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Detoxified Endotoxin Vaccine (J5dLPS/OMP) Protects Mice Against Lethal Respiratory Challenge with *Francisella tularensis* SchuS4

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Abstract

Francisella tularensis is a category A select agent. J5dLPS/OMP is a novel vaccine construct consisting of detoxified, O-polysaccharide side chain-deficient, lipopolysaccharide non-covalently complexed with the outer membrane protein of *N. meningitidis* group B. Immunization elicits hightiter polyclonal antibodies specific for the highly-conserved epitopes expressed within the glycolipid core that constitutes gram-negative bacteria (e.g., *F. tularensis*). Mice immunized intranasally with J5dLPS/OMP exhibited protective immunity to intratracheal challenge with the live vaccine strain, as well as the highly-virulent SchuS4 strain, of *F. tularensis*. The efficacy of J5dLPS/OMP vaccine suggests its potential utility in immunizing the general population against several different gram-negative select agents concurrently.

Keywords

tularemia; lungs; lipopolysaccharide

1. Introduction

Francisella tularensis is a highly pathogenic, gram-negative bacterium possessing attributes that favor its use as a weapon for bioterrorism [1]. *F. tularensis* is divided into two major subspecies: *tularensis* (type A), a highly virulent form most common in North America; and

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holarctica (type B), which is less virulent and responsible for most illnesses in Europe and Asia [2]. Inhalation of as few as 10 organisms can cause severe pneumonia and a high mortality rate [1]. Consequently, the CDC classifies *F. tularensis* as a category A select agent [1]. Immunization with the attenuated Live Vaccine Strain (LVS) derived from subspecies *holarctica* has been used for decades. While generally effective, vaccination provided human volunteers only partial protection against challenge with aerosolized type A *F. tularensis* [3]. Moreover, the need to vaccinate by scarification, an unclear understanding of the molecular basis for its attenuation, and a potential to revert to virulence negate its continued use [4].

Efforts to develop a safe and effective, alternative vaccine have focused on three approaches: heat- or chemically-inactivated whole cell, attenuated, and subunit vaccines. In the past, inactivated whole-cell preparations routinely failed to elicit protection in humans or animal models [5]. Recent studies, however, demonstrated varying degrees of protective immunity to respiratory infections in mice vaccinated with inactivated *F. tularensis* LVS that was: administered in conjunction with IL-12, targeted to Fc receptors via anti-*F. tularensis* LPS-specific monoclonal antibody, or adjuvanted with preformed immune-stimulating complexes suggesting promise of such a strategy [6-8]. Given the general effectiveness of *F. tularensis* LVS in preventing infections, attenuated microorganisms are a logical approach to vaccine development [9,10]. Despite their restricted ability to survive, replicate, and cause disease, however, attenuated microorganisms still represent considerable risk to immunocompromised individuals [11].

Subunit vaccines composed of a defined set of antigens offer a third approach to immunizing against *F. tularensis*. To date, only a limited number of subunit vaccines have proven effective; purified *F. tularensis* LPS is best described. Immunization with LPS provided protective immunity against systemic (not aerosol) challenge with type B *Francisella*, but negligible protection against challenge with type A strains regardless of route [12-15]. Immunity to infection appeared to derive from an elevated response to the O-polysaccharide component of LPS; free lipid A was an ineffective immunogen [14,16]. Besides LPS, a number of highly immunogenic outer membrane protein (OMPs) have been identified (e.g., FopA and TUL4); none of these has proven effective in eliciting protective immunity [12,17].

Both $CD4^+$ and $CD8^+$ T cells are essential to resolving primary and secondary tularemia infections [18,19]. The role of humoral immunity is also well-documented. Passive immunization with pooled human sera derived from vaccinated individuals protected mice against challenge with *F. tularensis* LVS [20]. Similarly, mice passively immunized with sera derived from animals previously infected with *F. tularensis* LVS resisted challenge with a lethal dose of LVS, but not type A *F. tularensis* (SchuS4) [13,21]. Notably, the protective antibodies comprising immune sera primarily recognized the LPS component of *F. tularensis* though antibodies reactive with a number of outer membrane and intracellular proteins are also present [20,22].

J5dLPS/OMP is a novel vaccine construct consisting of detoxified (de-O-acylated) LPS derived from *Escherichia. coli* 0111:B4, J5 (Rc chemotype), a mutant unable to attach the O-polysaccharide side chain to the outer core glycolipid [23-25]. The detoxified LPS (dLPS) core is complexed with the OMP of *Neisseria meningitidis* group B, a Toll-like receptor (TLR) 2 ligand that stabilizes the glycolipid structure [24,26,27]. In the absence of an immunodominant O side chain, immunization elicits a polyclonal antibody response to the highly-conserved epitopes expressed within the glycolipid core that constitutes gram-negative bacterial LPS [25,26]. Vaccine-induced antibody promotes polymicrobial clearance in rodents and protection against lethal infection in a number of experimental models of gram-negative sepsis [24-26, 28]. Additionally, mice vaccinated i.n. with J5dLPS/OMP resist lethal respiratory challenge with *Klebsiella pneumoniae* [29]. Here, we report that J5dLPS/OMP vaccinated mice resist

intratracheal (i.t.) challenge with type A *F. tularensis* SchuS4, as well as with type B *F. tularensis* LVS.

2. Materials and methods

2.1. Francisella tularensis

F. tularensis subspecies *holarctica* strain LVS was obtained, cultured, stored and used experimentally as we described previously [30]. *F. tularensis* subspecies *tularensis* strain SchuS4 was obtained from ATCC/BEI Resources (Manassas, VA). For experimentation, *F. tularensis* SchuS4 in stock vials stored at −80°C was thawed, grown and also used as previously reported [31]. Preliminary experiments established 1 LD_{50} equivalent to 1,280 CFUs *F. tularensis* LVS or 1-2 CFUs *F. tularensis* SchuS4 inoculated i.t. as described below.

The bacterial burden of the lungs, blood and livers of infected mice was calculated from the colonies that grew on supplemented Mueller-Hinton agar (*F. tularensis* LVS) or cysteine heart agar (*F. tularensis* SchuS4) plates in accordance with methods we described previously [30, 31]. All experiments involving *F. tularensis* LVS were conducted within BSL2 facilities located at Rhode Island Hospital (Providence, RI) or The University of Maryland School of Medicine (Baltimore, MD); experiments with *F. tularensis* SchuS4 were performed within the confines of the BSL3 at the New England Regional Center of Excellence (NERCE; Boston, MA).

2.2. Animals

Specific pathogen-free female, BALB/c mice, purchased from The Jackson Laboratories, Bar Harbor, ME were treated in accordance with protocols approved by the Rhode Island Hospital Animal Care and Use Committee and Harvard Medical School's (HMS) Standing Committee on Animals. Mice challenged i.t. with *F. tularensis* SchuS4 were housed and maintained in a specific pathogen-free facility utilizing TECHNIPLAST Sealsafe™ IVC cages (Exton, PA) at HMS.

2.3. Vaccination

Ten, 6- to 8-week-old mice were inoculated i.n. with 1 μg J5dLPS/OMP vaccine at 0, 2, and 4 weeks. Unmethylated cytidine-phosphate-guanosine (CpG) containing oligodinucleotide (ODN 10104; Coley Pharmaceutical Group, Wellesley, MA) was administered i.n. (25 μg/mouse) one hour prior to each vaccination. Control groups received CpG ODN only.

2.4. Antibody Responses

Sera and bronchoalveolar lavage (BAL) fluids were collected from non-infected mice on day 42 post-vaccination; core glycolipid-specific IgG and IgA antibodies were quantified by ELISA as previously described [25].

2.5. Respiratory challenge

Vaccinated mice were challenged i.t. on days 35 - 42 post-vaccination in accordance with methods previously described [30,32].

2.6. Myeloperoxidase

Myeloperoxidase activity in homogenates of the lungs was quantified spectrophotometrically by analyzing the H_2O_2-dependent oxidation of 3,3′5,5′-tetramethylbenzidine (Sigma-Aldrich) as we previously described [33].

2.7. RNA extraction, purification, and quantitative real-time RT-PCR

Total cellular RNA in representative tissue samples was extracted and purified using TRIzol (Invitrogen Corporation, Carlsbad, CA). Real-time RT-PCR was conducted using the methods and RNA primers we described previously [30].

2.8. Cytokine and chemokine analyses

The cytokines and chemokines present in representative tissue samples obtained from individual mice were quantified as we described previously using Bio-Plex cytokine assay kits and the Bio-Plex 200 system in accordance with the manufacturer's instructions (Bio-Rad Laboratories GmbH, Munich, Germany) [30].

2.9. Statistical analysis

The data were analyzed using the SigmaStat statistics program (Jandel Scientific, San Rafael, CA). Individual means were compared using a non-paired Student's *t* test, a Mann-Whitney Rank Sum test, or McNemar's paired and ranked non-parametric test. Survival differences were determined using Kaplan-Meier survival plots and analyzed by a LogRank test. $p < 0.05$ was considered statistically different.

2.10. Abbreviations used

BAL, bronchoalveolar lavage; CpG ODN, unmethylated cytidine-phosphate-guanosine containing oligodinucleotide; i.t., intratracheal; i.n., intranasal; J5dLPS/OMP, detoxified O-polysaccharide side chain-deficient *Escherichia coli* J5 mutant lipopolysaccharide non-covalently complexed with the outer membrane protein of *Neisseria meningitidis* group B; KC, keratinocyte-derived chemokine; LVS, live vaccine strain; MCP-1, monocyte chemotactic protein-1; OMP, outer membrane protein; TLR, toll-like receptor.

3. Results

3.1. J5dLPS/OMP vaccine elicits anti-J5 core glycolipid antibody production

J5dLPS/OMP vaccine administered in combination with CpG ODN stimulated the production of anti-J5 core glycolipid antibody. Glycolipid-specific IgG was ~1,000-fold greater in the sera and ~100-fold greater the BAL fluids obtained from mice immunized i.n. with J5dLPS/OMP vaccine and CpG ODN than those obtained from control mice administered CpG ODN alone (Figure 1A). Relative to the controls, the same sera and BAL fluids obtained from vaccinated mice contained 5- to 10-fold more anti-J5 core glycolipid-specific IgA (Figure 1B).

3.2. Vaccinated mice exhibit protective immunity against respiratory F. tularensis LVS infections

Mice immunized i.n. with J5dLPS/OMP vaccine and CpG ODN were subsequently challenged i.t. with a lethal dose *F. tularensis* LVS. Sixty percent of the vaccinated mice survived compared to 10% of the control mice administered CpG ODN alone (Figure 2). The elevated survival rate observed among vaccinated mice correlated inversely with the bacterial burden of tissues derived from these same animals. Two- to five-fold fewer organisms were recovered from the lungs, blood and livers of mice immunized with the J5dLPS/OMP vaccine and CpG ODN relative to the controls (Figures 3A-C).

3.3. Reduced cytokine/chemokine levels in the livers of vaccinated mice infected with F. tularensis LVS

The expression of proinflammatory cytokine messages, IL-12, IFN-γ and TNFα, was unchanged in the lungs of mice soon after respiratory challenge with *F. tularensis* LVS; at 4

hours post-infection, mice immunized with J5dLPS/OMP vaccine and CpG ODN were equivalent to those administered CpG ODN alone (Figure 4A-C). Additional analyses demonstrated no significant changes in the expression of IL-1β, IL-4, IL-6, IL-10, MIP-1α, MIP-1β, KC MIP-2 mRNAs and in the lungs of either the vaccinated or control group of mice at 4 hours postinfection (data not shown). These findings are supported by bead array analyses demonstrating equivalent protein concentrations of the same cytokines/chemokines in the lungs of both groups at 4 hours postinfection (Figure 4D). Notably, at 3 days post-challenge, the protein concentrations of these same cytokines/chemokines were elevated relative to those assessed in the lungs at 4 hours. There was no statistical difference, however, comparing levels in the lungs of vaccinated and control animals at this time (Figure 5A). In contrast, the concentrations of IL-12, TNF-α, IL-1β, IL-6, IL-10 and KC were significantly less in the livers of vaccinated, relative to control, mice at 3 days post-infection i.t. (Figure 5B).

3.4. Fewer neutrophils accumulate in the lungs of vaccinated mice infected with F. tularensis

A general reduction in cytokine/chemokine concentrations in the tissues of vaccinated mice at 3 days post-infection suggests a diminished inflammatory response. This suggestion is supported by a significant difference in myeloperoxidase activity (a correlate of neutrophil numbers) assessed in the lungs of vaccinated and control mice at 3 days (Figure 6).

3.5. J5dLPS/OMP vaccine protects mice against respiratory, F. tularensis SchuS4 challenge

Mice vaccinated with J5dLPS/OMP and CpG ODN resisted infection by type A *F. tularensis*. Sixty-five percent of vaccinated animals survived challenge i.t. with 10 LD_{50} *F. tularensis* SchuS4 (Figure 7). Notably, 100% of the mice vaccinated i.t. with a sublethal dose of *F. tularensis* LVS (positive control group) and 20% of mice that received CpG ODN alone (negative control), survived the same challenge. Inasmuch as the lethal dose of SchuS4 inoculated i.t. is only 1-2 CFUs, it seems unlikely that the surviving negative controls received a challenge dose.

4. Discussion

The majority of humans who survive primary exposure to *F. tularensis* possess specific serum antibodies at 8 years post-infection; LPS is the principal antigen recognized though its role in infection is unknown [20,34]. Similarly, LPS is the predominant antigen recognized by serum antibodies derived from mice infected sublethally with *F. tularensis* LVS [35]. Since it is intrinsically less toxic than LPS derived from enteric gram-negative bacteria [36-38], *F. tularensis* LPS represents a critical target for vaccine development. Indeed, the intact molecule and O-polysaccharide side chain have been screened as candidates [13-16]. While LPS-based vaccines demonstrate efficacy in mice challenged with type B strains of *F. tularensis*, immunity is incomplete; mice challenged with aerosolized type A *F. tularensis* succumb to infection. Mice vaccinated with J5dLPS/OMP, on the other hand, resisted respiratory challenge with both LVS and SchuS4 strains of *F. tularensis*.

J5dLPS/OMP vaccine stimulates the production of high antibody titers specific for the conserved glycolipid core of bacterial endotoxin [26,28,29]. These polyclonal antibodies recognize a broad range of LPS structures comprising heterologous gram-negative (but not gram–positive) bacteria. Previously, we reported that mice vaccinated i.n. with J5dLPS/OMP survived a lethal intratracheal challenge with *K. pneumoniae* [29]. Survival correlated with elevated core glycolipid-specific IgG and IgA class antibody titers in the sera and BAL fluids obtained from immunized mice, results comparable to those presented herein. The relevance of these antibodies to host resistance is supported by their ability to confer protection in a number of experimental models of sepsis; passive immunization with J5dLPS/OMP-specific antibody protected animals in a dose dependent manner [26,28]. Other investigators reported

similar findings in mice passively immunized with serum antibodies that primarily recognize the LPS component of *F. tularensis*, i.e., enhanced resistance to challenge with a lethal dose of *F. tularensis* LVS [13,20-22]. While the role of humoral immunity in host defenses to *F. tularensis* is well documented, the specific antibody function remains unclear. Previously we reported that core glycolipid-specific IgA and IgG class antibodies in sera and BAL fluids obtained from mice vaccinated with J5dLPS/OMP promoted the ingestion and killing of bacteria by alveolar macrophages [29]. In this regard, it is relevant to note that mice vaccinated with inactivated *F. tularensis* LVS coated with anti-LPS-specific mAb and targeted to Fc receptors expressed by alveolar macrophages exhibited IgA-dependent resistance to respiratory *F. tularensis* challenge [7].

The OMP component of the J5dLPS/OMP vaccine is a TLR2 ligand [26,28]. The literature suggests it plays a key role in the elevated resistance of J5dLPS/OMP-vaccinated mice challenged i.t. with *F. tularensis* SchuS4. TLRs expressed by antigen presenting cells are principally involved in innate host resistance [39]. Ligation up-regulates the production of proinflammatory cytokines/chemokines and the expression of cell-surface co-stimulatory molecules that foster the development of acquired immunity. The adjuvant-like activity of TLR ligands has lead investigators to suggest incorporating them into subunit vaccines in order to boost efficacy [40].

TLR2, heterodimerized with TLR1 or TLR6, recognizes a wide variety of microbial ligands [41]. While *F. tularensis* LPS is incapable of binding TLR2, two lipoproteins that can bind (TUL4 and FTT1103) are thought to initiate proinflammatory cytokine production during tularemia infections [42,43]. TLR2 expression is required for optimal host defenses against *F. tularensis*; TLR2-deficient mice exhibit increases in the bacterial burden of the lungs, spleen and liver, and a high rate of mortality [44]. These findings substantiate our conjecture that *N. meningitidis* OMP is a key factor contributing to the efficacy of J5dLPS/OMP vaccine. This conjecture is supported by a recent report demonstrating higher LPS-specific serum antibody titers and enhanced resistance to intranasal *F. tularensis* LVS challenge in mice immunized with purified *F. tularensis* LPS in combination with *N. meningitidis* Porin B (a TLR2 ligand) relative to mice immunized with LPS alone [45]. Similarly, we reported that both OMP and TLR2 were essential for an optimal response to J5dLPS/OMP vaccination. J5dLPS administered in the absence of OMP was poorly immunogenic [26]; serum glycolipid-specific IgG levels were significantly less in TLR2-deficient mice vaccinated with J5dLPS/OMP than in their vaccinated wild-type counterparts [46]. Notably, the serum antibody titers were higher in mice vaccinated i.n. with J5dLPS/OMP administered in conjunction with unmethylated CpG ODN (a TLR9 agonist with adjuvant-like properties) than in animals vaccinated with J5dLPS/OMP vaccine alone [29]. Other investigators reported similar findings: co-administration of CpG ODNs and vaccines by a respiratory route promoted a vigorous polyclonal B cell response, the production of antigen-specific IgA antibodies, and mucosal immunity [47].

CpG ODN administered alone exerts only a transient beneficial effect on innate host defenses to microbial infections [25]. Thus, it is unlikely the survival of control mice challenged with *F. tularensis* SchuS4 on day 35 following administration (shown in Figure 7) reflects the residual effects of CpG ODN. Rather, it seems more plausible that their survival represents a failure in protocol and our varied ability to challenge i.t. each animal uniformly with the exceedingly small number of organisms that constitutes a lethal dose. Assuming the same 22% rate of failure in challenging both the vaccinated and control groups, the actual survival rate of vaccinated mice challenged with a lethal *F. tularensis* dose would be 60% (9 out of 15), rather than the 65% shown in Figure 7.

Common mouse strains vary widely in their susceptibility to tularemia infections [48]. Relative to BALB/c, for example, C57BL/6 mice characteristically exhibit diminished resistance. In

particular, only BALB/c mice vaccinated intradermally with *F. tularensis* LVS were protected against an aerosol challenge with virulent *F. tularensis* Type B [49]. Similarly, in experiments undertaken in our laboratories, C57BL/6 mice vaccinated with J5dLPS/OMP and CpG ODN in accordance with the methods outlined here remained fully susceptible to intratracheal challenge with *F. tularensis* LVS (unpublished observation). Notably, C57BL/6 mice vaccinated i.p. with J5dLPS/OMP and CpG ODN exhibited marked increases in anti-core glycolipid-specific serum antibody [46]. Consequently, the failure of vaccinated C57BL/6 mice to resist intratracheal tularemia infection does not appear due to the absence of antibody production albeit the response to J5dLPS/OMP and CpG ODN administered mucosally remains to be determined.

Protective immunity and the decreased number of organisms found in the liver of J5dLPS/OMP vaccinated mice collected on day 3 post-infection i.t. with *F. tularensis* LVS correlated with a reduction in the cytokine/chemokine levels assessed. Other investigators reported similar findings, i.e., diminished levels of IL-1β, IL-6, IL-12p40, G-CSF, IFN-γ, TNF-α, MCP-1, and/or KC in the tissues of vaccinated, compared to non-vaccinated, mice challenged i.n. with *F. tularensis* [7,15,45]. Indeed, in a separate study, less MIP-2 (a neutrophil chemokine) and the accumulation of fewer neutrophils in the tissues of infected animals correlated directly with long-term survival [50]. Notably, the accumulation of neutrophils in the lungs of J5dLPS/OMP vaccinated, *F. tularensis* LVS-infected mice was reduced in the experiments reported here.

The findings presented herein suggest the potential use of J5dLPS/OMP vaccine to induce broad humoral immunity to a number of gram-negative select agents (e.g., *F. tularensis*, *Yersinia pestis*, *Burkholderia mallei* and *B. pseudomallei*) in the general population when the specific organism(s) speculated in an act of bioterrorism remains to be identified. The ability of J5dLPS/OMP vaccination to elicit protective immunity to other select agents including those listed immediately above is a matter of ongoing investigation in our laboratories.

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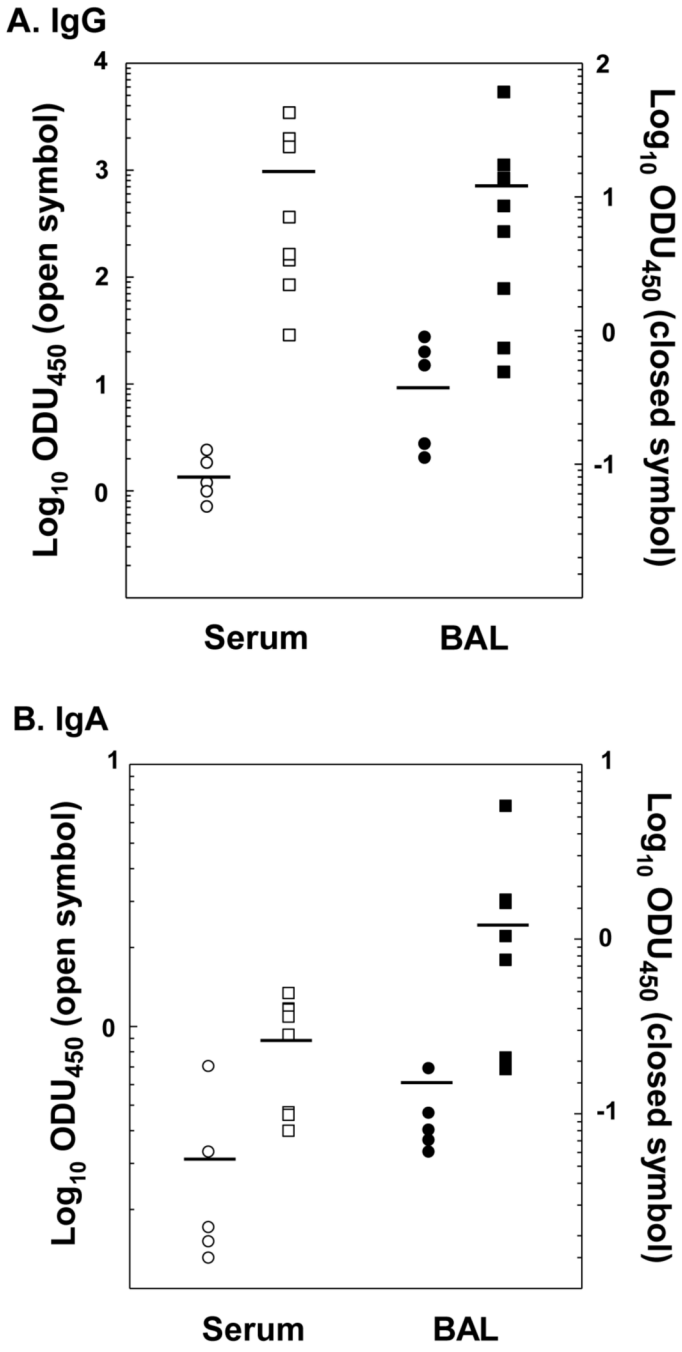

Fig. 1.

J5 core glycolipid-specific antibodies are elevated in mice immunized with J5dLPS/OMP vaccine. Groups of mice were inoculated i.n. with J5dLPS/OMP and CpG ODN (squares); control mice received CpG ODN alone (circles). Anti-J5 core glycolipid-specific IgG (A) and IgA (B) antibodies in the sera (open symbols) and BAL fluids (closed symbols) obtained on day 28 following the last inoculation were quantified. The pooled data derived from 2 separate experiments are shown. IgG and IgA anti-J5 core glycolipid-specific antibody concentrations in the sera and BAL fluids obtained from the vaccinated animals are significantly greater ($P<0.05$; non-paired Student's *t* test or Mann-Whitney Rank sum test).

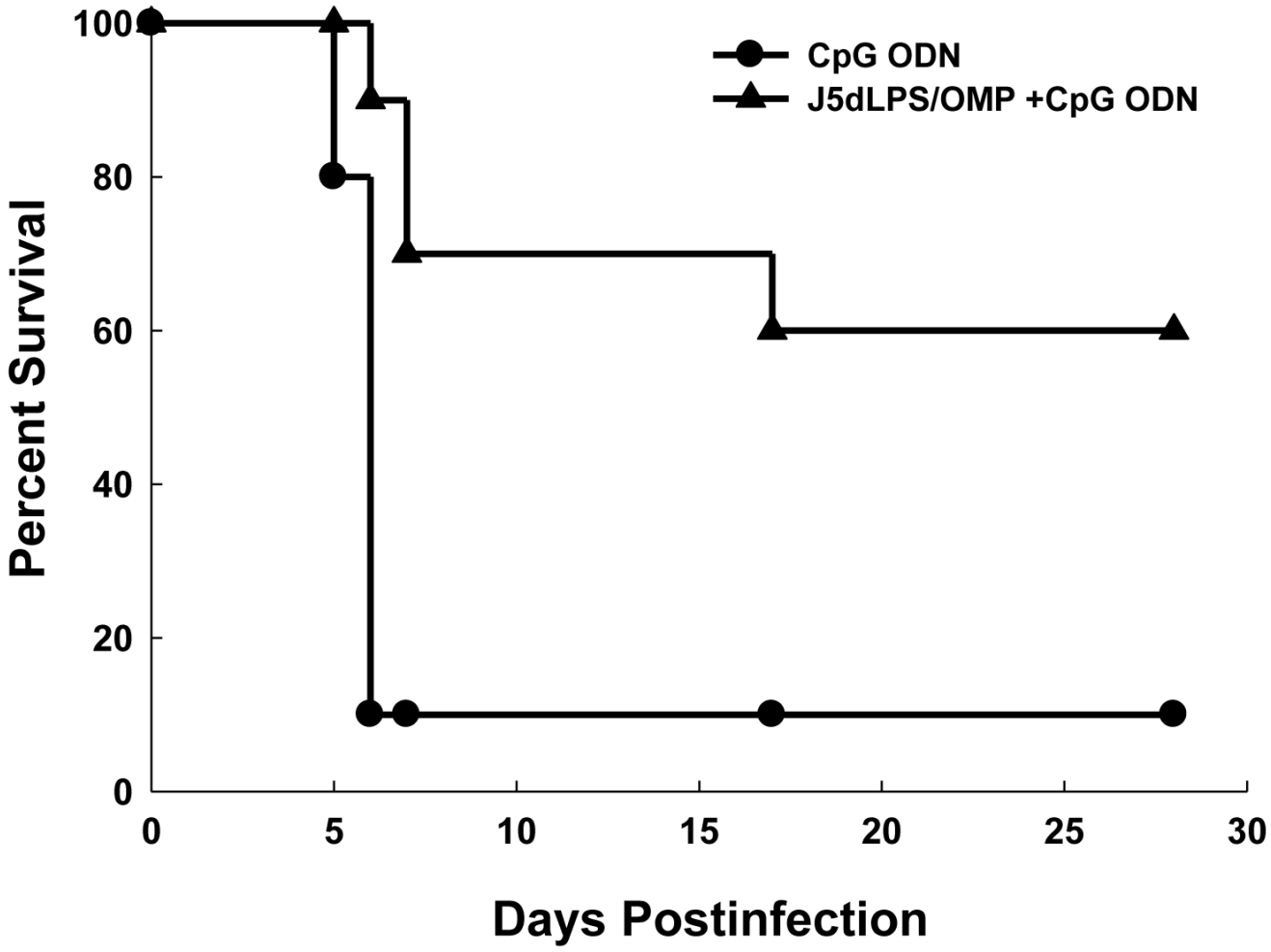

Fig. 2.

J5dLPS/OMP vaccine protects mice against F. tularensis LVS respiratory challenge. Groups of 10 control (CpG ODN alone) and 10 vaccinated (J5dLPS/OMP plus CpG ODN) mice were challenged i.t. with 9,800 CFUs *F. tularensis* LVS on day 28 post-vaccination. Data are derived from a single experiment representative of 4 separate experiments. J5dLPS/OMP vaccinated and CpG ODN control groups are significantly different; $P = 0.0006$ (LogRank test).

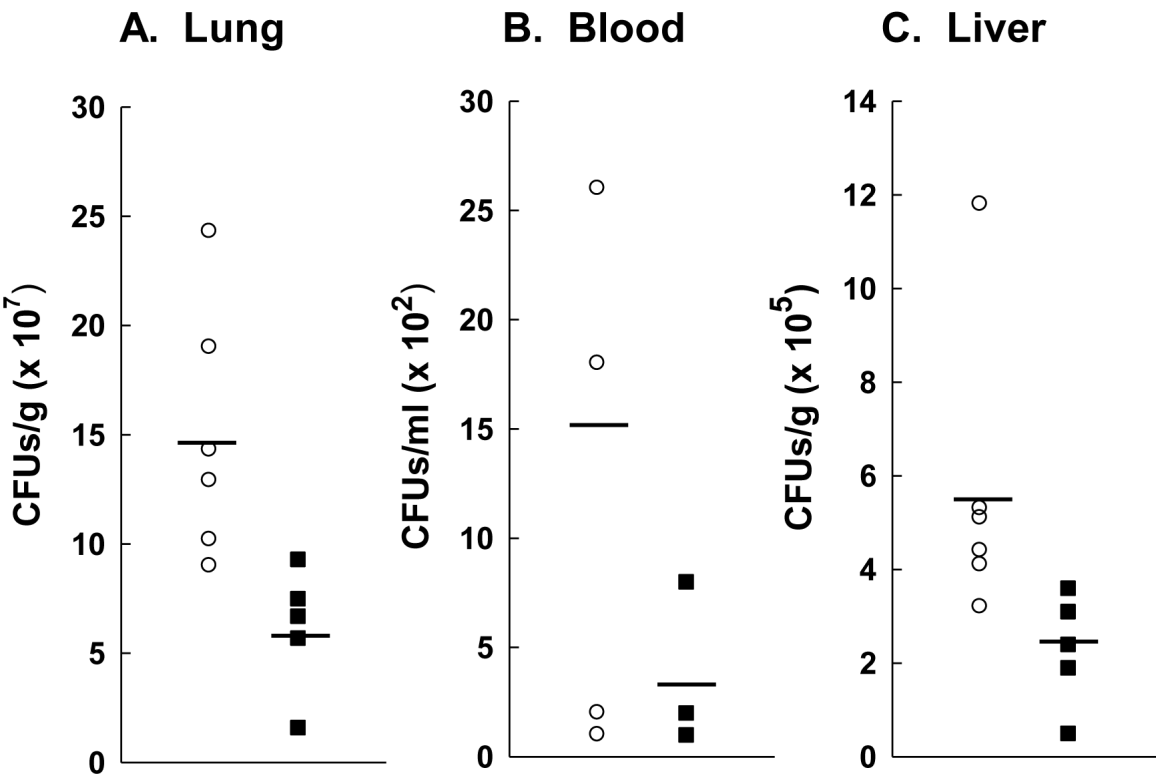

Fig. 3.

The bacterial burden is diminished in the tissues of vaccinated mice. Mice administered J5dLPS/OMP plus CpG ODN or CpG ODN alone were challenged i.t. with 6,800 CFUs (~5 LD_{50}) *F. tularensis* LVS on day 35 following final vaccine delivery. The lungs (A), blood (B) and livers (C) were collected on day 4 postinfection and the bacterial burden was determined. The pooled data derived from 2 separate experiments and a total of 5-6 mice per group are shown; each symbol represents a single mouse. The bacterial burden was significantly lower in all tissues derived from vaccinated animals ($p<0.05$, Student's *t* test).

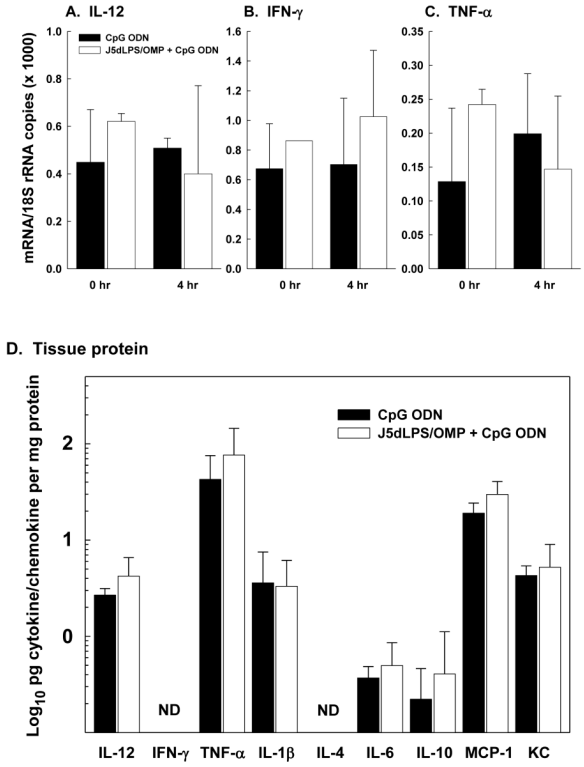

Fig. 4.

Proinflammatory cytokine/chemokine message and protein concentrations in the lungs are unchanged shortly following i.t. inoculation of F. tularensis LVS. Groups of control and vaccinated mice were challenged i.t. with 7,200 CFUs *F. tularensis LVS*. The lungs were dissected at 4 hours post-infection, and the expression of IL-12 (A), IFN-γ (B) and TNF-α (C) mRNAs quantified. The protein concentrations of the cytokines and chemokines listed were quantified (D). Data are the means ± SD derived from 4 mice treated similarly in each group. Second experiments yielded comparable results. ND = not detected. All differences between vaccinated and control mice were statistically insignificant; $P>0.05$ (Student's *t* test).

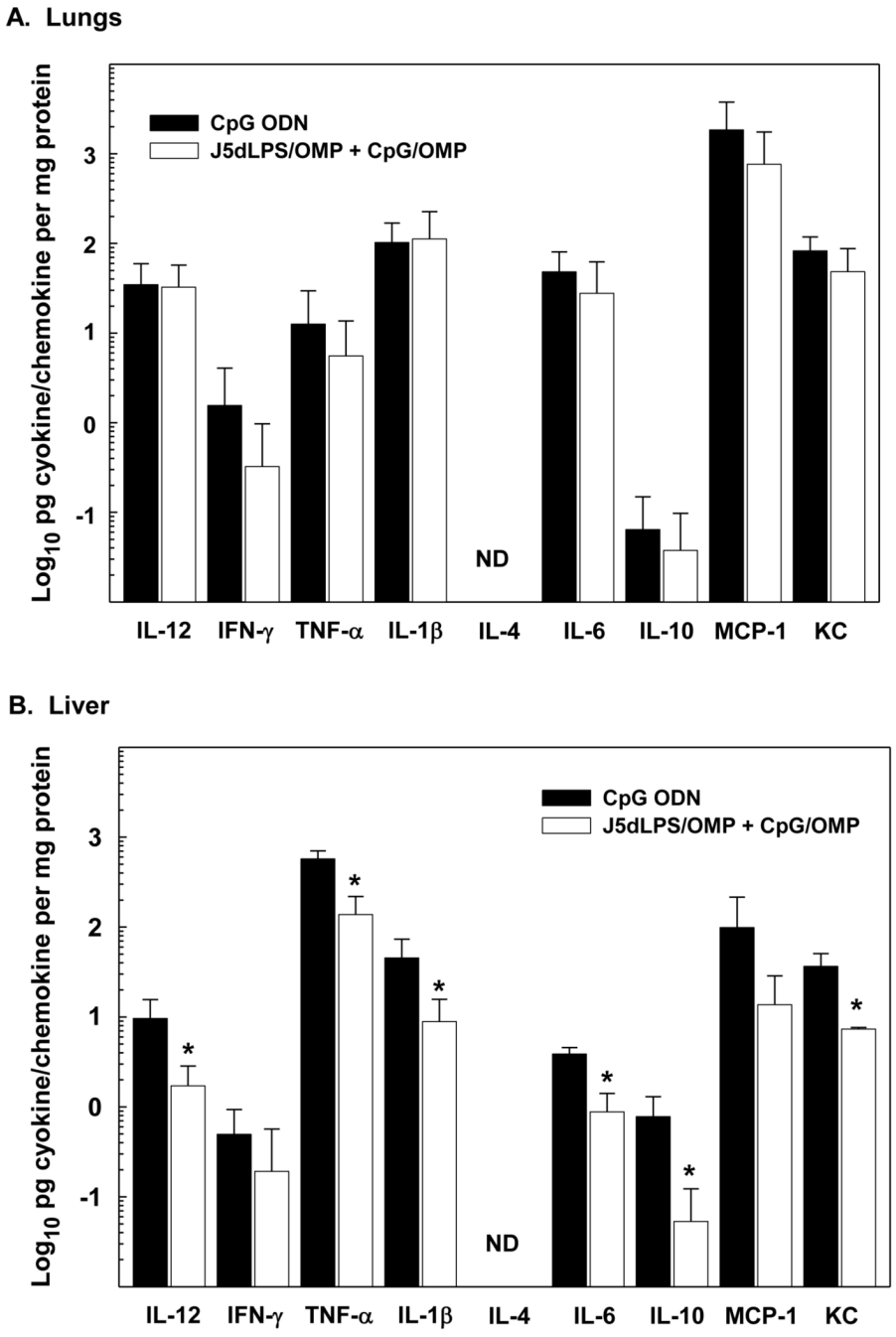

Fig. 5.

Proinflammatory cytokine/chemokine protein levels are diminished in the livers of vaccinated mice on day 3 post-challenged with F. tularensis LVS. Control (CpG ODN) and vaccinated (J5dLPS/OMP plus CpG ODN) mice were challenged i.t. with ~5 LD_{50} *F. tularensis* LVS. On day 3 post-infection, the lungs (top panel) and livers (bottom panel) were dissected; the cytokines and chemokines listed were quantified. Data are the means ± SD derived from 4 mice treated comparably. A second experiment yielded similar results. *Vaccinated group is significantly less than control: $P<0.05$ (Student's *t* test). ND = not detected.

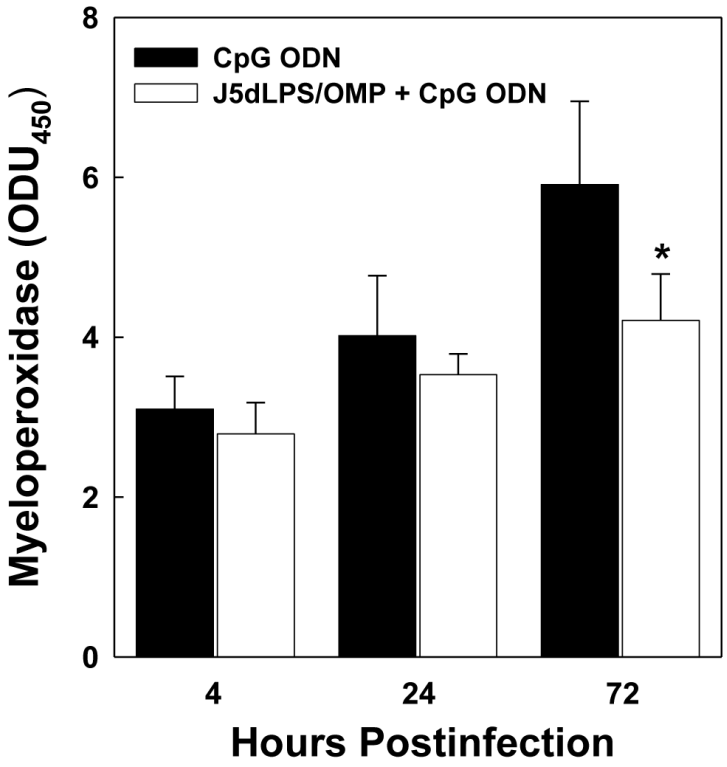

Fig. 6.

Vaccination suppresses the accumulation of neutrophils in the lungs subsequent to infection. Control and vaccinated groups of mice were challenged i.t. with 7,200 CFUs *F. tularensis* LVS. At 4, 24 and 72 hours post-infection, the lungs were dissected from four mice in each group and myeloperoxidase activity was quantified as a measure of neutrophil accumulation. *Significantly less than 72-hour group administered CpG ODN alone; $P<0.01$ (McNemar's test).

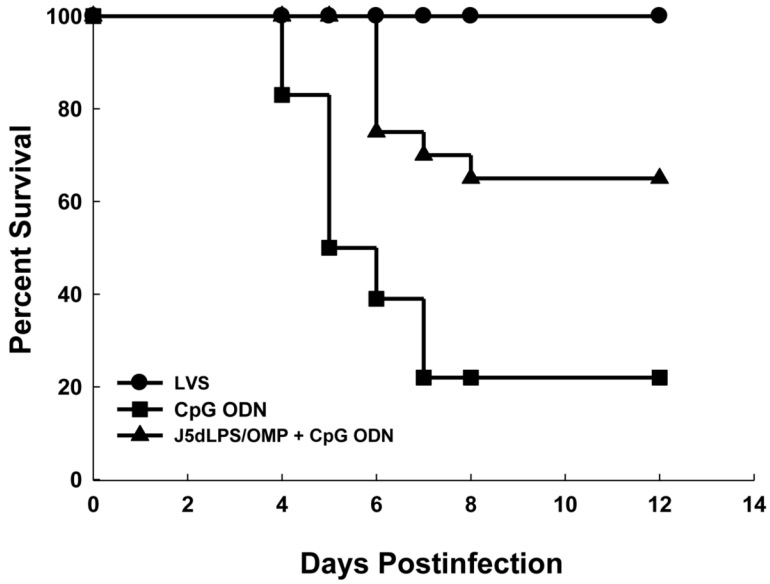

Fig. 7.

Vaccination with J5dLPS/OMP and CpG ODN protects mice against respiratory, F. tularensis SchuS4 challenge. Groups of mice were vaccinated with J5dLPS/OMP and CpG ODN; negative control mice received CpG ODN alone; positive control mice were immunized by infection i.t. with 100 CFUs *F. tularensis* LVS on day 0. All three groups were challenged i.t. with 10 CFUs *F. tularensis* SchuS4 on day 37 post-vaccination. Data are combined from 2 separate experiments and a total of 18-20 mice per group. J5dLPS/OMP vaccinated and CpG ODN control groups are significantly different; $P = 0.0013$ (LogRank test).