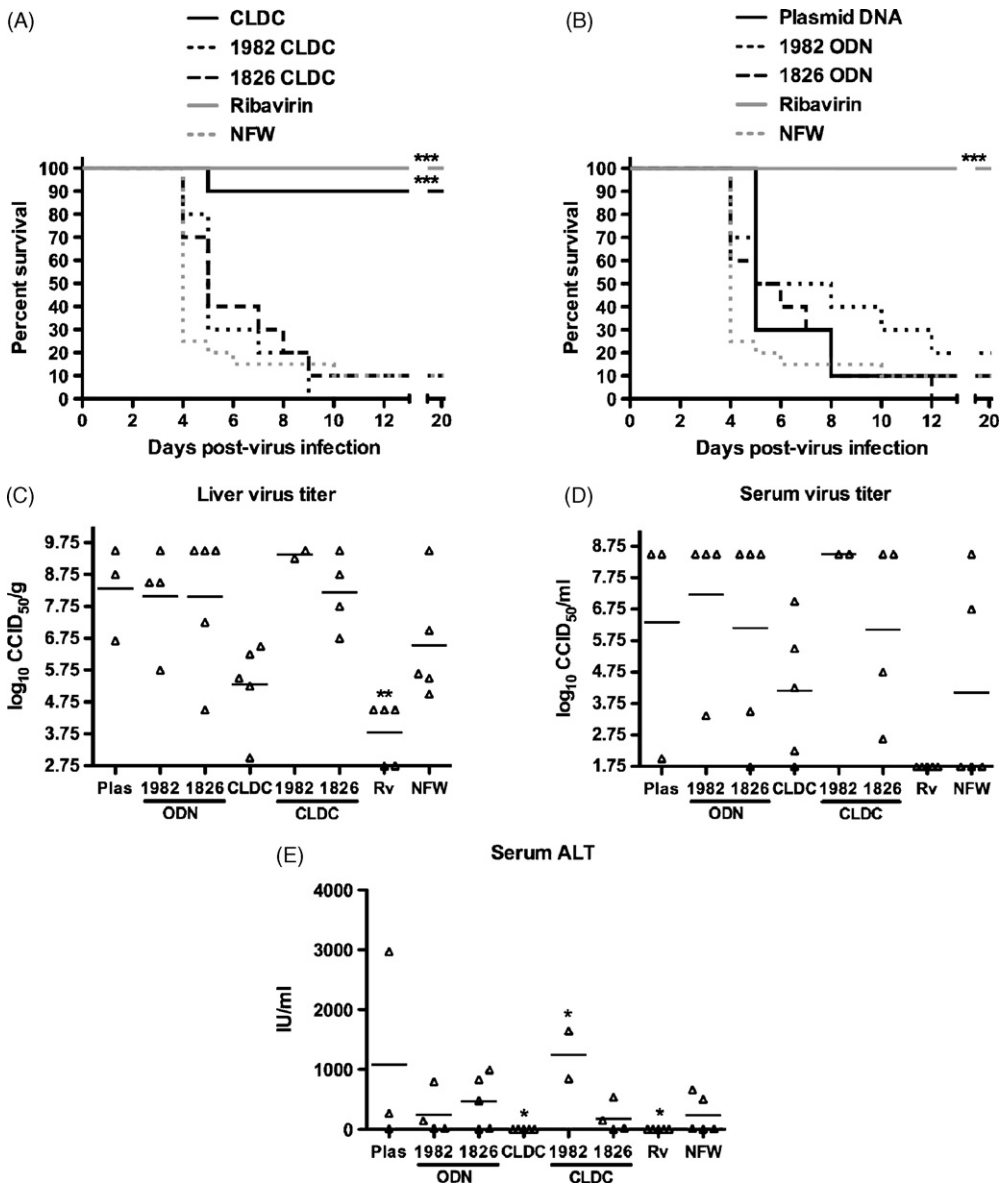

Fig. 2. Effect of mouse-reactive ODN-liposome complexes on the outcome of PTV infection in hamsters. Groups of 15 hamsters each (30 for the placebo group) were treated with a single i.p. injection of 30 μg of plasmid DNA and ODNs, alone, or complexed with liposomes, or NFW placebo, 4 h prior to PTV challenge. Ribavirin (40 mg/kg/day) was administered twice per day for 6 days starting 4 h before challenge. Ten hamsters per group (20 for the placebo) were observed daily and mortality was plotted over a 21-day period. Survival curves for hamsters receiving (A) complexed or (B) uncomplexed plasmid and ODNs are shown. The remaining five hamsters in each group (10 for the placebo) were sacrificed on day 4 of the infection for determination of (C) liver and (D) serum virus titers, and (E) serum ALT levels. Due to death prior to time of sacrifice, disease parameters could not be determined for 2, 1, 3, 1 and 5 hamsters from the plasmid DNA, 1982 ODN, 1982 CLDC, 1826 CLDC and NFW placebo groups, respectively. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ compared to NFW-treated animals. Mean virus titers and ALT levels for each group in graphs C–E are represented by horizontal lines. 1982 ODN, non-CpG control oligo; 1982 CLDC, 1982 ODN complexed with liposomes; 1826 ODN, CpG-containing mouse-reactive oligo; 1826 CLDC, 1826 ODN complexed with liposomes; nuclease-free water, NFW; plasmid, Plas; ribavirin, Rv.

tested mouse and human cross-reactive ODNs, as well as a plasmid devoid of CpG islands, for the ability to induce a protective effect. As in the initial experiment, standard CLDC afforded 50% protection from mortality, which was significantly better than the placebo treatment wherein only 5% of the hamsters survived (Fig. 3A). Notably, ribavirin, protected fewer animals than usual (80% survival instead of 90–100% commonly seen); however, the hamsters that died survived remarkably longer (11 days). Although not significant by the log-rank test, the 40% survival seen with the 2006 CLDC group was significant when comparing the total number of survivors to the placebo NFW group by the Fisher's exact test ($P = 0.0312$). In contrast to the extended survival time observed in the CLDC-treated hamsters that failed to survive the infection (7.6 ± 2.3 days), those treated with 2006 CLDC succumbed in a shorter period of time (4.3 ± 0.5 days; $P < 0.05$).

In the analysis of liver viral burden and disease present on day 4 of infection, CLDC and non-CpG plasmid nCLDC group values did not differ by much and were comparable to that of ribavirin (Fig. 3B and D). In contrast, liver disease and infectious virus loads were more dramatic with ODN CLDC treatment and comparable to the NFW placebo group. As alluded to previously, the analysis of the ODN CLDC and placebo groups is likely an underestimate of the magnitude of infection and disease since 1–2 animals in each group failed to survive. Consequently, mean serum viral burden and

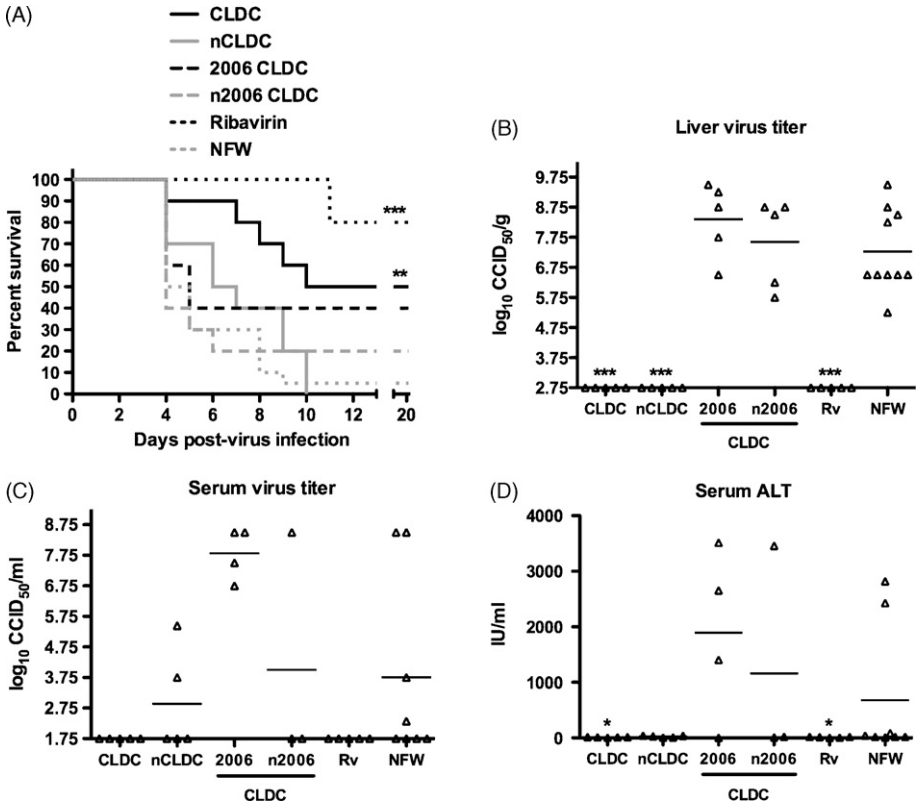

Fig. 3. Impact of CpG motifs on protective immunity elicited by CLDC. Groups of 15 hamsters each (30 for the placebo group) were treated 4 h prior to PTV challenge with a single i.p. injection of NFW (placebo), 30 μg of standard CLDC, or CLDC formulated with a non-CpG-containing plasmid (nCLDC), human-reactive ODN 2006 (2006 CLDC), or non-CpG ODN 2006 (n2006 CLDC). Ribavirin (40 mg/kg/day) was administered twice per day for 6 days starting 4 h before challenge. Ten hamsters per group (20 for the placebo) were observed daily for 3 weeks for (A) mortality. The remaining five hamsters in each group (10 for the placebo) were sacrificed on day 4 of the infection for determination of (B) liver and (C) serum virus titers, and (D) serum ALT levels. Due to death just prior to time of sacrifice, serum virus titers and ALT could not be determined for 1, 2 and 2 hamsters from the 2006 CLDC, n2006 CLDC, and NFW placebo groups, respectively. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ compared to NFW-treated animals. Mean virus titers and ALT levels for each group in graphs B–D are represented by horizontal lines. Nuclease-free water, NFW; ribavirin, Rv.

ALT levels did not include the sickest animals from these groups. Despite the slight hint of protection (40% survival) by 2006 CLDC in the treated animals evaluated for mortality, there was no apparent inhibition of virus replication or amelioration of liver disease (Fig. 3B–D).

3.4. Cytokine induction in hamsters following exposure to CLDC

Hamsters were treated i.p. with 30 μg of CLDC, and splenic transcript induction was assessed for a group of inflammatory cytokines including IFN-γ and IL-12, previously shown to be induced in mice stimulated with CLDC (Gowen et al., 2006a). As shown in Fig. 4A, IFN-γ was induced shortly after exposure, with maximal induction detected 3–4 h post-exposure, and transcript levels returning to normal by 12 h. Doubling the treatment dose of CLDC to 60 μg did not increase the highest observed IFN-γ gene expression levels seen at the 2- and 6-h time points. TNF-α was rapidly induced by CLDC with sustained elevated expression through 6 h post-exposure (Fig. 4B). The increased dosage of CLDC augmented TNF-α transcript levels at the 2-h time point; however, this elevation was not maintained out to the 6-h time point. Surprisingly, there was no apparent induction of IL-12 following CLDC treatment (data not shown), as was seen at the protein level in the murine system (Gowen et al., 2006a). IL-21 gene expression was dramatically elevated at 2 h with an abrupt decline by 3 h following exposure and returning to normal thereafter (Fig. 4C). The 60-μg CLDC injection evoked a robust induction of IL-21 when measured at 2 h, with return to baseline at 6 h. Overall, the data indicate that CLDC do impact gene expression of select inflammatory cytokines in the hamster system.

3.5. Expression analysis of CLDC- and CpG ODN-treated mice

Microarray experiments were conducted to gain insights into the mechanistic differences between CLDC and CpG ODN. Since no microarray sets for hamsters were available, mice ($n = 5$ per group) were either injected with PBS (control) or with 50 μg of CpG 1826 ODN, or with 20 μg of CLDC, all by the i.p. route. Six hours later, spleens were collected and RNA prepared for hybridization using a 36 K array. When CLDC-treated mice were compared to control animals, there were 17,599 differences out of a total of 36,177 transcripts, as defined by at least a two-fold change in the level of gene expression (Fig. 5A). For CpG ODN-treated mice versus controls, there were 12,328 differences in transcript expression noted (Fig. 5B). Of greatest interest, when CpG ODN- and CLDC-treated mice were compared, we noted 5727 differences in gene transcription (Fig. 5C), or differences in 16% of all genes expressed.

Next, differences in immune response genes in CLDC- versus CpG ODN-treated mice were examined. There were 64 (11%) immune response genes up-regulated in the mice receiving CLDC, relative to those that received CpG ODN, while 33 (5%) genes were down-regulated in CLDC-treated mice. Notable differences included up-regulation of type I and type II IFN responses, as well as

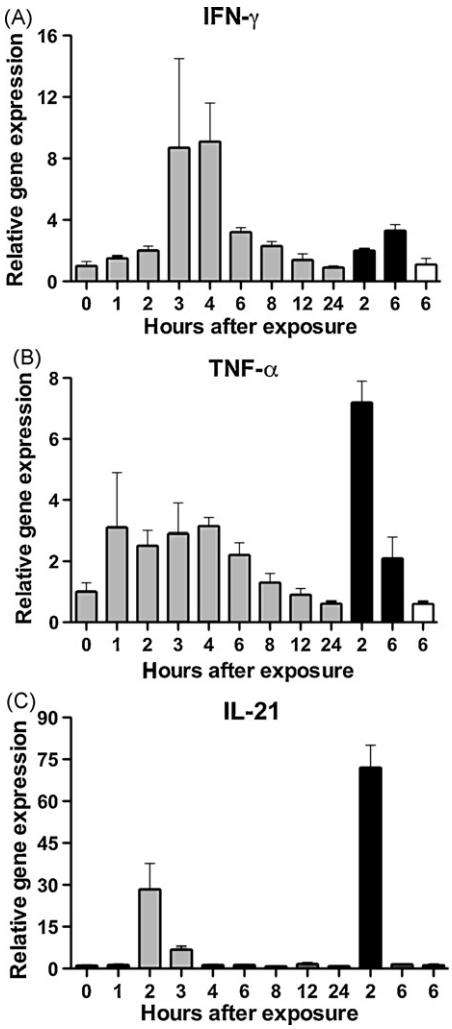

Fig. 4. Inflammatory cytokine gene induction in hamsters following exposure to CLDC. Hamsters were treated i.p. with a 30-μg dose of CLDC (grey bars) and transcript levels for IFN-γ (A), TNF-α (B) and IL-21 (C) were determined from spleens collected at 0, 1, 2, 3, 4, 6, 8, 12, 24 h after treatment. Also included in the analysis were hamsters exposed for 2 and 6 h to a 60-μg dose of CLDC (black bars) or 6 h to vehicle alone (white bars). Spleens were homogenized and total RNA was isolated for qRT-PCR analysis. Gene expression levels were normalized to γ-actin expression and the data are expressed as relative gene expression compared to the time 0 baseline value.

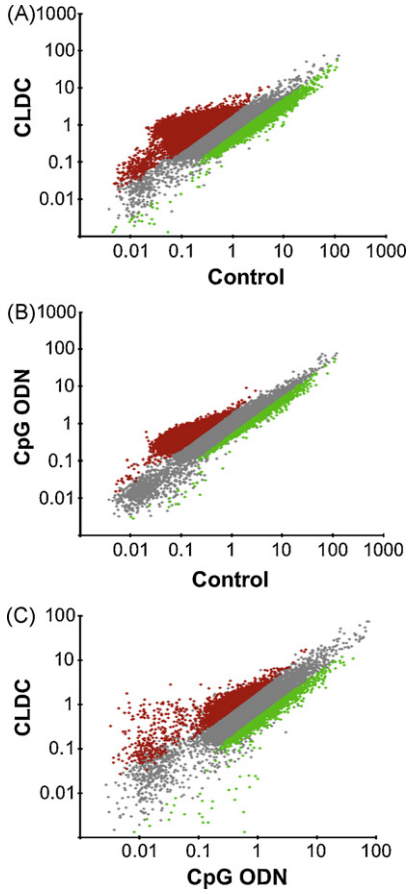

Fig. 5. Microarray analysis of differential gene induction following exposure to CLDC and CpG 1826 ODN. Mice ($n = 5$) were injected i.p. with 20 μg CLDC, 50 μg CpG 1826 ODN, or PBS control and 6 h later, RNA was prepared from spleens and subjected to microarray analysis. Data represent treatment with CLDC versus control (A), CpG ODN versus control (B), and CLDC versus CpG ODN (C). The grey color represents genes expressed in relatively equal amounts in the treatment groups (<2-fold change), the red color represents genes over expressed in mice treated as indicated on the *Y* axis and the green color represents genes over expressed in mice treated as indicated on the *X* axis.

IL-12 and IL-15 (Table 2). There was also strong up-regulation of a key co-stimulatory molecule responsible for generating $CD8^+$ T cell responses (CD70), as well as two chemokines that regulate monocyte (CCL2) and macrophage (M-CSF) recruitment and survival. One critical gene down-regulated in CLDC-treated mice was CD47, which is widely expressed on a variety of cell types and delivers a strong immunosuppressive signal via the signal regulatory protein alpha (SIRP-α). CD47/SIRP-α ligation has been shown to cause inhibition of cytokine production and phagocytosis by macrophages (Brown and Frazier, 2001; Latour et al., 2001). Down-regulation of the expression of this immunosuppressive signal could in part account for the enhanced activity of CLDC as an immunostimulant.

To confirm the IFN results from the microarray data, we conducted qRT-PCR on the same RNA samples. Although a difference was not suggested by microarray analysis we further analyzed IFN-β since it is believed to induce the production of IFN-α (Goodbourn et al., 2000). As shown in Fig. 6, induction of IFN-γ, -α, and -β was most prominent in mice exposed to CLDC. Notably, the fold change by qRT-PCR (Fig. 6A and B) was much higher than that of microarray data for IFN-γ and IFN-α (Table 2). While the microarray is a reliable method to determine relative expression differences it consistently underestimates expression levels by qRT-PCR and even misses some genes entirely (Dallas et al., 2005; Yuen et al., 2002), as was the case for IFN-β (Fig. 6C).

Table 2
Immune response genes up- and down-regulated[a] in CLDC-treated mice compared to CpG ODN.

Up-regulated in CLDC-treated mice	Down-regulated in CLDC-treated mice
IFN-α (5.17)	CD47 (2.37)
IFN-γ (7.16)	
IL-17 (2.92)	
IL-15 (2.01)	
IL-12 (p35) (2.06)	
CCL2 (3.31)	
M-CSF (2.23)	
CD70 (3.25)	

[a] The level of up- or down-regulation equivalent to fold change is indicated in parentheses.

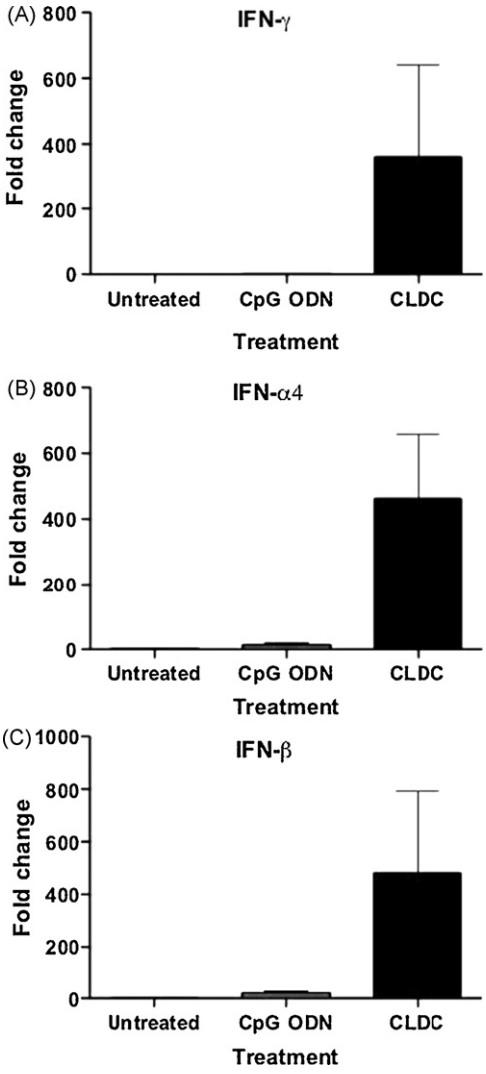

Fig. 6. Type I and II IFN gene induction in mice following exposure to CLDC. Mice were treated, and RNA was prepared, as described for the microarray analysis. Relative splenic messenger RNA levels of IFN-γ (A), IFN-α (B), and IFN-β (C) were determined by qRT-PCR. Gene expression was normalized to the HPRT1 housekeeping gene. The data are represented as fold change in IFN expression of treated (CpG ODN and CLDC) samples compared to untreated (PBS) samples.

4. Discussion

The synergistic combination of liposomes and plasmid DNA provides a unique and potent immunological stimulus, which activates substantial T_H1-mediated immune responses (Dow et al., 1999; Gowen et al., 2006a). The effect of CLDC on innate immune activation and subsequent cytokine production are directed through pathways including toll-like receptor 9 (TLR9) and potentially other immune response pathways (Yasuda et al., 2002, 2005a,b). Type I interferon (IFN-α/β) induction in plasmacytoid dendritic cells (DC), through activation of TLR9 is dependent on the transcription factor interferon regulatory factor 7 (IRF-7), which interacts with and is activated by myeloid differentiation factor 88 (MyD88) adaptor protein. Cationic liposomes markedly potentiate activation of innate immunity by TLR9 (bacterial DNA and CpG ODN) and TLR3 (poly I:C) agonists (Zaks et al., 2006). However, there is evidence suggesting that cationic liposome–DNA complexes can also activate the innate pathway through TLR9 independent mechanisms (Honda et al., 2005). The ability of cationic liposomes to potentiate activation of innate immunity by DNA is likely due to protection of the DNA from extracellular degradation, passive targeting to DC and macrophages, and to enhanced entry of DNA into the endosomal compartment, where TLRs are selectively expressed, thus allowing for increased immune activation (Akira and Takeda, 2004). The cationic liposomes may also increase the endosomal versus lysosomal retention of TLR9 agonists in conventional DC, leading to augmented IFN production (Honda et al., 2005).

When the immune stimulatory property of CLDC was first observed, it was initially assumed that increased potency was a primary difference between CLDC and CpG ODN. However, a series of recent studies have revealed that there are also important qualitative differences in the immune activation by these compounds (Honda et al., 2005; Stetson and Medzhitov, 2006; Takaoka et al., 2007). A key factor in understanding the differences between the two immune stimulatory compounds has been an increased understanding of the degree to which cytoplasmic sensors for nucleic acids contribute to activation of innate immunity. For example, these receptors, which include the cytosolic receptors for RNA molecules (RIG-I; retinoic acid-induced protein-I and mda-5; melanoma differentiation-associated gene-5) and for DNA (DAI; DNA-dependent activator of IFN-regulatory factors), have now been shown to be an important part of the signaling machinery for responding to nucleic acids. The DAI receptor was discovered by in vitro studies that revealed a different set of innate immune responses were activated when CpG ODN were delivered to DC and macrophages (Takaoka et al., 2007). These new observations suggest that combining cationic liposomes with nucleic acids fundamentally changes the immune stimulatory properties by altering intracellular trafficking.

Currently, there is a great need to develop effective prophylactic and therapeutic countermeasures for the prevention and treatment of RVF, particularly broad-spectrum drugs that address multiple targets on a long list of threat agents. We have previously demonstrated vigorous CLDC induction of antiviral cytokines and protective immunity in mice challenged with PTV, which serves as a model of RVF disease. Since immunomodulators act primarily through stimulation of cellular responses rather than direct disruption of the viral life cycle in order to exert their antiviral activity, demonstrating efficacy in multiple species is of great importance. In our experience with other immune response modifiers, efficacy in the mouse PTV infection model does not necessarily translate into comparable efficacy in hamsters (Gowen et al., 2008, 2006c). Taking this into consideration, the effective treatment of PTV infection in hamsters with CLDC reported here is encouraging.

It is conceivable that a higher dose of CLDC would demonstrate greater efficacy than seen in these three experiments. In mice, 1 μg of CLDC was found to be highly effective (Gowen et al., 2006a). On a per body weight basis, this would be equivalent to a dosage of approximately 10 μg in the size of hamster used in our studies. Since poly(I:C_{12}U) requires considerably more drug to be effective in the hamster PTV infection model than in the mouse (Gowen et al., 2006c), we chose to increase the dose of CLDC to 30 μg for the hamster studies. Future studies investigating the efficacy of higher doses or multiple dose treatment regimens may produce better results, as we may not have reached the optimal dosage in hamsters. This idea is supported by the hamster gene expression data of the inflammatory mediators, TNF-α and IL-21, wherein doubling the 30-μg dose augmented the levels of these cytokines induced in the spleens of treated animals.

It was hypothesized the CpG islands naturally present in the plasmid DNA used in the preparation of CLDC were principal components accounting for its immunostimulatory activity, presumably through signaling events resulting from recognition by TLR9 (Hemmi et al., 2000; Krieg, 2002). In experiments where

we used CLDC formulated with known mouse- or human-reactive ODNs containing CpG motifs instead of the standard plasmid DNA, very little, if any, antiviral activity was seen. Since species specificity has been observed with the recognition of CpG sequences (Bauer et al., 2001), it is possible that the hamster system is not responsive to the mouse- or human-reactive ODNs. This notion is consistent with the fact that CLDC prepared with plasmid devoid of CpG sequences failed to significantly protect hamsters from death, whereas the standard CLDC formulation was effective. Interestingly, there was a noticeable and significant reduction in day 4 viral loads and ALT levels suggesting that components other than CpG can evoke, to a lesser degree, a beneficial antiviral response. This finding was not surprising since previous reports indicate that other TLR9- and CpG-independent mechanisms likely contribute to the overall stimulatory properties of CLDC (Ishii et al., 2006; Stetson and Medzhitov, 2006).

The CpG dinucleotide sequences contained in plasmid DNA are known to be potent stimulants of antiviral defenses including the induction of type I IFN. Although the ability of CLDC to induce type I IFN has not been demonstrated in hamsters, intravenous administration evokes the rapid release of high levels of IFN-α in mice (Gowen et al., 2006a). Early appearance of, or treatment with, type I IFN has been shown to be effective in nonhuman primates challenged with RVFV (Morrill et al., 1989, 1990). Therefore, in its current formulation, the most likely use for CLDC as a countermeasure for RVF would be in instances of intentional release of aerosolized RVFV or in cases of accidental laboratory infections where the drug could be administered within a day or two after exposure. Post-exposure use in the event of intentional release would require rapid identification of the agent and timely administration of the drug. In future studies it will be important to define the window for both pre- and post-exposure prophylaxis. This is particularly important because delayed treatment might exacerbate infection through an immunopathological mechanism. Since the elaboration of exaggerated amounts of proinflammatory mediators is widely believed to contribute significantly to the pathogenesis of viral hemorrhagic fevers, treatment of advanced RVFV infection may actually worsen the disease state by accelerating the production of various cytokines and chemokines that increase disease severity and vascular dysfunction (Bray, 2005; Geisbert and Jahrling, 2004). Thus, CLDC treatment after the onset of illness may be contraindicated. Studies investigating therapeutic CLDC treatment in hamsters are needed to address this issue.

There were significant qualitative differences in immune gene expression in CLDC- versus CpG ODN-treated mice, with a number of important molecules necessary for effective antigen presentation, especially IL-12 and IL-15 and type I and II IFNs being up-regulated in CLDC-treated animals. Overall, in vitro and animal studies suggest that CLDC activate the innate immune system more broadly and at a higher level than CpGs. The microarray studies establish clearly that CLDC are a unique immune activator, which shares some similarities with CpG ODN, but also a number of important differences. Now we must add to this the possibility that fundamental mechanistic differences in immune activation separate these two modalities.

In the experiments reported here, CLDC were given prophylactically, which would support its potential application in preventing RVF disease in high-risk individuals such as herdsmen or others involved in livestock handling or processing, particularly in the setting of an epidemic, where use would be restricted to a limited window in time. Ultimately, studies in animal models employing authentic RVFV are needed to validate the efficacy seen in mice and hamsters challenged with PTV. From a practical standpoint, the potential use of CLDC as a prophylaxis to thwart natural outbreaks of RVF, which occur sporadically and sometimes in remote areas often far removed from modern medical facilities, will depend on cost and formulation of the drug. Since large-scale production of plasmid DNA can be done rather inexpensively, reduced manufacturing costs may make CLDC a viable option. However, unless the drug can be formulated for oral administration and without requirement for a cold chain, it is unlikely that CLDC will have any utility in a natural outbreak setting. Additional animal studies would be needed to define the window of protective immunity for extended prophylaxis following CLDC treatment.

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