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Treatment with cationic liposome–DNA complexes (CLDCs) protects mice from lethal Western equine encephalitis virus (WEEV) challenge

Christopher H. Logue[a,b,1], Aaron T. Phillips[b], Eric C. Mossel[a,b,*], Jeremy P. Ledermann[a], Thomas Welte[b], Steve W. Dow[b], Ken E. Olson[b], Ann M. Powers[a]

[a] Division of Vector-Borne Infectious Diseases, Centers for Disease Control, Fort Collins, CO 80521, USA
[b] Department of Microbiology, Immunology and Pathology, Colorado State University, Fort Collins, CO 80523, USA

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ABSTRACT

Having recently characterized a CD-1 outbred mouse model of pathogenesis for Western equine encephalitis virus, we examined the possible protective effects of cationic liposome–DNA complexes (CLDCs) against encephalitic arboviral infection. In this investigation, mice were pre-treated, co-treated, or post-treated with CLDC then challenged with a subcutaneous or aerosol dose of the highly virulent WEEV-McMillan strain, lethal in mice 4–5 days after inoculation. Pre-treatment with CLDCs provided a significant protective effect in mice, which was reflected in significantly increased survival rates. Further, in some instances a therapeutic effect of CLDC administration up to 12 h after WEEV challenge was observed. Mice treated with CLDC had significantly increased serum IFN-γ, TNF-α, and IL-12, suggesting a strong Th1-biased antiviral activation of the innate immune system. In virus-infected animals, large increases in production of IFN-γ, TNF-α, MCP-1, IL-12, and IL-10 in the brain were observed by 72 h after infection, consistent with neuroinvasion and viral replication in the CNS. These results indicate that strong non-specific activation of innate immunity with an immune therapeutic such as CLDC is capable of eliciting significant protective immunity against a rapidly lethal strain of WEEV and suggest a possible prophylactic option for exposed individuals.

1. Introduction

Previous studies have shown the antiviral potential of cationic liposome–DNA complexes (CLDCs) against members of the *Bunyaviridae* and *Hepadnaviridae* (Gowen et al., 2006, 2009; Morrey et al., 2008). Initially investigated as a gene delivery system in tumor immunotherapy, researchers observed that the administration of liposomes complexed with DNA elicited a significant activation of innate host immunity (Dow et al., 1999; Gursel et al., 2001; Lanuti et al., 2000; Whitmore et al., 1999, 2001; Zhu et al., 1993). CLDC-mediated stimulation of the innate immune system is due to the nature of the liposomes protecting DNA from extracellular degradation while presentation of non-methylated CpG oligonucleotides (ODN) is directed to the endosomal compartment of targeted cells such as plasmacytoid dendritic cells (DC) (Akira and Takeda, 2004; Krieg, 2002b; Takeshita et al., 2001; Zaks et al., 2006). These CpG-ODN motifs then stimulate the toll-like receptor (TLR) 9 signaling cascade to eventually induce type I interferon (IFN-α/β) and promote DC activation and maturation (Fitzgerald-Bocarsly, 2002; Ito et al., 2005) (Fig. 1). The induction IFN-α/β in DC through activation of TLR9 is dependent on activation of myeloid differentiation factor 88 (MyD88) adaptor protein by a cascade involving transcription factor interferon regulatory factor 7 (IRF-7). Production of type II interferon (IFN-γ), tumor necrosis factor alpha (TNF-α) and interleukin 12 (IL-12) by CLDC have been shown to involve the interaction of MyD88 with the NF-κB pathway (Akira and Takeda, 2004; Yasuda et al., 2005) (Fig. 1). As the immunologic pathways are further elucidated so too has the investigational use of CLDC as a potent vaccine adjuvant against viruses including Simian immunodeficiency virus (SIV) and Herpes simplex virus (HSV) (Bernstein et al., 2009; Fairman et al., 2009). Other TLR9-independent pathways, such as cytosolic DAI (DNA-dependent activator of IRFs) have also been shown to contribute to the immunostimulatory properties of CLDC (Hemmi et al., 2000; Honda et al., 2005; Ishii and Akira, 2006; Krieg, 2002a; Stetson and Medzhitov, 2006; Takaoka et al., 2007; Takaoka and Taniguchi, 2008; Wang et al., 2008) (Fig. 1).

Western equine encephalitis virus (WEEV) is a member of the *Alphavirus* genus of the family *Togaviridae*. WEEV has been responsible for periodic outbreaks of encephalitis in equines and humans and is a significant public-health concern in western/central North

* Corresponding author at: Department of Microbiology, Immunology and Pathology, 1492 Campus Delivery, Colorado State University, Fort Collins, CO 80523, USA. Tel.: +1 970 491 7350/5551; fax: +1 970 491 8323.
E-mail address: eric.mossel@colostate.edu (E.C. Mossel).
[1] Present address: Centre for Emergency Preparedness and Response, Health Protection Agency, Porton Down, Salisbury, SP4 0JG, UK.

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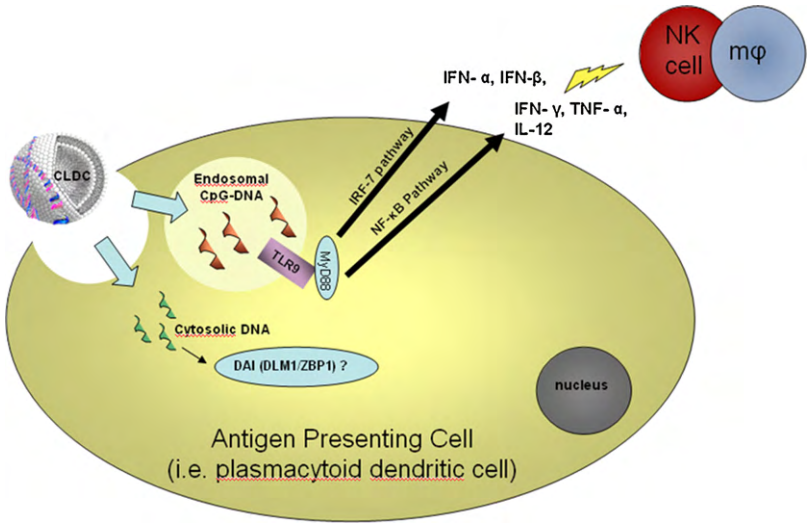

Fig. 1. Possible routes of processing of CLDC within a human host resulting in an enhanced activity of the innate immune response.

America (Calisher et al., 1988; Logue et al., 2009). In humans, WEEV causes signs and symptoms ranging from fever and headache to severe encephalitis that can result in the development of neurologic sequelae. Overall, the estimated case fatality rate in humans is 3–4%, but these rates have been reported to be as high as 8–15% during epidemics (Reisen et al., 2001; Reisen and Monath, 1988).

Although transmitted by the mosquito *Culex tarsalis* in its natural cycle, WEEV has also been shown to be infectious by aerosol. When aerosolized, WEEV causes high primate mortality (Reed et al., 2005) and is therefore considered a potential bioterrorism agent (Sidwell and Smee, 2003). Unfortunately, neither a human vaccine nor antiviral drugs are available for the prevention and treatment of WEEV infection.

In a previous investigation we characterized two WEEV isolates representing high (McMillan; McM) and low (Imperial-181; IMP-181) virulence phenotypes in an outbred murine model (Logue et al., 2009). In this investigation we examined the protective effects of administering CLDC to mice, pre-, co- and post-infection with a lethal dose of the established highly virulent McMillan strain of WEEV via four distinct infection routes. In order to ascertain the effects that CLDC had on virus propagation, the brain, spleen and lymph nodes were removed for virus titration over a 120 h period. Viremia and mean time of death were investigated, as were serum and CNS levels of the cytokines IFN-γ, IL-10, IL-12p40 and TNF-α, and the chemokine MCP-1. We found that CLDC treatment elicited protective immunity when administered prior to or shortly after WEEV challenge. Results of these studies in the new WEEV animal model may help direct future potential vaccine and therapeutic studies with the other encephalitic alphaviruses Venezuelan equine encephalitis virus (VEEV) and eastern equine encephalitis virus (EEEV).

2. Materials and methods

2.1. Virus strains

WEEV-McMillan isolate came from the Arbovirus Reference Collection at the Centers for Disease Control and Prevention, Fort Collins, Colorado, USA. Seed stocks for these experiments were made by infection of Vero cells (ATCC) grown in minimal essential medium with 10% fetal calf serum at a m.o.i. of ≤0.01. Cell culture supernatant was collected at 48 h post-infection (p.i.) and stored in aliquots at −80 °C. Virus titer was determined by plaque assay on Vero cells as previously described (Logue et al., 2009).

2.2. Preparation and administration of cationic liposome–DNA complexes

Cationic liposomes (100 mM DOTIM lipid + cholesterol) in 10% sucrose solution were provided by Juvaris BioTherapeutics (Pleasanton, CA). Cationic liposome–DNA complexes (CLDCs) were freshly prepared for each experiment as follows. Liposomes were diluted 1:5 in sterile Tris-buffered 5% dextrose water (pH 7.4). Non-coding plasmid DNA (pMB75.6 empty vector, 3 mg/ml) was then added to a final concentration of 0.1 mg/ml causing spontaneous formation of CLDC. The CLDCs were administered to mice within an hour of preparation.

2.3. Mouse virulence studies

The use of all animals was reviewed and approved by the Animal Care and Use Committee at Colorado State University. Care and handling of the mice was consistent with the PHS Policy and Guide for the Care and Use of Laboratory Animals. Outbred female CD-1 mice (The Jackson Laboratory, Bar Harbor, ME or Charles River Labs, Wilmington, MA) 5–7 weeks old were allowed to acclimate to the facility for 3–7 days. Subcutaneous (s.c.), intranasal (i.n.), and intravenous (i.v.) infections were performed at a dose of $1–5 \times 10^3$ plaque forming units (pfu) of WEEV stock isolates diluted in PBS. Subcutaneous injections were administered inside the left thigh unless noted. Intranasal inoculations were carried out by alternately dripping inocula onto the nostrils of lightly anesthetized mice until a volume of 20 μl was inhaled. Intravenous infections were performed via tail vein puncture. Inocula were titered by plaque assay on Vero cells to confirm dosage.

For aerosol infections, mice were exposed in a Middlebrook Airborne Infection Apparatus (Glas-Col, Terre Haute, IN). The nebulizer compartment was filled with a 5 ml suspension of virus at 10^6 pfu/ml. CD-1 mice were exposed to aerosol for 1 h then returned to cages. To determine the estimated inhalation dose, groups of 3 mice were sacrificed immediately after exposure and their lungs homogenized and plaque assayed, with a resulting 10^2 pfu/g.

CLDC-treated mice were injected subcutaneously in the inner left thigh with 250 μl CLDC 24 h prior to virus infection. All mice were observed twice daily for signs of morbidity. Moribund mice were euthanized by CO_2 inhalation. The day a moribund mouse was euthanized was considered the day of death for calculation of mean time to death (MTD). Survivorship was fol-

lowed for a period of 28 days (initial studies) or 14 days (later studies).

2.4. Tissue titer study

Tissues infected with McMillan were collected from three mice per group at 12, 24, 48, 72, 96 and 120 hours post-infection (hpi). The brain, spleen, popliteal and inguinal lymph nodes were extracted from each mouse. Samples were removed after a 5 min PBS perfusion by cardiac puncture to ensure all systemic blood was removed. Tissues were placed in pre-weighed 1 ml green bead tubes (Roche, Switzerland) containing 0.5 ml MEM and processed as described previously (Logue et al., 2009).

2.5. Viral nucleic acid extraction and TaqMan real-time PCR assay

Viral RNA was isolated from homogenized mouse tissue, serum, and virus seeds using the RNeasy 96 universal tissue kit (Qiagen, Valencia, CA). Total RNA was extracted from 25 μl of tissue homogenate diluted 1:1 in MEM and eluted from the kit columns to a final volume of 100 μl of elution buffer. WEEV TaqMan virus specific primers and probe were designed against IMP and McM strains with the Primer Select software program (DNASTAR, Madison, WI) (Logue et al., 2009). The TaqMan probe was labeled with a 5′ end FAM reporter dye and a 3′ end BHQ1 quencher dye. The QuantiTect probe RT-PCR kit (Qiagen, Valencia, CA) was used for the TaqMan assay. A 50 μl total reaction volume consisted of kit components, 10 μl of RNA, 0.4 μM of primer, and 0.15 μM of probe. The reactions were subjected to 45 cycles of amplification in an iQ5 real-time PCR detection system (BioRad, Hercules, CA) according to the recommended QuantiTect probe PCR kit RT-PCR conditions. A standard curve was used to quantify the viral nucleic acid in the mouse tissue samples. The standard curve was completed by serially diluting a WEEV stock, titrating each dilution, and then extracting the RNA according to the RNeasy protocol. A curve correlation coefficient of $\geq$0.950 and a 90–100% PCR efficiency was used to validate each detection assay.

2.6. Sample preparation for cytokine analysis

Mice were administered s.c. CLDC and/or challenged with WEEV-McMillan s.c. as described. At selected time points after treatment or challenge, mice were euthanized and blood, brain, and spleen were collected. Blood was allowed to clot at 4 °C overnight. Samples were centrifuged and the serum collected and stored at 4 °C short term, −80 °C long term. Spleens were placed into pre-weighed 2 ml MagNA Lyser™ (Roche Applied Science, Indianapolis, IN) tubes containing 1 ml sterile PBS. Brains were divided approximately in half. Each half was also placed in a MagNA Lyser™ tube with 1 ml PBS. Tubes were re-weighed to determine tissue weight. Tissues were homogenized with MagNA Lyser™ instrument two times for 45 s at 5000 rpm. Homogenates were clarified by centrifugation. Supernatants were pooled into fresh tubes. Brain supernatants from the same animal were pooled into one tube. Homogenates were stored at −80 °C until assay. Tissue homogenates and sera were assayed for murine IFN-γ, IL-12/IL-23p40, and MCP-1 with Quantikine® Colorimetric Sandwich ELISA Kits (R&D Systems, Inc., Minneapolis, MN) per manufacturer's protocol.

2.7. Cytokine profiling

Tissue homogenates and sera were assayed for murine IL-12/IL-23p40 with Quantikine® Colorimetric Sandwich ELISA Kits (R&D Systems, Inc., Minneapolis, MN) per manufacturer's protocol.

The Cytometric Bead Array (CBA) Mouse Inflammation Kit (BD Bioscience) was used for analysis of IL-10, IFN-γ, MCP-1, and TNF-α per manufacturer's instructions with the following deviation: after a final wash, the beads were fixed in 1% paraformaldehyde in PBS for 24 h at 4 °C, centrifuged, and re-suspended in FACS buffer, and stored at 4 °C until analyzed. Flow cytometry was performed with a CyAn ADP Analyzer flow cytometer using Summit software (Beckman Coulter, Fullerton, CA).

2.8. Statistical analyses

Viremia titers in mice were compared using the Kruskal–Wallace non-parametric ANOVA. Survival was assessed using Kaplan–Meier survival curves, followed by statistical comparison using the log-rank test. These statistical analyses were done using GraphPad Prism v4.06 (GraphPad Software, San Diego, CA, USA). Statistical significance of titers in organs infected with McM and IMP-181 via aerosol or subcutaneously in addition to those pre-treated/untreated with CLDCs was calculated using a generalized linear model (S-Plus, Insightful Software, USA). The model included $\log_{10}$ (titer) as the response and strain, route, tissue, and time as explanatory variables. Correlation between measurements on the same mouse was included in the model. In addition to the four main effects, two-way interactions between strain and tissue, between route and tissue, and between time and tissue were included in the model. A three-way interaction between strain, route, and tissue was also included.

3. Results

3.1. CLDC administration protects mice from subcutaneous WEEV infection

To examine the potential of CLDC immunotherapy in the treatment of WEEV-McMillan infection, mice were treated with a single subcutaneous 250 μl (0.25 μg DNA) dose of CLDC 24 h prior to WEEV-McMillan challenge administered by the four routes described in Section 2.3. Fig. 2A and B show survival curves of untreated and treated animals, respectively. CLDC treatment had no effect on the outcome of WEEV-McMillan infection by the aerosol route. Thus, all CLDC-treated and untreated animals (n = 10 per group) succumbed to challenge by the fourth day post-infection. Similarly, CLDC treatment had no protective effect in mice subjected to WEEV-McMillan infection by the i.n. route: 100% and 90% mortality and a mean time to death (MTD) of 4.4 and 4.6 days in CLDC-treated and untreated mice, respectively (Table 1). Some protective efficacy of CLDC treatment was noted in mice infected with WEEV-McMillan by the i.v. route. Survival was significantly improved from 10% to 30% (p = 0.02) and MTD improved from 6.1 to 7.3 days with CLDC administration.

The greatest benefit from CLDC treatment was observed in mice infected with WEEV-McMillan by the s.c. route. In untreated animals, 100% of the animals succumbed to infection by 5 dpi with a

Table 1
Mean times to death (MTD) of WEEV-McMillan infected CD-1 mice treated/untreated with s.c. CLDC.

Route	CLDC	Survival (%)	MTD
s.c.	–	0	4.3
s.c.	+	83	5.5
i.v.	–	10	6.1
i.v.	+	30	7.3
i.n.	–	10	4.6
i.n.	+	0	4.4
Aero	–	0	4
Aero	+	0	4

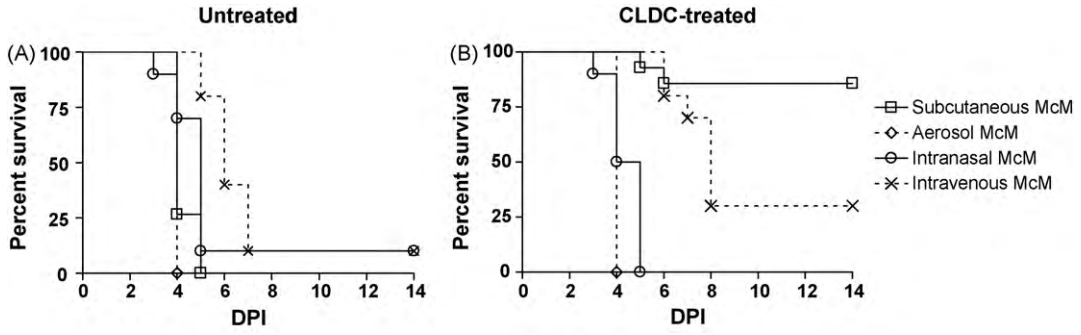

Fig. 2. Survival of CD-1 mice infected with WEEV-McMillan via four routes (s.c.: subcutaneous, i.v.: intravenous, i.n.: intranasal and Aero: aerosol). (A) Untreated, (B) treated with CLDC subcutaneously 24 h prior to WEEV infection.

MTD of 4.3 dpi. However, in the CLDC-treated animals, there was significant improvement in the survival rate (87%; $p < 0.0001$), and a MTD of 5.5 dpi. No morbidity was observed among surviving animals.

Since 24 h pre-treatment with CLDC elicited significant protection in the s.c. challenge model, survival improvement following CLDC treatment at the same time as exposure or after exposure to WEEV-McMillan was assessed. Therefore, mice inoculated with WEEV-McMillan by the s.c. route were then treated with CLDC 24 h pre-infection, at the same time as infection, or 24 h post-infection (hpi; Fig. 3). Treatment with CLDC 24 hpi did not produce an increase in survival. Moreover, CLDC treatment at the time of infection also did not produce a significant increase in survival (Fig. 3). When the site of CLDC administration was moved dorsally to the cervical spine, treatment at the time of infection resulted in 100% survival, a significant increase over untreated animals ($p = 0.005$; Fig. 4). Additionally, treatment at this site 12 hpi also resulted in a significant 75% increase in survival ($p = 0.045$) relative to untreated animals, thus demonstrating a benefit to post-exposure CLDC treatment in the CD-1/WEEV-McMillan model of infection. These results indicated that the site of CLDC administration was an important variable in determining treatment efficacy.

3.2. Distribution of WEEV in CLDC-treated mice

Virus titers in various tissues of CLDC-treated and untreated mice were measured to determine whether CLDC treatment was associated with a reduction in viral titers. Mice were treated with CLDC administered s.c. to the inner thigh 24 h prior to s.c. or aerosol WEEV-McMillan infection as described in Section 2.3. At the indicated time points, serum was collected, tissues were harvested, and viral titers were determined by real-time RT-PCR. Correlation of the genomic equivalents to a standard curve was made to determine viral pfu.

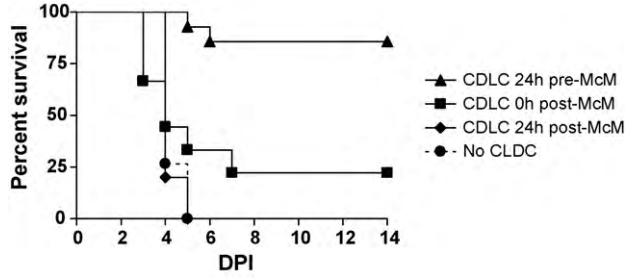

Fig. 3. Survival of CD-1 mice treated by subcutaneous CLDC administration at the inner thigh 24 h prior to infection, at the same time as infection (0 h), or 24 h post-infection with WEEV-McMillan delivered by subcutaneous injection at the inner thigh.

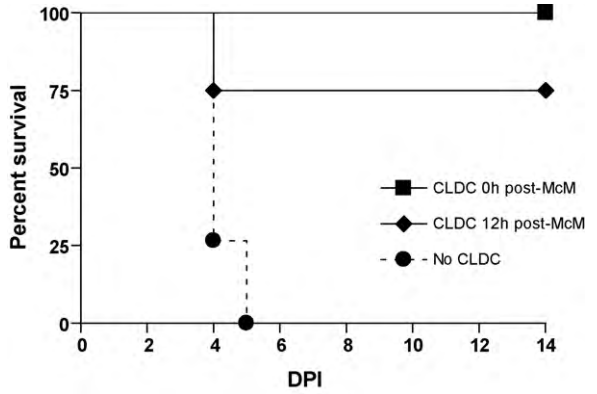

Fig. 4. Survival of CD-1 mice treated by subcutaneous CLDC administration at the back of the neck at the same time as infection, or 24 h post WEEV-McMillan infection delivered by injection at the inner thigh.

CLDC treatment significantly suppressed viremia in mice infected with WEEV-McMillan by either the s.c. or aerosol routes ($p = 0.032$ and 0.002, respectively). Virus titers in the serum of animals infected with s.c. WEEV-McM were up to 10-fold higher at each time point in untreated relative to CLDC-treated mice (Fig. 5). Further, in the CLDC treatment group, 10/18 measurements of viral titers over the course of the experiment were below the limit of detection (LOD; LOD ~1 pfu/mL). Thus, CLDC treatment

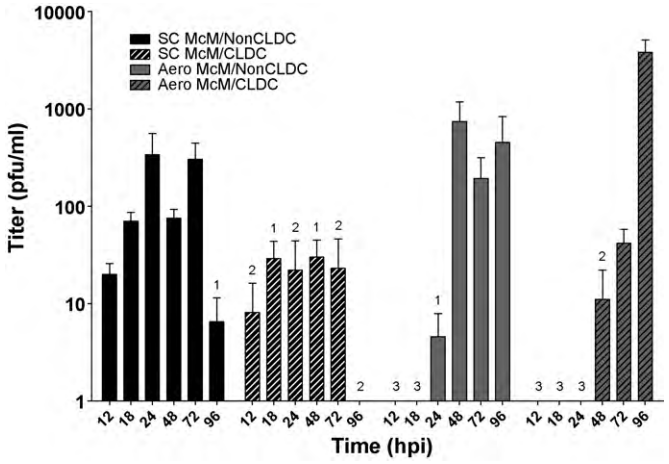

Fig. 5. Viremia of WEEV-McMillan infected mice, protected and unprotected by CLDCs administered s.c. at the inner thigh 24 h prior to infection via the subcutaneous and aerosol routes. Numbers over the bars indicate the number of determinations (out of 3) below the limit of detection. Virus titers were determined by real-time RT-PCR relative to a standard curve of stock virus for pfu conversion.

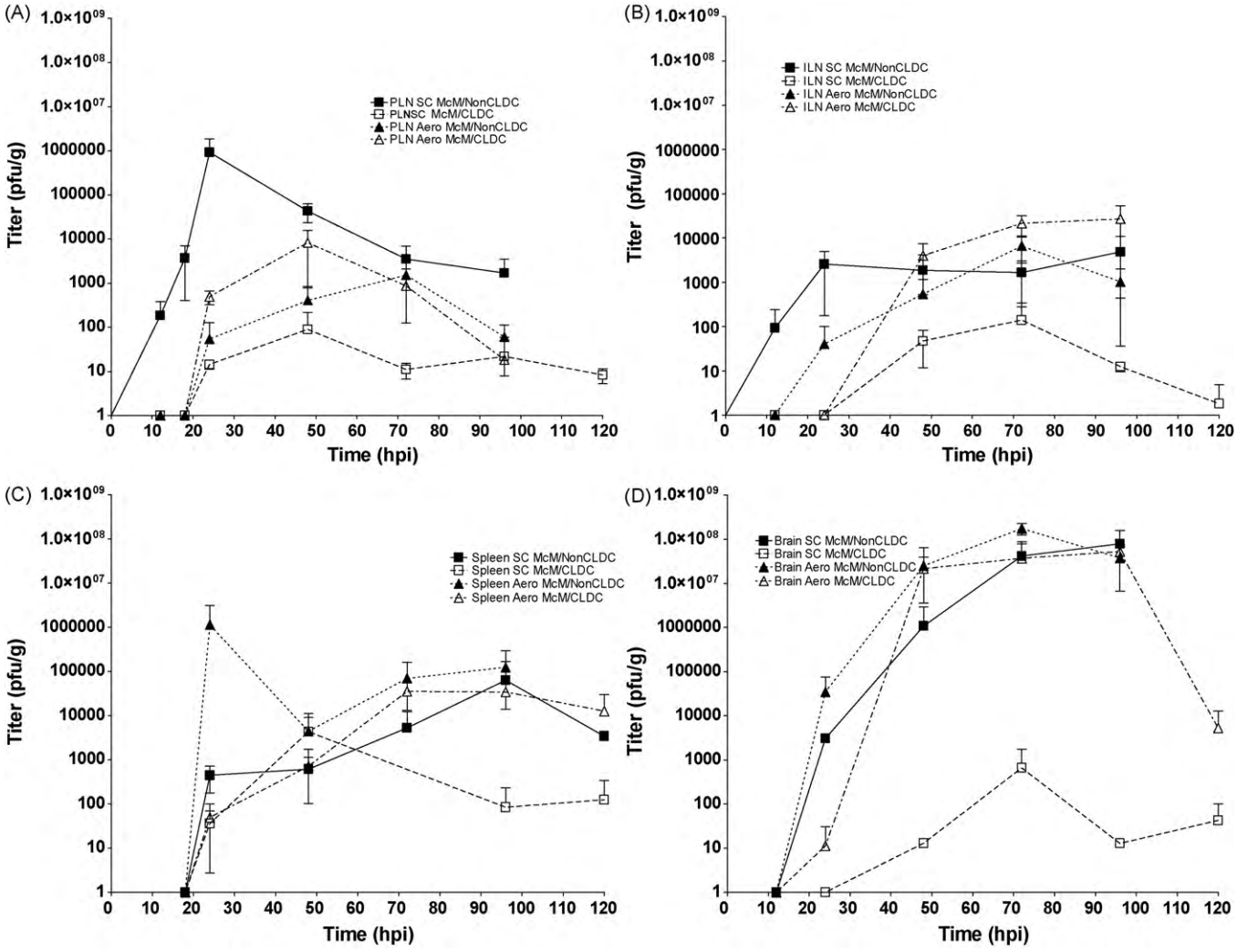

Fig. 6. Titers of WEEV-McMillan detected in the (A) popliteal lymph nodes, (B) inguinal lymph nodes, (C) spleen, and (D) brain when infected via the subcutaneous and aerosol routes and treated/untreated subcutaneously at the inner thigh with CLDC. Virus titers were determined by real-time RT-PCR relative to a standard curve of stock virus for pfu conversion.

significantly suppressed viremia in s.c. challenge model of WEEV-McMillan infection ($p = 0.032$).

CLDC treatment also significantly suppressed viremia following aerosol WEEV-McM infection ($p = 0.002$). Detectable viremia appeared in the untreated animals at 24 hpi, while in the CLDC-treated group detectable viremia did not appear until 48 hpi (Fig. 5). In both groups, all animals became viremic by 72 hpi. Titers among untreated mice were maximal 48 hpi ($\sim 10^3$ pfu/ml) and remained high until death at about 96 hpi. Viral titers in the CLDC-treated mice tended to rise from their appearance at 48 hpi until death at about 96 hpi, reaching $\sim 5 \times 10^3$ pfu/ml.

Virus titers were also determined over time for the inguinal and popliteal lymph nodes, spleen, and brain in mice challenged by the s.c. route. The site of injection on the inner thigh of s.c. infected mice suggested that the inguinal lymph nodes of the groin area and/or the popliteal lymph nodes of the knee joint might be of particular importance in virus dissemination from that site. Therefore, viral titers in these tissues were determined. Virus titers in the inguinal lymph nodes following s.c. infection increased through 24 hpi and were then maintained at a mean of 10^3–10^4 pfu/g through 96 hpi in untreated animals (Fig. 6B). In CLDC-treated mice, virus was undetectable in the inguinal lymph nodes until 48 hpi, a delay that might be expected based on local suppression of virus replication by cytokines elicited from the CLDC treatment. Titers were maximal at 100 pfu/g 72 hpi and decreased over the next 48 h. Similar, but more pronounced trends were observed in the popliteal lymph nodes of s.c.-infected animals (Fig. 6A). Virus titers were detectable in untreated animals by 12 hpi. Titers reached 10^6 pfu/g 24 hpi and then decreased until death. Titers in CLDC-treated animals were not detectable until 24 hpi, again delayed relative to untreated animals. From 24 hpi through 120 hpi virus titers remained <10–100 pfu/g.

Virus was not detectable following aerosol WEEV-McM infection until 24–48 hpi, as might be expected based on the distal location relative to the site of infection. Virus titers increased in the inguinal lymph nodes through 72 hpi to about 10^4 pfu/g then remained constant in CLDC-treated animals and decreased slightly in untreated animals. Titers in the popliteal lymph nodes were maximal 48–72 hpi at 10^3–10^4 pfu/g, decreasing over the next 24–48 h. Virus titers in the inguinal and popliteal lymph nodes following aerosol infection were not modulated by CLDC administration.

WEEV-McM titers in the spleen were similar for all four infection/treatment groups through 48 hpi (Fig. 6C). An outlying data point (3.4×10^7 pfu/g) resulted in the titer spike observed 24 hpi in the aerosol-infected, untreated group. With the exception of that single animal, both aerosol-infected groups and the untreated s.c.-infected group presented titer profiles that increased though 96 hpi to 10^5 pfu/g. Titers in surviving mice decreased slightly over the next 24 hpi. After 48 hpi, virus titers in the spleens of

s.c.-infected, CLDC-treated mice decreased to 10^2 pfu/g at 96 and 120 hpi.

The most striking differences in measured virus titer were observed in the brain (Fig. 6D). Brain virus titers in untreated, s.c. infected mice rose rapidly after 24 hpi and peaked 72–96 hpi at 10^8 pfu/g. Virus titer in the brains of CLDC-treated mice was undetectable until 48 hpi. Except for a single determination of 1.9×10^3 pfu/g at 72 hpi, measured titers were less than 1.1×10^2 pfu/g. As might be expected by the similar outcomes to infection, virus titers in the brains of mice infected by the aerosol route were independent of CLDC treatment. For both treatment groups, titers rose rapidly from 24 to 72 hpi. From 72 to 96 hpi, time of death for most animals, titers were 10^7–10^8 pfu/g.

3.3. Induction of cytokines/chemokines by CLDC stimulation and/or WEEV infection

To characterize the cytokine profile of CLDC-treated uninfected CD-1 mice over time, serum and whole brains were collected at the indicated time points and assayed for cytokine concentrations (Fig. 7). As expected, serum concentrations of IFN-γ, IL-12, and TNF-α were significantly upregulated in response to CLDC administration, all peaking at 36 h post CLDC administration (12 h time point in Fig. 7 as this is relative to inoculation of infected animal groups). CLDC treatment resulted in decreased serum levels of IL-10 within the first 72 h followed by a rapid increase at 96 h post administration. The serum concentration of MCP-1 remained unchanged by the CLDC treatment.

When animals were challenged with WEEV 24 h after CLDC treatment, serum IFN-γ was significantly reduced, and, interestingly, untreated infected animals showed no significant induction of serum IFN-γ at any time point. There was no evidence suggesting encephalitis in the brains of CLDC-treated animals as indicated by the lack of increased levels of MCP-1, IFN-γ, IL-10, IL-12, or TNF-α. Untreated, virus-infected animals, however, showed marked increases in MCP-1, IFN-γ, IL-12, IL-10, and TNF-α expression in the brain at 72 h post-infection. Treatment with CLDC did not significantly alter the levels of MCP-1, IFN-γ, IL-10, or TNF-α in the brain. Therefore, brain cytokine expression is assumed to have resulted from neuroinvasion and virus replication in untreated animals, as reflected in the virus titers of brains in treated and untreated animals (Fig. 6D). These results support the hypothesis that the CLDC treatment effectively cleared the virus from the periphery and thus did not allow for neuroinvasion and subsequent encephalitis.

4. Discussion

We have used a highly lethal model of WEEV infection to evaluate the utility of CLDC as a prophylactic immunotherapy, capable of protecting mice from WEEV encephalitis. We found with this model that CLDC treatment may also have therapeutic potential for the post-exposure treatment of WEEV infection. These results might further extend to other encephalitic alphaviruses such as VEEV and EEEV.

It is likely that the acute virulence of the challenge virus used in our model affected our ability to observe protection using CLDC immunotherapy. Following s.c. infection, the incubation period is approximately 3–4 days, after which animals progress to severe clinical illness within 24–48 h. As indicated, we saw no example in these studies of CLDC treatment facilitating the recovery from clinical illness. This contrasts with the human infection, which by the natural route may have an incubation time of 5–10 days. For those who progress to severe disease, it can still be up to a week from the time clinical symptoms appear until death. The longer disease course in humans may provide a larger therapeutic window, allowing for more successful post-exposure treatment, than we have with the current model. Using a wild-type virus strains such as Montana-64, with 70% mortality and MTD of 6.9 days (Logue et al., 2009), two days longer than McMillan, may give a better representation of the human condition and improve the success of post-exposure treatment.

Despite the challenge presented by the virulence of this model, by changing the site of CLDC administration from the thigh to the neck, a significant treatment benefit was observed. It is not clear from the current data whether administering the CLDC at the back of the neck resulted in a more potent systemic immunostimulatory effect or whether the effect is graduated with immunostimulatory effect decreasing as distance from the site of administration increases. If the latter is true, then the most potent effect was localized more closely to the head and brain, which may have led to more efficient prevention of neuroinvasion. Further studies on the role of the site of treatment are warranted to fully evaluate this positional phenomenon.

In addition to the survival analyses, we also sought to gain an understanding of the immune response to WEEV infection in the presence and absence of CLDC treatment. IL-12 and IFN-α are produced in response to many viral infections, primarily by plasmacytoid DCs. Alphaviruses have been shown to block the induction of type I interferon, thus limiting upregulation of dependent antiviral gene products (Barry et al., 2009; Burke et al., 2009). Further, signaling through the IFN-α/β receptor was shown to be necessary for the induction of the antiviral state and protection from VEEV challenge following treatment with viral replicon particles (VRP) (Konopka et al., 2009). As with VRP, CLDC administration, and subsequent type I IFN production, allows for an established antiviral state in advance of disseminating infection, and circumvents the requirement for the infected cell to produce IFN-α/β. Although alphaviruses can vary in their sensitivity to an established antiviral state generated through IFN-α/β priming (Simmons et al., 2009), WEEV has been shown to be exquisitely sensitive to interferon in vivo (Julander et al., 2007). However, virulence can vary greatly among isolates of WEEV (Logue et al., 2009). It is possible that this virulence variation could involve viral suppression of both type I and type II interferon responses through host macromolecular shutoff and/or inhibition of Jak/STAT phosphorylation, as shown with the prototypic alphavirus Sindbis (Ryman et al., 2007). Despite this, infection with acutely lethal WEEV-McMillan strain was found to be controlled through the immunomodulatory effects of CLDC treatment.

The possibility for other TLR agonists to provide similar protection remains open. It is curious that administration of poly(I:C), a TLR3 agonist, was previously shown to elicit very low levels of IFN-γ and IL-12 when compared to that of CLDC (Dow et al., 1999). However, in that study the poly(I:C) was not complexed to liposomes, thus limiting the presentation of the TLR agonist from the endosomal compartments where TLR3 is found. When poly(I:C) was complexed to liposomes it was equally efficient to CLDC in cross-priming CD8+ T cells (Zaks et al., 2006). When poly(I:C) was complexed to the same liposome formulation as used for the CLDC in this study, up to 80% of animals were protected from WEEV-McMillan infection. As with CLDC, this protection was dependent on time and route of poly(I:C) administration (Phillips and Olson, unpublished data).

Among the cytokines induced by CLDC treatment, IFN-γ presents great potential for aiding in the control of WEEV infection. We observed two different IFN-γ responses in our study. CLDC-treated animals produced increased serum IFN-γ relative to untreated animals. This may play a role in the prevention of the neuroinvasion and acute encephalitis and/or the resolution of any neuroinvasion that does occur. Untreated animals showed increased IFN-γ in the CNS relative to CLDC-treated animals. That

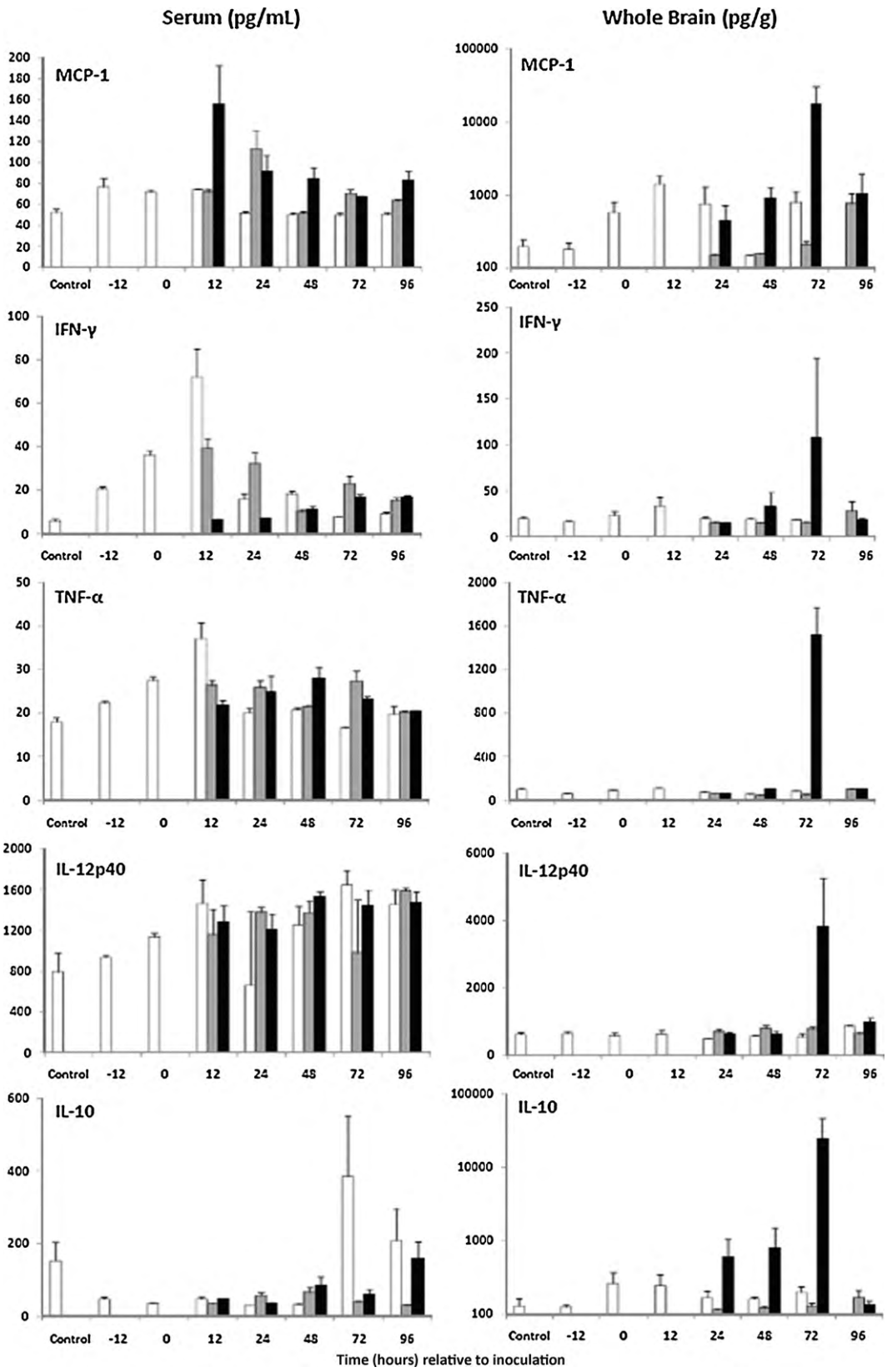

Fig. 7. Cytokine expression following exposure to CLDC and/or WEEV. Mice were treated s.c. on the inner thigh with a 25-μg dose of CLDC and challenged with 1×10^3 pfu WEEV-McM at 24 h after the CLDC treatment (grey bars), or challenged without CLDC treatment (black bars), or only treated and not challenged (white bars). Whole brains were extracted and homogenized and serum was collected at −12, 0, 12, 24, 48, 72, and 96 h relative to virus inoculation. IFN-γ, TNF-α, MCP-1, IL-10, and IL-12 concentrations were determined by either ELISA or cytometric bead assay and flow cytometry. The mean cytokine concentration (±SE) was plotted for each treatment group at each time point. Also included in the analysis are sham-injected control mice.

this peaked at the same time that virus titers in the brain peaked suggests that it is in response to the neuroinvasion and virus replication within the CNS. Several lines of evidence imply a role for IFN-γ in resolution of both peripheral and CNS WEEV infection. IFN-γ was shown to have direct antiviral effects that appear in advance of the adaptive immune response (Schroder et al., 2004). These antiviral properties include reduced viral protein synthesis, inhibited viral transcription, and recovery of host protein synthesis (Burdeinick-Kerr and Griffin, 2005). IFN-γ was shown to aid in the noncytolytic clearance of alphavirus infection from neurons (Burdeinick-Kerr and Griffin, 2005), an observation critically relevant to WEEV, as neuronal death is a prominent feature in WEEV pathogenesis. Further, cellular protein synthesis in neurons can be restored by an IFN-γ-dependent mechanism (Burdeinick-Kerr et al., 2009). Finally, the critical role of IFN-γ in the protective effects of CLDC to infection by other intracellular pathogens (Goodyear et al., 2009) suggest that the mechanistic details of this cytokine's actions may provide many avenues of study of WEEV pathogenesis and therapeutics.

Upregulated IFN-stimulated genes have previously been utilized as IFN response markers (Satoh et al., 2006) and may provide insight into the effects of CLDC treatment and the host response to WEEV infection. MCP-1 was dramatically upregulated in virulent SINV infection of mouse brains (Johnston et al., 2001), as well as the brain tissue of untreated infected animals of the current study. MCP-1 is highly induced in a variety of diseases that feature monocyte-rich cellular infiltrates and was shown to be critical in protection from intracellular pathogens (Goodyear et al., 2010). Monocyte chemotactic proteins were shown to inhibit the production of IL-12 by macrophages but are ineffective for inhibition in DCs, thus suggesting that different G-protein-coupled receptors are involved in the regulation of IL-12 production by DCs and by phagocytes (Aliberti et al., 2000; Trinchieri, 2003). IL-12 induces T cells and NK cells to produce cytokines such as GM-CSF and TNF-α and is remarkably efficient at inducing the production of IFN-γ (Murphy et al., 1994). The particular immunomodulation and physiological effects of high MCP-1 expression require further investigation, and the contribution of MCP-1 expression to WEEV pathogenesis in the CNS, remains to be elucidated.

The contribution of IL-10 to the overall outcome of WEEV infection of the CNS requires further investigation. T_H1 responses generally suppress T_H2 responses through the production of cytokines such as IL-12 and IFN-γ, and that IL-10 is involved in limiting the effectiveness of the T_H1 response. IL-10 and IFN-γ are antagonistic; with IL-10 acting through suppression of IL-12 (Fiorentino et al., 1989). Conversely, IFN-γ inhibits IL-10 production from monocytes which leads to upregulation of TNF-α (Chomarat et al., 1993; Donnelly et al., 1995). Therefore, IL-10 is a potent inhibitor of monocyte/macrophage function and anti-inflammatory with regard to its antagonism IL-12. Interestingly, IL-10 was shown to increase MCP-1 expression in blood mononuclear cells, however this effect on MCP-1 expression is activation state and cell type dependent (Seitz et al., 1995). It is interesting to speculate whether the increase in IL-10 observed in the brains of untreated animals is being produced in a vain effort to dampen the effects of the inflammatory cytokines present.

In addition to furthering our understanding of WEEV, and potentially VEEV and EEEV pathogenesis, clarifying the immunostimulatory effects of CLDC and which of those effects are important in protection/recovery from WEEV infection is essential to the refinement of CLDC as a therapeutic. The ultimate feasibility of CLDC as a potential therapeutic for WEEV infection remains to be determined. In these studies, the therapeutic window was relatively short and CLDC failed to protect from aerosol challenge. However, that CLDCs were able to offer protection in such a challenging model, suggests that additional study is warranted. It is possible that the antiviral activity of CLDC could be enhanced in a pathogen non-specific manner by coupling it with another immunomodulatory compound or in a more pathogen-specific fashion with a targeted antiviral therapeutic.

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