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Mucosal immunotherapy for protection from pneumonic infection with *Francisella tularensis*

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Abstract

Previous studies have demonstrated that systemically administered immunotherapy can protect mice from systemic challenge with the bacterial pathogen *Francisella tularensis*. However, for protection from inhalational challenge with this bacterium, we wondered if mucosally administered immunotherapy might be more effective. Therefore, we administered cationic liposome–DNA complexes (CLDC), which are potent activators of innate immunity, intranasally (i.n.) and assessed the effectiveness of protection from lethal inhalational challenge with *F. tularensis*. We found that pretreatment by i.n. administration of CLDC 24 h prior to bacterial challenge elicited nearly complete protection of BALB/c mice from lethal challenge with *F. tularensis* LVS strain. We also observed that mucosal CLDC immunotherapy provided a statistically significant increase in survival time in mice challenged with the highly virulent *F. tularensis* Schu4 strain. Protection was associated with a significant reduction in bacterial burden in the lungs, liver, and spleen. Mucosal administration of CLDC elicited significantly increased expression of IL-12, IFN- γ , TNF- α , IFN- β and IFN- α genes in the lung as detected by real-time quantitative PCR. In vitro treatment of *F. tularensis* infected macrophages with CLDC-elicited cytokines also significantly suppressed intracellular replication of *F. tularensis* in infected macrophages. In vivo, depletion of NK cells prior to administration of CLDC completely abolished the protective effects of CLDC immunotherapy. CLDC-elicited protection was also dependent on induction of IFN- γ production in vivo. We conclude therefore that activation of local pulmonary innate immune responses is capable of eliciting significant protection from inhalational exposure to a virulent bacterial pathogen.

Keywords

Bacterial; Mucosa; Lung; Cytokines; Rodent

1. Introduction

Francisella tularensis is a Gram-negative facultative intracellular bacterium that causes the dangerous and occasionally fatal disease tularemia. While cases of infection in humans are

relatively rare, concern over the use of *F. tularensis* as a biological weapon has brought renewed interest in this pathogen [1]. Inhalation of as few as 10 organisms has been shown to cause an acute and fatal disease in mice [2]. The high infectivity and virulence of inhaled *F. tularensis* has resulted in the organism being classified as a category A priority pathogen. Despite the importance of the inhaled route of infection, immune responses associated with protection from pneumonic tularemia are not well understood [3].

Two *F. tularensis* subspecies cause the majority of human infections: *tularensis* and *holarctica*, also referred to as 'type A' and 'type B,' respectively. A third subspecies *novicida* is attenuated in humans but is studied as a model pathogen of mice. A live vaccine strain (LVS) was derived from *F. tularensis* ssp. *holarctica* during the 1940s and 1950s by sequential passage on agar plates followed by passage in mice [4]. LVS is attenuated in humans but retains virulence for mice, although it is less virulent in mice than wild-type A and B strains. Because LVS causes a disease in mice which mimics virulent disease in humans, it has been studied extensively as a model intracellular pathogen [5,6]. However, LVS is not licensed as a vaccine for widespread use due to concerns regarding immune reactivity and a lack of information on the molecular basis for attenuation. Thus the lack of an effective licensed vaccine has resulted in a need for alternative immunotherapeutic approaches to prevent pneumonic tularemia.

Though *F. tularensis* is susceptible to treatment with several different antibiotics, non-specific immunotherapeutics that stimulate mucosal innate immunity for defense against *F. tularensis* would be desirable for several reasons. Appropriate innate immune responses have the potential to provide immediate and potent defense at mucosal sites of infection. In addition, such innate immune responses are often pathogen non-specific and capable of providing protection against a broad range of different organisms. This is particularly desirable in a biodefense situation in which the identity of an outbreak-causing pathogen may not be known. The potential for stimulation of innate immunity to provide host defense against *F. tularensis* was demonstrated by Elkins et al. who found that parenteral administration of immunostimulatory CpG oligonucleotides protected mice from intraperitoneal challenge with *F. tularensis* LVS [7,8]. However, to provide defense against respiratory infection, it may be advantageous and more effective to stimulate mucosal as opposed to systemic immune responses. For example, two research groups demonstrated that IL-12 administered intranasally to mice 24 h prior to challenge provided IFN- γ -dependent protection from lethal i.n. challenge with *F. tularensis* LVS or *F. tularensis* ssp. *novicida* [9,10]. However, repeated administration of high doses of recombinant cytokines is not likely to be an effective or easily administered option for immunotherapy of inhaled bacterial infections. In addition, a synthetic TLR4 agonist administered intranasally to mice provided protection from *F. tularensis* ssp. *novicida* but did not address whether this agonist could provide protection from *F. tularensis* type A and B strains [11]. Thus, it appears possible to control or prevent pneumonic tularemia using mucosal immunotherapy. However, an effective immunotherapeutic must be easily and safely administered and must demonstrate efficacy against *F. tularensis* type A and B strains.

Therefore, we evaluated the use of an immunotherapeutic (cationic lipid–DNA complexes) with a demonstrated record of safety in multiple mammalian species including humans (J. Fairman, personal communication) that was capable of potently and broadly activating host innate immunity with potential to be applied to mucosal immunotherapy for *F. tularensis*. Previous studies have shown that combining cationic liposomes with nucleic acids (e.g., with CpG ODN, plasmid DNA, or synthetic RNA) markedly increases immune stimulation when administered systemically or as a vaccine adjuvant [12–15]. Moreover, we have recently demonstrated that mucosally administered CLDC can protect mice from pathogenic *Burkholderia* species [16]. Therefore, we wondered if administration of CLDC to the airways might be effective as an immunotherapeutic for pneumonic tularemia.

To address this question, we administered CLDC immunotherapy to mice and assessed the effectiveness of protection from inhalational challenge with *F. tularensis* using infection models with *F. tularensis* LVS and the highly virulent *F. tularensis* ssp. *tularensis* Schu4 strain. In particular, we investigated the influence of route of administration and timing of CLDC administration on protection from pneumonic tularemia. In addition, immunological mechanisms of protection were investigated, with an emphasis on mucosal mechanisms of protection. We report here that mucosally administered immunotherapy can be used to effectively prevent or delay infection with virulent *F. tularensis*. Thus, mucosal immunotherapy may provide an additional option for non-specific control or prevention of inhaled pneumonic bacterial pathogens.

2. Materials and methods

2.1. Mice

Specific pathogen-free 6–8-week-old BALB/cJ, 129SvEv, and ICR mice were purchased from the Jackson Laboratory (Bar Harbor, ME), Taconic (Hudson, NY), and Harlan (Indianapolis, IN), respectively. IFN- $\gamma^{-/-}$ mice in BALB/cJ background were purchased from the Jackson Laboratory. Mice lacking the type I interferon receptor (IFN- $\alpha/\beta R^{-/-}$) on the 129SvEv background were kindly provided by Dr. Phillipa Marrack (National Jewish, Denver, CO) and Dr. Michel Auget (Swiss Institute for Biomedical Research, Lucerne). All studies involving mice were approved by the Institutional Animal Care and Use Committee at Colorado State University.

2.2. Preparation and administration of cationic lipid-DNA complexes

Cationic liposomes (100 mM DOTIM lipid + cholesterol) in 10% sucrose solution were provided by Juvaris BioTherapeutics (Pleasanton, CA). Cationic liposome–DNA complexes (CLDC) 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 0.2 mg/ml causing spontaneous formation of CLDC. For intranasal administration of CLDC, mice were deeply anesthetized by intraperitoneal injection of 100 mg/kg ketamine (Fort Dodge Animal Health, Overland Park, KS) with 10 mg/kg xylazine (Ben Venue Labs, Bedford, OH) and 20 μ l CLDC was rapidly placed in sequential droplets on alternating nares, allowing mouse to inhale the droplets. Intraperitoneal and subcutaneous injection of CLDC was accomplished with 200 μ l injected with an 18-gauge needle. Subcutaneous injection was given in the skin dorsal to the cervical region of the mouse spine. Intravenous injection of 100 μ l 0.5X (half of the lipid and DNA concentration) CLDC was given through the lateral tail vein.

2.3. Francisella tularensis challenge model

Frozen stocks of *F. tularensis* LVS and strain Schu4 were prepared as previously described [17,18]. Bacteria were thawed just before use, diluted in PBS and kept on ice prior to challenge. For intranasal challenge, mice were first deeply anesthetized with 100 mg/kg ketamine plus 10 mg/kg xylazine administered intraperitoneally. Then 20 μ l of diluted bacteria was placed in sequential droplets on alternating nares, allowing inhalation of the droplets. The 20 μ l challenge inoculum contained either 10^5 colony forming units (CFU) *F. tularensis* LVS or 40 CFU Schu4. All mice were monitored twice daily for disease symptoms and euthanized at a pre-determined humane endpoint.

2.4. Enumeration of bacterial burden in organs

Lungs, spleen and liver were collected and homogenized in 5 ml sterile PBS using a stomacher (Teledyne Tekmar, Mason, OH). Bacterial counts were determined by plating 100 μ l of serial

10-fold dilutions of organ homogenate on modified Mueller–Hinton agar and incubating at 37 °C with 5% CO₂ for 72 h. The lower limit of detection using this method was 50 CFU per organ.

2.5. Cytokine gene expression analysis

Individual mouse tissues were placed in 1 ml Trizol Reagent (Invitrogen, Carlsbad, CA) and frozen at –80°C. Tissues were thawed and homogenized using a Polytron PT10-35 rotorstator homogenizer (Kinematica, Lucerne, Switzerland). RNA was extracted following the manufacturer's instructions for Trizol Reagent. Any remaining DNA was then eliminated by treatment with DNase I Amplification Grade (Invitrogen) using 4U in a 100 µl volume. RNA preparations were further purified using the RNeasy Mini Kit (Qiagen, Valencia, CA) RNA cleanup protocol and quantified using a SmartSpec 3000 Spectrophotometer (Bio-Rad, Hercules, CA) at OD₂₆₀.

For qRT-PCR, 4 µg RNA was reverse transcribed to cDNA using the SuperScript III First-Strand Synthesis Kit for RT-PCR (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions using the provided oligo dT to prime reverse transcription. Cytokine cDNA was quantified using specific primers and FAM dye-labeled Taq-Man probes purchased from Applied Biosystems Inc., Foster City, CA. Primer and probe sequences for IFN-α4 and IFN-β were published previously [19]. All other primers and probes were designed by Applied Biosystems Inc. Reactions were prepared with Taqman Universal PCR Master Mix (Applied Biosystems Inc.) and run in 96-well format on an iCycler (Bio-Rad, Hercules, CA). Cytokine mRNA expression was normalized to abundance of the hypoxanthine guanine phosphoribosyl transferase 1 (HPRT1) housekeeping gene to control for different levels of mRNA recovery between samples. The ΔΔCt method of relative quantification was used to calculate fold change in transcript abundance of treated samples compared to untreated samples.

2.6. NK cell depletion

NK cells were systemically depleted from mice by intraperitoneal administration of 50 µl anti-asialo-GM1 antibody (Wako, Osaka, Japan) diluted to 200 µl in sterile PBS. Anti-asialo-GM1 antibody was administered 24 h prior to CLDC treatment and then administered again 5 days later to assure continued NK cell depletion. A control group of mice received i.p. injection of 50 µg ChromPure rabbit IgG antibody (Jackson ImmunoResearch, West Grove, PA) diluted to 200 µl in PBS.

2.7. In vitro infections

Spleen supernatants used for in vitro experiments were produced using ICR mice. Mice were given 200 µl CLDC by intravenous injection. At 3 h post-treatment, mice were euthanized and spleens were removed. Single-cell suspensions were prepared from spleens by mechanical disruption and screening through a 100 µm nylon mesh screen (BD Biosciences, San Jose, CA) followed by NH₄Cl lysis. Cells were resuspended in 24-well plates at 5 × 10⁶ cells per ml in complete MEM (Invitrogen, Carlsbad, CA) containing 10% FBS (Gemini Bio-Products, West Sacramento, CA), 2mM L-glutamine (Invitrogen), 1X non-essential amino acids (Invitrogen), 0.075% sodium bicarbonate (Fisher Scientific, Pittsburgh, PA), 100U/ml penicillin, and 100 µg/ml streptomycin (Invitrogen). After 20 h incubation at 37 °C, supernatants were removed, centrifuged to remove cells, combined and stored in frozen aliquots at –80 °C. Each aliquot was thawed for one use and then discarded. For some experiments IFN-γ and TNF-α in spleen supernatant were neutralized by the addition of anti-IFN-γ (clone R4.6A2) or anti-TNF-α (clone TN3-19.12) antibodies (eBioscience, San Diego, CA). Isotype antibodies for anti-IFN-γ (clone eBRG1) and anti-TNF-α (clone eBio299Arm) were used as controls (eBioscience). Antibodies were incubated at a concentration of 10 µg/ml in spleen supernatant at 37 °C for 30 min prior to treatment of cells.

Mouse alveolar macrophage AMJ.2 cells were pre-treated prior to infection by plating 200,000 cells per well of a 24-well plate in antibiotic-free complete media (see previous paragraph) containing dilutions of CLDC or control spleen supernatant. Cells were incubated 16 h at 37 °C in 5% CO₂, supernatants were removed and cells were washed with 1 ml sterile PBS. To infect AMJ.2 cells, 500 µl antibiotic-free media containing *F. tularensis* LVS at a multiplicity of infection (MOI) of 50 CFU per cell was added to each well. Plates were centrifuged at 2400 × *g* for 2 min and then incubated at 37 °C for 1.5 h. Infection medium was removed and cells were washed twice with sterile PBS. To remove remaining extracellular bacteria, 1 ml of media containing 50 µg/ml gentamicin (Sigma–Aldrich, St. Louis, MO) was added and cells were incubated for 1 h at 37 °C. The supernatant was then removed, cells were washed with sterile PBS and 1 ml media containing 10 µg/ml gentamicin was added to cells to prevent extracellular bacterial growth during incubation. Cells were incubated for 48 h at 37 °C in 5% CO₂ to allow for intracellular growth of *F. tularensis* LVS. To quantify intracellular bacteria, cells were washed three times with sterile PBS and then lysed in 1 ml sterile de-ionized H₂O. Cell lysates were serially diluted 10-fold in PBS, plated on modified Mueller-Hinton agar and incubated for 72 h at 37 °C in 5% CO₂. In addition, to account for any remaining extracellular bacteria, the final PBS wash fluid was also serially diluted and plated. Intracellular *F. tularensis* LVS CFU/well was calculated by subtracting the final PBS wash CFU/well from the cell lysate CFU/well.

2.8. Statistical analysis

All statistical analyses were conducted using Prism v. 5.0 (Graph-Pad Software, La Jolla, CA). Kaplan–Meier survival and log rank analysis was used to compare survival times. In survival analyses involving comparisons of more than 2 groups, the Bonferroni corrected threshold was applied to determine the appropriate level of significance. For experiments involving comparison of two groups, Student's two-tailed *t* test was used for statistical analysis. Comparison of multiple treatment groups was performed using ANOVA, followed by Tukey's multiple means comparison test. Serial mouse weight measurements were analyzed by two-way ANOVA with repeated measures and Bonferroni post-tests. Group differences were considered statistically significant for *p* < 0.05.

3. Results

3.1. Delivery of cationic lipid–DNA complexes (CLDC) to the lung mucosa protects mice from lethal inhalational challenge with *F. tularensis*

We used a high-dose *F. tularensis* LVS inhalational challenge model to determine whether CLDC administered directly to the nasal and lung mucosa could generate rapid protective immunity. For intranasal (i.n.) delivery of CLDC, BALB/c mice were anesthetized and given 20 µl of CLDC (10 µl per nostril). This volume of CLDC contained 4 µg non-coding plasmid DNA. Control mice were administered 20 µl of the 5% dextrose water diluent used to prepare CLDC. At 24 h post-treatment, mice (BALB/c, *n* = 5 per group) were challenged with 10⁵ colony forming units *F. tularensis* LVS via the i.n. route. We and others have found that this dose consistently results in 100% lethality for BALB/c mice [18,20–22]. Infected mice were monitored twice daily for disease symptoms and euthanized at a pre-determined humane endpoint. Untreated and diluent-treated mice all developed severe infection and were euthanized at days 7–8 post-challenge (Fig. 1a). In contrast, all mucosal CLDC-treated mice were protected from disease. Organ bacterial burdens were determined at day 6 post-challenge for diluent and CLDC-treated mice. CLDC treatment resulted in a greater than one to two log decrease in bacterial burden in lungs, liver and spleen (Fig. 1b). These data indicate that mucosal CLDC treatment provides innate immune protection from pneumonic *F. tularensis* LVS.

To determine whether CLDC-treated mice cleared *F. tularensis* LVS infection, CLDC-treated mice surviving challenge were euthanized at day 40 and lungs, liver and spleen were homogenized and plated on modified Mueller–Hinton agar. In two separate experiments ($n = 5$ mice per group), we observed that all organs were free of bacteria, suggesting that CLDC-treated mice effectively and completely cleared the *F. tularensis* infection.

We also investigated whether clearance of the initial challenge dose was associated with development of long-lasting adaptive immunity to *F. tularensis* which might protect against future infection. Therefore, survivors from the initial CLDC-treated mice were subjected to re-challenge on day 40 after the first challenge. The mice (BALB/c, $n = 5$ per group) were challenged i.n. with 10^5 or 10^6 CFU *F. tularensis* LVS. In two separate experiments, all mice ($n = 5$ per group) survived re-challenge at 10^5 and 10^6 CFU with no disease symptoms during 40 days of observation (data not shown). The ability of previously CLDC-treated and infected mice to resist re-challenge with *F. tularensis* LVS at a 10-fold higher dose than the initial 100% lethal dose with no apparent signs of disease strongly suggests that these mice mounted a protective adaptive immune response. Thus, CLDC-treated and challenged mice were able to develop effective adaptive immune responses against subsequent re-challenge with *F. tularensis*.

3.2. Route of administration determines the effectiveness of CLDC immunotherapy

Next, we assessed the impact of route of delivery on the effectiveness of immunotherapy with CLDC. Mice ($n = 5$ per group) were pre-treated 24 h before infection with CLDC administered by the i.n. route (20 μ l), the i.p. route (200 μ l), the s.c. route (200 μ l) and the i.v. route (100 μ l). These doses of CLDC were chosen because they elicit significant immune stimulation, without toxicity (Dow, S., unpublished observations). Twenty-four hours after administration of CLDC, mice were challenged i.n. with 10^5 CFU *F. tularensis* LVS. We observed that immunotherapy with CLDC administered via the i.v., i.p. and s.c. routes did not significantly increase survival time compared to untreated controls (Fig. 2a). In contrast, i.n. administration of CLDC 24 h before challenge elicited complete protection from lethal infection (Fig. 2a). Thus, mucosal administration of CLDC immunotherapy was much more effective than systemic treatment for generating protection from pneumonic *F. tularensis* infection.

3.3. Effects of timing of administration on generation of protective immunity

We next investigated the effects that timing of CLDC administration had on generating protective immunity. Mice ($n = 5$ per group) were treated with 20 μ l CLDC administered i.n. 72 h prior to challenge, 24 h prior to challenge, 10 min after challenge (0 h time point) or 24 h after challenge. Whereas pre-treatment 24 h prior to challenge resulted in 100% protection from lethal infection, CLDC immunotherapy administered 72 h before challenge resulted in only 60% survival (Fig. 2b). Administration of CLDC concurrently with infection (0 h time point) or administration 24 h after infection resulted in 40% and 60% survival, respectively (Fig. 2b). These findings suggest that there is a window of maximal activity for mucosal CLDC immunotherapy, which diminishes partially with increasing time of administration before or after challenge.

3.4. Mucosal delivery of CLDC elicits pro-inflammatory cytokine gene expression in the lung

Studies were conducted next to characterize the CLDC-induced innate immune responses in the lung. Systemically administered CLDC have been shown previously to provoke a strong Th1 type immune response with production of pro-inflammatory cytokines [13,15]. Several of these cytokines including IFN- γ , IL-12 and TNF- α have been demonstrated to function in host defense against pneumonic *F. tularensis* [9,23]. Thus, we sought to determine whether mucosal administration of CLDC resulted in increased expression in the lung of these important protective cytokines. In addition, we examined expression of the type I interferons IFN- α and

IFN- β in the lung in response to mucosal CLDC immunotherapy. Recent studies suggest that type I IFNs can play an important role in either increasing or decreasing the susceptibility of animals to bacterial infections [24–31]. Moreover, CLDC are known to be potent inducers of type I IFN production [32,33].

Cytokine gene expression in lung tissues following mucosal administration of CLDC was determined using quantitative realtime PCR (qRT-PCR). Induction of cytokine gene expression by CLDC treatment was assessed by comparing mRNA levels in lungs from CLDC-treated mice to lungs from untreated mice, resulting in a relative measure of the fold change in mRNA expression. At 24 h after CLDC treatment, lungs from treated mice ($n = 4$ mice per group) had significant increases in expression of IFN- γ , IL-12b(p40), TNF- α , IFN- $\alpha 4$ and IFN- β (Fig. 3). For example, cytokine induction in lungs of CLDC-treated mice ranged from a high of 46-fold induction for IFN- γ to 4-fold for IFN- $\alpha 4$ (Fig. 3). These data demonstrate that mucosal CLDC treatment resulted in the upregulation of key pro-inflammatory cytokine genes with potential antibacterial activity in the lung. The role of these key cytokines in CLDC-induced protection was examined next.

3.5. IFN- γ is required for CLDC-induced protection

Previous studies have highlighted the critical importance of IFN- γ for defense against *F. tularensis* [23,34–36]. In addition, CLDC have been shown in earlier studies to potently induce production of IFN- γ by NK cells [13]. Therefore, we hypothesized that IFN- γ production might be a key factor in the protection elicited by mucosally administered CLDC. This possibility was tested by assessing the ability of CLDC immunotherapy to protect mice unable to produce IFN- γ against *F. tularensis* challenge. Wild-type BALB/c mice and IFN- $\gamma^{-/-}$ mice ($n = 5$ per group) on the BALB/c background were administered CLDC by the i.n. route, then subjected to lethal i.n. challenge with 10^5 CFU *F. tularensis* LVS 24 h after CLDC treatment. We observed that CLDC immunotherapy was completely ineffective in protecting IFN- $\gamma^{-/-}$ mice from *F. tularensis* challenge, with survival times in CLDC-treated IFN- $\gamma^{-/-}$ mice similar to those of untreated wild-type mice (Fig. 4a). In addition, CLDC treatment of IFN- $\gamma^{-/-}$ mice failed to reduce their bacterial burdens below the levels present in infected but sham-treated wild-type mice (Fig. 4b). These data indicate that CLDC-elicited production of IFN- γ is required for protection against lethal *F. tularensis* pulmonary challenge.

3.6. Role of NK cells in protection generated by CLDC immunotherapy

We have found previously that NK cells are the primary source of IFN- γ production in CLDC-treated animals [13]. Thus, we hypothesized that NK cells might play an important role in protection mediated by mucosally delivered CLDC. This possibility was examined by means of systemic NK depletion experiments. Mice were depleted of NK cells by i.p. administration of 50 μ l of anti-asialo-GM1 antibody, a treatment which we have found results in >80% depletion of lung NK cells at 24 h (data not shown). Additionally, other investigators have demonstrated that systemic NK cell depletion after administration of the asialo-GM1 antibody lasts for at least 5 days [37–39]. Wild-type BALB/c mice ($n = 5$ per group) were administered anti-asialo-GM1 antibody or an equivalent amount of control rabbit IgG 24 h prior to CLDC treatment. The mice were administered CLDC i.n. and then subjected to i.n. challenge 24 h later with 10^5 CFU *F. tularensis* LVS. Five days after the first NK cell depletion, the mice were treated with a second dose of NK depleting or control antibody to assure continued NK depletion. Mucosal immunotherapy with CLDC treatment generated complete protection in mice pre-treated with irrelevant rabbit IgG and in non pre-treated mice (Fig. 5a). In contrast, NK cell depletion eliminated the protective effect of CLDC treatment. For example, all NK cell-depleted and CLDC-treated mice were euthanized on approximately the same days as diluent treated mice (Fig. 5a). In addition, suppression of bacterial replication and dissemination from the lungs to spleen and liver by CLDC immunotherapy was also

significantly abrogated in the NK cell depleted mice (Fig. 5b). Thus, these data indicate a key role for NK cells in mediating effective control of bacterial growth and dissemination in response to mucosally administered CLDC immunotherapy.

3.7. Type I interferons play a role in host defense against *F. tularensis* but are not necessary for CLDC-induced protection

While the type I interferons (IFN), IFN- α and IFN- β , have been classically understood to provide broad defense against many viral infections, it has more recently become appreciated that type I interferons also may play a role in defense against certain bacterial pathogens [26, 28] or have a pathogenic role in other bacterial infection [24,25,27,29]. At least one previous report indicated that mice lacking IFN- α/β receptor were more resistant to *F. tularensis* [3]. However, IFN- β is also required for activation of the host-protective inflammasome in response to *F. tularensis* infection [40–42]. Thus we hypothesized that type I IFN production may modulate pneumonic *F. tularensis* infection. To address this question, we conducted challenge studies in mice which lack the common receptor for type I interferon (IFN- α/β receptor^{-/-}). Intranasal challenge of wild-type 129 mice ($n = 5$ per group) demonstrated that this mouse strain is highly resistant to *F. tularensis* LVS. Wild-type 129 mice challenged with up to 10⁶ CFU all survived to day 40 without developing severe disease (Fig. 6a and data not shown). However, when isogenic IFN- α/β R^{-/-} mice were challenged with 10⁵ CFU *F. tularensis* LVS, they succumbed to disease within 9 days of challenge (Fig. 6a). Thus, these results suggest that type I IFNs may play a protective role in immunity against *F. tularensis* infection in the context of the highly resistant 129 mouse model.

Recent studies have also demonstrated that CLDC are potent inducers of type I IFN production [32,33]. Since the preceding experiments suggested a potential role for type I IFNs in protection from *F. tularensis* infection, we next assessed the role of type I IFNs in CLDC-mediated protection. When IFN- α/β R^{-/-} mice were treated i.n. with CLDC 24 h prior to challenge with 10⁵ CFU *F. tularensis* LVS, we observed that they were significantly protected compared to sham-treated IFN- α/β R^{-/-} mice (Fig. 6b). Thus, induction of type I IFN responses do not appear to be necessary for CLDC-induced protection.

3.8. CLDC-induced IFN- γ promotes resistance of alveolar macrophages to infection with *F. tularensis* LVS

Having established the importance of IFN- γ for CLDC-induced protection from *F. tularensis*, we next sought to further characterize the mechanism of cytokine induced protection. Previous studies have established that macrophages and dendritic cells are important primary target cells for infection with *F. tularensis* [18,43–45]. In addition, it has been shown that IFN- γ and TNF- α can induce macrophage intracellular killing of *F. tularensis* [46–50]. Therefore, we investigated the effects of CLDC-elicited cytokines on intracellular inhibition of *F. tularensis* LVS, using an in vitro system. Alveolar macrophages from the AMJ.2 cell line were pre-treated for 24 h with supernatants from CLDC-stimulated spleen cells, then infected with *F. tularensis* and the effects on numbers of viable intracellular bacteria determined 48 h later, as described in Section 2.

We found that pre-treatment of AMJ cells with supernatants from CLDC-stimulated spleen cells resulted in significant reduction in intracellular growth of *F. tularensis*, compared to untreated cells or cells pre-treated with supernatants from control spleens (Fig. 7). The inhibitory activity was also present even when supernatants were diluted as much as 1:100. This result indicated that soluble factors induced by CLDC promoted cellular resistance to *F. tularensis* LVS intracellular growth.

To identify the specific factor(s) responsible for inhibiting *F. tularensis* intracellular growth, we assessed the effects of neutralizing IFN- γ or TNF- α activity (or both) on the ability of supernatants to inhibit replication of *F. tularensis* in macrophages. Neutralization of IFN- γ activity resulted in nearly complete elimination of the *F. tularensis* suppressive activity in supernatants (Fig. 7), which indicated that IFN- γ was the primary CLDC-induced factor responsible for inhibiting *F. tularensis* LVS growth. Neutralization of TNF- α activity induced a small but not significant reduction in *F. tularensis* suppressive activity (Fig. 7), indicating that TNF- α was not the major active cytokine. However, neutralization of both IFN- γ and TNF- α activity completely abrogated the inhibitory effects of CLDC-stimulated spleen supernatants, suggesting a possible cooperative effect between the two cytokines. These findings suggest that one effect of mucosal CLDC immunotherapy may be to inhibit the replication of *F. tularensis* in an early target cell population of alveolar macrophages in an IFN- γ dependent fashion.

3.9. Mucosal CLDC immunotherapy generates partial protection against virulent *F. tularensis* Schu4

The preceding results indicated that mucosal immunotherapy was effective in generating rapid protection against *F. tularensis* LVS challenge. However, *F. tularensis* LVS, while fully virulent in mice, is significantly attenuated in humans [4,51]. Therefore, results of experiments conducted with *F. tularensis* LVS, while mechanistically informative, may not fully predict outcomes in humans exposed to more virulent strains of *F. tularensis*. Therefore, we next evaluated the effectiveness of CLDC immunotherapy in a challenge model employing the highly virulent Schu4 strain of *F. tularensis* [2].

BALB/c mice ($n = 5$ per group) were treated with CLDC or diluent administered by the i.n. route. Twenty-four hours later, the mice were challenged with an LD100 dose of 40 CFU *F. tularensis* Schu4, administered by the i.n. route. We observed that all diluent treated mice succumbed to disease by 4.5 days post-challenge (Fig. 8a). In contrast, mice treated with CLDC survived significantly longer ($p = 0.0027$) than control mice, though they ultimately did succumb to infection. We also assessed the impact of CLDC immunotherapy on body weight changes in infected mice and found that CLDC-treated mice had a significant reduction in *F. tularensis*-induced weight loss, compared to control mice (Fig. 8b). In addition, CLDC treatment resulted in a more than 10-fold reduction in bacterial burdens in the lung, liver and spleen at day 3 after bacterial challenge (Fig. 8c). Collectively, these data demonstrate that mucosal CLDC treatment is capable of eliciting at least partial protection even against a highly virulent strain of *F. tularensis*. These results suggest that additional refinements to the CLDC immunotherapy platform, or the use of combination therapy, could result in greater protective effects.

4. Discussion

In this study we found that delivery of immunostimulatory cationic lipid-DNA complexes to the lung mucosa protected mice from pulmonary challenge with *F. tularensis*. In the *F. tularensis* LVS challenge model, mucosal CLDC treatment provided complete protection from lethal pulmonary challenge. In the case of the highly virulent *F. tularensis* Schu4 strain, mucosal CLDC treatment resulted in a statistically significant increase in survival time and decrease in bacterial burden. CLDC-protected mice surviving LVS challenge were immune to subsequent high dose pulmonary challenge. Thus, in the LVS challenge model innate immune protection with mucosal CLDC was not sterilizing and allowed the development of an adaptive immune response which was host-protective.

While previous studies have demonstrated that vaccination and other strategies utilizing antigen-specific immunotherapeutic approaches can provide protection from inhaled *F.*

tularensis [4,21,52–56], fewer studies have examined the ability of pathogen non-specific innate immunity to generate protection from inhalational challenge [9–11]. Previous work has established that systemic immunotherapy can protect mice from systemic challenge with *F. tularensis* [7,8]. However, in the case of inhalational challenge, we found that only mucosally administered CLDC immunotherapy provided complete protection while systemic routes of immunotherapy provided incomplete protection. Targeting of CLDC to the lung mucosa likely has several advantages over systemic delivery for protection from pulmonary *F. tularensis*. By directing immunotherapy to the initial site of infection, the immune response has an opportunity to limit bacterial growth in the lung prior to systemic dissemination of the infection. In this study we demonstrated that CLDC promoted a pro-inflammatory environment in the lung characterized by increased gene expression of IL-12, IFN- γ , TNF- α , IFN- α and IFN- β . We also found that CLDC-elicited cytokines induced alveolar phagocytes to resist *F. tularensis* intracellular growth in vitro. Alveolar phagocytes are important initial target cells for *F. tularensis* infection in the lung [3,18,45,57], and therefore CLDC-induced activation of these cells may serve to help restrict initial infection. In contrast, our own recent studies and those of others suggest that *F. tularensis* pulmonary infection typically results in an immunosuppressive phenotype in the lung characterized by a lack of pro-inflammatory cytokines and an increase in the immunosuppressive cytokine TGF- β which likely favors bacterial growth and dissemination [17,18,58,59]. Thus mucosal CLDC immunotherapy promotes a pro-inflammatory environment in the lung which favors bacterial killing and likely at least partially counteracts the immunosuppressive environment induced by *F. tularensis*.

The timing of mucosal CLDC administration was critical in influencing the outcome of *F. tularensis* LVS challenge. Mucosal CLDC provided complete protection when given 24 h prior to challenge while administration at later times concurrent with and following challenge only provided partial protection. Thus pre-exposure prophylaxis with mucosal CLDC would likely be very effective. Pre-exposure prophylaxis might be practical and useful in situations in which a threat of exposure is known such as in the early stages of an epidemic when only a few individuals have become infected but many more could be at risk of infection. The partial efficacy of therapy 24 h after challenge also suggests that this therapy might potentially be beneficial for post-exposure treatment. For post-exposure therapy, mucosal CLDC might be used in combination with other therapies to provide augmented protection. The greater efficacy of pre-exposure prophylaxis may in part be explained by *F. tularensis* infection causing an immunosuppressive phenotype in the lung [17,18,58,59]. Pre-exposure prophylaxis would therefore provide the opportunity for mucosal CLDC to generate a pro-inflammatory anti-bacterial Th1 type response in the lung prior to *F. tularensis*-induced immunosuppression. In contrast, post-exposure CLDC treatment may not be able to fully counteract the already established *F. tularensis*-induced immunosuppression.

In this study we found that mucosal CLDC treatment induced Th1 cytokine gene expression in the lung including IL-12, IFN- γ , TNF- α , IFN- α and IFN- β . Of the tested genes, IFN- γ was the most up-regulated. The important role of IFN- γ in defense against *F. tularensis* is very well established [9,23,34–36,60]. As previously described [9,23], we found that IFN- $\gamma^{-/-}$ mice were highly susceptible to inhaled challenge with *F. tularensis* LVS resulting in a high day 6 bacterial burden (approximately 10^9 CFU per lung, spleen or liver). We found that treatment of IFN- $\gamma^{-/-}$ mice with mucosal CLDC did not increase survival and importantly did not alter bacterial burden in any tested organ. Thus it appeared that IFN- γ was absolutely necessary for mucosal CLDC-induced protection. We further investigated the role of cytokines in defense against *F. tularensis* using an in vitro model of LVS infection in alveolar macrophages. Pre-treatment of cells with CLDC spleen supernatant resulted in a two log reduction in intracellular *F. tularensis* growth. Antibody depletion of IFN- γ present in CLDC spleen supernatant resulted in nearly complete restoration of *F. tularensis* intracellular growth, suggesting that IFN- γ is the major CLDC-induced component responsible for induction of cellular resistance.

Numerous studies corroborate this data by showing that IFN- γ induces an anti-*F. tularensis* state in phagocytes [46,48,50]. Cellular killing of *F. tularensis* by IFN- γ -activated phagocytes is typically dependent on nitric oxide (NO) [48,50,61], although one study found that IFN- γ -induced killing of *F. tularensis* by murine alveolar macrophages was NO independent [45].

We assessed the role of NK cells in mucosal CLDC-induced protection by systemically depleting NK cells from BALB/c mice with anti-asialo-GM1 antibody. NK cell depletion resulted in a significant and marked loss of CLDC-induced protection. Thus, we concluded that NK cells were required for mucosal CLDC-induced protection. We have previously found that NK cells are the source of the majority of systemic CLDC-induced IFN- γ in spleen and lung [13]. Therefore, it is likely that NK cells are the major source for IFN- γ production in response to mucosal CLDC as well. However, we cannot exclude the possibility that IFN- γ might also be produced by another cell type such as CD8⁺ T cells or that alternative IFN- γ -independent mechanisms may play a role in CLDC-induced protection from *F. tularensis* growth and dissemination. Nonetheless, the effect of NK cell depletion on CLDC-treated mouse survival implicates an important role for NK cells in CLDC-induced protection.

CLDC are potent inducers of the type I interferons, IFN- α and IFN- β [32,33]. While type I interferons have classically been understood to provide host defense against viruses, more recently it has been demonstrated that type I interferons can also have important impacts on bacterial infections as well [26]. For some bacteria, type I interferons exert host-protective effects [28,30,31] while in other bacterial systems they have been shown to enhance pathogenesis [24,25,27,29]. In *F. tularensis* infection, IFN- β appears to play a key role in activation of the inflammasome for innate immune defense [40–42]. However, at least one report has demonstrated that BALB/c mice lacking IFN- α/β receptor were more resistant to *F. tularensis* LVS [3]. In contrast, our finding that IFN- α/β receptor^{-/-} mice on a 129SvEv background were more susceptible to inhaled *F. tularensis* LVS infection supports the idea that type I interferons may play a protective role in defense against pneumonic tularemia. The basis for the discrepancy in results between this study and that of Metzger et al. [3] is not known. It is possible that a difference in the mouse strain studied may influence the role of type I interferons in protection/pathogenesis. For example, we found that 129SvEv mice survived challenge with an inhaled dose of *F. tularensis* LVS which was 10-fold higher than the LD₁₀₀ for BALB/c mice. The basis for this high level of natural resistance is not known and would be intriguing to investigate. Regardless of the effect of type I interferons on infection, we found that type I interferons were not necessary for mucosal CLDC-induced protection, suggesting that CLDC-induced IFN- γ responses are likely sufficient for protection elicited by mucosal CLDC treatment.

In summary, we found that mucosal CLDC immunotherapy provided protection against lethal pulmonary *F. tularensis*. Mucosal administration of CLDC resulted in a lung innate immune response characterized by upregulation of pro-inflammatory cytokines. We found that CLDC-induced protection was dependent on the ability of mice to produce IFN- γ . Additionally, the ability of CLDC to limit *F. tularensis* growth in alveolar macrophages was highly dependent on IFN- γ . NK cell depletion experiments demonstrated that NK cells also play an important role in CLDC-induced protection. To our knowledge no truly antigen non-specific innate immunity has previously demonstrated the ability to protect mice against lethal challenge with the virulent Schu4 strain of *F. tularensis*. Therefore, our finding that mucosal CLDC provides partial protection against pulmonary Schu4 challenge represents a step forward in the development of immunotherapeutics to combat virulent pulmonary *F. tularensis*. New formulations of CLDC which combine additional pattern recognition receptor agonists may provide enhanced potency. While immunotherapy alone may not be sufficient to provide complete protection from highly virulent pulmonary *F. tularensis*, CLDC immunotherapy might potentially be used to augment or enhance post-exposure vaccine or antibiotic therapy.

We are currently studying whether CLDC-based immunotherapy might be effective in enhancing the potency of antibiotic therapy or allowing delayed initiation of antibiotic therapy.

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References

1. Oyston PC, Sjøstedt A, Titball RW. Tularaemia: bioterrorism defence renews interest in *Francisella tularensis*. *Nat Rev* 2004;2(December (12)):967–978.
2. Conlan JW, Chen W, Shen H, Webb A, KuoLee R. Experimental tularemia in mice challenged by aerosol or intradermally with virulent strains of *Francisella tularensis*: bacteriologic and histopathologic studies. *Microb Pathog* 2003;34(May (5)):239–248. [PubMed: 12732472]
3. Metzger DW, Bakshi CS, Kirimanjeswara G. Mucosal immunopathogenesis of *Francisella tularensis*. *Ann NY Acad Sci* 2007 June;1105:266–283. [PubMed: 17395728]
4. Conlan JW. Vaccines against *Francisella tularensis*—past, present and future. *Exp Rev Vaccines* 2004;3(June (3)):307–314.
5. Elkins KL, Cowley SC, Bosio CM. Innate and adaptive immunity to *Francisella*. *Ann NY Acad Sci* 2007 June;1105:284–324. [PubMed: 17468235]
6. Tarnvik A, Eriksson M, Sandstrom G, Sjøstedt A. *Francisella tularensis*—a model for studies of the immune response to intracellular bacteria in man. *Immunology* 1992;76(July (3)):349–354. [PubMed: 1526645]
7. Elkins KL, Rhinehart-Jones TR, Stibitz S, Conover JS, Klinman DM. Bacterial DNA containing CpG motifs stimulates lymphocyte-dependent protection of mice against lethal infection with intracellular bacteria. *J Immunol* 1999;162(February (4)):2291–2298. [PubMed: 9973506]
8. Elkins KL, Colombini SM, Krieg AM, De Pascalis R. NK cells activated in vivo by bacterial DNA control the intracellular growth of *Francisella tularensis* LVS. *Microb Infect/Institut Pasteur* 2009;11(January (1)):49–56.
9. Duckett NS, Olmos S, Durrant DM, Metzger DW. Intranasal interleukin-12 treatment for protection against respiratory infection with the *Francisella tularensis* live vaccine strain. *Infect Immun* 2005;73(April (4)):2306–2311. [PubMed: 15784575]
10. Pammit MA, Budhavarapu VN, Raulie EK, Klose KE, Teale JM, Arulanandam BP. Intranasal interleukin-12 treatment promotes antimicrobial clearance and survival in pulmonary *Francisella tularensis* subsp. novicida infection. *Antimicrob Agents Chemother* 2004;48(December (12)):4513–4519. [PubMed: 15561819]
11. Lembo A, Pelletier M, Iyer R, Timko M, Dudda JC, West TE, et al. Administration of a synthetic TLR4 agonist protects mice from pneumonic tularemia. *J Immunol* 2008;180(June (11)):7574–7581. [PubMed: 18490759]
12. Zaks K, Jordan M, Guth A, Sellins K, Kedl R, Izzo A, et al. Efficient immunization and cross-priming by vaccine adjuvants containing TLR3 or TLR9 agonists complexed to cationic liposomes. *J Immunol* 2006;176(June (12)):7335–7345. [PubMed: 16751377]
13. Dow SW, Fradkin LG, Liggett DH, Willson AP, Heath TD, Potter TA. Lipid-DNA complexes induce potent activation of innate immune responses and antitumor activity when administered intravenously. *J Immunol* 1999;163(August (3)):1552–1561. [PubMed: 10415059]
14. Gursel I, Gursel M, Ishii KJ, Klinman DM. Sterically stabilized cationic liposomes improve the uptake and immunostimulatory activity of CpG oligonucleotides. *J Immunol* 2001;167(September (6)):3324–3328. [PubMed: 11544321]
15. Whitmore M, Li S, Huang L. LPD lipopolyplex initiates a potent cytokine response and inhibits tumor growth. *Gene Therapy* 1999;6(November (11)):1867–1875. [PubMed: 10602382]

16. Goodyear A, Kelliham L, Bielefeldt-Ohmann H, Troyer R, Propst K, Dow S. Protection from pneumonic infection with burkholderia species by inhalational immunotherapy. *Infect Immun* 2009;77(April (4)):1579–1588. [PubMed: 19179415]
17. Bosio CM, Bielefeldt-Ohmann H, Belisle JT. Active suppression of the pulmonary immune response by *Francisella tularensis* Schu4. *J Immunol* 2007;178(April (7)):4538–4547. [PubMed: 17372012]
18. Bosio CM, Dow SW. *Francisella tularensis* induces aberrant activation of pulmonary dendritic cells. *J Immunol* 2005;175(November (10)):6792–6801. [PubMed: 16272336]
19. Karaghiosoff M, Steinborn R, Kovarik P, Kriegshauser G, Baccarini M, Donabauer B, et al. Central role for type I interferons and Tyk2 in lipopolysaccharide-induced endotoxin shock. *Nat Immunol* 2003;4(May (5)):471–477. [PubMed: 12679810]
20. Fortier AH, Slayter MV, Ziemba R, Meltzer MS, Nacy CA. Live vaccine strain of *Francisella tularensis*: infection and immunity in mice. *Infect Immun* 1991;59(September (9)):2922–2928. [PubMed: 1879918]
21. Kirimanjeswara GS, Golden JM, Bakshi CS, Metzger DW. Prophylactic and therapeutic use of antibodies for protection against respiratory infection with *Francisella tularensis*. *J Immunol* 2007;179(July (1)):532–539. [PubMed: 17579074]
22. Sebastian S, Dillon ST, Lynch JG, Blalock LT, Balon E, Lee KT, et al. A defined O-antigen polysaccharide mutant of *Francisella tularensis* live vaccine strain has attenuated virulence while retaining its protective capacity. *Infect Immun* 2007;75(May (5)):2591–2602. [PubMed: 17296751]
23. Chen W, KuoLee R, Shen H, Conlan JW. Susceptibility of immunodeficient mice to aerosol and systemic infection with virulent strains of *Francisella tularensis*. *Microb Pathog* 2004;36(June (6)):311–318. [PubMed: 15120157]
24. Auerbuch V, Brockstedt DG, Meyer-Morse N, O’Riordan M, Portnoy DA. Mice lacking the type I interferon receptor are resistant to *Listeria monocytogenes*. *J Exp Med* 2004;200(August (4)):527–533. [PubMed: 15302899]
25. Carrero JA, Calderon B, Unanue ER. Type I interferon sensitizes lymphocytes to apoptosis and reduces resistance to *Listeria* infection. *J Exp Med* 2004;200(August (4)):535–540. [PubMed: 15302900]
26. Decker T, Muller M, Stockinger S. The yin and yang of type I interferon activity in bacterial infection. *Nat Rev Immunol* 2005;5(September (9)):675–687. [PubMed: 16110316]
27. MancaCTsenovaL BergtoldAFreemanSToveyMMusserJMVirulence of a Mycobacterium tuberculosis clinical isolate in mice is determined by failure to induce Th1 type immunity and is associated with induction of IFN-alpha/beta. *Proc Natl Acad Sci U S A* 2001; 98May (10)57525757 [PubMed: 11320211]
28. Mancuso G, Midiri A, Biondo C, Beninati C, Zummo S, Galbo R, et al. Type I IFN signaling is crucial for host resistance against different species of pathogenic bacteria. *J Immunol* 2007;178(Mar (5)):3126–3133. [PubMed: 17312160]
29. O’Connell RM, Saha SK, Vaidya SA, Bruhn KW, Miranda GA, Zarnegar B, et al. Type I interferon production enhances susceptibility to *Listeria monocytogenes* infection. *J Exp Med* 2004;200(August (4)):437–445. [PubMed: 15302901]
30. Schiavoni G, Mauri C, Carlei D, Belardelli F, Pastoris MC, Proietti E. Type I IFN protects permissive macrophages from *Legionella pneumophila* infection through an IFN-gamma-independent pathway. *J Immunol* 2004;173(July (2)):1266–1275. [PubMed: 15240719]
31. Weigent DA, Huff TL, Peterson JW, Stanton GJ, Baron S. Role of interferon in streptococcal infection in the mouse. *Microb Pathog* 1986;1(August (4)):399–407. [PubMed: 2469934]
32. Sellins K, Fradkin L, Liggitt D, Dow S. Type I interferons potently suppress gene expression following gene delivery using liposome(-)DNA complexes. *Mol Ther* 2005;12(September (3)):451–459. [PubMed: 15950543]
33. Gowen BB, Fairman J, Smee DF, Wong MH, Jung KH, Pace AM, et al. Protective immunity against acute phleboviral infection elicited through immunostimulatory cationic liposome-DNA complexes. *Antiviral Res* 2006;69(March (3)):165–172. [PubMed: 16430975]
34. Elkins KL, Rhinehart-Jones T, Nacy CA, Winegar RK, Fortier AH. T-cell independent resistance to infection and generation of immunity to *Francisella tularensis*. *Infect Immun* 1993;61(March (3)):823–829. [PubMed: 8432603]

35. Elkins KL, Rhinehart-Jones TR, Culkin SJ, Yee D, Winegar RK. Minimal requirements for murine resistance to infection with *Francisella tularensis* LVS. *Infect Immun* 1996;64(August (8)):3288–3293. [PubMed: 8757866]
36. Leiby DA, Fortier AH, Crawford RM, Schreiber RD, Nacy CA. In vivo modulation of the murine immune response to *Francisella tularensis* LVS by administration of anticytokine antibodies. *Infect Immun* 1992;60(January (1)):84–89. [PubMed: 1729199]
37. Byrne P, McGuirk P, Todryk S, Mills KH. Depletion of NK cells results in disseminating lethal infection with *Bordetella pertussis* associated with a reduction of antigen-specific Th1 and enhancement of Th2, but not Tr1 cells. *Eur J Immunol* 2004;34(September (9)):2579–2588. [PubMed: 15307190]
38. Habu S, Fukui H, Shimamura K, Kasai M, Nagai Y, Okumura K, et al. In vivo effects of anti-asialo GM1.I. Reduction of NK activity and enhancement of transplanted tumor growth in nude mice. *J Immunol* 1981;127(July (1)):34–38. [PubMed: 7240748]
39. Kasai M, Iwamori M, Nagai Y, Okumura K, Tada T. A glycolipid on the surface of mouse natural killer cells. *Eur J Immunol* 1980;10(March (3)):175–180. [PubMed: 6966574]
40. Cole LE, Santiago A, Barry E, Kang TJ, Shirey KA, Roberts ZJ, et al. Macrophage proinflammatory response to *Francisella tularensis* live vaccine strain requires coordination of multiple signaling pathways. *J Immunol* 2008;180(May (10)):6885–6891. [PubMed: 18453609]
41. Henry T, Brotcke A, Weiss DS, Thompson LJ, Monack DM. Type I interferon signaling is required for activation of the inflammasome during *Francisella* infection. *J Exp Med* 2007;204(May (5)):987–994. [PubMed: 17452523]
42. Mariathasan S, Weiss DS, Dixit VM, Monack DM. Innate immunity against *Francisella tularensis* is dependent on the ASC/caspase-1 axis. *J Exp Med* 2005;202(October (8)):1043–1049. [PubMed: 16230474]
43. BenNasr A, Haithcoat J, Masterson JE, Gunn JS, Eaves-Pyles T, Klimpel GR. Critical role for serum opsonins and complement receptors CR3 (CD11b/CD18) and CR4 (CD11c/CD18) in phagocytosis of *Francisella tularensis* by human dendritic cells (DC): uptake of *Francisella* leads to activation of immature DC and intracellular survival of the bacteria. *J Leukocyte Biol* 2006;80(October (4)):774–786. [PubMed: 16857732]
44. Fortier AH, Leiby DA, Narayanan RB, Asafoadjei E, Crawford RM, Nacy CA, et al. Growth of *Francisella tularensis* LVS in macrophages: the acidic intracellular compartment provides essential iron required for growth. *Infect Immun* 1995;63(April (4)):1478–1483. [PubMed: 7890413]
45. Polsinelli T, Meltzer MS, Fortier AH. Nitric oxide-independent killing of *Francisella tularensis* by IFN-gamma-stimulated murine alveolar macrophages. *J Immunol* 1994;153(August (3)):1238–1245. [PubMed: 8027551]
46. Anthony LS, Morrissey PJ, Nano FE. Growth inhibition of *Francisella tularensis* live vaccine strain by IFN-gamma-activated macrophages is mediated by reactive nitrogen intermediates derived from L-arginine metabolism. *J Immunol* 1992;148(March (6)):1829–1834. [PubMed: 1541823]
47. Cowley SC, Elkins KL. Multiple T cell subsets control *Francisella tularensis* LVS intracellular growth without stimulation through macrophage interferon gamma receptors. *J Exp Med* 2003;198(August (3)):379–389. [PubMed: 12885873]
48. Fortier AH, Polsinelli T, Green SJ, Nacy CA. Activation of macrophages for destruction of *Francisella tularensis*: identification of cytokines, effector cells, and effector molecules. *Infect Immun* 1992;60(March (3)):817–825. [PubMed: 1541555]
49. Green SJ, Nacy CA, Schreiber RD, Granger DL, Crawford RM, Meltzer MS, et al. Neutralization of gamma interferon and tumor necrosis factor alpha blocks in vivo synthesis of nitrogen oxides from L-arginine and protection against *Francisella tularensis* infection in *Mycobacterium bovis* BCG-treated mice. *Infect Immun* 1993;61(February (2)):689–698. [PubMed: 8423095]
50. Lindgren H, Stenman L, Tarnvik A, Sjöstedt A. The contribution of reactive nitrogen and oxygen species to the killing of *Francisella tularensis* LVS by murine macrophages. *Microb Infect/Institut Pasteur* 2005;7(March (3)):467–475.
51. Eigelsbach HT, Downs CM. Prophylactic effectiveness of live and killed tularemia vaccines. I. Production of vaccine and evaluation in the white mouse and guinea pig. *J Immunol* 1961 October; 87:415–425. [PubMed: 13889609]

52. Klimpel GR, Eaves-Pyles T, Moen ST, Taormina J, Peterson JW, Chopra AK, et al. Levofloxacin rescues mice from lethal intranasal infections with virulent *Francisella tularensis* and induces immunity and production of protective antibody. *Vaccine* 2008;26(December (52)):6874–6882. [PubMed: 18930100]
53. Wayne Conlan J, Shen H, Kuolee R, Zhao X, Chen W. Aerosol-, but not intradermal-immunization with the live vaccine strain of *Francisella tularensis* protects mice against subsequent aerosol challenge with a highly virulent type A strain of the pathogen by an alphabeta T cell-and interferongamma-dependent mechanism. *Vaccine* 2005;23(March (19)):2477–2485. [PubMed: 15752834]
54. Wu TH, Hutt JA, Garrison KA, Berliba LS, Zhou Y, Lyons CR. Intranasal vaccination induces protective immunity against intranasal infection with virulent *Francisella tularensis* biovar A. *Infect Immun* 2005;73(May (5)):2644–2654. [PubMed: 15845466]
55. Jia Q, Lee BY, Clemens DL, Bowen RA, Horwitz MA. Recombinant attenuated *Listeria monocytogenes* vaccine expressing *Francisella tularensis* IgIC induces protection in mice against aerosolized Type A *F. tularensis*. *Vaccine* 2009;27(February (8)):1216–1229. [PubMed: 19126421]
56. Bakshi CS, Malik M, Mahawar M, Kirimanjeswara GS, Hazlett KR, Palmer LE, et al. An improved vaccine for prevention of respiratory tularemia caused by *Francisella tularensis* SchuS4 strain. *Vaccine* 2008;26(September (41)):5276–5288. [PubMed: 18692537]
57. Hall JD, Craven RR, Fuller JR, Pickles RJ, Kawula TH. *Francisella tularensis* replicates within alveolar type II epithelial cells in vitro and in vivo following inhalation. *Infect Immun* 2007;75(February (2)):1034–1039. [PubMed: 17088343]
58. Andersson H, Hartmanova B, Kuolee R, Ryden P, Conlan W, Chen W, et al. Transcriptional profiling of host responses in mouse lungs following aerosol infection with type A *Francisella tularensis*. *J Med Microbiol* 2006 Mar;55(Pt 3):263–271. [PubMed: 16476789]
59. Chase JC, Celli J, Bosio CM. Direct and indirect impairment of human dendritic cell function by virulent *Francisella tularensis* Schu S4. *Infect Immun* 2009;77(January (1)):180–195. [PubMed: 18981246]
60. Collazo CM, Sher A, Meierovics AI, Elkins KL. Myeloid differentiation factor-88 (MyD88) is essential for control of primary in vivo *Francisella tularensis* LVS infection, but not for control of intra-macrophage bacterial replication. *Microb Infect/Institut Pasteur* 2006;8(March (3)):779–790.
61. Lindgren H, Shen H, Zingmark C, Golovliov I, Conlan W, Sjostedt A. Resistance of *Francisella tularensis* strains against reactive nitrogen and oxygen species with special reference to the role of KatG. *Infect Immun* 2007;75(March (3)):1303–1309. [PubMed: 17210667]

Fig. 1. Mucosally administered CLDC immunotherapy generates rapid protection against *F. tularensis* pneumonia. BALB/c mice ($n = 5$ per group) were administered CLDC by the i.n. route, as described in Section 2, or were sham-treated with diluent (5% dextrose water) alone. Twenty-four hours later, mice were subjected to i.n. challenge with 10^5 CFU *F. tularensis* LVS, as described in Section 2. In (a) survival times for each group of mice were plotted using a Kaplan–Meier curve. In (b), organ bacterial burdens were determined on day 6 after bacterial challenge, as noted in Section 2. Bacterial burden in all 3 organs assessed were significantly lower in CLDC-treated mice than diluent treated mice as assessed by Student’s two-tailed t test (** $p < 0.001$). Group means are plotted ($\pm$ SD). Each experiment was repeated once with similar results.

Fig. 2.

Effects of route and timing of CLDC delivery on protection. In (a) mice ($n = 5$ per group) were treated with established immunostimulatory doses of CLDC via four different routes, as described in Section 2. Treated mice were then challenged 24 h later i.n. with a lethal dose of *F. tularensis* LVS. Survival was evaluated by Kaplan–Meier survival curves, followed by log-rank analysis using the Bonferroni corrected threshold for multiple comparisons. Only mice treated with CLDC by the i.n. route had a statistically significant increase in survival ($p = 0.0015$; Bonferroni corrected threshold for significance: $p < 0.0125$), compared to control mice. In (b) the effects of timing of CLDC mucosal administration on protection from *F. tularensis* LVS were evaluated. Groups of mice ($n = 5$) were administered CLDC by the i.n. route at the times noted (relative to the time of challenge). Mice were subjected to lethal i.n. challenge with *F. tularensis* LVS. CLDC treatment 24 h prior to challenge provided complete protection ($p = 0.0015$; Bonferroni corrected threshold for significance: $p < 0.0125$) while treatment at 24 h after challenge provided partial protection, with a significant increase in survival time compared to untreated controls ($p = 0.0088$; Bonferroni corrected threshold for significance: $p < 0.0125$). CLDC treatment 72 h prior to challenge and 0 h after challenge did not significantly increase survival time ($p = 0.0807$, $p = 0.0160$, respectively; Bonferroni corrected threshold for significance: $p < 0.0125$). Each experiment was repeated once with similar results.

Fig. 3.

Induction of cytokine production in the lungs by mucosal administration of CLDC. Cytokine gene expression in lung tissues of untreated control mice and mice ($n = 4$ per group) treated with i.n. administration of CLDC 24 h prior to sacrifice was assessed by quantitative real-time PCR, as described in Section 2. The fold increase in lung mRNA expression in CLDC-treated versus control mice was plotted for IFN- γ (a), IFN- α 4 (b), IFN- β (c), IL-12b (d) and TNF- α (e). Statistical significance was assessed using Student's two-tailed t test (** $p < 0.01$, * $p < 0.05$). Results are expressed as group means $\pm$ SD and are representative of at least two repeated experiments.

Fig. 4.

IFN- γ is required for mucosal CLDC protection. Wild-type and IFN- $\gamma^{-/-}$ mice ($n = 5$ per group) were treated with i.n. CLDC or diluent 24 h prior to *F. tularensis* LVS i.n. challenge. (a) All CLDC-treated wild-type mice survived, whereas there were no survivors among the CLDC-treated IFN- $\gamma^{-/-}$ mice. In (b), organ bacterial burdens were assessed at day 6 after challenge. In wild-type mice, CLDC treatment resulted in a significant decrease in bacterial burden in all organs compared to sham-treated mice (*** $p < 0.001$, one-way ANOVA with Tukey's multiple comparison test). However, in IFN- $\gamma^{-/-}$ mice ($n = 5$ per group), CLDC treatment failed to produce a significant change in bacterial burdens. Bars represent group means $\pm$ SD. Each experiment was repeated once with similar results.

Fig. 5.

NK cells are necessary for mucosal CLDC protection from *F. tularensis* challenge. NK cells were depleted from wild-type BALB/c mice ($n = 5$ per group) by i.p. injection of anti-asialo-GM1 antibody 24 h prior to CLDC treatment and again 5 days later, as described in Section 2. Another group of mice was treated with irrelevant rabbit IgG instead of asialo-GM1 antibody. Mice were treated with CLDC or sham-treated with diluent and 24 h later challenged i.n. with *F. tularensis* LVS. In (a), non-depleted mice and mice pre-treated with control rabbit antibody and then treated with CLDC all survived *F. tularensis* challenge. However, NK cell depleted mice treated with CLDC failed to survive the *F. tularensis* challenge. In (b), organ bacterial burdens were determined 6 days after *F. tularensis* challenge as described in Section 2. CLDC treatment alone resulted in a significant ($***p < 0.001$) reduction in bacterial burden in all organs evaluated. Control antibody treatment prior to CLDC treatment did not alter the effect of CLDC treatment on bacterial burden. In contrast, NK cell depletion prior to CLDC treatment significantly increased bacterial dissemination to the spleen and liver ($***p < 0.001$, one-way ANOVA with Tukey's multiple means comparison test), compared to mice treated with CLDC alone. Bars represent group means $\pm$ SD. Each experiment was repeated once with similar results.

Fig. 6.

Type I interferons provide partial protection from *F. tularensis* LVS challenge but are not required for CLDC-induced protection. Wild-type 129 mice ($n = 5$ per group) and IFN- α/β receptor^{-/-} mice on the 129 background were challenged with 10^5 CFU *F. tularensis* LVS, as noted in Section 2. In (a), mice lacking IFN- α/β R expression were all euthanized on day 9 while wild-type 129 mice were completely resistant to death from *F. tularensis* challenge. Kaplan-Meier survival curve differences assessed by log-rank analysis demonstrated a significant increase in survival time ($p = 0.0027$) in wild-type mice compared to IFN- α/β R^{-/-} mice. Similar results were found in one additional experiment. In (b), i.n. administration of CLDC to IFN- α/β R^{-/-} mice 24 h prior to challenge resulted in a significant increase in survival time ($p = 0.0358$) compared to sham-treated IFN- α/β R^{-/-} mice. These results are representative of two independent experiments.

Fig. 7.

CLDC-induced cytokines inhibit *F. tularensis* LVS growth in infected macrophages in vitro. Mouse alveolar macrophage cells (AMJ cell line) in triplicate wells were incubated 16 h with supernatant from spleens of CLDC or sham-treated mice, as described in Section 2. The cells were then infected with *F. tularensis* LVS and the effects of pre-treatment on intracellular replication of *F. tularensis* in infected macrophages 48 h later were assessed. Pre-treatment of cells with CLDC supernatant resulted in a nearly two log decrease in intracellular growth of *F. tularensis* in infected macrophages. Similar results were obtained in three additional experiments. The effects of neutralizing IFN- γ or TNF- α activity in CLDC supernatant were assessed in the same in vitro infection assay. To neutralize IFN- γ and TNF- α activity, supernatants were pre-incubated for 30 min with specific neutralizing antibodies. Control irrelevant antibodies were also included for specificity. Addition of anti-IFN- γ antibody nearly eliminated the *F. tularensis* inhibitory activity present in CLDC supernatant, whereas addition of anti-TNF- α antibody did not result in a significant change. Statistical significance was assessed by one-way ANOVA with Tukey's multiple means comparison test (*** p < 0.001, ** p < 0.01). Bars represent group means $\pm$ SD. These results are representative of two independent experiments with similar results.

Fig. 8.

Mucosal CLDC provides protection against virulent *F. tularensis* Schu4 challenge. Mice (BALB/c, $n = 5$ per group) were pre-treated with i.n. CLDC (or sham treated with diluent) and then challenged i.n. 24 h later with 40 CFU *F. tularensis* Schu4. In (a), survival was assessed using Kaplan–Meier survival curves, followed by log-rank analysis. Mucosal administration of CLDC to mice resulted in a significantly greater survival time than in sham-treated mice ($p = 0.0027$). In (b), body weight was monitored twice daily in CLDC and sham-treated mice infected with *F. tularensis* Schu4 and the mean percent of starting weight ($\pm$ SD) for each mouse for each day was plotted. The body weight data was analyzed by two-way ANOVA with repeated measures and Bonferroni post-tests. The percentage change in body weights was

calculated for the time points until the first mice in each treatment group were euthanized (day 4.5 for diluent group, day 5.5 for CLDC group). Diluent treated mice had significantly greater weight loss on days 3.5 through 4.5 ($***p < 0.001$, $*p < 0.05$) compared with CLDC-treated mice. In (c), organ bacterial burdens were determined at day 3 post-challenge and compared using Student's two-tailed t test. Bars represent group means $\pm$ SD. CLDC-treated mice had significantly lower bacterial burden than diluent treated mice for all organs examined ($***p < 0.001$, $**p < 0.01$, $*p < 0.05$). Each experiment was repeated once with similar results.