Published in final edited form as:

Vaccine. 2010 September 14; 28(40): 6562–6572. doi:10.1016/j.vaccine.2010.07.072.

Long lived protection against pneumonic tularemia is correlated with cellular immunity in peripheral, not pulmonary, organs

Rebecca V. Anderson*, Deborah D. Crane*, and Catharine M. Bosio*,†

* Laboratory of Intracellular Parasites, Rocky Mountain Laboratories, NIAID, NIH, Hamilton, Montana

Abstract

Protection against the intracellular bacterium *Francisella tularensis* within weeks of vaccination is thought to involve both cellular and humoral immune responses. However, the relative roles for cellular and humoral immunity in long lived protection against virulent *F. tularensis* are not well established. Here, we dissected the correlates of immunity to pulmonary infection with virulent *F. tularensis* strain SchuS4 in mice challenged 30 and 90 days after subcutaneous vaccination with LVS. Regardless of the time of challenge, LVS vaccination protected approximately 90% of SchuS4 infected animals. Surprisingly, control of bacterial replication in the lung during the first 7 days of infection was not required for survival of SchuS4 infection in vaccinated mice. Control and survival of virulent *F. tularensis* strain SchuS4 infection within 30 days of vaccination was associated with high titers of SchuS4 agglutinating antibodies, and IFN- γ production by multiple cell types in both the lung and spleen. In contrast, survival of SchuS4 infection 90 days after vaccination was correlated only with IFN- γ producing splenocytes and activated T cells in the spleen. Together these data demonstrate that functional agglutinating antibodies and strong mucosal immunity are correlated with early control of pulmonary infections with virulent *F. tularensis*. However, early mucosal immunity may not be required to survive *F. tularensis* infection. Instead, survival of SchuS4 infection at extended time points after immunization was only associated with production of IFN- γ and activation of T cells in peripheral organs.

Keywords

Tularemia; lung; vaccine

1.0 Introduction

Successful immunization regimes directed against many intracellular bacterial pathogens consist of vaccinating with live attenuated strains of the pathogen of interest or closely related, less virulent, isolates. For example, *Mycobacterium bovis* strain Bacillus Calmette Guérin, *Brucella abortus* strain 19 and *Francisella tularensis* Live Vaccine Strain (LVS) can protect against low doses of related virulent bacteria [1–3]. The protection engendered by these viable, attenuated organisms is believed to hinge on the ability of the vaccine strains to elicit the broad immunity, e.g. memory CD4⁺ and/or CD8⁺ T cells and antibody responses,

†Corresponding Author: Catharine M. Bosio, Ph.D., Laboratory of Intracellular Parasites, RML/NIAID/NIH, 903 S. 4th Street, Hamilton, Montana 59840, bosioc@niaid.nih.gov, Phone: 406-363-9425.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

required to eliminate intracellular bacteria. However, the specific mechanism by which these vaccines work is largely undefined. Understanding the correlates of immunity in the immune host would significantly contribute toward the development and implementation of novel diagnostics.

F. tularensis is a Gram negative, facultative intracellular, bacterium and is the causative agent of Tularemia. There are five primary forms of Tularemia that are largely distinguished by either the route of inoculation and/or presentation of disease [as reviewed, [4]]. Ulceroglandular and ocularglandular Tularemia occur following inoculation into the skin following the bites of an infected arthropod or direct infection of the eye. Oropharyngeal Tularemia occurs following infection of the tonsils and/or adenoid tissues. All three of these forms of Tularemia are characterized by severe lymphadenopathy of the lymph nodes draining the site of infection. The enlargement of these lymph nodes is reminiscent of buboes commonly associated with *Yersinia pestis* infections. This shared pathology between *Y. pestis* and *F. tularensis* infection resulted in the early characterization of Tularemia as a “plague-like illness” before isolation and identification of the causative agent [5]. The last two forms of Tularemia are typhoidal and pneumonic. Typhoidal Tularemia occurs following ingestion of the bacterium and is typically marked by severe diarrhea. Pneumonic Tularemia occurs following inhalation of *F. tularensis*. Pneumonic Tularemia is characterized as an atypical pneumonia since infection and replication of the bacterium in the lungs often occurs in the absence of detectable pulmonary pathology during the early phases of infection.

At the turn of the 20th century Tularemia was a serious, wide spread, human disease, causing significant morbidity and mortality in both the lay populations and laboratory staff working directly with the pathogen [6]. With the advent of antibiotics and an increase in our knowledge of Tularemia, the incidence of *F. tularensis* infection in the United States decreased dramatically. However, *F. tularensis* was developed by both the former Soviet Union and United States as a highly effective aerosol bioweapon [7]. Thus, interest in the pathology of Tularemia infections and the physiology of the bacterium continues today. One goal in past and present Tularemia research is development of novel vaccines and diagnostics that are especially effective against the pneumonic form of this disease or can aid in predicting vaccine efficacy against Tularemia, respectively.

Vaccine development against Tularemia has been addressed by scientists since the identification of *F. tularensis* as a human pathogen in the early 1900's [8]. In 1956 a live vaccine was developed following attenuation of an isolate of *F. tularensis* subspecies *holarctica* [2]. This strain was designated live vaccine strain (LVS). Although LVS protects against very low doses of virulent *F. tularensis*, it was soon realized that LVS failed to fully protect against even median doses, i.e. ≥ 200 bacteria, of virulent *F. tularensis* [9]. Furthermore, protection against a low dose challenge of virulent *F. tularensis* engendered by LVS waned over time [10]. To further complicate matters, the small number of studies that have addressed requirements and correlates of immunity against virulent *F. tularensis*, have focused on responses generated within a week of the host clearing LVS (approximately 21–28 days after vaccination) when a strong effector phase dominates the host response [11–13]. Thus, development of a more effective, long lived, vaccine directed against *F. tularensis* is dependent on identifying not only the correlates of immunity present early after vaccination, but also those that persist in the host after the effector phase has ended.

In this report, we define the correlates of immunity engendered by LVS vaccination for protection against pulmonary infection with virulent *F. tularensis* strain SchuS4 during early and late time points after immunization.

2.0 Materials and Methods

2.1 Bacteria

F. tularensis strain SchuS4 was provided by Jeannine Peterson, Ph.D. (Centers for Disease Control, Fort Collins, CO.) *F. tularensis* strain holarctica Live Vaccine Strain was provided by Jean Celli, Ph.D. (Rocky Mountain Laboratories, Hamilton, MT). SchuS4 and LVS were cultured in modified Mueller-Hinton (MMH) broth at 37°C with constant shaking overnight, aliquoted into 1 ml samples, frozen at -80°C and thawed just prior to use as previously described [14]. Frozen stocks were titered by enumerating viable bacteria from serial dilutions plated on modified Mueller-Hinton agar as previously described [15,16]. The number of viable bacteria in frozen stock vials varied less than 5% over a 10 month period.

2.2 Generation of whole cell lysate (WCL)

SchuS4 and LVS were grown in MMH broth as described above. Following overnight culture, bacteria were centrifuged for 15 min at 8000 × g. Bacteria were resuspended in breaking buffer (50 mM Tris/HCl, 0.6 µg/ml DNase, 0.6 µg/ml RNase, 1 mM EDTA [all from Sigma] and 1 Complete EDTA free tablet [Roche]) and centrifuged again for 15 min at 8000 × g. Bacteria were resuspended in breaking buffer and added to Fast Prep Lysing Matrix B tubes (MPBiomedical, Solon, OH). Bacteria were lysed by processing samples in a FastPrep24 (MPBiomedical) for 10 cycles of 45 seconds with a rest period of 2 min on ice in between each cycle. The resulting slurry was centrifuged at 10,000 rpm for 10 min. The supernatant was designated WCL. Aliquots of WCL were irradiated to render them sterile, and frozen at -80°C prior to use.

2.3 Mice

Specific-pathogen-free, 6–8 week old female Balb/c mice (wild type) (n = 3–10/group) were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were housed in sterile microisolator cages in the ABSL-3 facility at the NIAID/Rocky Mountain Laboratories (RML). All research involving animals was conducted in accordance with Animal Care and Use guidelines and animal protocols were approved by the Animal Care and Use Committee at RML.

2.4 Immunization of mice

LVS was serially diluted in PBS to a concentration of 1×10^3 CFU/ml. Mice were immunized subcutaneously (s.c.) with 200 CFU LVS injected in a volume of 200 µl. Actual inoculum concentration was confirmed by plating a portion of the inoculum onto MMH agar plates, incubating plates at 37°C/5%CO₂, and enumerating colonies. Mice were bled via the tail vein for serum at the indicated time points. All mice were challenged with *F. tularensis* SchuS4 30 or 90 days after the last vaccination as described below.

2.5 Titration of SchuS4 specific antibodies

Sera from vaccinated mice were analyzed for IgG, IgG1, IgG2a, and IgM antibodies directed against SchuS4 WCL by modified ELISA. Briefly, 100 µl WCL (1 µg/ml) in 0.1 mM sodium bicarbonate buffer (pH 9.6) was incubated overnight at 4°C in wells of Immulon 2HB plates (Fisher Scientific, Waltham, MA). Wells were washed three times with PBS, 0.05% Tween 20 (PBST). Then, wells were incubated with 1% BSA in PBS for 1 h at room temperature and washed with PBST. Sera were serially diluted in PBS and 100 µl of each sample added to the WCL coated plates to incubate for 1 h at room temperature. Plates were washed with PBST again. Bound IgG or IgM was detected following addition of either goat anti-mouse IgG, IgG1, IgG2a or IgM antibodies (Jackson ImmunoResearch) followed by horseradish peroxidase (HRP) conjugated donkey anti-goat antibodies (Jackson

ImmunoResearch). Bound antibodies were visualized using 1-step TMB ELISA substrate solution. Color development was stopped with 1 N HCL and absorbance of each well was assessed at 450 nm using a MRX Revelation and Revelation Software (Dynex Technologies, Chantilly, Virginia). Titers were determined as absorbance units two times greater than negative controls (serum from unvaccinated mice).

2.6 Agglutination assay

Sera was collected as described above and serially diluted in PBS. SchuS4 was diluted to 5×10^8 /ml in PBS. Fifty microliters of diluted serum was mixed with 50 μ l diluted SchuS4 in round bottom 96 well plates. Sera and SchuS4 were incubated 1 h at 37°C, followed by overnight incubation at 4°C. Agglutination was observed by light microscopy and is indicated in the results section as the last dilution at which agglutination was observed.

2.7 Infection of mice with *F. tularensis* SchuS4

Mice were infected with *F. tularensis* SchuS4 30 or 90 days after the last vaccination as previously described [17]. Briefly, mice were anesthetized via intraperitoneal injection with 100 μ l of ketamine (12.5 mg/ml) + xylazine (3.8 mg/ml) solution and intranasally infected with 25 CFU SchuS4 diluted in a final volume of 25 μ l of PBS. Inoculating doses were confirmed by plating inoculum on MMH agar. This inoculum routinely results in 100% mortality and a mean time to death of 5 days following infection in naive animals.

2.8 Collection of lung and spleen cells

Lung cells and splenocytes were collected as previously described with the following modifications (17). Briefly, lungs were excised, minced and incubated in PBS supplemented with 0.35 mg/ml Blendzyme 3 (Roche, Nutley, NJ) for 45 minutes at 37°C/5%CO₂. Tissues were triturated using a 5 ml syringe and 18G needle. Then, cells were pelleted by centrifugation at 1200 rpm for 5 min and red blood cells (RBC) were lysed with NH₄Cl. Remaining cells were washed in PBS and passed through a 70 μ m nylon screen. Spleens were excised, gently pressed through a 70 μ m nylon screen and pelleted by centrifugation. RBC were lysed as described above and cells were washed in PBS. Lung cells and splenocytes were resuspended in FACS buffer prior to flow cytometric analysis or RPMI supplemented with 10% heat-inactivated fetal calf serum (FCS), 0.2 mM L-glutamine, 1 mM HEPES buffer and 0.1 mM nonessential amino acids (all from Invitrogen, Carlsbad, CA) (cRPMI) prior to addition to tissue cultures. Total live cells from the lungs and spleens were enumerated using trypan blue and a hemacytometer.

2.9 Flow cytometry and analysis of lung and spleen cells

Lung and splenocyte populations were assessed by flow cytometry. Directly conjugated antibodies for these analyses were purchased from BD Biosciences (San Jose, CA). The following antibodies in various combinations were used for flow cytometric analysis: PECy7 CD4 (RM4-5), PerCPCy5.5 CD8 (53–6.8), and FITC CD44 (IM7). Staining with directly conjugated antibodies was done in FACS buffer at 4°C. Following staining, cells were washed and fixed in 1% paraformaldehyde for 30 minutes at 4°C. Cells were washed a final time, resuspended in FACS buffer and stored at 4°C until analyzed. Samples were collected using a Partec ML flow cytometer (Partec, Swedesboro, NJ). Analysis gates were set on viable unstained cells and were designed to include all viable cell populations. Approximately 100,000 events were analyzed for each sample. Isotype control antibodies were included when analyses and panels were first being performed to assure specificity of staining, but were not routinely included with each experiment. Data was analyzed using FlowJo software (Treestar, Ashland, OR).

2.10 In vitro stimulation of lung and spleen cells

Spleens and lungs were harvested from mice (n= 5) 30 or 90 days after vaccination or 4 days after SchuS4 challenge. Unvaccinated, unchallenged mice (n = 4–5) and unvaccinated SchuS4 challenged mice (n=5) served as controls. Resting peritoneal macrophages (pMØ) were obtained from naïve, uninfected mice and were plated at 2×10^5 cells/well in a 96-well plate and were incubated overnight with 10µg/ml WCL. pMØ cultured in the absence of WCL (no Ag) served as negative controls. At the indicated time points, single cell suspensions of lung cells and splenocytes were obtained as described above. Splenocytes or lung cells were resuspended in cRPMI and added at a ratio of 1:1 to pMØ. Splenocytes and lung cells from naïve, uninfected animals and naïve, SchuS4 infected mice served as controls. For analysis of secreted cytokines, cultures were incubated for 72 hrs at 37°C/ 5%CO₂ at which time supernatants were collected for analysis of cytokines. For analysis of intracellular cytokines, cells were incubated at 37°C/7% CO₂ for 4 h in the presence of 10 µg/mL of brefeldin A (Invitrogen) and stained for surface receptors and intracellular cytokines as described below.

2.11 Detection of Secreted Cytokines

Culture supernatants were assessed for the presence of IFN-γ, IL-12p70, IL-5 and IL-10 by Cytometric Bead Array (CBA) Flex Sets according to the manufacturer's instructions (BD Biosciences).

2.12 Intracellular Cytokine Staining

Intracellular cytokines were detected by flow cytometry as previously described [18]. Following incubation conditions described above, cells were washed once and resuspended in FACS buffer and stained for CD4, CD8 or CD49b as described above. Then cells were fixed in 2% paraformaldehyde in PBS for 10 min at 37°C/7% CO₂ and washed twice more in perm buffer (FACS buffer supplemented with 0.25% saponin [Sigma-Aldrich]). Cells were incubated for 20 min at room temperature with anti-mouse IFN-γ (APC; clone XMG1.2) or APC conjugated rat IgG (isotype control) (both from BDBiosciences). Cells were washed twice in perm buffer, fixed in 1% paraformaldehyde for 30 min, and then resuspended in FACS buffer and stored at 4°C until analysis. Cells were acquired and analyzed using a CyFlow ML flow cytometer and FlowJo Software (Treestar).

2.13 Statistical Analysis

Statistical differences between two groups were determined using an unpaired t test with the significance set at p<0.05. For comparison between three or more groups, analysis was done by one-way ANOVA followed by Tukey's multiple comparisons test with significance determined at p<0.05. Significance in survival was determined using log-rank (Mantel-Cox) test with significance set at p<0.05.

3.0 Results

3.1 LVS provides long lived protection against pulmonary SchuS4 infection

LVS engenders complete protection against a low dose intranasal challenge with virulent *F. tularensis* strain SchuS4 when mice are exposed within 30 days of vaccination [10,19,20]. However, it has been reported that LVS provides only median protection in mice challenged greater than 70 days after vaccination [10]. Thus, we first tested the ability of our isolate of LVS to protect mice at different time points after vaccination. Mice were immunized subcutaneously with LVS and then were challenged intranasally with approximately 25 CFU of SchuS4 30 or 90 days after vaccination. As expected, within 30 days after vaccination, nearly all mice (9/10) that received LVS survived SchuS4 challenge (Figure 1). Similar to

mice challenged 30 days after vaccination, 90% of mice that received LVS 90 days prior to challenge survived pulmonary SchuS4 infection (Figure 1). Thus, subcutaneous vaccination with LVS provided sustained immunity against intranasal SchuS4 challenge.

3.2 Control of SchuS4 replication and dissemination in vaccinated animals

We next determined if the consistent protection afforded by vaccination with LVS was associated with equivalent control of SchuS4 replication and dissemination in two important target organs, the lung and spleen. Mice challenged with SchuS4 30 days after LVS vaccination had significantly fewer bacteria in the lungs 3 and 4 days after infection compared to unvaccinated animals ($p < 0.05$) (Figure 2). Although LVS vaccinated mice did not completely inhibit SchuS4 replication in the lung, bacterial numbers in this organ were modest (approximately 10^3 CFU/lung) up to 7 days after SchuS4 infection (Figure 2). The control of SchuS4 replication in the lungs of mice that received LVS 30 days prior to challenge correlated with delayed dissemination of SchuS4 to the spleen. Specifically, spleens of LVS vaccinated mice had significantly fewer bacteria up to 4 days after challenge compared to unvaccinated controls ($p < 0.05$) (Figure 2). Within 3 weeks of infection, vaccinated mice had no detectable SchuS4 in the lungs or spleens.

In contrast to mice vaccinated with LVS 30 days prior to challenge, mice infected with SchuS4 90 days after vaccination did not have significantly lower numbers of bacteria in the lungs compared to unvaccinated controls for the first 4 days after infection. Further, mice challenged with SchuS4 90 days after LVS vaccination did not appear to control bacterial replication in the lung up to 7 days after infection (Figure 2). However, these vaccinated animals did have significantly fewer bacteria in the spleen 3 and 4 days after infection compared to unvaccinated animals ($p < 0.05$) (Figure 2). In contrast to mice challenged 30 days after vaccination, small numbers of bacteria were still detected in the spleens and lungs 21 days after infection of mice challenged 90 days after vaccination. This suggested that immunity in both the lung and spleen was not as effective in mice challenged 90 days after vaccination compared to those challenged 30 days after vaccination. However, all mice (regardless of the time of challenge) cleared SchuS4 in the lung and spleen within 30 days of infection. Together, these data suggested that LVS vaccination engendered strong immunity that resulted in rapid clearance of SchuS4 in the lung and spleen 30 days after vaccination. In contrast, rapid control in the lung was not present in mice challenged 90 days after vaccination.

3.3 Humoral response in vaccinated animals

Presence of antibodies specific for *F. tularensis* have been used as a potential correlate of protection in immunized hosts [22,23]. It has also been suggested that antibodies may contribute to control of virulent *F. tularensis* infections. For example, passive transfer of hyper-immune serum can aid in control of *F. tularensis* infections in humans [21]. Thus, control and survival of SchuS4 infection in vaccinated animals in the present study may be correlated with the presence of SchuS4 specific antibody in LVS vaccinated animals. Therefore, we quantified SchuS4 reactive IgG and IgM present in the serum of animals. Thirty days after vaccination, mice had detectable titers of IgM directed against SchuS4 WCL ranging from $1:10^4$ – $1:10^5$ (Figure 3A). Ninety days after vaccination, titers of SchuS4 reactive IgM were still detectable, but had dropped to titers ranging from $1:10^3$ – $1:10^4$ (Figure 3A). Titers of SchuS4 specific IgG were initially lower than IgM titers in LVS vaccinated mice 30 days after vaccination. However, titers of SchuS4 specific serum IgG did not change between 30 and 90 days after vaccination (Figure 3A). We also examined the sub-classes of IgG present in LVS vaccinated animals. Serum collected from animals 30 and 90 days after immunization had similar titers of IgG2a and IgG1 directed against SchuS4

antigens. Presence of these two sub-classes of IgG suggested LVS engendered a mixed Th1 and Th2 type immune response in vaccinated mice.

Detection of antibodies by ELISA only indicates the presence of pathogen specific antibody. This type of analysis does not measure the functional properties of antibodies. One function of antibodies is to agglutinate pathogens which, in turn, can aid in clearance of bacteria. Thus, we next determined the titer of serum antibodies capable of agglutinating viable SchuS4. Serum from mice collected 30 days after vaccination had uniform agglutination titers of 1:200. Ninety days after vaccination, agglutinating titers in mice that had received LVS dropped to an average of approximately 1:75. Thus, high titers of agglutinating antibody were associated with control of SchuS4 replication in the lungs of mice vaccinated with LVS 30 days prior to challenge. Furthermore, the drop in agglutinating titers observed in mice 90 days after LVS vaccination corresponded to poor control of SchuS4 replication in the lung. However, the drop in titers of agglutinating antibodies did not correlate with the sustained protection against SchuS4 infection observed in LVS vaccinated animals.

3.4 Presence of SchuS4 reactive IFN- γ producing cells after vaccination

In models utilizing attenuated strains of *F. tularensis*, protection is largely dependent on development of cellular immunity which requires CD4⁺ or CD8⁺ T cells [16,24]. Additional studies have shown that depletion of either CD4⁺ or CD8⁺ T cells or IFN- γ in vaccinated animals interferes with protective immunity against SchuS4 challenge at early time points after vaccination [13]. However, the correlation of cellular immunity and/or IFN- γ in hosts vaccinated with LVS greater than 90 days prior to challenge is not known. Furthermore, it has not been determined if there are differences in the strength of cellular responses in specific target organs among vaccinated animals. Thus, we next determined if vaccination with LVS generated SchuS4 reactive cells capable of secreting IFN- γ . We also assessed IL-12p70, IL-5 and IL-10. Prior to challenge, splenocytes and lung cells harvested 30 days after LVS vaccination secreted significantly higher amounts of IFN- γ in response to SchuS4 WCL compared to cells from unvaccinated mice ($p < 0.05$) (Figure 4). Cells harvested from mice 90 days after vaccination had a more divergent response. Lung cells harvested from mice 90 days after vaccination failed to secrete significantly different amounts of IFN- γ compared to unvaccinated controls ($p < 0.05$) (Figure 4). In contrast, splenocytes from mice vaccinated 90 days earlier were still capable of secreting IFN- γ in response to SchuS4 antigens (Figure 4). Splenocytes, but not lung cells, from LVS vaccinated animals also secreted significantly more IL-10 in response to SchuS4 WCL than cells from unvaccinated mice ($p < 0.05$) (Figure 4). Despite the ability of cells from vaccinated mice to secrete IFN- γ in response to SchuS4 antigens, we did not detect significant differences in secretion of IL-12p70 among the different groups of mice (Figure 4). No IL-5 was detected in splenocyte cultures, and there was no difference in production of IL-5 in lung cell cultures (Figure 4). In agreement with the presence of SchuS4 reactive IgG1 and IgG2a in LVS vaccinated mice, the presence of cells that secrete both IFN- γ and IL-10 in response to SchuS4 antigens suggested that LVS promoted a mixed Th1 and Th2 type immune response in these animals. Furthermore, our data also suggests that the cellular component of this immune response was retained in the spleen but not the lung.

3.5 IFN- γ production in the lung following SchuS4 infection

Mice challenged 30 days after vaccination controlled replication of SchuS4 in the lung within 4 days of infection (Figure 2). This protection was correlated with IFN- γ secreting cells (Figure 4). Both NK cells and T cells represent important sources of IFN- γ . Thus, we first assessed the ability of NK cells present in the lungs and spleens of vaccinated mice to produce IFN- γ after SchuS4 infection. Mice challenged with SchuS4 30 days after vaccination had significantly more SchuS4 specific IFN- γ ⁺ NK cells in the lungs compared

to unvaccinated controls ($p < 0.05$) (Figure 5). However, mice vaccinated 90 days prior to SchuS4 infection did not have significantly different numbers of IFN- γ producing NK cells in the lungs compared to controls. There were no statistical differences in SchuS4 specific IFN- γ^+ NK cells in the spleens of mice tested at any time point (Figure 5). Thus, IFN- γ producing NK cells present in the lung correlated with control of SchuS4 replication in this organ in mice challenged 30, but not 90, days after infection. Furthermore, IFN- γ producing NK cells were not correlated with control of infection in the spleen at any time point.

3.6 Presence of splenic CD4⁺IFN- γ^+ and CD8⁺IFN- γ^+ cells after SchuS4 challenge are correlated with protection

In addition to NK cells, activated T cells are also capable of producing IFN- γ in a pathogen specific manner in immunized animals. Therefore, activated T cells could also represent a source of SchuS4 specific IFN- γ in immunized animals. Thus, we next examined if T cells in the lungs and spleens of vaccinated animals were activated and produced IFN- γ in response to SchuS4 challenge. T cell activation was monitored by examining increased expression of CD44 on the surface of CD4⁺ and CD8⁺ T cells. There were no detectable differences in T cell activation among any mice before day 4 of SchuS4 infection (data not shown). However, on day 4 of SchuS4 infection, mice that received LVS 30 days prior to challenge had significantly higher numbers of CD4⁺CD44^{hi} and CD8⁺CD44^{hi} in the lungs and spleens compared to uninfected, unvaccinated controls ($p < 0.05$) (Figure 6). However, mice challenged 90 days after vaccination failed to activate CD4⁺ or CD8⁺ T cells in their lungs (Figure 6). In contrast to the absence of activated T cells in the lungs, mice infected with SchuS4 90 days after vaccination had significantly higher numbers of activated CD4⁺ and CD8⁺ T cells in the spleen ($p < 0.05$) (Figure 6). This suggested that waning control of infection in the lungs of mice challenged 90 days after vaccination was associated with the absence of activated CD4⁺ and CD8⁺ T cells in this organ.

We next assessed the ability of T cells present in the lungs and spleens of vaccinated, SchuS4 challenged mice to produce IFN- γ . Surprisingly, we did not observe significantly more IFN- γ producing CD4⁺ or CD8⁺ T cells in the lungs of mice infected with SchuS4 30 or 90 days after vaccination compared to naïve, SchuS4 infected mice or naïve, uninfected animals (Figure 7). In contrast, significantly more SchuS4 specific IFN- γ producing CD4⁺ and CD8⁺ T cells were detected in the splenocyte samples from vaccinated mice that were challenged 30 days after immunization compared to all other groups of mice (Figure 7). Despite the ability of splenocytes harvested from mice 90 days after vaccination (but before challenge) to secrete IFN- γ , we did not observe significantly different numbers of IFN- γ producing T cells from the spleens of these mice 4 days after SchuS4 challenge compared to naïve, SchuS4 infected animals or naïve, uninfected mice (Figure 7). Together, these data suggest that superior control of SchuS4 replication was correlated with the ability of these animals to activate T cells and for these cells to produce IFN- γ after infection. Further, the survival of vaccinated mice was correlated with activated, IFN- γ producing CD4⁺ and CD8⁺ T cells in the spleen, but not the lung, in response to SchuS4 infection.

4.0 Discussion

Given the ability of LVS to protect both mice and humans against infection with virulent *F. tularensis*, this strain has become a gold standard by which other, experimental Tularemia vaccines are measured [2,9,25]. However, LVS as a vaccine has several points of concern including its unexplained mechanism of attenuation, spontaneous phase shift which nullifies its protective efficacy, documentation of adverse events following vaccination, and our minimal understanding of the mechanism of protection afforded by this vaccine [2,26]. Due to these concerns, there has been a renewed effort to develop novel vaccines for Tularemia.

One major hurdle in development of novel Tularemia vaccines is that the correlates of immunity, which could be used to measure their efficacy, are poorly defined. To date, many of the studies designed to determine these correlates have examined mice vaccinated and re-challenged with LVS rather than a virulent isolate of *F. tularensis* [16,24,27]. In those studies, immunity to LVS was complex and included roles for IFN- γ , TNF- α , IL-12, CD4⁺ and CD8⁺ T cells and B cells [15,16,28–37]. However, with the exception of IFN- γ , T cells and immune sera many of these parameters have remained unexplored with regard to their importance or correlation in protection against infection with Type A subspecies of *F. tularensis* [12,13]. Thus, it is not known if immunity generated against an attenuated strain would be the same as that directed against virulent organisms. Furthermore, even in cases where a virulent isolate of *F. tularensis* is used as the challenge strain, correlates are typically assessed within 30 days of vaccination with LVS [13]. Since LVS is cleared from the host between 21–28 days after vaccination, this 30 day time point likely includes strong effector responses still present in the vaccinated host as opposed to true memory responses ([38], Supplemental Figure 1). Thus, requirements and correlates for long lived immunity against *F. tularensis* infections are not known.

The primary goal of the study presented herein was to determine if correlates of immunity present shortly after animals had cleared LVS (30 days) were similar to those present during later time points (90 days) after vaccination. Interestingly, mice challenged 30 or 90 days after vaccination had similar survival rates. This observation is in contrast to previously published reports in which intranasal vaccination, presumably enhancing pulmonary immune responses, was required for survival of virulent *F. tularensis* infection in mice. Our data is also in conflict with the observation that mice challenged more than 70 days after vaccination were not protected against intranasal SchuS4 challenge [10,39]. One explanation for this discrepancy may lie in the isolate of LVS used to vaccinate animals in those studies. The doses of LVS used in the study presented herein are in agreement with doses used in original manuscripts published by Eigelsbach and colleagues. For example, the LD50 of LVS in mice was approximately 1000 colony forming units (CFU). Furthermore, the subcutaneous immunizing dose that provided full protection against aerosol infections with SchuS4 was approximately 200 CFU [2,11]. More recent manuscripts describing the failure of LVS delivered subcutaneously or intradermally to protect against pulmonary challenge with virulent *F. tularensis* utilized a minimum immunizing dose of 10⁵ CFU. This number of bacteria is 100 times the LD50 and nearly 1000 times more than the vaccinating dose previously reported by Eigelsbach and colleagues [10,39]. Clearly, the isolates of LVS used in studies in which s.c. vaccination failed to protect against pulmonary challenge were far more attenuated than those used in the original reports, as well as the isolate used herein. Highly attenuated strains of LVS fail to protect against virulent *F. tularensis* infections [2]. Thus, it is possible that the poor survival of virulent *F. tularensis* infections observed in other reports following s.c. or intradermal vaccination and challenge with LVS at time points later than 30 days after vaccination could be a result of using strains of LVS that have undergone further attenuation. A direct comparison of the different LVS isolates via comparative genomics, and their ability to engender SchuS4 specific immunity in various organs would provide critical insight into the requirements and correlates of protection against virulent *F. tularensis* infections.

Despite the equivalent survival of mice challenged either 30 or 90 days after vaccination our study revealed clear differences in the ability of these groups of mice to control SchuS4 infection in the lung. Animals challenged 30 days after vaccination were able to modestly control SchuS4 replication in the lung during the first 7 days of infection, whereas those challenged 90 days after vaccination did not (Figure 2). Both groups of animals had similar control of SchuS4 replication in the spleen. This suggested immunity in the lung among mice vaccinated 30 and 90 days prior to challenge was different. To determine the correlates

of immunity that were associated with the difference in control of SchuS4 replication in vivo we first examined humoral responses in vaccinated mice.

Both groups of mice had high titers of antibodies that recognized SchuS4 antigens. Titers of serum IgG directed against SchuS4 did not dramatically change overtime, thus it is unlikely that changes in IgG are responsible for the loss of early control of SchuS4 replication in the lungs of mice challenged 90 days after vaccination. SchuS4 specific IgM was also present in vaccinated mice. By 90 days after vaccination, IgM titers had dropped modestly and were similar to titers observed for SchuS4 specific IgG (Figure 3). It seems unlikely that this small drop in SchuS4 specific IgM could account for the failing immunity observed in the lung. Thus, presence of SchuS4 reactive IgG, IgG1 or IgG2a antibodies correlates with survival of SchuS4 infection, but does not correlate with the control of SchuS4 replication in target organs, i.e. the lung. These results extend observations made in previous reports. In earlier studies the presence of high titers against LVS were directly correlated with protection and uniform control of replication of LVS [27]. Furthermore, transfer of anti-LVS antibodies provided complete protection against subsequent LVS challenge [27]. In contrast, administration of LVS immune serum extended the mean time to death of mice infected with SchuS4, but did not increase the number of surviving animals [12]. The ability of LVS immune serum to control replication of SchuS4 in specific target organs was not addressed in those studies. Thus, it is possible that Immune serum may have aided in control of SchuS4 replication in the spleen but failed to have an effect in the lung, which ultimately contributed to the death of infected animals.

In addition to assessment of SchuS4 specific antibodies by ELISA, we also assessed the presence of agglutinating antibodies. Presence of antibodies capable of agglutinating *Francisella* have been used as a diagnostic for exposure and/or infection and vaccine efficacy. Indeed, mice had high titers of agglutinating antibodies 30 days after vaccination. However, despite 90% of vaccinated animals surviving SchuS4 infection when challenged 90 days after vaccination, the titer of agglutinating antibodies had dropped significantly. Therefore, high titers of agglutinating antibodies did not correlate with the ability of animals to survive infection. Rather, together these data suggest that presence of IgG antibodies may serve to predict the ability of the host to survive *Francisella* infection, but assessment of these SchuS4 specific antibodies cannot predict the relative control of bacterial replication in specific target organs.

In contrast to the modest correlation of anti-*Francisella* antibodies, we observed consistent, significantly different, responses associated with cellular immunity in vaccinated animals compared to unvaccinated controls. Lung cells and splenocytes harvested from mice 30 days after vaccination produced significantly more IFN- γ compared to unvaccinated mice (Figure 4). Whereas significant differences in IFN- γ production from cells examined 90 days after vaccination was only observed in splenocyte, but not lung cell, cultures (Figure 4). The importance of IFN- γ and T cells, for survival of LVS infections is well documented [16,24,40]. Similarly, it has also been shown that IFN- γ and CD4⁺ and CD8⁺ T cells are also required for survival of virulent *F. tularensis* infections 30 days after vaccination [13]. However, none of these studies determined if the presence of these cells was required at the site of infection (the lung) and/or in other organs. Thus, our data confirm and extend the correlation of these important cells in mediating strong protective immunity directed against virulent *F. tularensis* in three different ways.

First, our data demonstrates that the primary IFN- γ secreting cells in the lung are different from those present in the spleen in mice challenged 30 days after vaccination. Surprisingly, the only pulmonary cell type found to secrete IFN- γ in a SchuS4 specific fashion were NK cells (Figure 5). It has been shown that NK cells contribute to protection against infections

with LVS, but their role in SchuS4 infections has not been explored [41]. Our data suggest that these cell types may serve a crucial role in controlling SchuS4 replication in the pulmonary compartment. This hypothesis is further supported by examining IFN- γ production by NK cells in mice challenged 30 versus 90 days after vaccination. As discussed above, mice challenged 30 days after vaccination with LVS controlled replication of SchuS4 in the lungs, whereas animals challenged 90 days after vaccination did not (Figure 2). In light of our data demonstrating presence of SchuS4 specific NK cells in lungs of mice challenged 30 days after vaccination, it is tempting to speculate that these cells play an important role in rapid control of SchuS4 infection in the lung. Additional studies designed to determine the specific role of these cells following intranasal infection with SchuS4 are currently underway in our laboratory.

The second critical observation was that, in contrast to NK cells acting as the source of IFN- γ in the lung among mice challenged 30 days after vaccination, the source of splenic IFN- γ was predominately CD4⁺ and CD8⁺ T cells. This suggests divergent mechanisms at work in the lung and spleen for production of *F. tularensis* specific IFN- γ following subcutaneous vaccination with LVS.

Perhaps the most surprising finding from our study was that survival of pulmonary SchuS4 infection in mice challenged 90 days after vaccination was not associated with control of bacterial replication in the lung during the first 7 days of infection. In contrast, survival of these vaccinated mice was directly correlated with control of SchuS4 replication in the periphery, i.e. the spleen within the first 4 days of infection. Unlike many pulmonary pathogens, such as *Pseudomonas aeruginosa* and Influenza virus, *F. tularensis* readily disseminates from the lung to colonize other organs. It is widely accepted that mortality in Tularemia is a direct result of both destruction of peripheral organs and septicemia, rather than exclusive consolidation of the lung [6,42,43]. This is evident in earlier studies in humans where therapeutics that were effective in patients with severe, but somewhat contained Tularemia, routinely failed in septic patients [21]. While early limitation (during the first 7 days of infection) of bacterial replication in the lung undoubtedly contributes to superior control of SchuS4 replication and dissemination observed 30 days after vaccination, our data clearly demonstrates that it is not required to survive Tularemia. Rather, strong cellular responses in the periphery are correlated with survival of SchuS4 infection. Obviously, bacterial replication must be limited in the lung at some point. However, in the setting of immunity in the periphery, the host has the luxury of time to recruit and/or activate cells that can limit SchuS4 replication in the lung at a later point in the infection.

By extension our data suggests that novel diagnostics developed to assess vaccine efficacy should target presence of peripheral cellular immunity. During the early years of Tularemia research a skin test was developed that measured delayed type hypersensitivity (DTH) responses among infected humans [44]. Later, this skin test was assessed for its ability to predict vaccine efficacy in experimental animals and humans [45,46]. In those studies, laboratory animals and humans were evaluated within a few weeks of vaccination. However, those data suggested that the skin test showed promise as a good predictor for the presence of effective anti-Francisella immunity. DTH responses rely, at least in part, on CD4⁺ and CD8⁺ T cells [47–49]. Therefore, in consideration of the data presented in our report, a skin test in vaccinated individuals may serve to aid in determination of the presence of protective immunity better than the presence of agglutinating antibodies.

Virulent *F. tularensis* is a bacterium with surprising ability to both evade and suppress host immune responses [14,18]. Thus, generation of effective, long lasting, immunity directed against these organisms has many unique challenges not observed in disease caused by other pathogens. Identification of the correlates and requirements of immunity associate with

survival of infections with these highly virulent bacteria will aid in the development of novel vaccines and therapeutics. Evidence provided in this study suggests assessment of cellular responses in the periphery, rather than humoral responses or cellular response in the lung, is the best indicator of effective protection against pneumonic Tularemia.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the Intramural Research Program of the National Institutes of Health, National Institute of Allergy and Infectious Diseases. The authors would like to thank Dr. Rong Wang, Ms. Jennifer Chase, and Ms. Robin Ireland for their excellent technical help in the execution of experiments described herein. We also thank Dr. Harlan Caldwell for his comments and suggestions pertaining to this manuscript.

6.0 References

1. Bosseray N. Vaccine and serum-mediated protection against brucella infection of mouse placenta. *Br J Exp Pathol.* 1983 Dec; 64(6):617–25. [PubMed: 6419766]
2. 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 Oct. 87:415–25. [PubMed: 13889609]
3. Orme IM, Collins FM. Protection against *Mycobacterium tuberculosis* infection by adoptive immunotherapy. Requirement for T cell-deficient recipients. *J Exp Med.* 1983 Jul 1; 158(1):74–83. [PubMed: 6602861]
4. Nigrovic LE, Wingerter SL. Tularemia. *Infect Dis Clin North Am.* 2008 Sep; 22(3):489–504. ix. [PubMed: 18755386]
5. McCoy GW, Chapin CW. *Bacterium tularense* the cause of a plague-like disease of rodents. *Journal of Infectious Diseases.* 1912; 10(1):17–23.
6. Jellison, WL. Tularemia in North America, 1930–1974. Missoula: University of Montana, University of Montana Foundation; 1974.
7. Dennis DT, Inglesby TV, Henderson DA, Bartlett JG, Ascher MS, Eitzen E, et al. Tularemia as a biological weapon: medical and public health management. *JAMA.* 2001 Jun 6; 285(21):2763–73. [PubMed: 11386933]
8. Wherry WB, Lamb BH. Infection of man with *Bacterium tularense*. *Journal of Infectious Diseases.* 1914; 15:331–40.
9. McCrumb FR. Aerosol Infection of Man with *Pasteurella Tularensis*. *Bacteriol Rev.* 1961 Sep; 25(3):262–7. [PubMed: 16350172]
10. Chen W, Shen H, Webb A, KuoLee R, Conlan JW. Tularemia in BALB/c and C57BL/6 mice vaccinated with *Francisella tularensis* LVS and challenged intradermally, or by aerosol with virulent isolates of the pathogen: protection varies depending on pathogen virulence, route of exposure, and host genetic background. *Vaccine.* 2003 Sep 8; 21(25–26):3690–700. [PubMed: 12922099]
11. Eigelsbach HT, Hunter DH, Janssen WA, Dangerfield HG, Rabinowitz SG. Murine model for study of cell-mediated immunity: protection against death from fully virulent *Francisella tularensis* infection. *Infect Immun.* 1975 Nov; 12(5):999–1005. [PubMed: 1193736]
12. Kirimanjeswara GS, Olmos S, Bakshi CS, Metzger DW. Humoral and cell-mediated immunity to the intracellular pathogen *Francisella tularensis*. *Immunol Rev.* 2008 Oct; 225(1):244–55. [PubMed: 18837786]
13. 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 interferon gamma-dependent mechanism. *Vaccine.* 2005 Mar 31; 23(19):2477–85. [PubMed: 15752834]

14. Bosio CM, Bielefeldt-Ohmann H, Belisle JT. Active suppression of the pulmonary immune response by *Francisella tularensis* Schu4. *J Immunol.* 2007 Apr 1; 178(7):4538–47. [PubMed: 17372012]
15. Bosio CM, Elkins KL. Susceptibility to secondary *Francisella tularensis* live vaccine strain infection in B-cell-deficient mice is associated with neutrophilia but not with defects in specific T-cell-mediated immunity. *Infect Immun.* 2001 Jan; 69(1):194–203. [PubMed: 11119506]
16. 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 Aug; 64(8):3288–93. [PubMed: 8757866]
17. Chase JC, Bosio CM. The presence of CD14 overcomes evasion of innate immune responses by virulent *Francisella tularensis* in human dendritic cells in vitro and pulmonary cells in vivo. *Infect Immun.* 2010 Jan; 78(1):154–67. [PubMed: 19841074]
18. Chase JC, Celli J, Bosio CM. Direct and indirect impairment of human dendritic cell function by virulent *Francisella tularensis* Schu S4. *Infect Immun.* 2009 Jan; 77(1):180–95. [PubMed: 18981246]
19. 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 Sep 26; 26(41):5276–88. [PubMed: 18692537]
20. Qin A, Scott DW, Thompson JA, Mann BJ. Identification of an essential *Francisella tularensis* subsp. *tularensis* virulence factor. *Infect Immun.* 2009 Jan; 77(1):152–61. [PubMed: 18981253]
21. Foshay L. Tularemia: A summary of certain aspects of the disease including methods for early diagnosis and the results of serum treatment in 600 patients. *Medicine.* 1940; 19:1–83.
22. Burke DS. Immunization against tularemia: analysis of the effectiveness of live *Francisella tularensis* vaccine in prevention of laboratory-acquired tularemia. *J Infect Dis.* 1977 Jan; 135(1):55–60. [PubMed: 833449]
23. Koskela P, Herva E. Cell-mediated and humoral immunity induced by a live *Francisella tularensis* vaccine. *Infect Immun.* 1982 Jun; 36(3):983–9. [PubMed: 7095859]
24. Yee D, Rhinehart-Jones TR, Elkins KL. Loss of either CD4+ or CD8+ T cells does not affect the magnitude of protective immunity to an intracellular pathogen, *Francisella tularensis* strain LVS. *J Immunol.* 1996 Dec 1; 157(11):5042–8. [PubMed: 8943413]
25. Saslaw S, Eigelsbach HT, Prior JA, Wilson HE, Carhart S. Tularemia vaccine study. II. Respiratory challenge. *Arch Intern Med.* 1961 May; 107:702–14. [PubMed: 13746667]
26. Eigelsbach HT, Tulis JJ, Overholt EL, Griffith WR. Aerogenic immunization of the monkey and guinea pig with live tularemia vaccine. *Proc Soc Exp Biol Med.* 1961 Dec; 108:732–4. [PubMed: 13889610]
27. 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 Jul 1; 179(1):532–9. [PubMed: 17579074]
28. Anthony LS, Kongshavn PA. H-2 restriction in acquired cell-mediated immunity to infection with *Francisella tularensis* LVS. *Infect Immun.* 1988 Feb; 56(2):452–6. [PubMed: 3257461]
29. Conlan JW, Sjostedt A, North RJ. CD4+ and CD8+ T-cell-dependent and -independent host defense mechanisms can operate to control and resolve primary and secondary *Francisella tularensis* LVS infection in mice. *Infect Immun.* 1994 Dec; 62(12):5603–7. [PubMed: 7960142]
30. 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 Aug 4; 198(3):379–89. [PubMed: 12885873]
31. Cowley SC, Sedgwick JD, Elkins KL. Differential requirements by CD4+ and CD8+ T cells for soluble and membrane TNF in control of *Francisella tularensis* live vaccine strain intramacrophage growth. *J Immunol.* 2007 Dec 1; 179(11):7709–19. [PubMed: 18025217]
32. 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 Apr; 73(4):2306–11. [PubMed: 15784575]

33. Elkins KL, Colombini SM, Meierovics AI, Chu MC, Chou AY, Cowley SC. Survival of secondary lethal systemic *Francisella* LVS challenge depends largely on interferon gamma. *Microbes Infect.* 2010 Jan; 12(1):28–36. [PubMed: 19781659]
34. Elkins KL, Cooper A, Colombini SM, Cowley SC, Kieffer TL. In vivo clearance of an intracellular bacterium, *Francisella tularensis* LVS, is dependent on the p40 subunit of interleukin-12 (IL-12) but not on IL-12 p70. *Infect Immun.* 2002 Apr; 70(4):1936–48. [PubMed: 11895957]
35. Ray HJ, Cong Y, Murthy AK, Selby DM, Klose KE, Barker JR, et al. Oral live vaccine strain-induced protective immunity against pulmonary *Francisella tularensis* challenge is mediated by CD4⁺ T cells and antibodies, including immunoglobulin A. *Clin Vaccine Immunol.* 2009 Apr; 16(4):444–52. [PubMed: 19211773]
36. Sjøstedt A, Sandstrom G, Tarnvik A. Humoral and cell-mediated immunity in mice to a 17-kilodalton lipoprotein of *Francisella tularensis* expressed by *Salmonella typhimurium*. *Infect Immun.* 1992 Jul; 60(7):2855–62. [PubMed: 1612751]
37. Stenmark S, Lindgren H, Tarnvik A, Sjøstedt A. Specific antibodies contribute to the host protection against strains of *Francisella tularensis* subspecies *holarctica*. *Microb Pathog.* 2003 Aug; 35(2):73–80. [PubMed: 12901846]
38. Fortier AH, Slayter MV, Ziemba R, Meltzer MS, Nacy CA. Live vaccine strain of *Francisella tularensis*: infection and immunity in mice. *Infect Immun.* 1991 Sep; 59(9):2922–8. [PubMed: 1879918]
39. 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 May; 73(5):2644–54. [PubMed: 15845466]
40. 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 Jan; 60(1):84–9. [PubMed: 1729199]
41. Lopez MC, Duckett NS, Baron SD, Metzger DW. Early activation of NK cells after lung infection with the intracellular bacterium, *Francisella tularensis* LVS. *Cell Immunol.* 2004 Nov–Dec; 232(1–2):75–85. [PubMed: 15922718]
42. Conlan JW, North RJ. Early pathogenesis of infection in the liver with the facultative intracellular bacteria *Listeria monocytogenes*, *Francisella tularensis*, and *Salmonella typhimurium* involves lysis of infected hepatocytes by leukocytes. *Infect Immun.* 1992 Dec; 60(12):5164–71. [PubMed: 1452350]
43. Forestal CA, Malik M, Catlett SV, Savitt AG, Benach JL, Sellati TJ, et al. *Francisella tularensis* has a significant extracellular phase in infected mice. *J Infect Dis.* 2007 Jul 1; 196(1):134–7. [PubMed: 17538893]
44. Foshay L. Tularemia: Accurate and earlier diagnosis by means of the intradermal reaction. *J Infect Dis.* 1932; 51:286–91.
45. Claflin JL, Larson CL. Infection-immunity in tularemia: specificity of cellular immunity. *Infect Immun.* 1972 Mar; 5(3):311–8. [PubMed: 4564560]
46. Tarnvik A. Nature of protective immunity to *Francisella tularensis*. *Rev Infect Dis.* 1989 May–Jun; 11(3):440–51. [PubMed: 2665002]
47. Huber B, Devinsky O, Gershon RK, Cantor H. Cell-mediated immunity: delayed-type hypersensitivity and cytotoxic responses are mediated by different T-cell subclasses. *J Exp Med.* 1976 Jun 1; 143(6):1534–9. [PubMed: 1083891]
48. Mody CH, Paine R 3rd, Jackson C, Chen GH, Toews GB. CD8 cells play a critical role in delayed type hypersensitivity to intact *Cryptococcus neoformans*. *J Immunol.* 1994 Apr 15; 152(8):3970–9. [PubMed: 8144964]
49. Vadas MA, Miller JF, McKenzie IF, Chism SE, Shen FW, Boyse EA, et al. Ly and Ia antigen phenotypes of T cells involved in delayed-type hypersensitivity and in suppression. *J Exp Med.* 1976 Jul 1; 144(1):10–9. [PubMed: 1084399]

Figure 1.

Protection of vaccinated mice against pulmonary SchuS4 infection. Mice were immunized (n=10/group) subcutaneously once with LVS (200 CFU). Unvaccinated mice (n=5/group) served as negative controls. Animals were intranasally challenged 30 or 90 days after the last vaccination with approximately 25 CFU *F. tularensis* strain SchuS4 and survival of infection was monitored. * = $p < 0.05$. Data is representative of three experiments of similar design.

Figure 2.

Replication and dissemination of SchuS4 in the lungs and spleens of vaccinated animals. Mice (n=5/group) were vaccinated with LVS and challenged as described above. Unvaccinated, SchuS4 challenged mice (n=5/group) served as negative controls. At the indicated time points lungs and spleens were assessed for bacterial loads following serial dilution of organ homogenates on MMH agar plates. * = significantly different from unvaccinated (p<0.05). Error bars represent SEM. Data is representative of two experiments of similar design.

Figure 3.

SchuS4 specific antibodies in vaccinated mice. Mice were vaccinated with LVS as described above. Unvaccinated mice served as negative controls. At the indicated time points, serum was collected from mice (n=3–5/group) via the lateral tail vein. (A) Serum was assessed for IgM, IgG, IgG1 and IgG2a directed against SchuS4 whole cell lysate (WCL) by CBA. (B) Serum was assessed for agglutination of viable SchuS4. Each symbol represents individual mice. Bars represent median titer. Data is representative of three experiments of similar design.

Figure 4.

SchuS4 specific production of cytokines in vaccinated animals. Mice were vaccinated with LVS (n=5/group) as described above. Unvaccinated mice (n=5/group) served as negative controls. Thirty and 90 days after the last vaccination lung and spleen cells from vaccinated and naïve mice were added to cultures of resting peritoneal macrophages that had been exposed to SchuS4 WCL the night before. Lung cells and splenocytes added to macrophages that did not receive SchuS4 WCL (no Ag) served as negative controls. Culture supernatants were collected 72 hours later and assessed for IFN- γ , IL-12p70, IL-5 and IL-10 by ELISA. * = significantly different from unvaccinated animals ($p < 0.05$). Error bars represent SEM. Data is representative of two experiments of similar design.

Figure 5.

IFN-γ producing NK cells in the lungs and spleens of vaccinated mice after SchuS4 challenge. Mice (n=5/group) were immunized with LVS as described above. Unvaccinated, uninfected mice (naïve) and unvaccinated, SchuS4 infected mice (unvaccinated) served as negative controls. Mice were infected with approximately 25 CFU SchuS4 30 or 90 days after vaccination. Three days after infection splenocytes were collected and were added, in the presence of brefeldin A (10μg/ml) for 5 hours, to cultures of resting peritoneal macrophages that had been exposed to SchuS4 WCL the night before. Cells were stained for surface expression of CD49b and intracellular IFN-γ and analyzed by flow cytometry. * = significantly unvaccinated controls (p<0.05). Bars represent SEM. Data is the result of two independent experiments pooled together.

Figure 6.

Presence of activated T cells in vaccinated animals after SchuS4 challenge. Mice (n=5/group) were immunized with LVS as described above. Unvaccinated, uninfected mice (n=5) and unvaccinated SchuS4 infected mice (-) (n=5) served as negative controls. Mice were infected with approximately 25 CFU SchuS4 30 and 90 days after the last vaccination. Four days after infection, lungs and spleens were assessed for presence of CD4⁺CD44^{hi} and CD8⁺CD44^{hi} cells by flow cytometry. * = significantly different from controls (p<0.05). Symbols represent individual mice. Data is representative of two experiments of similar design.

Figure 7.

IFN- γ producing T cells in the lungs and spleens of vaccinated mice after SchuS4 challenge. Mice (n=5/group) were immunized with LVS as described above. Unvaccinated, uninfected mice (n=4) and unvaccinated, SchuS4 infected mice (-) (n=5) served as negative controls. Mice were infected with approximately 25 CFU SchuS4 30 and 90 days after the last vaccination. Four days after infection, lung cells and splenocytes were collected and, in the presence of brefeldin A (10 μ g/ml), were added to cultures of resting peritoneal macrophages that had been exposed to SchuS4 WCL the night before. Five hours later, cells were stained for surface expression of CD4 and CD8 and intracellular IFN- γ and analyzed by flow cytometry. * = significantly different from controls (p<0.05). Symbols represent individual mice. Bars represent mean number of cells positive for both the indicated cell surface receptor and IFN- γ . Data is representative of two experiments of similar design.