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Antibody-mediated protection against pulmonary infection with virulent type A Francisella tularensis

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ANTIBODY-MEDIATED PROTECTION AGAINST PULMONARY INFECTION WITH VIRULENT TYPE A *FRANCISELLA TULARENSIS*

BY

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DISSERTATION

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DEDICATION

To my mother, Mara-Koosham Chinnu bai- the ultimate epitome of sacrifice and pristine love. An eternal and unfathomable gratitude for this life and ***LIFE***.

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ANTIBODY-MEDIATED PROTECTION AGAINST PULMONARY INFECTION

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ABSTRACT

Respiratory infections with the virulent type A strains of *Francisella tularensis (Ft)* cause pneumonic tularemia, an illness which results in high case fatalities in the absence of timely antibiotic treatment. Although immunization is an effective countermeasure, vaccines against pulmonary tularemia are currently unavailable. A better understanding of protective immune components is therefore needed to develop licensable vaccines against pulmonary tularemia. While the indispensability of cell-mediated immunity in protection against this gram-negative intracellular bacterium is well-documented, the protective role of antibodies (Abs) is unclear. Hence we examined the role of humoral

immunity by developing a passive immunization model of respiratory tularemia in Fischer 344 (F344) rats. Naïve F344 rats treated prophylactically with immune rat serum (IRS) or purified immune IgG obtained from rats vaccinated subcutaneously with the attenuated live vaccine strain (LVS) were protected against a lethal intratracheal infection with *Ft* type A strain SCHU S4. The resistance conferred by IRS was dependent on both anti-serum volume and SCHU S4 challenge dose, and was abrogated when IRS treatment was delayed beyond 48 h after the infection. Ab-mediated protection correlated with decreased dissemination to liver and spleen where the histopathology was less severe and depended on CD8 T cells. *In vitro* and *in vivo* bacterial burden analyses implicated other protective mechanisms of IRS in addition to enhancing phagocytosis and delaying bacterial growth. Further experimental analyses showed decreased levels of inflammatory cytokines and tissue damage markers in the sera and tissues of IRS-treated rats, which also showed markedly decreased apoptosis in their lungs. At the peak of infection, unlike the lung-associated lymph nodes (LALN) of normal serum treated rats, LALN of IRS-treated rats had lower SCHU S4 burden and higher number of viable dendritic cells, T cells, B cells and IFNγ –secreting cells. These results therefore demonstrate the ability of Abs to limit inflammation and cell death during pulmonary

SCHU S4 infection to generate protective immunity and have important implications for understanding SCHU S4 virulence and development of Ab-based vaccines and therapeutics.

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CHAPTER 1: INTRODUCTION

Mammalian host defenses

Mammals are persistently exposed to microbes and foreign particles due to their cohabitation. The consequences of a viable entry could be dire- disrupting the homeostasis to result in morbidity and death. In order to detect, combat, and overcome the invasion of 'non-self' antigens and survive the deleterious consequences, the mammalian host has evolved immune defenses broadly categorized as innate and adaptive or acquired immune systems(1, 2). Constituents of the innate immunity are at the vanguard of the immune response continuum and are the first to sense the entry of foreign entity in to the host after they break through the physical barriers in place (skin, mucus). The resulting inflammatory response is rapid and acute and is initiated by the engagement of germline-encoded specialized transmembrane proteins called pattern recognition receptors (PRR) expressed constitutively on phagocytes, fibroblasts, endothelial and epithelial cells(3-5). PRRs by virtue of broad specificity recognize unique molecular signatures called pathogen-associated molecular patterns (PAMPs), which are highly conserved structures critical for pathogen survival. In addition, PRRs can sense endogenous danger signals termed damage-associated molecular patterns (DAMPs), released from damaged cells (2, 6, 7). Successful detection of danger at the host's doorstep triggers signaling cascades, which result in the development of inflammatory responses characterized by the prototypical transcriptional upregulation of proinflammatory cytokines like tumor necrosis factor (TNF), interleukin-6 (IL-6), and interleukin-1 (IL-1) (8). These have pleiotropic effects and orchestrate the activation of several downstream pathways that contribute significantly towards overwhelming the pathogen. The early inflammatory responses are vital for controlling pathogen

multiplication and/or dissemination but are inadequate to eliminate the pathogen and clear the infection. Adaptive immune responses may therefore be necessary for pathogen clearance.

Adaptive immunity spearheaded by B and T lymphocytes gains maximal effect 7-10 days after the initial innate immune activation and is activated by B- and T-cell receptors' binding to their cognate antigenic epitopes derived from pathogens in the regional secondary lymphoid organs. The receptors recognize antigenic epitopes with remarkable specificity and are expressed after their germline-encoded gene segments undergo extensive rearrangements during lymphocyte development. Upon stimulation, antigen-specific T and B cells undergo clonal expansion and resolve the infection completely. B lymphocytes exert their control primarily through antibody (Ab) production. Both CD4 and CD8 T cells secrete cytokines that activate several downstream pathways that help control the infection. In addition, CD8 T cells also have the ability to lyse infected cells and limit the infection. Repeat infections with pathogens are hence comparatively limited due to the heightened and brisk response of memory T and B cells. The regulated and combined actions of both innate and adaptive immune arms control and eliminate infections.

Stimulation of specific PRRs has been shown to significantly affect the phenotype, magnitude, and longevity of antigen-specific B cell responses (9, 10). Thus innate immune cells dictate the specific nature of adaptive immune responses and *vice versa* (11, 12). Cytokine release by $CD4^+$ T cells activates macrophages and dendritic cells (DCs) to enhance antigen processing and peptide presentation in the grooves of major histocompatibility (MHC) molecules of antigen presenting cells (APCs). In

addition, B cell activation and differentiation is affected by the T cell cytokine profiles in the microenvironment. B cells also express the necessary costimulatory molecules for T cell activation and also secrete specific Abs which may activate the innate classical pathway of complement. Once activated, the adaptive immune responses amplify and enhance innate immune system functions by activating the various components of the innate immune system (13, 14). Host actions resulting in the development of immunity therefore exist in a web of interdependent pathways layered in complexity, and require well-orchestrated and synergistic actions of different arms of the immune response to clear the infection. The aforementioned basic tenets of the host immune response form the core of design and efficacy of vaccines and adjuvants and are integral to the current thesis.

Vaccines

Vaccines have come to signify major public health interventions of the 20th century and continue to be instrumental in saving millions of lives and dollars by preventing several diseases, which otherwise pose a serious threat of unimaginable proportions to global health. Small pox was successfully wiped out and the number of polio cases has been brought down to near-eradication levels due to vaccines (15, 16). The benefits of vaccine administration is further illustrated by the steep drop in the number of cases of several vaccine-preventable diseases like diphtheria, measles, and diarrheal diseases with increase in the vaccinated population percentage across all the continents (15). In view of vaccines' success and the burden of several emerging and re-emerging infectious diseases to the exchequer and general public health, efforts to

develop vaccines against these pathogens are a priority. Development of most of the currently licensed vaccines however remains the greatest irony, since they are all empirical derivatives, developed with the minimal understanding of vaccine *modus operandi* in the context of human immune system. However, current licensure of vaccines and other biological products for biothreats and emerging infections, which are not ethical or feasible for human testing, requires efficacy testing in animal models under 'the Animal Rule' drafted by the FDA (17). Therefore, understanding the basis for protection elicited by novel vaccine candidates in animal models is vital for developing licensed vaccines that are safe and provide long-lasting efficacy to humans against dangerous pathogens for which vaccines are not yet available. This protection relies mainly on the sustained and adequate responses of the acquired immune system.

Abs can thwart infection by binding to the epitopes on pathogens' surface and blocking their interaction with the host cell surface receptors. Extracellular virulence factors like toxins are also neutralized by Abs. Due to their purported presence and activity only in the extracellular milieu, Abs were hence deemed to be critical for protection only against extracellular microbes and a few intracellular pathogens which predominantly cause virulence due to secretion of toxins which circulate outside the cells. Cellular adaptive immunity, mediated by T cells, was thought to be indispensible against intracellular bacteria and viruses. However, evidence arguing against the rigidity of this immunological division of labor has been increasing (18, 19).

Immune system duality and Abs' role against intracellular pathogens

Pathogens can escape the potency of Ab functions through their inherent obligate intracellular lifestyle, immune exclusion, or by secreting virulence factors. Engulfed pathogens enter either MHC I or II antigen processing pathways and thereby come under the influence of CD8 and CD4 T cells, respectively. Proteins of pathogens sampled by APCs in to the phagosomes are processed in to peptides and presented to CD4 T cells in complex with MHC II molecules. CD8 T cells recognize their cognate peptide-MHC I complexes which are formed from processed antigens of cytosolic bacteria and viruses known to synthesize proteins and replicate in the cytosol. The pathogen load is significantly decreased after the T cells expand clonally and differentiate to amplify their effector functions (20). Thus, intracellular pathogens that are able to evade Ab functions are controlled by T cells. The failure to demonstrate the critical role of Abs and the growth of experimental evidence supporting the indispensability of T cells in protection against intracellular pathogens gave rise to the immunological duality paradigm(19).

The failure of Abs to protect the host against an intracellular pathogen does necessarily not rule out the benefit they provide. For, Ab specificity, dose, isotype, idiotype, inoculum strain and dose, and host characteristics have all been shown to alter the outcome of infection when Abs were tested (21-24). The interdependence of both T and B cells in facilitating a potent immune response has further blurred the lines between humoral and cell-mediated arms of the immune system and the scope of benefit they provide against intra- and extracellular pathogens respectively. In addition, pathogens hitherto thought to be intracellular were shown to be present outside the cells, thereby making them susceptible to Ab actions. For example, specific Abs against *Erhlichia*

chaffensis, an obligate intracellular bacterium, protected both normal and SCID mice. This pathogen was later shown to be present extracellularly (25, 26). However, Ab activity is not restricted to the extracellular environment alone. Intracellular growth of *Listeria monocytogenes* in infected macrophages is significantly controlled by mAbs against listeriolysin O (LLO), a pore-forming toxin necessary for the bacterial escape from the phagosome in to the cytosol (27). The mAbs neutralized LLO intracellularly and restricted the bacterial number independent of FcγRs, IFNγ-signaling, or reactive oxygen species (ROS) and reactive nitrogen species (RNS) production. Intracellular neutralization of viruses by IgA during transcytosis and targeting viruses for proteasomal degradation in the cytosol by IgG has also been shown (28, 29). Hence Abs alone, or in conjunction with CMI, may benefit the host in protection against intracellular pathogens. The protective role of Abs against virulent type A strains of *F. tularensis*, a facultative intracellular pathogen, during a respiratory infection is unclear and is the principal objective of this thesis.

Francisella tularensis: strains, life cycle and virulence

Discovered 100 years ago in the rodents of Tulare County, California, *F. tularensis* is a 0.2 µm x 0.2-0.7 µm-sized coccobacillus, which infects a wide range of hosts across different phyla (30-32). Humans contract tularemia through direct contact with infected animals and vector bites in addition to ingesting and inhaling contaminated material and infectious aerosols, respectively (33-36). After an incubation period of 3-5 days, patients develop flu-like symptoms with swollen lymph nodes and the illness may protract or relapse in the absence of prolonged treatment with aminoglycosides (37, 38).

Infection by the oral, ocular, respiratory routes causes oropharyngeal or gastrointestinal, oculoglandular, and pneumonic or respiratory tularemia respectively. Disease severity is dependent on the route of infection, inoculum dose and strain (31). Irrespective of the route of entry, *Francisella* disseminates to several tissues such as lungs, lymph nodes, spleen, liver, kidneys, and intestines, where it multiplies.

F. tularensis is divided in to three subspecies based on the geographical distribution and virulence in humans (39). *F. tularensis* subsp. *mediasiatica* found in Central Asia is nonpathogenic while *F. tularensis* subsp. *holarctica* (type B or biovar B) causes widespread recoverable mild disease in the European populations and is highly virulent in murine infections. Of the three subspecies, *F. tularensis* subsp. *tularensis* (type A or biovar A) endemic to North America is the most virulent in humans. Epidemiological studies have recently classified these subspecies further in to three clades Ala, A1b, and A2 based on differences in virulence in humans (40, 41). Airway infection with as few as 10 type A strain bacilli may result in a productive infection and could be fatal in one-third of patients in the absence of treatment (42). Investigators routinely employ three different strains in their studies-live vaccine strain (LVS), SCHU S4, and U112. SCHU S4 is a type A strain of subsp. *tularensis* while LVS is an attenuated form of subsp. *holarctica* developed through serial passages and the current vaccine gold standard. U112 is a strain of *F. novicida*, a closely related species of *Francisella*, which shares a high degree of homology to subsp. *holarctica* and *tularensis*. It is avirulent in humans but causes lethal disease in rodents and is also used as a model for *F. tularensis* due to the ease of genetic manipulation and less restrictive

biocontainment requirements. The virulence, target cells, and immunogenicity of the strains differ significantly from each other (Table I).

Table I. Comparison of virulence characteristics of different Francisella strains ((43-54).

Strain	Humans	Mice (C57BL/6J)	Rats (F344)	Target cells[a]	Phenotypic activation of APC	Cell Death
Type A1a/b *Ft*	Highly virulent, can be lethal	LD_{50}<10 by all routes[b]	LD_{99}<100 IT, $>10^5$ SC	AMs/DCs> Neutrophils	Inhibited in mice during respiratory infection	Apoptosis
Type A2 *Ft*	Causes morbidity but rarely lethal	LD_{50}<10 by all routes	ND	ND	ND	ND
Type B *Ft*	Causes morbidity but rarely lethal	LD_{50}<10 by all routes	LD_{50} 10^5 IT, <10 IP	ND	ND	ND
LVS	Vaccine strain, Attenuated type B, Productive infection by airways and skin	LD_{50} 10^3IN, 10^6 ID, <10 IP or IV, Sublethal doses used for vaccination	LD_{50} 10^5 IT, IP $>10^8$, Sublethal doses used for vaccination	AMs/DCs> Neutrophils	Seen in both human and mouse macrophages *in vitro*	Pyroptosis, apoptosis
F. novicida	Infections rare, Cases in immunocompromised individuals	LD_{50} <10 IP, IV, or IN, 10^3ID, Sublethal doses used for vaccinating mice	LD_{50} 5 x 10^6	Neutrophils> AMs/DCs	ND	Pyroptosis, apoptosis

[a] Cells targeted during i.n. challenge
[b] Approximate lethal dose values expressed as CFU
ND, Not determined; APC, antigen presenting cells; AMs, alveolar macrophages; DCs, dendritic cells; IT, intra-tracheal; IP, intra-peritoneal; ID, intradermal; IV, intravenous; SC, sub-cutanaeous.

F. tularensis infects a wide range of mammalian cells which include mononuclear phagocytes, neutrophils, hepatocytes, erythrocytes, B cells, and fibroblasts, epithelial and endothelial cells (52, 55-61). Neutrophil and DC uptake of *Francisella* is opsonin-dependent while macrophages also phagocytose the pathogen through opsonin-independent mechanisms by 'looping phagocytosis' (Figure 1) (62-64). Opsonization with freshly isolated naïve serum facilitates increased bacterial engulfment predominantly by complement receptor CR3 (CD11b/CD18) (62, 65). Serum opsonization also results in *Francisella* uptake by the scavenger receptor class A (SRA), FcγRs, nucleolin, and lung surfactant protein A (SPA) (62, 66, 67). Non-opsonized *Francisella* is phagocytosed through the mannose receptor (68). The ingested bacteria reside for a short time period in the phagosome and interact with the early and late endocytic compartments (65, 69-71). The phagosome does not acquire Cathepsin D and other lysosomal tracers and hence, fails to mature in to a phagolysosome (65, 72, 73). Subsequent acidification of the phagosome has been shown to activate the virulence genes necessary for the phagosome membrane disruption and escape in to the cytosol (70, 74). Phagosome disruption and cytosolic entry from the vesicular components is strategically critical for *Francisella* survival since infection of macrophages with phagosome escape-deficient mutants results in decreased bacterial numbers (71, 72, 75). Once in the cytosol, *Francisella* proliferates during the next 24h and is released from the cells through caspase-dependent lysis (52, 76-78). Thus phagosome escape followed by cytosolic replication and induction of host-cell apoptosis may explain part of the pathogenesis caused by all *Francisella* strains.

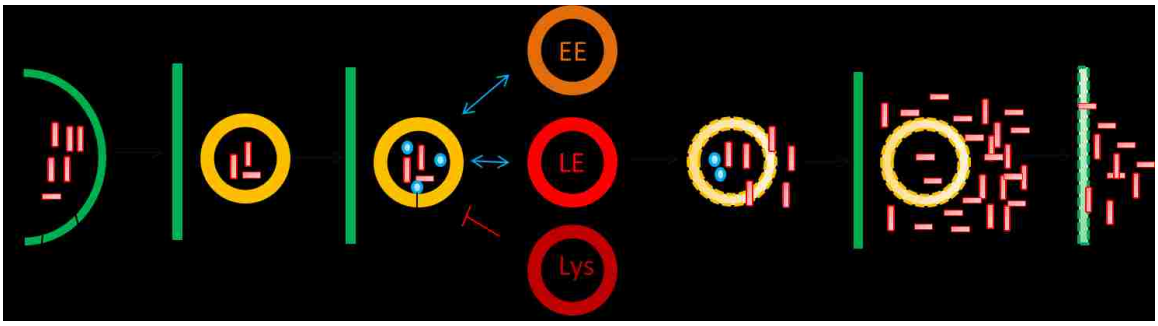

Figure 1. *Intracellular lifestyle of Francisella sp. in the macrophages*. (1) *Francisella* enters macrophages by 'looping phagocytosis' using different receptors. (2) The engulfed bacteria reside transiently in *Francisella*-containing phagosome (FCP). (3) The FCP interacts with early endosome (EE) and late endosomes (LE) but the bacteria inhibit fusion with the lysosomes (Lys). Phagosome acidification triggers protein synthesis necessary for phagosomal disruption and escape in to the cytosol. (4) Post-phagosome disruption, the bacteria escape in to the host cell cytosol. (5)Replication in the cytosol. (6) Host cell death by apoptosis or pyroptosis releases the bacteria which go on to infect other cells (79-81).

The genomic sequences of LVS, SCHU S4, and U112 are >97% identical (82). Nonetheless, only type A and B strains are pathogenic to humans and only infections with subsp. *tularensis* result in severe morbidity and fatalities. The difference in outcomes may be due to varied virulence determinants between the three strains. All three strains encode the *Francisella* pathogenicity island (FPI), a ~30kb locus encoding 17 genes required for phagosome escape and intracellular multiplication (83-86). The pathogenic strains encode two copies of FPI while *F. novicida* has only one copy (87). However, it has not been determined whether the FPI copy number has any correlation with the virulence. During an intranasal (i.n) infection, *F. novicida* targets the lung neutrophils and AMs before multiplying in the neutrophils while the pathogenic strains preferentially infect the AMs and later proliferate in AMs, DCs, and PMNs (88). The LPS of *F. novicida* differs from that of the pathogenic strains in having two novel sugars in the repeating carbohydrate tetramer of O antigen (89, 90).

The virulence of type A strains stems from their ability to survive and proliferate to significant levels undetected during the first 48h in the host cells (91-94). Facilitating its virulence is the tetra-acylated nature of type A strain's LPS, which is distinct from the hexa-acylated LPS of *E. coli* and *Salmonella* spps (95, 96) and does not induce an innate immune response. The lack of two acyl groups makes it a poor stimulatory agent of TLR4 unlike *E. coli* LPS, a potent TLR4 agonist. The preferential tropism of SCHU S4 to AMs and DCs during pulmonary infection may also render it undetectable by the host cells due to their lack of CD14 leading to increased virulence (97). This co-receptor enhances binding of microbial ligands to TLR2 and was shown to be associated with increased production of TNF-α and IL-6 and bacterial control when administered

exogenously. Human myeloid dendritic cells (mDCs) directly infected with SCHU S4 as well as bystander cells are impaired in their ability to respond to the pathogen as well as secondary stimuli (98). In addition, DCs of mice infected with SCHU S4 aerosols are not activated and show decreased expression of co-stimulatory molecules, MHC II, and inflammatory molecules (55). On the contrary, the infected animals produced the anti-inflammatory tumor growth factor-β (TGFβ) and did not respond to LPS administered i.n. Early studies in aerogenically infected tularemic patients also reported similar unresponsiveness to *S. typhosa* and *E. coli* endotoxins (99). Together these mechanisms act to increase the 'stealth' nature type A *Ft* strains.

Other inherent mechanisms contribute towards SCHU S4 virulence. The virulent strains utilize the CR3 under opsonizing conditions and mannose receptors (MRs) in the absence of opsonins to invade the macrophages (68). This provides a strategic advantage to the pathogen as the entry through these receptors is devoid of respiratory burst. On the other hand, FcγR-mediated uptake triggers the bacteria-damaging ROS production, but the virulent strains have also developed a mechanism to combat this host defense. SCHU S4, but not LVS, binds to the host serine protease plasmin to degrade the binding Ab and thereby prevents opsonization, phagocytosis, and the associated TNF-α and IL-6 production (100). Biovar A strains can also weather the bactericidal effects of ROS through efficient regulation of iron metabolism. Their relatively low iron content and stringent regulation of iron uptake and storage makes them more resistant to H_2O_2 by preventing the formation of bactericidal hydroxyl radicals and anions (101). In addition to catalase, the virulent strains also have other unidentified mechanisms to neutralize the host ROS (102). An array of factors, host-derived and inherent to the pathogen, hence

contributes towards the virulence of type A strains. Therefore, host immune components that protect even in the presence of pathogen's virulent factors are crucial for effective treatments.

Innate immunity against respiratory *F. tularensis* infections

The improved understanding of *Francisella* lifestyle and virulence is the result of numerous *in vitro* studies with mononuclear phagocytes since these cells are converted to safe niches for their growth through several previously described subversive mechanisms. Both pathogenic strains multiply exponentially in macrophages and dendritic cells isolated from peripheral blood, lungs, peritoneal cavity, bone marrow of several species (45, 97, 98, 101-105). *In vitro* model systems that examine intra-phagocyte *Francisella* growth have been developed and are routinely used to identify protective immune correlates and effector molecules induced by vaccines and therapeutics (69, 106, 107). The intracellular bacterial proliferation has been shown to ultimately result in macrophage apoptosis during a pulmonary SCHU S4 infection (52). This has led researchers to postulate that the death of these innate immune cells prevents adequate development of protective immunity (51, 52). On the other hand, elimination of these cells could be eliminating *Ft* growth foci, since *Francisella* preferentially infects and multiplies in the phagocytes. This immune cell destruction may therefore benefit the host. However, the absence of macrophage-deficient models makes their protective role currently unclear. Similarly, although altered DC function during murine pulmonary infection has been suggested, their role in enabling host survival has not been directly

examined (55, 94). Type A strains also target other innate immune cells and their importance in protection is beginning to be understood.

Neutrophil response to infection is rapid and is a result of neutrophil extracellular traps (NETs), bactericidal granules, and the highly reactive superoxide radicals. During a murine pulmonary infection with SCHU S4, a dose effect in neutrophil recruitment to the lungs is observed (88, 108). Neutrophil influx and bacterial uptake by itself did not provide any benefit to the host, since neither neutrophil depletion nor early recruitment decreased the bacterial burden or improved the survival, respectively (108, 109). These innate immune cells are in turn converted to safe havens by the virulent strains which inhibit NADPH oxidase assembly in the phagosome and escape in to the cytosol (110). However, knockout studies revealed a limited role for this enzyme suggesting other crucial mechanisms at play (108).

Mouse pneumonic infections with LVS suggest the important function of IFNγ production by NK cells during the first week of infection (111-113). Their effect is pronounced in liver where they were shown to be indispensible for pyrogranuloma formation and thereby restricting hepatic bacterial spread (111). But the benefit of elevated IFNγ secretion is not translated in to an improved outcome against SCHU S4, since NK cell-depletion did not alter the resistance significantly (104). SCHU S4 infection of murine hepatic NK cells also increased IFNγ production when supplemented with recombinant IL-12 and IL-18 *in vitro* (114). However, their protective *in vivo* function(s) during respiratory infection with SCHU S4 and cell lysing function have not been studied yet.

Alveolar epithelial type I (ATI) and type II (ATII) cells line the pulmonary mucosal cavity, making them highly susceptible for interaction with *Francisella* during a pulmonary infection. In support of this hypothesis, SCHU S4 stimulated human primary ATII cells secreted chemokines through NF-κB activation to induce transmigration of neutrophils and DCs across primary human pulmonary microvasculature endothelial cells (58). Although the protective role of mast cells through IL-4 secretion has been reported during pulmonary LVS infection, their role with regard to SCHU S4 is yet to be studied (107, 115, 116).

T cell adaptive immunity against respiratory *F. tularensis* infections

T cells are central to vaccine-induced protection by inducing anamnestic responses upon infection with several intracellular pathogens. A vaccinated state that protects against subsequent infections is induced in humans naturally infected with *Francisella* despite developing illness (117). Safe vaccines that generate sustained protective immune responses are necessary to limit casualties from intentional aerosol release of type A strains. Several vaccination protocols in murine models consistently implicate the critical role of T cells against pulmonary tularemia caused by type A biovars (118-121). Of these, the respiratory route of vaccination with LVS and SCHU S4-mutants provided reliable protection against lethal pulmonary infection, which was mediated by CD4, CD8 and NK T cells (118, 121). Data from the *in vitro* model systems developed by Elkins & co-workers conclude that the IFNγ production by primed $CD4^+$ T cells is paramount for activating macrophages and controlling intracellular *Francisella*

growth (43, 122, 123). On the other hand, CD8 T cells produce the same aforementioned effect through TNFα (124). In the absence of both T cell subsets, primary i.n. LVS infection results in a long-term chronic infection due to their inability to clear the bacteria (125). Compared to parenteral routes, vaccinating the airways with LVS induced the higher multiplication and accumulation of DN T cells in the lungs and correlated with improved outcome (125). These MHC-I restricted DN T cells aid *in vivo* by producing IL-17 (122, 125). Several groups have reported significant increase in IL-17 during airway inoculation of mice (91, 125-128). This pro-inflammatory cytokine, produced significantly higher by DN T cells than the other two subsets, has been shown to exert its influence by facilitating rapid and increased development of Th1 immunity and decreasing intracellular bacterial growth in macrophage through synergistic action with IFNγ (125, 127). IL-17 mediated control in bacterial growth in the lungs was also observed during a secondary pulmonary challenge with SCHU S4 (91).

The relative robustness of pulmonary vaccination compared to other routes of vaccination with LVS seen in mice has also been demonstrated in humans (129). But humans are currently vaccinated in the US under Special Immunization Protocol by intradermal scarification with LVS. These vaccinees develop T cell-mediated immune responses (130-132). Although the direct roles of the T cell subsets during severe pulmonary tularemia have not been tested in humans, and perhaps never will be, *in vitro* assays with PBMCs from naturally infected patients and LVS vaccinees produce significantly high levels of IFNγ, TNFα, IL-17, and IL-2 (133-139). Results from *in vitro* intracellular SCHU S4 growth assay from our lab further attest to the protective role of T cells and IFNγ in LVS-vaccinated humans (Hahn, 2011, manuscript in preparation).

Depletion of CD4 and CD8 T cells from the PBMCs of LVS vaccinees resulted in the loss of intracellular SCHU S4 growth restriction. LVS-specific T cell responses are rapid and effector CD4 and CD8 memory T cell populations were found in the vaccinated cohort even after scores of years (132, 139-141).

Pulmonary tularemia by type A strains and humoral immunity

Adaptive B cell responses do not correlate with T cell immunity development in vaccinated humans. These responses, measured through the estimation of LVS-specific serum IgM, IgG, and IgA titers using microagglutination assay and ELISA are detected ~2 weeks after vaccination (130, 132). The titers were shown to peak within 2 months and sustain for more than a decade before waning (130, 132, 133, 142). But their significant presence does not parallel the protective outcome typically associated with T cell responses. The killed Foshay vaccine used in the pre-antibiotic era which stimulated a strong *Francisella*-specific Ab response in the vaccines had relatively higher incidence of tularemia in vaccinated lab workers than those vaccinated with LVS (143). Other studies also found dissociation between serum Ab titers and protective capabilities when killed or inactive vaccines were used (42, 144-147). The protective relevance of Abs against virulent strains was thus questioned by these studies.

Ab protection studies in mice have yielded similar results with SCHU S4 pulmonary infections. The common finding of these studies was that despite their presence Abs are protective only against pulmonary infection with the less virulent LVS or type B but not biovar A strains (109, 148-152). As a result, Abs' protective role against virulent respiratory tularemia was considered clinically unimportant until two

recent studies brought it back to the fore. The oral vaccination study with *F. novicida* and the investigation involving passive transfer of immune serum from mice cured of SCHU S4-induced pulmonary tularemia through laevofloxacin treatment suggested Ab-mediated protection (44, 153). However, these studies did not explore Ab protective mechanisms against SCHU S4. Hence Abs' role against virulent strains is still unclear.

Fischer 344 rat model of pulmonary tularemia

In mice, the limited or no protection mediated by Abs against respiratory infections with type A strains does not rule out their benefit completely. This is due to certain inadequacies of the mouse models. All the inbred strains of mice to date display an extreme inherent susceptibility to even less virulent strains of *Francisella*-a trait not seen in immunocompetent humans. While the mouse model is an essential tool to dissect immunopathological mechanisms of tularemia, it may not be extrapolated to humans. This is more so with regard to virulent respiratory tularemia. The F344 rat model of pulmonary tularemia developed in our lab adequately complements the mouse models (48). Like humans, the F344 rats are highly resistant to LVS vaccination by several routes and show poor survival against i.t. challenged SCHU S4 unless vaccinated with LVS, which was shown to increase resistance significantly. The factors contributing towards protection are not yet determined. The extracellular phase of SCHU S4 reported recently brings up the possibility of Abs being able to access the pathogen and thereby influence the infection outcome (59, 154).

CHAPTER 2: HYPOTHESIS AND AIMS

Hypothesis and aims

Type A strains of *F. tularensis* can be easily aerosolized, and due to high infectivity and extreme virulence by the respiratory route, pose a serious threat to public health in the event of intentional bioterrorist release (155). Although antibiotics are currently used to treat infected individuals, their usage is deterred by reports of engineered antibiotic-resistant strains, development of bacterial resistance, occasional relapse, and financial constraints (37). Vaccines, on the other hand, have limited drawbacks and can offer long-term protection. LVS is currently used to vaccinate at-risk individuals as an investigational drug but is unlikely to be licensed by the FDA due to safety concerns. Characterization of protective immune components is therefore needed to develop new licensable vaccines against pulmonary tularemia. The protective mechanism of Abs from murine models is still unclear. The overall objective my research project was to determine the role of Abs in protection against pulmonary tularemia caused by type A strains using the F344 rat model. I hypothesized that ***Francisella-specific Abs protect F344 rats against a lethal respiratory infection with F. tularensis type A strain***.

AIM 1: Determine if LVS-specific Abs can protect naïve F344 rats against a lethal i.t. infection with SCHU S4. A protocol for LVS-immunization, immune serum transfer, and SCHU S4 challenge was developed to explore the protection conferred by immune Abs induced by LVS vaccination in F344 rats.

AIM 2: Evaluate the role of different T lymphocyte subsets in influencing the survival in passively immunized rats. Passive immunization experiments with LVS

suggest the critical role of T cells in the protection mediated by Abs (150, 151). This is perhaps due to the interdependence of T and B cells for optimal effector functions. Therefore, we examined the role of T cells in the context of our passive immunization model.

AIM 3: Examine the potential mechanism(s) of LVS-specific Abs'-mediated protection against a lethal i.t. SCHU S4 challenge. The dynamics of interactions between LVS-specific Abs and SCHU S4 in the context of F344 rat as the host and the resulting outcomes were explored. Ab-mediated SCHU S4 uptake and the resulting consequences were studied both *in vitro* and *in vivo*. We also tested if prophylactic Ab administration was immunomodulatory in ultimately limiting apoptosis and inflammation to enable adaptive immunity development.

CHAPTER 3: MATERIALS AND METHODS

Rats

Female F344 rats and athymic rnu/rnu rats were purchased from The National Cancer Institute at Frederick (Frederick, MD). The animals were housed in a specific-pathogen free facility at the University of New Mexico Animal Resource Facility. All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee and the Biosafety Committee at the University of New Mexico.

Bacteria

F. tularensis strains LVS and SCHU S4 were obtained from DynPort Vaccine Company LLC (Frederick, MD). The original stock was expanded in Chamberlain's broth (Teknova; Hollister, CA) at 37^{o}C for 48 h with gentle shaking and aliquots of the culture were stored at -80^{o}C without any preservative.

Vaccination and serum collection

Rats were lightly anesthetized with isoflurane (Abbott Laboratories; Chicago, IL) and vaccinated s.c. between the shoulder blades with 5 x 107 CFU LVS in 100 µl of PBS. 4 wks after vaccination, rats were euthanized by CO2 overexposure and immune serum was collected and pooled. Normal serum was collected in a similar manner or purchased from Charles River Laboratories (Wilmington, MA). Both normal and immune sera were heated to 55° C for 30 min to inactivate complement, filter-sterilized through 0.22 µm syringe tip-filter, (Millipore; Billerica, MA) and stored at -80° C.

Purification of serum IgG and IgM

Serum IgG and IgM were purified using Melon Gel purification kit (Thermo Scientific Pierce Protein Research; Rockford, IL) and Capture Select IgM affinity matrix (BAC B.V.; Naarden, Netherlands) respectively, following the manufacturer's instructions with a few modifications. Briefly, normal and immune sera were diluted 10-fold in PBS and then precipitated slowly with 50% ammonium sulfate overnight at 4^{o}C. The precipitate was separated from the supernatant by centrifugation at 3,000*g* for 20 min and was resuspended in Melon Gel Purification Buffer for IgG purification or in PBS for IgM purification. The suspensions were subsequently dialyzed 3 times against 300 volumes of Melon Gel Purification Buffer for IgG or PBS for IgM using Slide-A-Lyzer dialysis cassette with 10kDa molecular weight cutoff (Thermo Scientific Pierce Protein Research; Rockford, IL). IgG was purified from the dialyzed samples following the manufacturer's microcentrifuge spin-column protocol using a Melon gel volume that is 1.25 times the undiluted serum volume. IgM was purified using IgM affinity matrix resin that is 0.5 times the undiluted serum volume. Before analyzing the purity and subsequent treatment of rats, purified IgG and IgM were dialyzed against PBS as described above. The concentration of total IgG and IgM was interpolated from a standard curve generated with commercially available IgG and IgM of known concentration (Sigma-Aldrich; St. Louis, MO) and the titer of anti-LVS IgG and IgM as well as contaminating Ab isotypes was determined using LVS-specific ELISA as described below. The purity was analyzed by 10% SDS polyacrylamide gel electrophoresis (Biorad; Hercules, CA).

ELISA for anti-LVS Ab titer

Maxisorp 96-well microtiter plates (Nunc; Rochester, NY) were coated with 2.5-5 x 106 CFU/ml of heat-killed LVS in PBS overnight at 4°C. After blocking with 5% non-fat dry milk/PBS for 1 h at 37°C, 100 µl of rat serum were added in 2-fold serial dilutions and incubated at 37°C for 1 h. Abs class and subclasses were detected by incubation with horseradish peroxidase-conjugated goat Abs against rat IgG (Calbiochem; San Diego, CA), IgG1, IgG2a, IgG2b, IgG2c, IgM and IgA (Thermo Scientific Pierce Protein Research Products; Rockford, IL) for 45 min at 37oC. Between each step, the plates were washed 5 times with 0.05% Tween 20/PBS. The plates were developed with a solution of 3, 3', 5, 5'-tetramethylbenzidine (TMB) and the reaction was stopped with 1.8N H2SO4 and the optical density (OD) was read at 450nm. Ab titer was defined as the reciprocal of the highest dilution of immune serum that had a mean OD value that is at least 3 standard deviations higher than the mean OD of normal serum at the same dilution.

Passive immunization and intratracheal challenge

Rats were passively immunized by i.p. injection of 250 µl of serum, unless otherwise indicated in selected experiments. Purified IgG and IgM was used in some experiments; the volume given contained the equivalent amount of LVS-specific IgG as in 250 µl of LVS-immune serum. 24 h after passive immunization, rats were infected i.t. as described previously (48)(73). Briefly, rats were immobilized on an inclined platform (Alpha Lab Supply; Albuquerque, NM) and intubated with a 20-gauge i.v. catheter

(Terumo Medical Products; Somerset, NJ) with the help of a small animal laryngoscope with a fiber optic light source (Penn-Century, Inc; Philadelphia, PA). In some experiments 50μl inoculum pre-mixed with self-illuminating quantum dots (Zymera; San Jose, CA) was delivered using a blunt-ended needle and followed by 50μl of Coelenterazine and a burst of >500μl of air to ensure the delivery of the inoculum. The infected rats were imaged *in vivo* using the IVIS 100 Optical Imaging System (Caliper Life Sciences; Hopkinton, MA). The health of the infected animals was monitored daily along with weight loss and survival. The clinical scores of infection were assigned as follows: 0 = active, bright and alert, responsive to handling; 1 = slight lethargy and weight loss; 2 = decreased responsiveness to handling, clear piloerection, more pronounced weight loss; 3 = definite decreased activity, ruffled coat, rapid and shallow breathing, hunched posture, eyes half closed and may have porphyrin secretion; and 4 = inactive and unresponsive to handling, weak and/or ataxic, severe weight loss, eyes completely closed with a large amount of porphyrin secretion. Animals that succumbed to infection were given a maximum score of 4.

Bacterial burden analysis

To quantify the deposited bacteria, lungs were aseptically removed 1 h after infection and homogenized in PBS using a hand-held or multi-sample homogenizer fitted with disposable plastic homogenizing probes (Omni International; Marietta, GA). Lung homogenates were plated neat or at appropriate dilutions onto selective cysteine heart agar plates with 5% rabbit blood, 100U/ml penicillin G, and 100U/ml polymyxin B

(Remel; Lexena, KS) and bacterial colonies were quantified 4-5 days later using Qcount (Spiral Biotech; Bethesda, MD). A similar procedure was followed to determine the bacterial burden in lungs, spleen, and liver over the course of infection. When no organism was found, a value equal to the limit of detection was used to calculate the mean bacterial burden.

Histopathological evaluation

Following i.t. challenge, rats were euthanized by i.p. injection of 150µl of Sleepaway (Fort Dodge Animal Health; Fort Dodge, IA) (>100mg/kg) on day 1, 3, 5, 7, 10, 14, and 21 post-challenge. Lungs were removed from the thorax en bloc and inflated with 10% neutral buffered formalin (NBF) via a tracheal cannula. Lungs, spleen, liver, and tracheo-bronchial lymph nodes were fixed in 10% NBF for 24-72 hours and subsequently trimmed for paraffin embedding. Paraffin-embedded tissues were sectioned at 5 µm and stained with hematoxylin and eosin for histological analysis by a board certified veterinary pathologist. Lesions were graded in a blinded manner on a semi-quantitative scale based upon the severity and distribution of lesions (minimal = 1, mild = 2, moderate = 3, and marked = 4).

In vivo T-cell depletion and flow cytometry

The hybridoma clones OX-8 (mouse anti-rat CD8; IgG1), OX-38 (mouse anti-rat CD4; IgG2a), 55-6 (mouse anti-HIV-1 gp120; IgG2a) were obtained from European Collection of Cell Cultures (Salisbury, United Kingdom) and TS2/18.1.1 (mouse anti-

human CD2; IgG1) was from American Type Culture Collection (Manassas, VA). Ascites fluids were generated in female ICR SCID mice and the IgG concentrations were determined by high-performance liquid chromatography (Taconic; Albany, NY). For CD4 T cell depletion, rats were injected i.p. with 5mg/kg of CD4 T cell-depleting Ab OX-38 or isotype control Ab 55-6 for five consecutive days and then 1mg/rat twice a week. For CD8 T cell depletion, 1mg/rat of CD8 T-cell depleting Ab OX-8 or isotype control Ab TS2/18.1.1 Abs were administered once a week. One week after the start of Ab treatment, the depletion efficiency was confirmed by flow cytometric analyses of PBMCs collected by lateral tail vein bleed. In addition, the effect of CD4 T cell depletion on the population of NK cells, B cells, and CD8 T cells in the spleen, liver, and blood was examined. Rats were euthanized by CO2 overexposure and exsanguinated by cutting the inferior vena cava. Blood collected from the chest cavity was mixed with an equal volume of PBS supplemented with 50U/ml of heparin and layered over Lympholyte-M density separation medium (Cedarlane; Burlingtion, NC) following manufacturer's instructions. The lymphocytes were collected at the interface of the density gradient medium. To isolate splenocytes, spleens were homogenized between ground glass slides and passed through 70µm nylon screen (BD Biosciences; San Jose, CA). To isolate liver cells, the right lobes were homogenized through a 200-gauge stainless steel mesh, resuspended in 40% Percoll and layered over a 70% Percoll solution. The samples were centrifuged at 836 x g for 20 min. at 4˚C without break. Cells were harvested from the Percoll interface. All the cell preparations were treated with RBC lysis buffer (0.15M NH4Cl, 1mM KHCO3, 0.1mM Na2 EDTA) and washed with PBS and resuspended in RPMI-10 (RPMI 1640 medium supplemented with 10% heat-

inactivated fetal bovine serum, 1 mM non-essential amino acids, 1 mM L-glutamine, 1 mM sodium pyruvate) before staining. Cells were stained with biotinylated anti-CD8b (mouse IgG1, κ, clone 341) and anti-CD161a (mouse IgG1, κ, clone 10/78), fluorescein isothiocyanate (FITC)-conjugated anti-CD45R (mouse IgG2b, κ, clone HIS24) and allophycocyanin (APC)-conjugated anti-CD3 (mouse IgM, κ, clone iF4) Abs from BD Biosciences (San Jose, CA) and phycoerythrin (PE)-conjugated anti-CD8b (mouse IgG1, κ, clone eBio341) and FITC-conjugated anti-CD4 Abs (mouse IgG2a, clone OX-35) Abs from eBiosciences (San Diego, CA). Biotinylated cells were detected using peridinin-chlorophyll-protein (PerCP)-conjugated streptavidin (Biolegend; San Diego, CA). Before staining the cells, non-specific Ab binding was blocked by incubating with anti-rat CD32 (mouse IgG1, κ, clone D34-485) according to the manufacturer's instructions (BD Biosciences; San Jose, CA). Appropriate isotype controls were used. The stained cells were fixed in 0.5% paraformaldehyde and analyzed by flow cytometry on a FACS calibur (BD Immunocytometry Systems; San Jose, CA). The flow cytometry data was analyzed using Winlist (Verity; Topsham, ME).

Lymph node harvest and staining

Rats were euthanized with CO_2 and lung-associated lymph nodes (LALNs) were excised in to staining buffer (2% FBS, 0.2mM EDTA in PBS) before being homogenized between ground slides. Mononuclear cells were isolated by ficoll density gradient centrifugation (GE Healthcare, Waukisha, WI). Viable cells were enumerated by trypan blue exclusion method and 1 x 10^6 cells per sample were stained in the same manner as

described above. Abs used were APC-conjugated anti-CD3 (mouse IgM, κ, clone iF4), PerCP-conjugated anti-RT1B (mouse IgG1, κ, clone OX-6) from BD Biosciences, APC-conjugated anti-CD45RA from Biolegend (mouse IgG1, κ, clone OX-33) and PE-conjugated anti-CD103 from eBiociences (mouse IgG1, clone OX-62). $CD3^+$ cells were designated as T cells while B cells were $CD3^-$ $CD45RA^+$ $RTIB^+$ and DCs were $CD3^-$ $CD45RA^-$ $RTIB^+$ $CD103^+$.

Intracellular bacterial growth assay

Peripheral blood from naïve F344 rats was collected in to an equal volume of PBS with 50U/ml of heparin. Mononuclear cells from the blood were enriched using Lympholyte-M density separation medium according to the manufacturer's instructions (Cedarlane, Burlington, NC). Cells were enumerated by trypan blue exclusion method and 2.5×10^5 cells were plated per well and incubated overnight at 37° C. SCHU S4 was opsonized in RPMI-10 with heat-inactivated NRS or IRS for 20 min at 37° C by shaking and added to triplicate wells at a multiplicity of infection of 10. The final serum volume per well was 0.1%. After 2h, cells were pulsed with 50μg/ml of gentamicin for 45-60 min and washed 3 times with warm PBS before adding RPMI-10 and incubated till the harvesting point. At the indicated time points (~4 and 24 h) after infection, cells were lysed with 0.1% sodium deoxycholate and serial dilutions of the lysates were plated on chocolate agar plates (Remel, Lenexa, KS) to determine the intracellular bacterial load.

Luminex analysis

Cytokine concentrations in the tissue homeogenate supernatants were measured using Ab-linked beads that recognize 13 different cytokines (Invitrogen; Carlsbad, CA & Millipore; Billerica, MA) and a Luminex 100 reader (Luminex Corporation; Austin, TX). The raw data were analyzed with StatLIA statistical analysis program (Brendan Scientific; Carlsbad, CA).

Blood chemistry

Blood samples were collected from rats sacrificed at 7 days p.i. with SCHU S4. Sera were collected and filter-sterilized using 0.2μm syringe filters. Sterility was confirmed by plating undiluted serum samples on cysteine heart agar plates with 5% rabbit blood and selective antibiotics. Sera were analyzed for blood urea nitrogen (BUN) and alanine aminotransferase (ALT) at Tricore Reference Laboratories (Albuquerque, NM).

IFNγ ELISPOT assay

IFNγ secreting cells in the LALNs of SCHU S4- and mock-infected rats were estimated on days 3, 5, and 7 p.i. using an ELISPOT assay by following the manufacturer's instructions (Mabtech, Cincinnati, OH). Briefly, cell culture plates were coated with 1.5 μg/well of monoclonal Ab specific for rat IFNγ in PBS and incubated overnight at 4 ˚ C. Excess Ab was washed away and the plate was blocked with RPMI-

10 for at least 30 min at room temperature before washing the plate again and adding 2.5 x 10^5 single cell suspensions of LALN in 250 µl of RPMI-10 per well without any Ags for stimulation. After overnight incubation at 37 ˚ C, the plates were washed and the biotinylated detection Ab was added to incubate the plate at room temperature for 2 h. Spots were visualized using streptavidin-alkaline phosphatase and BCIP (5-bromo-4-chloro-3-indolyl-phosphate)/ NBT (nitro blue tetrazolium) as substrate. The spots were enumerated using ViruSpot reader (Cell Technology, Mountain View, CA). Mock-infected mice did not produce any spots while wells with cells stimulated with Conconavalin A (Sigma-Aldrich, St. Louis, MO) served as positive controls.

TdT-mediated dUTP-biotin nick end labeling (TUNEL) staining

Apoptotic cells in the tissue sections were detected by TUNEL of cleaved double-strand DNA using ApopTag peroxidase *in situ* apoptosis detection kit (Millipore, Billerica, MA) following manufacturer's instructions. Briefly, deparaffined and rehydrated tissue sections were treated with 3% hydrogen peroxide to quench the endogenous peroxidases and subsequently permeabilized with proteinase K. Sections were developed with diaminobenzidine after sequential treatment with TdT and anti-digoxigenin conjugate.

Statistics

Kaplan-Meier survival curves were analyzed by log-rank test and SCHU S4 burden following multiple serum treatments was analyzed by two-way ANOVA. Total cell populations from the tissues of isotype control-treated and CD4-depleting Ab-treated animals were compared using two-tailed unpaired *t* test. These and other analyses were performed using GraphPad Prism version 5.01 software (GraphPad Software; San Diego, CA) as described in the figure legends. SCHU S4 growth kinetics in experiments with single prophylactic serum treatment were analyzed by fitting two-way ANOVA with interaction using general linear model in SAS (SAS Institute Inc.; Cary, NC).

CHAPTER 4: ANTIBODIES CONTRIBUTE TO EFFECTIVE VACCINATION AGAINST RESPIRATORY INFECTION BY TYPE A *FRANCISELLA TULARENSIS* STRAINS

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Summary

Pneumonic tularemia is a life-threatening disease caused by inhalation of the highly infectious intracellular bacterium *Francisella tularensis*. The most serious form of the disease associated with the type A strains can be prevented in experimental animals through vaccination with the attenuated Live Vaccine Strain (LVS). The protection is largely cell-mediated, but the contribution of Abs remains controversial. We addressed this issue in a series of passive immunization studies in Fischer 344 (F344) rats. Subcutaneous LVS vaccination induced a robust serum Ab response dominated by IgM, IgG2a and IgG2b Abs. Prophylactic administration of LVS immune serum or purified immune IgG reduced the severity and duration of disease in naïve rats challenged intratracheally with a lethal dose of the virulent type A strain SCHU S4. The level of resistance increased with the volume of immune serum given, but the maximum survivable SCHU S4 challenge dose was at least 100-fold lower than that shown for LVS vaccinated rats. Protection correlated with reduced systemic bacterial growth, less severe histopathology in the liver and spleen during the early phase of infection and bacterial clearance by a T cell-dependent mechanism. Our results suggest that treatment with immune serum limited the sequelae associated with infection, thereby enabling a sterilizing T cell response to develop and resolve the infection. Thus, Abs induced by LVS vaccination may contribute to the defense of F344 rats against respiratory infection by type A strains of *F. tularensis*.

Introduction

Pneumonic tularemia is a highly debilitating disease caused by the gram-negative coccobacillus *Francisella tularensis*. Strains classified under subspecies *tularensis* (type A) are the most virulent and pose the biggest challenge from a clinical perspective (41). The mortality rate of pneumonic tularemia caused by the type A strains has been estimated to exceed 30% in untreated patients (31). Prophylactic vaccination is the best countermeasure and there is good historical evidence that pneumonic tularemia can be prevented by vaccination with the attenuated *F. tularensis* Live Vaccine Strain (LVS) (42). However, LVS is unlikely to be licensed for mass vaccination because the mechanism of attenuation has not been defined. Due to the potential of a major public health threat, there is an urgent need to understand the protective mechanisms associated with an effective immune response so that novel vaccines can be developed.

Protective immunity against *F. tularensis* infection is usually attributed to an effective T cell response. However, *F. tularensis* has a significant extracellular phase, which makes it accessible to humoral immune responses (154). Indeed, there is ample evidence that B cells and Abs are necessary for mice to develop their natural resistance to primary and secondary LVS infections. Purified lipopolysaccharide (LPS) from LVS induced a population of B1-a cells within 2-3 days of administration that protected mice against intraperitoneal (i.p.) LVS challenge (156-158)(181-183). Consistent with these results, μMT mice lacking mature B cells exhibited increased susceptibility to primary intradermal (i.d.) LVS infection and delayed bacterial clearance (159, 160). μMT mice were also more susceptible to secondary i.p. LVS infection and this defect was corrected by reconstitution with LVS primed B cells (132). The contribution of Abs has been

addressed repeatedly in passive immunization experiments, which showed that immune serum from humans and mice vaccinated with live or inactivated LVS protected naïve mice against challenges with LVS or other low virulence strains given by a variety of routes (149, 151, 161-164). The dominant Ab response was directed at LPS, but Abs against protein antigens have also been found (160, 165-167). Monoclonal Abs specific for LPS or the outer-membrane protein FopA provided significant protection against LVS challenge when given either prophylactically (168) or therapeutically (168). Together, these results suggest that Abs contribute towards effective control of attenuated or low virulence *F. tularensis* strains.

It has been much more difficult to demonstrate Ab-mediated protection against type A strains in mice (152, 168, 169), even though they express most of antigens recognized by LVS immune serum (161). This is not surprising given the historical difficulties in generating protective immunity against type A strains in this animal model (170). However, Ray et al. recently showed that oral LVS vaccination protected mice against a pulmonary SCHU S4 challenge in an Ab-dependent manner (44). Klimpel et al. also reported a similar finding using immune serum from mice cured of a lethal i.n. SCHU S4 infection with levofloxacin in a passive immunization model (153). Thus, the protective effects of Abs appear not to be restricted only to low virulence strains but may also contribute to the protection against highly virulent type A strains.

To further characterize the mechanism of Ab-mediated protection, we utilized the recently characterized Fischer 344 (F344) rat model (48). Since F344 rats developed much stronger resistance to respiratory SCHU S4 challenge after LVS vaccination than

previously observed in mice, we speculated that Abs may provide better protection in this model and allow us to define their protective mechanism more thoroughly. We now show in a passive immunization model that serum Abs from LVS-vaccinated rats conferred protection against a lethal intratracheal SCHU S4 challenge. Protection correlated with reduced systemic bacterial growth and less severe histopathology during the early phase of infection and bacterial clearance by a T cell-dependent mechanism. Thus, Abs contribute to but are not sufficient for the effective control of respiratory infections by fully virulent type A strains. Our studies provide valuable insights into the protective mechanisms of Abs that will guide future development of tularemia vaccine candidates.

Results

Development of serum Ab response after s.c. LVS vaccination

We showed previously that Fischer 344 rats cleared a vaccine inoculum of 5×10^7 LVS within two weeks of subcutaneous vaccination and were protected when challenged two weeks later with SCHU S4 i.t. (48). To determine the Ab response over this 4-week period, the serum concentration of LVS-specific IgM, IgG, and IgA were measured. Subcutaneous LVS vaccination induced a robust serum IgM and IgG response in F344 rats (Figure 2). The average IgM and IgG titers after 7 days were 1:64,000 and 1:16,000, respectively. The IgM titer peaked one week later at 1:32,000 and declined thereafter. The IgG titer remained relatively stable at 1:16-32,000 over the 4-week period. Serum IgA was detected in all vaccinated rats, but the titer never exceeded 1:800 (data not shown).

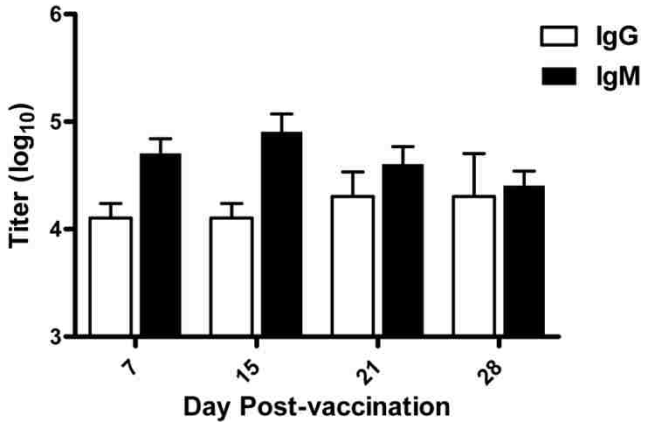

Figure 2. *Subcutaneous LVS vaccination induces a robust IgM and IgG serum Ab response*. F344 rats (n = 3) were vaccinated s.c. with 5 X 10^7 CFU of LVS. Sera were collected on days 7, 15, 21, and 28 d post-inoculation and the total LVS-specific IgM, IgG and IgA Ab titer was determined for each rat by ELISA using heat-killed LVS as the capture antigen. Ab titer was defined as the reciprocal of the highest dilution of immune serum that had a mean OD value that is greater than 3 standard deviations above than the mean OD of normal serum at the same dilution. The data show the geometric mean ± SD.

Passive immunization with immune serum protects F344 rats against pneumonic tularemia

To determine whether LVS-specific serum Abs can protect F344 rats against i.t. SCHU S4 challenge, naïve F344 rats were treated prophylactically with immune rat serum (IRS) and then challenged i.t. with SCHU S4. Immune sera were pooled from several F344 rats 28 days after LVS vaccination when no trace of LVS could be detected systemically. The predominant Abs were IgM, IgG2a and IgG2b and the titers of IgG1, IgG2c and IgA were at least 10-fold less (Figure 3). Naïve F344 rats were treated i.p. with an arbitrary volume of 250 µl IRS, a volume which constituted ~7 % of the total serum volume in a recipient F344 rat weighing ~150 g. 24 h after serum transfer, the passively immunized rats were challenged i.t. with a small but lethal dose of ~250 CFU, which was selected intentionally to reduce the threshold required to detect any protective effect brought about by the LVS-immune serum. Rats treated with normal rat serum (NRS) and LVS-vaccinated rats were used as negative and positive controls, respectively. All rats were monitored for survival, weight loss, and clinical signs for 5 weeks.

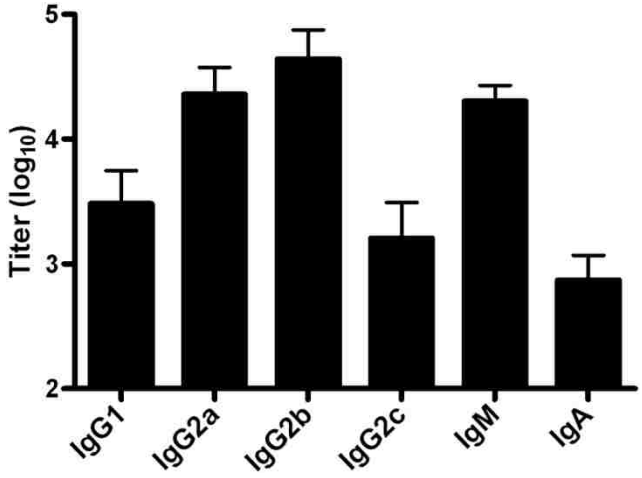

Figure 3. *Ab composition in the immune serum used for passive immunization*. F344 rats (n = 3-4) were vaccinated s.c. with 5 x 10^7 CFU of LVS. Sera were collected 28 d post-vaccination and analyzed by ELISA for the presence of LVS-specific Abs of the indicated isotypes and subclasses using heat-killed LVS as the capture antigen. Ab titer was defined as described in Fig. 1. The data represent the geometric mean ± SD of samples combined from two independent experiments.

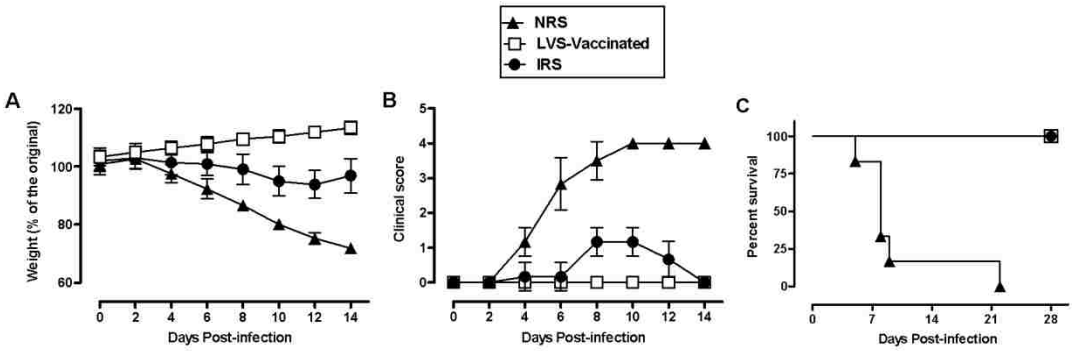

Figure 4. *Passive immunization with IRS protects naïve rats against a lethal i.t. SCHU S4 challenge*. Groups (n=6) of LVS-vaccinated rats and naïve F344 rats were treated i.p. with 250 µl of heat-inactivated NRS or IRS and then challenged i.t. 24h later with 240 CFU of SCHU S4. The challenge dose represents the actual lung deposition determined within 1 h of infection. The infected rats were monitored daily for (A) weight, (B) clinical signs, and (C) survival. In panel A, the results are presented as a percent relative to the body weight measured 24h before challenge. A value of 100% indicates no weight change and points above and below 100% represents weight gain and loss, respectively. In panel B, the disease severity was scored based on the criteria described in Materials and Methods. Data represents the average of all survivors in each group ± SD.

The first indication of illness in the NRS-treated rats was a slight weight loss and decreased alertness 3-4 days post-infection (p.i.). The disease progressed very quickly over the next 48-72 h and was characterized by rapid loss up to 30% of body weight (Figure 4A) and development of severe lethargy, ruffled coat, eyelid ptosis, and hunched posture (Figure 4B). At the peak of disease severity, the rats were extremely weak and unresponsive and their eyes were completely closed surrounded by a large amount of ocular discharge. Most infected rats died within 2 weeks of infection (Figure 4C). Although the IRS-treated rats exhibited some of the early signs of infection, weight loss occurred more gradually and rarely exceeded 10% of the initial body weight. All signs of illness resolved within 2 weeks of infection and the rats remained outwardly healthy for the remaining 2-3 weeks of monitoring. LVS vaccinated rats never lost weight or exhibited any overt signs of disease. In five independent experiments, at a SCHU S4 challenge dose of 218-240 CFU, 27 of 29 rats treated with IRS survived while 29 of 30 NRS-treated rats died (Table II). These results suggested that serum Abs are capable of mediating protection against a lethal respiratory SCHU S4 infection.

Table II. Summary of survival results of F344 rats challenged intra-tracheally with SCHU S4 after prophylactic treatment with 250 µl of serum.

SCHU S4 Challenge Dose (CFU)[a]	Immune Rat Serum		Normal Rat Serum	
	Survival ratio (No. alive/total)	MTD[c] (days)	Survival ratio (No. alive/total)	MTD (days)
130	6/6	-	1/5	7
218-240[b]	27/29	11	1/30	9
360	5/6	12	0/5	8
727	5/6	14	0/6	7
1496	1/6	11		
3525	0/6	13	0/6	8
10083	0/6	10	0/6	4

[a] Bacteria recovered from the lungs 1 h after infection.
[b] Includes 5 separate experiments using a challenge dose within this range.
[c] Mean time to death

Purified IgG is sufficient for protection against SCHU S4 infection

To verify that LVS-specific Abs were responsible for the serum-mediated protection, IgG and IgM were purified from normal and immune sera. The purification process reduced the titer of contaminating Ab isotypes to < 1:100 and removed most contaminating proteins, except for a prominent 75 kDa protein in the purified IgG fraction that has not been identified (Figure 5A and B). For passive immunization, F344 rats were injected with an amount of purified IgG and/or IgM that is equivalent to that contained in 250 µl of serum. Similar to IRS, purified immune IgG provided significant protection against i.t. SCHU S4 challenge (Figure 5C). In contrast, purified immune IgM offered no protection and the treated rats succumbed to SCHU S4 infection at the same time as rats treated with normal serum. These results indicated that LVS-specific IgG is the principal protective component in the immune serum. Since IRS and purified immune IgG provided similar level of protection, IRS was used in all experiments described hereafter.

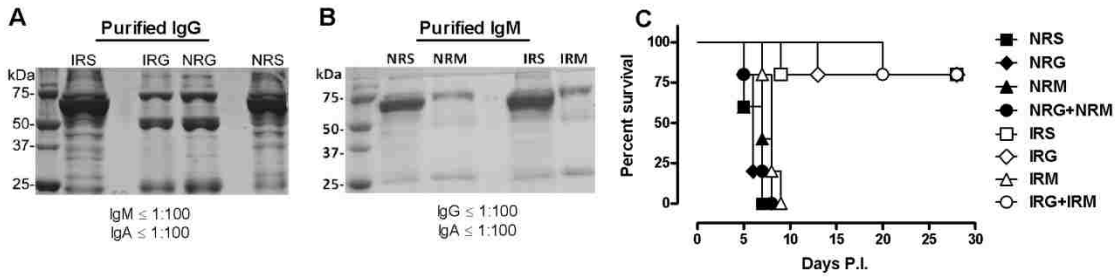

Figure 5. *Passive transfer of purified LVS-immune IgG, but not IgM, protects naïve rats against a lethal i.t. SCHU S4 challenge.* IgG and IgM were purified from pooled normal rat serum (NRS) and immune rat serum (IRS) collected 28 days after s.c. LVS vaccination. The purity of enriched IgG (A) and IgM (B) preparations was analyzed by SDS/PAGE gels stained with Coomassie blue dye. IRG, immune IgG; NRG, normal IgG; IRM, immune IgM; NRM, normal IgM. The titer of LVS-specific IgM, IgG, and IgA was determined by ELISA as indicated in Figure 2 and the titer of contaminating Ab isotypes in the enriched preparations was < 1:100. (C) Groups of 5 F344 rats were treated with an amount of purified IgG and IgM that is equivalent to 250 µl of serum and challenged i.t. 1 day later with 810 CFU of SCHU S4. Survival was monitored daily.

Immune serum treatment of F344 rats limits SCHU S4 growth

Since IRS treatment provided significant protection against pulmonary SCHU S4 infection, we next investigated the effect of this treatment on SCHU S4 growth. IRS-treated rats exhibited a pattern of SCHU S4 growth and dissemination that was intermediate between the NRS-treated rats and the LVS-vaccinated rats. Bacterial expansion in the first two days following infection was identical between the IRS- and NRS-treated rats: in both groups, the number of lung bacteria increased to 10^7 CFU and systemic dissemination to the liver and spleen had occurred in the majority of animals (Figure 6). The two groups started to diverge on day 3 when fewer bacteria were recovered from the IRS-treated rats. The bacterial burden in NRS-treated rats peaked on day 7 p.i. shortly before they died with $8x10^8$ CFU in the lungs, $4x10^8$ CFU in the liver, and $2x10^7$ CFU in the spleen. In contrast, the bacterial burden in the IRS-treated rats increased at a slower rate to peak on day 10 p.i. By day 14, the infection began to clear in the IRS-treated rats and the bacterial load in all three tissues had dropped from their peak. These results suggest that IRS may contribute to protection of naïve F344 rats by limiting bacterial growth and facilitating development of an immune response that eventually cleared the infection.

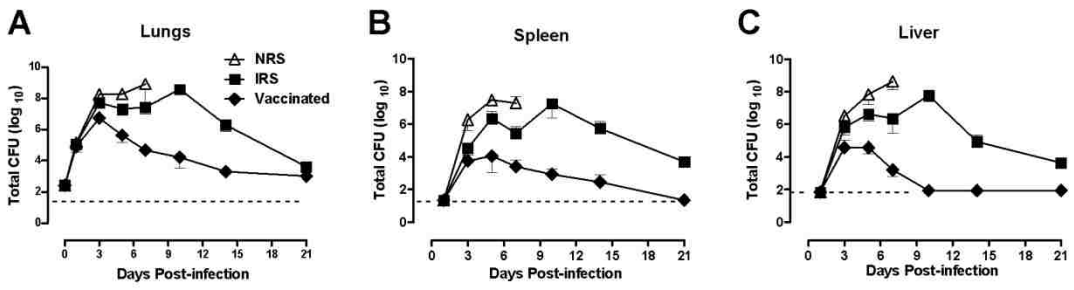

Figure 6. *IRS-treated rats exhibit a pattern of SCHU S4 growth intermediate between NRS-treated rats and LVS-vaccinated rats*. LVS-vaccinated F344 rats and naïve rats treated with 250 µl of heat-inactivated normal rat serum (NRS) or heat-inactivated immune rat serum (IRS) were challenged i.t. 1 d after serum treatment with 260 CFU of SCHU S4. 3-4 rats were euthanized from each group on days 0, 1, 3, 5, 7, 10, 14, and 21 post-challenge and to determine the total SCHU S4 burden in the lungs, spleen, and liver. The numbers of lung bacteria on day 0 reflects the actual lung deposition determined within 1 h of infection and the dashed lines represent the limit of detection for each organ. Each data point represents mean ± SD.

Protection by IRS is dependent on the SCHU S4 challenge dose and the volume of IRS

To further characterize the potency of IRS, titrations of the SCHU S4 challenge dose and the IRS volume were performed. A single treatment with 250 µl of IRS provided long-term protection to over 90% of naïve rats challenged with up to ~700 CFU SCHU S4 (Table II). The mortality rate increased when the challenge dose was increased to over 1.5 x 10^3 CFU and all of the infected rats died between 10 and 13 days after challenge. There was little correlation between the challenge dose and the time-to-death. The cumulative results from 7 independent experiments suggested that the i.t. LD_{50} of SCHU S4 in F344 rats treated with 250 µl of IRS was in the range of 700 and 1500 CFU; this is at least 100-fold less than the LD_{50} associated with s.c. LVS vaccination (48). Reducing the IRS volume had a dose-dependent effect on the level of protection against a fixed challenge dose. When the IRS volume was reduced to 25 µl, the treatment prolonged the survival of rats challenged i.t. with 360 CFU by 4-5 days, but they eventually succumbed to infection (Figure 7). All protective effects were eliminated when the IRS volume was further reduced to 2.5 and 0.25 µl. Increasing the IRS volume to 1 ml did not substantially delay disease onset, improve clinical signs, or accelerate resolution compared to the 250 µl treatment (data not shown). Since the effectiveness of a single IRS treatment may be limited by the availability of target organisms at or around the time of administration, rats were given repeated IRS injections in an attempt to match the increasing bacteria numbers due to proliferation over the course of infection. F344 rats were either treated with 250 µl of IRS once before SCHU S4 challenge or multiple times on days -1, 3, 6, 9, and 12 relative to challenge. The total bacterial burden in the lungs, spleen, and liver of rats was determined on days 3, 5, 7, 10, and 14 after challenge.

As shown in Figure 8, multiple Ab treatments did not alter the general pattern of SCHU S4 growth and dissemination compared to a single treatment ($p > 0.05$ for all three organs).

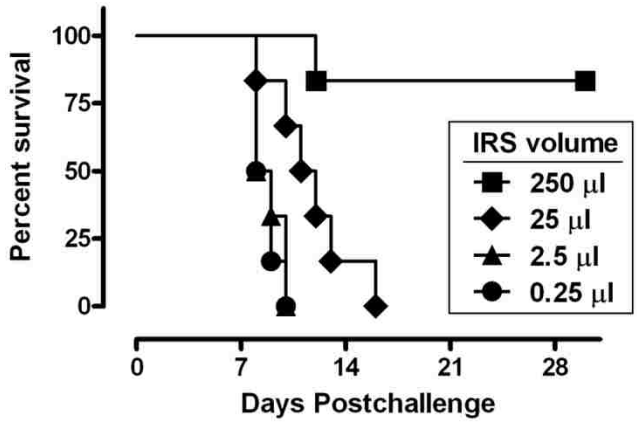

Figure 7. *IRS-mediated protection is dose-dependent*. Groups of F344 rats (n = 6) were treated i.p. with the indicated volumes of heat-inactivated IRS and 1 d later challenged i.t. with 360 CFU of SCHU S4. Four additional groups of rats were treated with the equivalent volumes of NRS. All but one rat treated with 250 µl died by day 10 of infection.

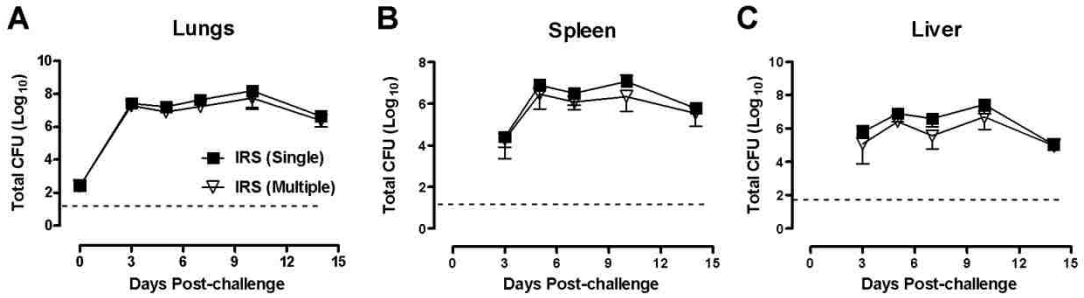

Figure 8. *Multiple IRS treatments have the same effect on SCHU S4 growth kinetics as a single treatment*. Naïve F344 rats were either treated with a single dose of 250µl of heat-inactivated IRS 1 d before challenge (day -1) or with multiple 250 µl doses on days -1, 3, 6, 9, and 12. One day after the first treatment, rats were challenged i.t. with 420 CFU of SCHU S4. On the indicated days, the bacterial burdens in the lungs and spleens and liver were determined from 3 rats per group. The dashed lines indicate the detection limit for each tissue and each data point represents mean ± SD. There was no significant difference in the bacterial burden between the two groups ($p > 0.05$).

Taken together, these results showed that a single IRS treatment was sufficient to protect F344 rats against i.t. challenge of up to ~700 SCHU S4 organisms. This protection required a minimum IRS volume of 250 µl and any amount beyond this volume threshold given in a single or multiple treatments provided little additional benefit.

Histopathology of serum-treated rats after i.t. challenge with SCHU S4

In order to determine how the IRS-treated rats survived a lethal pulmonary SCHU S4 challenge despite an extremely high bacterial burden, we next evaluated whether IRS treatment limited the histopathology in the infected tissues. Lung lesions in both IRS- and NRS-treated rats were first detected on day 3 p.i., and consisted of neutrophilic and histiocytic inflammation within alveoli, bronchioles, and bronchi, and in the perivascular spaces of adjacent blood vessels. Over the next 4 days, the lung inflammation became progressively more necrotizing in both exposure groups. Lung lesions severity was similar in the IRS- and NRS-treated rats during the first 7 days p.i., after which time the NRS-treated rats did not survive. For comparison, the lung lesions in LVS-vaccinated rats were similar in nature and severity to the IRS- and NRS-treated rats during the first 5 days p.i., but gradually decreased in severity starting at day 7 p.i.

Lesions in the liver and spleen were first detected in the IRS- and NRS- treated rats on day 3 p.i.. Splenic and hepatic lesions consisted of multifocal, random neutrophilic and histiocytic inflammation on day 3 p.i., and frequently progressed to necrotizing inflammation by day 5 p.i. In both the IRS- and NRS-treated rats, maximal

hepatic and splenic lesion severity was achieved on day 5 p.i. However, the maximal severity score for splenic and hepatic lesions in the IRS-treated rats was less than for the NRS- treated rats. Furthermore, the lesion severity decreased for the IRS- treated rats by day 7 p.i., but not for the NRS- treated rats. For comparison, in the vaccinated rats, lesions were sparse to non-existent at all the time points examined (Figure 9A-F). These results demonstrate that IRS treatment reduced the inflammation in tissues to which *F. tularensis* is known to disseminate.

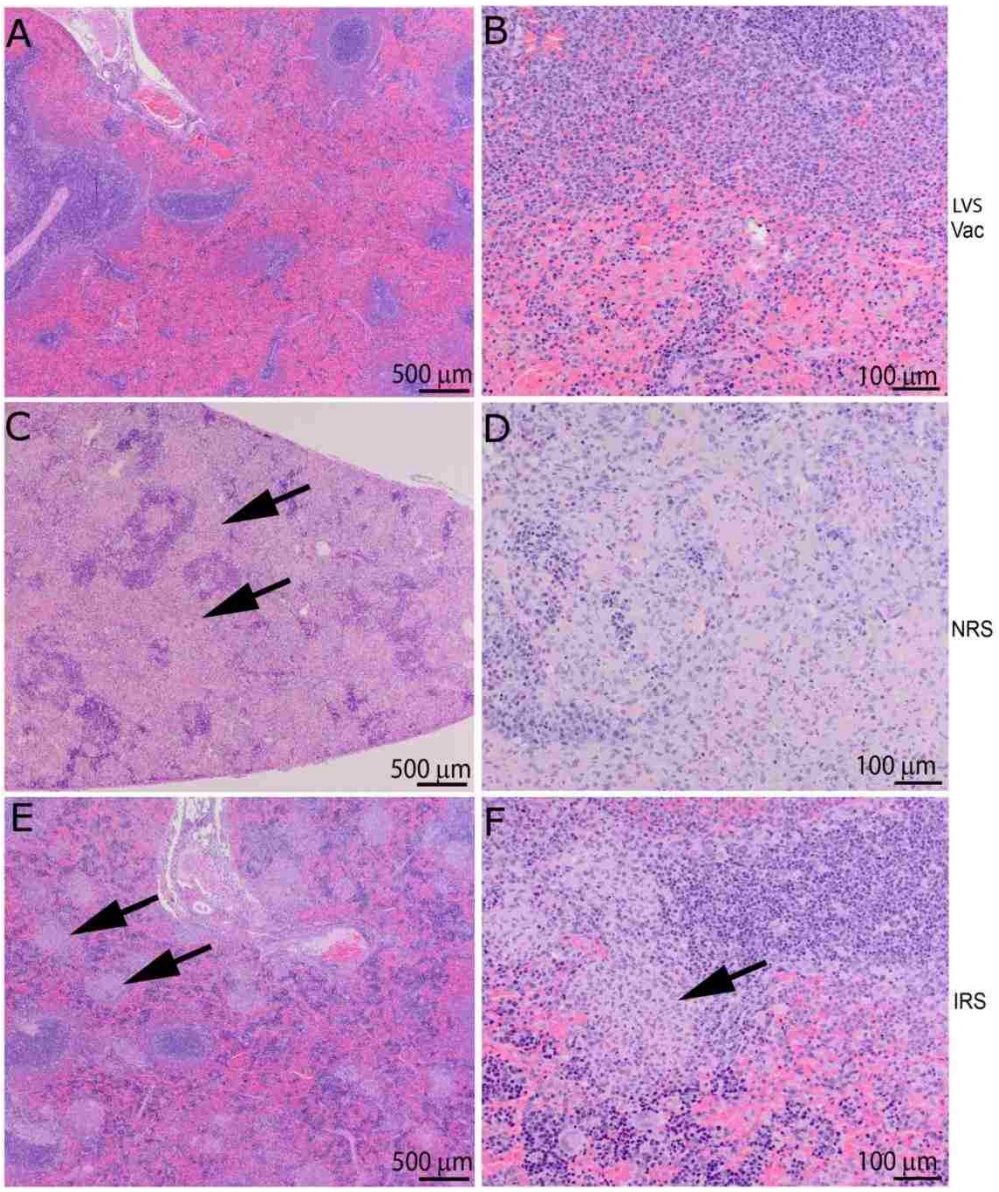

Figure 9. *IRS-treated rats develop less severe splenic inflammation than NRS-treated control rats at day 7 p.i. with SCHU S4*. The spleens of LVS-vaccinated (LVS vac), or IRS- and NRS-treated rats were examined histologically at day 7 after SCHU S4 challenge. The spleens of vaccinated rats were without detectable lesions (A and B). The spleens of the NRS-treated rats exhibited nearly complete effacement of the red pulp with neutrophilic and histiocytic inflammation (C and D), while the spleens of the IRS-treated rats exhibited only multifocal neutrophilic and histiocytic inflammation in the red pulp (E and F). Arrows point to paler appearing areas of inflammation in the splenic red pulp.

T cells are critical for Ab-mediated protection

The importance of T cells in Ab-mediated protection was determined in T cell-deficient athymic nude rats. Nude rats were derived from a heterogeneous genetic background and have normal B cell function, but an increased NK cell population. Nude rats were more resistant to SCHU S4 infection and lived 2-3 days longer than similarly infected F344 rats. Prophylactic treatment with 250 µl of IRS significantly prolonged the survival of infected nude rats by 3 days ($p < 0.01$) but all the infected animals died by day 16 p.i. (Figure 10A). These results suggested that a T cell-independent mechanism can temporarily modify the disease process, but T cells are critical for the long-term protection associated with immune serum.

To determine the requirement for CD4 and CD8 T cells in passively immunized F344 rats, we developed very effective *in vivo* depletion regimen using the anti-CD8 Ab, OX-8, and the anti-CD4 Ab, OX-38. These Ab treatment regimens reduced and maintained the peripheral blood CD4 and CD8 T cell populations to < 5 and < 1 % of normal levels, respectively, over the course of infection. As shown in Figure 10B, depletion of CD8 T cells completely abolished the ability of IRS to protect against SCHU S4 challenge. Depletion of CD4 T cells had only a partial effect. This may be related to a slight (<2-fold), but not statistically significant, increase in the total number of CD8 T cells in the spleen, liver and blood; no difference in B cells ($CD45R^+$) or NK cells ($CD161^+$) were observed (data not shown). Indeed, the mortality rate of rats depleted of both CD4 and CD8 T cells was similar to the animals depleted of CD8 T cells alone. Thus, these results suggest that CD8 T cells are essential for IRS to protect rats against pulmonary infection with SCHU S4.

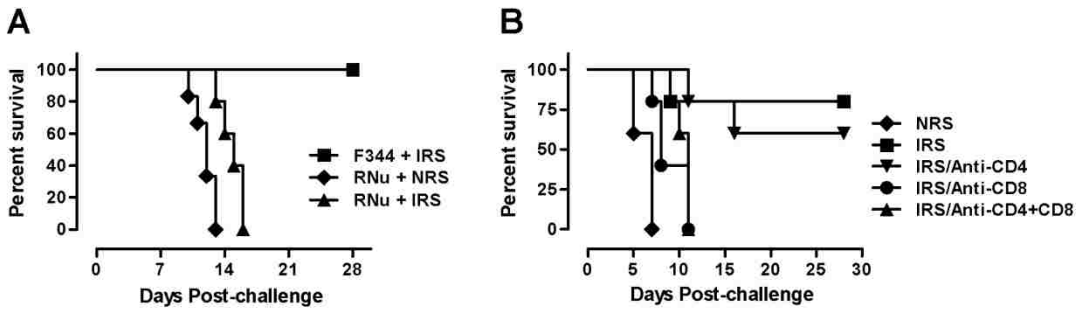

Figure 10. *IRS-mediated protection is dependent on T cells.* (A) T cell-deficient athymic (RNu) nude rats and immunocompetent Fischer 344 rats (n = 6 per group) were treated with 250µl of heat-inactivated IRS or NRS and 1 day later challenged i.t. with 465 CFU SCHU S4. (B) Naïve F344 rats (n=5) were depleted of CD4, CD8 or both CD4 and CD8 T cells by i.p. injection of depleting Abs OX-38 (CD4) and OX-8 (CD8). T cell depletion was maintained over the course of infection with additional treatments with depleting Abs. The T cell-depleted rats were treated with 250µl of heat-inactivated IRS or NRS and 1 day later challenged i.t. with 810 CFU of SCHU S4. The rats were monitored daily for survival and clinical signs of illness.

Discussion

It is widely accepted that tularemia vaccines such as LVS must induce a potent cell-mediated immunity to be effective against highly virulent type A strains of *F. tularensis*. LVS vaccination also induces a strong Ab response, but its role in protection has not been thoroughly addressed. We now show that the serum Abs provided significant protection against a lethal, respiratory SCHU S4 challenge in the F344 rat passive immunization model. These results suggest that Abs may be an important component in the overall defense against pneumonic tularemia.

We previously showed that the F344 rat is a good animal model for studying human pneumonic tularemia (48). In the present study, F344 rats were vaccinated s.c. to reproduce human vaccination by the scarification method currently used under the Special Immunization Program at USAMRIID. Similar to humans vaccinated by scarification (131, 132), the s.c. vaccinated rats developed a strong IgM and IgG response within a week of vaccination. Further studies showed that only purified serum IgG but not IgM mediated protection against i.t. SCHU S4 challenge in the rat. We have not ruled out the potential contribution of IgA to protection, especially in the lungs or other mucosal surfaces. In fact, the majority of humans (131, 132) and mice developed positive serum IgA titers following scarification or i.n. vaccination, respectively. The protective capabilities of IgA has been demonstrated repeatedly in vaccine studies with IgA-deficient mice, which did not develop complete resistance against respiratory infections with LVS (171, 172) and SCHU S4 (44). A significant amount of IgA was also found in the immune mouse serum that passively transferred immunity against i.n.

LVS infection (151). Thus, in addition to IgG, IgA may also play a role in protection against respiratory tularemia caused by type A strains.

It has been suggested that the morbidity and mortality associated with pneumonic tularemia is caused by the damage inflicted on the extrapulmonary tissues (173). An extension of this idea is that control of bacterial dissemination and growth outside of the lungs would offer considerable survival advantage. Our results are consistent with this idea and suggest that passive immunization modified the course of acute infection by enhancing the innate immune response to disseminated bacteria. The presence of LVS-specific Abs appeared to be most critical during the early phase of infection because IRS failed to rescue infected rats when the treatment was delayed by 48 h (data not shown) and increasing the amount of serum or the treatment frequency did not improve the level of protection. IRS treatment had little, if any, quantitative or qualitative impact on the bacterial burden and the histopathology in the lungs. Rather, the effect of IRS on bacterial growth was most clearly observed in the infected liver and spleen. The liver is a major site of *F. tularensis* colonization and replication and pneumonic tularemia is associated with hepatocellular damage in multiple experimental animal models, including the F344 rat (48, 173). Although the level of damage falls short of liver failure and may be reversible, such damage may nevertheless compromise the liver's ability to perform essential metabolic functions and to utilize its many innate immune mechanisms to control systemic bacterial growth. In contrast, IRS treatment reduced the bacterial burden and histopathology in liver and, in doing so, may have preserved more liver functions in the infected rat. Similar passive immunization studies in mice with LVS point to a process that involves Fc receptor and IFNγ (151, 164) and the liver is one of the

richest sources of NK cells that are capable of producing IFNγ in response to *F. tularensis* infection (111). This may also explain the ability of immune serum to delay the death of athymic nude rats and mice, which express higher NK cell activity than wild-type animals. By controlling the rapid growth rate and dissemination of SCHU S4 and limiting the pathological damage associated with infection, IRS may have enabled the host to survive long enough to develop an effective T cell response that eliminated the infection. Indeed, LVS-vaccinated rats with both *F. tularensis*-specific Abs and immune T cells have the lowest bacterial burden and show the least histological changes following SCHU S4 infection.

T cells are required for the long-term protective effects of passive immunization. In the absence of T cells, IRS only delayed the death of athymic nude rats by several days. CD8 T cells appeared to play a more critical role than CD4 T cells since depletion of CD8 T cell rendered rats completely susceptible to SCHU S4 infection while only a fraction of the rats depleted of CD4 T cells succumbed. A possible explanation for the partial effect of CD4 T cell depletion is that the level of depletion may have been slightly different for each rat and the survivors had more residual cells. It is also possible that challenge dose varied slightly for each rat and the survivors were challenged with a lower dose.

With the growing awareness of the importance of Abs for effective immunity against pneumonic tularemia, new vaccine designs are beginning to incorporate Abs or elements that induce stronger Ab responses. For example, Abs against LPS were used to enhance antigen presentation by targeting inactivated LVS to Fc receptors on myeloid cells (172,197, 198, 199,). Cholera toxin B and a LVS O-polysaccharide-tetanus toxoid

glycoconjugate (174) were used to induce a better Ab response to augment the cellular immunity generated with inactivated or mutant LVS. Several groups have also used protein microarrays (160, 165) and immunoproteomic approaches (166, 167) to identify immunoreactive Abs in serum from humans and mice with previous exposure to *F. tularensis*. In order to further improve these novel vaccine designs and to develop Abs into potential ancillary therapeutic agents, it will be necessary to characterize the protective response associated with any potential Ab candidate. The fact that F344 rats were consistently protected by immune serum alone suggests that the F344 rat model will be a valuable tool not only to test the protective effect of these Abs but also to characterize their mechanism of protection.

In conclusion, our studies showed that LVS vaccination induced serum Abs that were protective against a lethal respiratory SCHU S4 infection. The protective responses defined in these studies provide valuable insights into the mechanism of Ab-mediated protection and will help guide the rational design of novel tularemia vaccines that induce not only a robust cellular immunity but also a strong humoral immunity.

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CHAPTER 5: PASSIVE IMMUNIZATION WITH LVS-SPECIFIC ANTIBODIES CONTROLS INFLAMMATION AND ENABLES THE DEVELOPMENT OF PROTECTIVE IMMUNITY AGAINST PULMONARY INFECTION BY VIRULENT TYPE A STRAINS OF *FRANCISELLA TULARENSIS*

Summary

Type A *Ft* strains cause fatal illness when infected by the aerosol route. The protective mechanisms of Abs during pulmonary infection, hitherto unexplored, were studied using the F344 passive immunization model. Naïve F344 rats were killed by lethal i.t. SCHU S4 challenge when the Abs specific for *Ft* LPS were absent and also upon delaying immune Ab administration for >48 h after infection. Opsonization with Abs significantly increased SCHU S4 uptake by PBMCs and controlled their intracellular growth rate but the bacterial numbers remained significantly high when the direct effects of Abs were studied *in vitro*. IRS treatment in the SCHU S4 infected animals was associated with reduced hyperinflammatory cytokine profiles, apoptosis, and injury in the sampled tissues. In contrast to the NRS-treated rats where the immune cell viability and IFNγ secretion was severely compromised in the LDLNs by 7 days p.i., IRS-treatment might have enabled survival against both primary and secondary challenges against SCHU S4 by acting early in the infection to allow for timely and adequate development of adaptive immunity. These studies offer several clues to explore the potential actions of Abs early in the infection to alter the disease processes while providing scope for designing Ab-based therapeutics to mitigate the severity associated with pulmonary tularemia.

Introduction

Type A strains of *F. tularensis* can endanger human lives when they infect the airways due to their proven high infectivity and extreme virulence through this route of exposure. The resulting illness can prolong for weeks in the absence of timely treatment

with antibiotics and may lead to case fatalities as exemplified by the outbreaks of pneumonic tularemia on Martha's Vineyard (175, 176). The development of prophylactic and therapeutic medical interventions is therefore necessary to minimize fiscal and human losses. Since vaccines represent cheap, yet potent, means of circumventing the adverse effects of pulmonary infection with SCHU S4, delineating the interplay between the pathogen and host as well as characterizing the protective immune components capable of subverting the damage inflicted during inhalational tularemia are essential for designing effective vaccines.

Three distinct virulence attributes are commonly encountered during infection with type A *F. tularensis*. First, the pathogen multiplies unbridled, to very high levels at the infection site and in all the organs to which it disseminates to adversely impact their normal functioning (43). Second, a sepsis-like condition, characterized by dysregulated host immune response with elevated cytokines and chemokines, ensues a few days after infection. Sepsis with accompanying manifestation of inflammatory mediators has been reported in both murine models and humans (52, 53, 91, 177-181). Finally, extensive tissue damage and multiple organ failure with concomitant death of innate and adaptive immune cells, renders infection with type A *F. tularensis*, life-threatening. Specimens from autopsy samples of humans who succumbed to type A *F. tularensis* showed extensive necrotizing lesions in the lungs, lymph nodes, kidneys, livers, and spleens (182). Plasma samples consistently show significantly high levels of liver enzymes and lactate dehydrogenase, which is indicative of tissue damage (181, 183-185). Severe loss in liver macrophage viability and T cell destruction in the thymus and T-dependent regions of peripheral lymphoid tissues of SCHU S4-infected animals have been reported

(52, 186). Hence the immune responses targeting the pathogen may not only have to be effective in eliminating the pathogen, but also be balanced at the same time to prevent the collateral damage from becoming fatal.

A measured host response that enables the timely development of adaptive immunity that can clear SCHU S4 may hence be critical for survival. The protective role of Abs, which has remained ambiguous, was addressed through the development of passive immunization model using F344 rats. As described in Chapter IV, LVS-specific Abs provided long-term protection in F344 rats infected with a lethal dose of SCHU S4 with the help of CD8 T cells. Even in the absence of T cells, Abs significantly prolonged the MTD. The current chapter aims to identify the potential mechanism(s) through which Abs prove to be beneficial to the host. This chapter describes the investigations in to the protective actions of Abs. The direct role of Abs in arresting SCHU S4 growth in infected rat PBMCs was probed. Furthermore, the immunomodulatory effect of LVS-immune Abs administered both before and after pulmonary SCHU S4 infection was studied. The kinetics of adaptive immune response development as well as population dynamics of different cells which potentiate these adaptive immune responses were examined in the context of LALNs.

Results

SCHU S4 opsonization with IRS enhances phagocytosis and controls its intracellular growth rate

Our previous experiments suggested that the SCHU S4 growth kinetics *in vivo* (Figures 6 and 8) could be influenced by factors other than immune Abs, the direct effects of Abs on intracellular SCHU S4 growth kinetics were studied in the rat PBMCs in order to gain further insight in to protective actions of IRS. Compared to NRS, opsonization with IRS significantly enhanced the SCHU S4 uptake when the intracellular bacteria was plated and enumerated ~4hrs after infection (Figure 11A). As little as 0.1% of IRS added to PBMCs consistently enabled phagocytosis of SCHU S4 by at least a log higher than NRS. 1 d later the intracellular SCHU S4 increased to > 10^4 CFU in both the treatments and the IRS-treated wells still harbored significantly higher bacteria (Figure 11A). However, when the change in intracellular SCHU S4 between the initial and final time points was calculated, the fold increase in IRS-treated wells was significantly lower than NRS treatments (Figure11B). These results therefore suggest that enhanced phagocytosis by IRS results in decreased intracellular multiplication of SCHU S4.

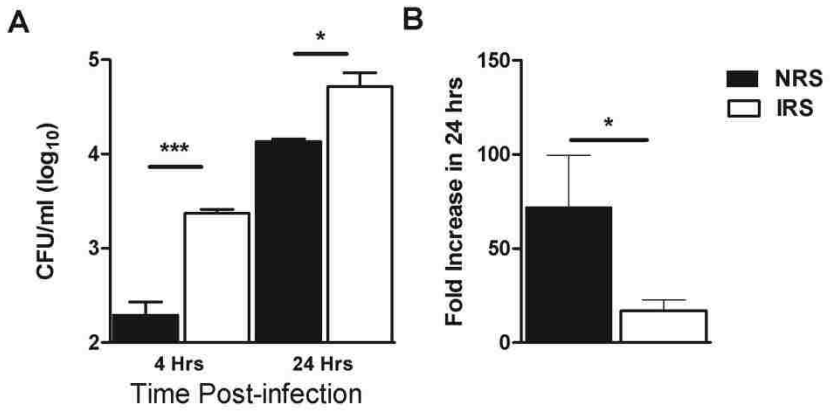

Figure 11. *Opsonization with IRS enhances phagocytosis while limiting the intracellular SCHU S4 growth*. Rat PBMCs were isolated from blood collected from naïve rats through lateral tail vein bleeding. Isolated PBMCs were plated in triplicates and infected with NRS- or IRS-opsonized SCHU S4 (0.1% serum; 10 MOI) using the gentamicin protection assay described in Materials and Methods. PBMCs were lysed at the indicated time points and the bacteria were enumerated after making appropriate dilutions and plating the lysates. Data shown in (A) are mean +/- SD of results from triplicate wells and are representative of four independent experiments of similar design. In (B), the fold-increase for each treatment was calculated by dividing the average CFU of the triplicates at 24 h p.i. by the average CFU of the triplicates at 4 h p.i. The results of four independent and similar experiments were combined and represented as mean +/- SEM. Asterisks denote statistically significant differences according to one-tailed student's t-test (*, $p<0.05$; ***, $p<0.0001$).

Multiple, but not single, IRS treatments protect naïve rats against i.t. challenge with a high dose of SCHU S4

Experiments described previously showed that IRS-treated rats harbor $>10^7$ CFU of SCHU S4 till 2 weeks p.i. in their reticuloendothelial organs (Figures 6 and 8). This high level of bacteria is lethal to a naïve rat. Furthermore, multiple IRS treatments did not reduce the bacterial burden in the sampled tissues significantly (Figure 8). In addition, the gentamicin protection assay with rat PBMCs showed that IRS-mediated uptake failed to decrease intracellular SCHU S4 burden since the bacterial numbers remained significantly high 24 h p.i. Since these data suggested that IRS mediates protection through mechanisms in addition to controlling bacterial growth, we wanted to see if repeated Ab administration protects naïve F344 rats against i.t. challenge with a high dose of SCHU S4. One group of naïve F344 rats were treated once with 250µl of IRS 24h before challenge while another group received additional 250µl-IRS treatments on days 1, 2, 3, 5, 7, 9, and 11 p.i. These groups were challenged i.t. with a high dose of SCHU S4 while the other two groups given a single treatment with 250µl of NRS or IRS and challenged with a low-dose SCHU S4 challenge served as controls. Multiple IRS treatments significantly enhanced the survival against a high-dose SCHU S4 challenge (~66% survival) (Figure 12). Moreover, the MTD was significantly delayed in the 2 rats that died from this group. In contrast, in the group that received a single prophylactic IRS treatment, 5 out 6 rats succumbed to infection. These results therefore demonstrate the benefit of repeated IRS treatments in enhancing protection even against high SCHU S4 challenge doses.

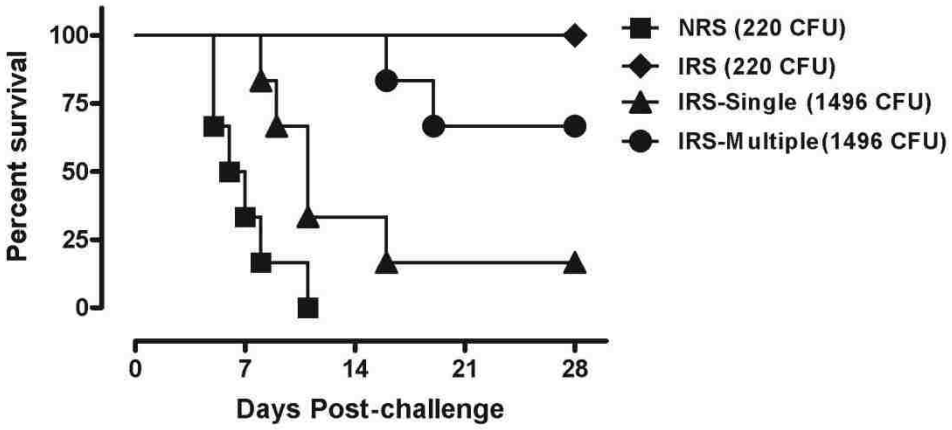

Figure 12. *Multiple IRS treatments provide protection to naïve F344 rats against a high dose i.t. SCHU S4 challenge*. Groups (n=6) of naïve F344 rats were given a single treatment with 250µl of heat-inactivated NRS or IRS 24h before challenge (-1) or multiple 250µl IRS-treatments on days -1 and 1, 2, 3, 5, 7, 9, and 11. One day after the first treatment, the animals were challenged i.t. with the indicated dose of SCHU S4 and monitored daily for survival.

Post-challenge treatment with IRS rescues F344 rats from i.t. SCHU S4 challenge

Experimental data presented so far showed that prophylactic IRS treatment provided protection against i.t. SCHU S4 challenged. To determine if the delayed Ab treatment affected the survival of SCHU S4-infected animals, naïve F344 rats were challenged i.t. with a lethal dose of SCHU S4 and different groups were treated with 250μl of IRS at 1, 24, 48, 72, and 96 h post-infection. F344 rats prophylactically treated with IRS served as positive controls. Rats that received IRS at 1, 24, and 48 h after SCHU S4 challenge were significantly protected (Figure 13A). Only 1 rat died from the group which received IRS ~1h after challenged. When the IRS treatment was delayed beyond 48h, none of the rats were protected (72 and 96h p.i.). It must be mentioned that the i.t. infection becomes bacterimic by 48 h after infection (data not shown). Rats in these groups also showed sharp weight loss. In the rats that survived the lethal challenge, the weight loss was distinctly more in the rats injected with Abs 48 h p.i. than those animals which received Abs earlier (-24, 1, and 24h p.i.). As a proof of therapeutic potential of LVS-immune Abs, these results show that IRS can provide protection when administered up to 48h post-challenge and any further delay in IRS treatment significantly compromises the survival.

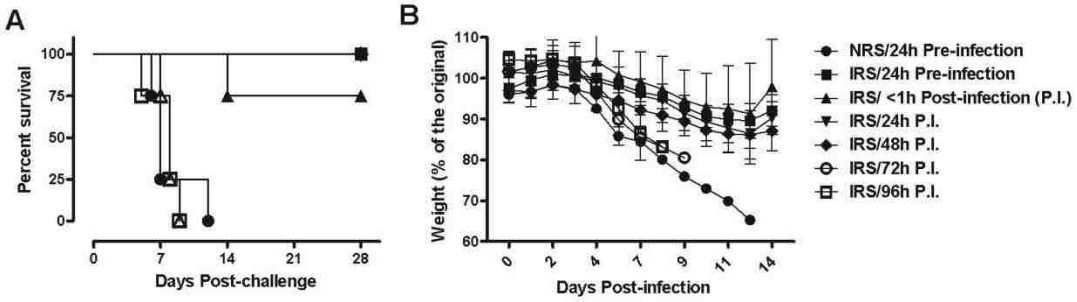

Figure 13. *Post-challenge treatment with IRS for up to 48 h provides protection*. Groups (n=4) of naïve F344 rats were challenged i.t. with 428 CFU of SCHU S4 treated with 250µl of heat-inactivated IRS or NRS at the indicated time points. The challenge dose represents the actual lung deposition determined 1h after infection. The infected rats were monitored daily for survival (A) and weight loss (B). A value of 100% indicates no weight change, and points above and below 100% represent weight gain and loss, respectively. Data represents mean +/- SD of all the survivors.

IRS-treated rats show decreased inflammation profile and damage in SCHU S4 target organs

In vitro and *in vivo* bacterial growth data as well as survival results of post-challenge IRS treatment suggested additional factors apart from control in bacterial dissemination contributed towards protection of IRS-treated rats. To this end, a series of experiments were conducted to ascertain the potential factors contributing towards survival during a lethal i.t. pulmonary infection. The cytokine and chemokine profiles in the sera and lung, spleen, and liver homogenates of SCHU S4-infected rats at various time points were evaluated by multi-analyte analysis. At the early time points (2-3 days p.i.), the cytokine levels in the IRS-treated rats were not significantly different from NRS-treated rats in any tissue homogenates, which suggested that IRS treatment did not initiate an early response to SCHU S4 infection (data not shown). At 5 days p.i., the levels of pro-inflammatory IL-1α, IL-6, TNFα, IL-12p40, IFNγ in the NRS-treated lungs and livers began to rise above the passively immunized rats and reached significantly higher levels than IRS-treated rats by 7 days p.i. (Figures 14 and 15). The spleens of the infected animals showed highest cytokine levels at day 5 p.i. and dropped by 7 days p.i. At 5 days p.i. IRS-treated rat-spleens had 2-3 times lower levels of cytokines (Figure 16). In addition, the sera of NRS-treated rats had significantly elevated amounts of cytokines at both 5 and 7 days after i.t. infection with SCHU S4 (Figure 17). The effect of upregulated inflammatory mediators on tissue damage was next evaluated by blood biochemical analysis of sera from both infected and uninfected rats. Suggestive of decreased liver and kidney injury, IRS-treated rats showed significantly lower levels of alanine aminotransferase (ALT) and blood urea nitrogen (BUN) in the circulation at 7

days p.i. (Figure 18). Taken together, these results indicated that IRS treatment is associated with decreased inflammatory cytokines and tissue injury despite harboring a high bacterial burden.

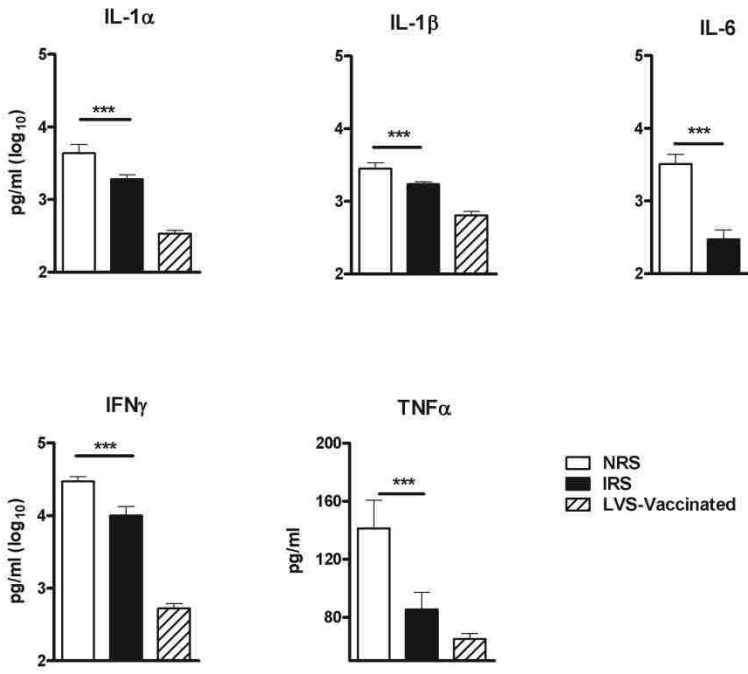

Figure 14. *IRS-treated lungs show decreased inflammatory cytokine profiles*. Groups (n=3-4) of naïve and LVS-vaccinated F344 rats were challenged i.t. with ~240 CFU of SCHU S4 after being treated with 250µl of heat-inactivated IRS or NRS 1 d earlier. Lungs were excised at days 2, 5, and 7 days postchallenge and homogenized in PBS. Supernatants collected from the homogenates were stored at -80 ° C before they were subjected to luminex analyses. Data shown represents mean +/- SD of duplicate samples of 3-4 rats from each group at 7 days p.i. from two experiments. Statistical significance of the indicated cytokines was calculated using one-tailed *t* test (***, $p < 0.0001$).

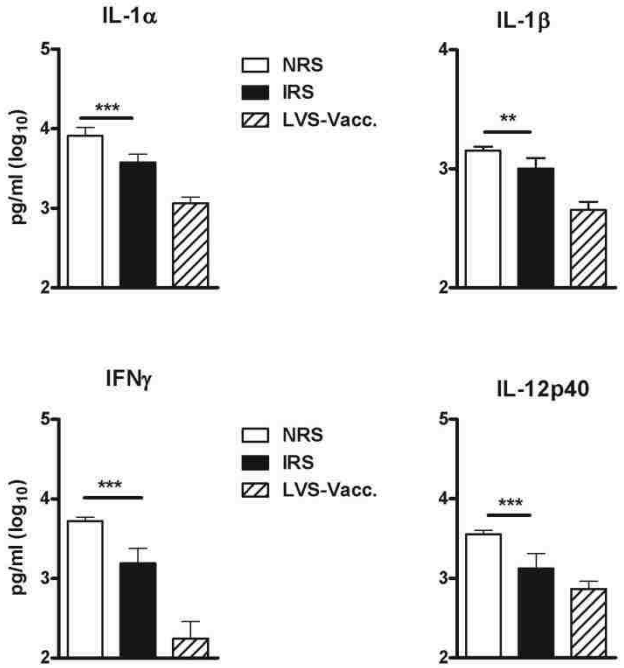

Figure 15. *IRS-treatment is associated with decreased inflammatory cytokine profiles in the liver*. Groups (n=3-4) of naïve and LVS-vaccinated F344 rats were challenged i.t. with ~240 CFU of SCHU S4 after being treated with 250µl of heat-inactivated IRS or NRS 1 d earlier. Livers were excised at days 2, 5, and 7 days postchallenge and homogenized in PBS. Supernatants collected from the homogenates were stored at -80 ° C before they were subjected to luminex analyses. Data represents mean +/- SD of duplicate samples of 3-4 rats from each group at 7 days p.i. from two independent experiments. Statistical significance of the indicated cytokines was calculated using one-tailed *t* test (**, $p < 0.001$; ***, $p < 0.0001$).

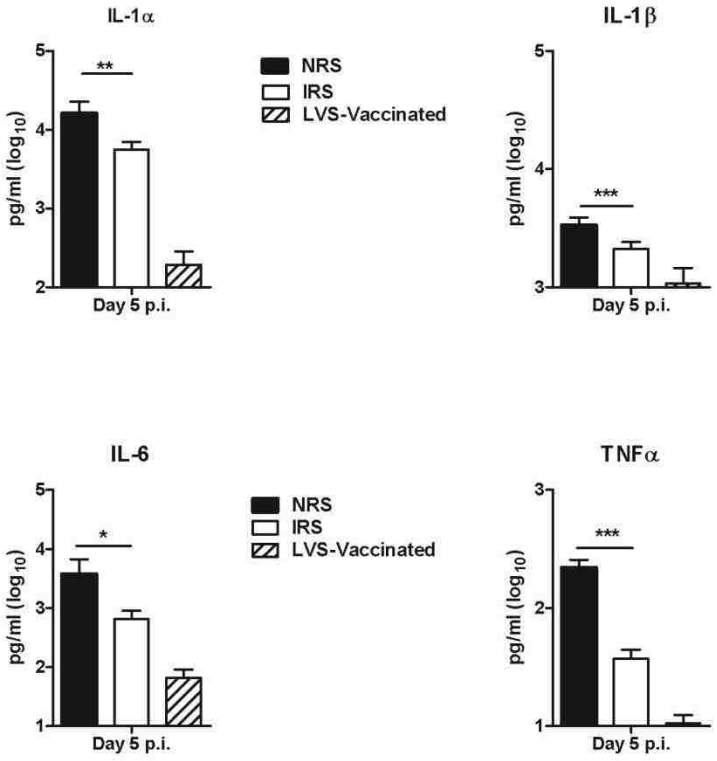

Figure 16. *Spleens of IRS-treated show decreased level of hypercytokinemia*. Groups (n=3-4) of naïve and LVS-vaccinated F344 rats were challenged i.t. with ~240 CFU of SCHU S4 after being treated with 250µl of heat-inactivated IRS or NRS 1 d earlier. Spleens were excised at days 2, 5, and 7 days postchallenge and homogenized in PBS. Supernatants collected from the homogenates were stored at -80 ° C before they were subjected to luminex analyses. Results shown are mean +/- SD of duplicate samples of 3-4 rats from each group at day 5 p.i. from two independent experiments. Statistical significance of the indicated cytokines was calculated using one-tailed t test (**, $p < 0.001$; ***, $p < 0.0001$).

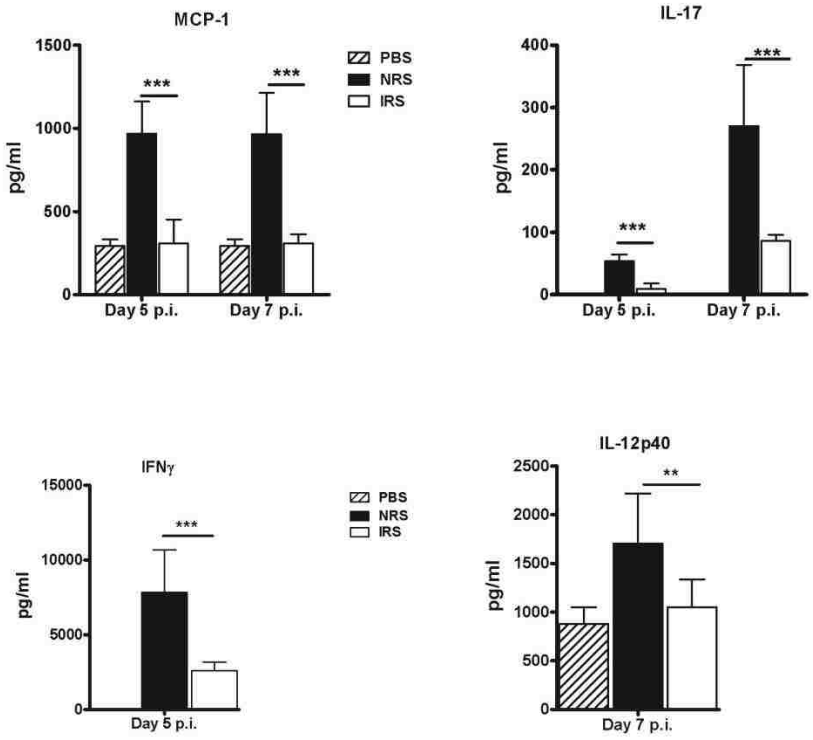

Figure 17. *Decreased cytokine levels are seen in sera of IRS-treated animals*. Groups (n=3-4) of naïve F344 rats treated with 250µl of PBS or heat-inactivated IRS or NRS. 24h later, the rats were mock-infected with PBS or challenged i.t. with ~310 CFU of SCHU S4. Sera were collected at 3, 5, and 7 days postchallenge stored at -80 ° C before they were subjected to luminex analyses. Results shown are mean +/- SD of duplicate samples of 3-4 rats from each group at the indicated time points from two independent experiments. Statistical significance was calculated using one-tailed *t* test (**, $p < 0.001$; ***, $p < 0.0001$).

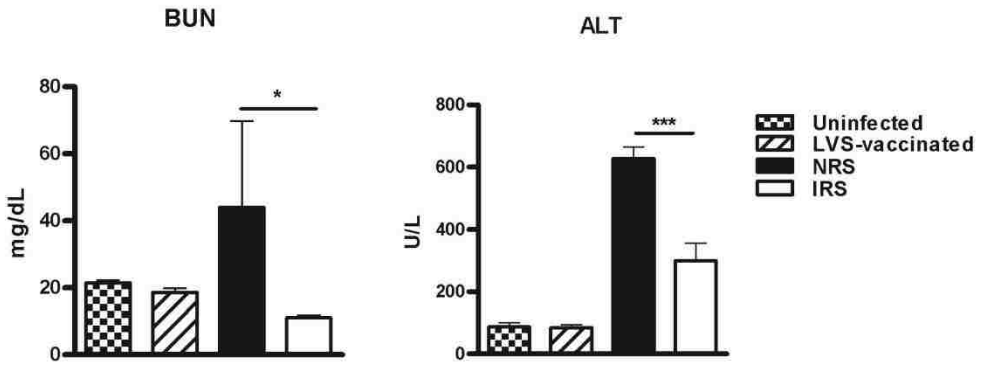

Figure 18. *IRS-treated rats show decreased liver and kidney damage*. Groups (n=4) of naïve and LVS-vaccinated F344 rats were challenged i.t. with ~260 CFU of SCHU S4 after being treated with 250µl of heat-inactivated IRS or NRS 1 d earlier. Sera were collected at 7 days postchallenge stored at -80 ° C before analyzing their blood chemistry. Results shown are mean +/- SD from one experiment. Statistical significance was calculated using one-tailed *t* test (*, $p < 0.05$; ***, $p < 0.0001$).

Prophylactic IRS treatment is associated with decreased apoptosis in the lungs

Pulmonary infection of mice with type A strains of *Ft* have been shown to result in aberrant upregulation of cytokines accompanied with increased apoptosis in the lungs and systemic organs (53). In our passive immunization studies, histopathological analysis in SCHU S4 infected rats revealed extensive tissue destruction (Figure 9). Hence a TUNEL assay was performed on tissue sections processed at different time points subsequent to i.t. SCHU S4 infection. The number of apoptosing cells in livers and spleens of both NRS- and IRS-treated rats did not differ significantly at 7 days p.i. However, the lungs of NRS-treated rats showed distinctly large foci with TUNEL positive cells at 5 days p.i. (Figure 19A), which diffused and enlarged by 7 days p.i. (Figure 19D). In contrast, lung tissue sections of IRS-treated rats had far less number of sparsely distributed TUNEL positive cells (Figure 19C) which remained constant over the next two days (Figure 19F). LVS-vaccinated rats on the other hand, showed minimal apoptosis on both 5 and 7 days p.i (Figure 19B and E).

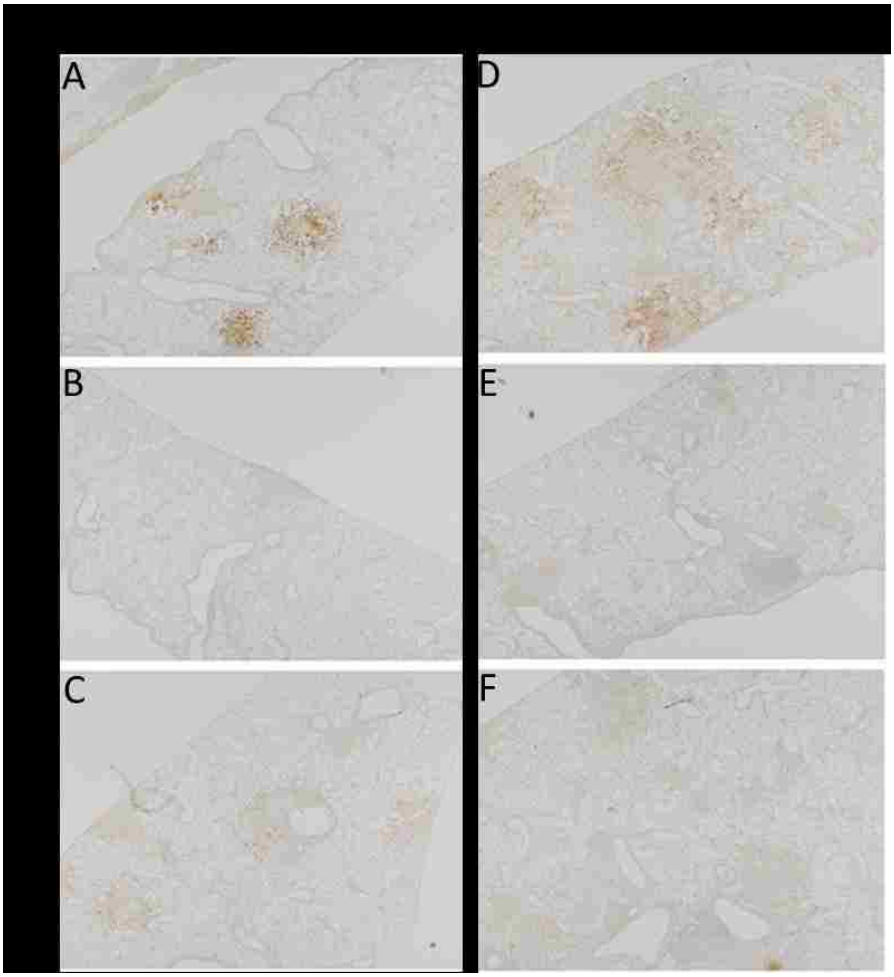

Figure 19. *Lungs of IRS-treated F344 rats show decreased apoptosis during pulmonary SCHU S4 infection*. Lungs from naïve F344 rats treated with NRS or IRS and LVS-vaccinated rats were harvested at 5 and 7 days p.i. with SCHU S4 and processed and stained as described in Materials and Methods. *In situ* TUNEL was used for indirect detection of apoptotic cells. The lungs of NRS-treated rats had large TUNEL-positive foci (A and D), while the lungs of IRS-treated rats had fewer and dispersed TUNEL-positive cells (C and F). LVS-vaccinated rats had minimal or no apoptotic cells (B and E).

Immune cell viability in LALNs of IRS-treated F344 rats is not compromised during pulmonary SCHU S4 infection

Lymphocytes and APCs home to the regional LNs in response to an infection and initiate adaptive immune responses. To further explore the effect of increased inflammation on viable cell number and the development of immune responses, LALNs were analyzed after i.t. SCHU S4 infection. Viable T, B, and DC numbers were quantified at different time points post-infection to evaluate if the cell death seen in other tissues also extended to the LALNs. This analysis would also help assess the differences in cell trafficking kinetics to the LALN between rats that survived and those that succumbed to SCHU S4 infection. As shown in Figure 20, no differences in the viable T and DC numbers were observed until 7 days p.i., when the NRS-treated rats showed a precipitous drop. Viable B cells in the IRS-treated rats on the other hand, increased to significantly higher levels at after 5 days p.i.

The critical role of IFNγ, produced mainly by T cells and to some extent by NK cells, in protection against *Ft* infections has been widely documented (119, 164, 187). To determine if the viable cell numbers in the LALN (Figure 20) translated in to IFNγ production, IFNγ ELISPOT was done using single cell suspensions obtained by density gradient separation. At both 3 and 7 days p.i. IRS-treated rats had significantly higher number of IFNγ-producing cells in the LALNs (Figure 21). These results demonstrate that LALN of IRS-treated rats have significantly high number of IFNγ-secreting cells early in the SCHU S4 infection.

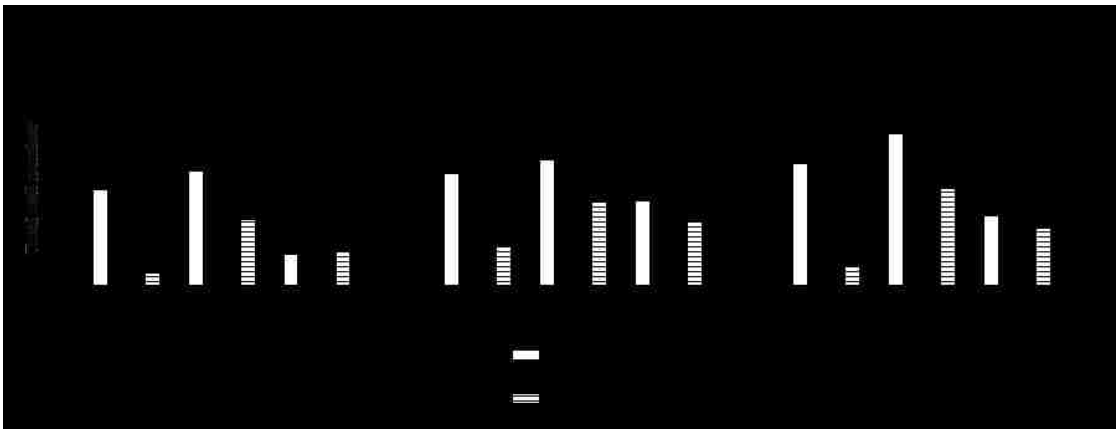

Figure 20. *IRS-treated rats do not show decreased viable immune cell numbers in in their LALNs after a lethal i.t. SCHU S4 infection*. Groups of naïve F344 rats were treated with 250µl of heat-inactivated IRS or NRS and infected i.t. with 210 CFU of SCHU S4 after 24h. Naïve rats treated with PBS and mock-infected with PBS served as controls. At the indicated time points, LALNs were excised and single cell suspensions were prepared. Total viable cell number in the LALN of each rat were counted by trypan blue exclusion before being stained with fluorchrome-conjugated Abs and analyzed by flow cytometry. Data represents mean +/-SD of 4 animals/grp for each time point. The results represent two experiments for T and B cells and DC kinetics are from one experiment of similar design. Statistical significance was calculated by one-tailed unpaired *t* test. (*, $p < 0.05$; **, $p < 0.001$).

Figure 21. *IRS-treated rats show higher number of IFNγ-secreting cells in the LALN than NRS-treated rats.* Naïve F344 rats were treated with 250µl of heat-inactivated IRS or NRS and infected i.t. with 210 CFU of SCHU S4 the next day. Naïve rats treated with PBS and mock-infected with PBS served as controls. At the indicated time points, LALNs were excised and single cell suspensions were prepared. The total number of IFNγ-secreting cells was determined by IFNγ ELISPOT without any antigenic stimulation as described in Materials and Methods. Data represents mean +/-SD of 4 animals/grp for each time point. Results from one xperiment are shown. Statistical significance was calculated by one-tailed unpaired *t* test. ($p < 0.001$; ***, $p < 0.0001$). SFCs, spot forming cells.

IRS-treated rats show decreased SCHU S4 burden in the LALNs

The effect of increased cell viability and IFNγ-secreting cells on SCHU S4 burden in the LALNs was next investigated. Since LVS was shown to utilize the migratory behavior of immune cells to seed and replicate in the mediastinal LNs during an i.n. infection (188), the effects of Ab treatment on SCHU S4 growth in LALN was next investigated. This assay, in addition, would help in understanding the manifestation of increased cell viability and IFNγ-secreting cells in IRS-treated rats. At 3 days p.i. the SCHU S4 burden in the LALNs in the rats of both serum-treated groups was similar (Figure 22), which suggested that IRS treatment did not prevent bacterial dissemination to LALN unlike liver and spleen (Figure 6). However, unlike the NRS-treated rats which showed a more or less constant SCHU S4 burden in the LALNs, the bacterial load in the LALNs of IRS-treated rats dropped to significantly low levels by day 7 p.i. When the humoral responses in the form of SCHU S4-specific IgM and IgG were tested by ELISA in the sera at 7 days p.i., the binding titers were not greater than the background in both NRS-and IRS-treated rats (data not shown). Hence, these data show that IRS treatment brought about decreased SCHU S4 growth in the LALNs.

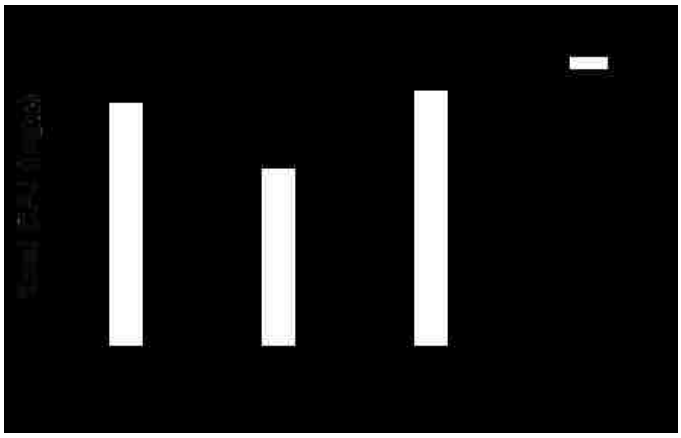

Figure 22. *IRS treatment controls SCHU S4 growth in LALNs*. Groups of naïve F344 rats were treated with 250µl of heat-inactivated IRS or NRS and infected i.t. with 210 CFU of SCHU S4 after 24h. At the indicated time points, LALNs were harvested and homogenized using ground slides before the burden was determined by dilution plating on to cysteine heart agar plates. Results represents mean +/-SD of 4 animals/grp for each time point. One-tailed unpaired *t* test was used to calculate the statistical significance (*, $p < 0.05$).

IRS treatment generates protective immunity against a high-dose i.t. SCHU S4 challenge

Bacterial burden analyses (Figures 6 and 22) in the IRS-treated rats suggested a possible development of protective immunity after the low-dose i.t. infection with SCHU S4. To determine if it did occur, protective immunity development was tested *in vivo* by re-infecting the rats with a high dose (2.1×10^4 CFU) of SCHU S4. At this dose, all the passively immunized rats were shown to succumb to SCHU S4 infection (Table II). Five weeks after surviving the initial challenge of 240 CFU of SCHU S4, all the IRS-treated rats were protected upon re-infection with a high-dose of SCHU S4 (Figure 23). The protection was similar to both the groups of LVS-vaccinated rats. These results suggest that the IRS treatment enables the development of protective immunity after respiratory infection with SCHU S4.

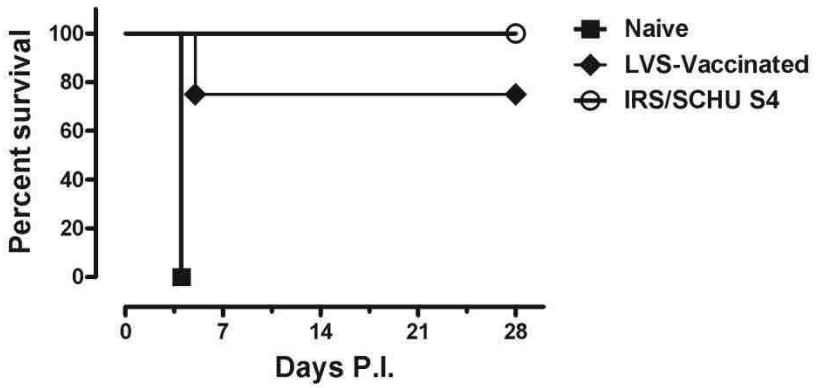

Figure 23. *IRS-treated rats develop protective immunity to survive i.t. re-infection with high dose of SCHU S4*. Groups of (n=4-6) naïve and LVS-vaccinated F344 rats along with IRS-treated rats that survived a lethal i.t. SCHU S4 infection for ~ 5 weeks were infected i.t. with 2.1 x 104 CFU of SCHU and monitored daily for survival.

Discussion

Although there is evidence of exotoxin secretion by type A *F. tularensis*, Abs may be protective for a multitude of reasons. These may include altering the repertoire of infected cells during lung infection, limiting the SCHU S4 growth rate, lowering the magnitude and slowing the course of dissemination, preventing the animals from developing an irreversible pathological state through adequate adaptive immune response development. The survival data from the therapeutic Ab treatment study suggest that the initial 48 h of infection may be decisive in determining the outcome of the infected animal (Figure 17). This is similar to the observations made by Foshay (177, 178) in tularemic patients where the poor clinical outcome correlated with a delay in passive Ab therapy. Similar to our study, delayed Ab administration 2 days after i.n. infection with LVS killed BALB/c mice (151). The ready availability of Abs and other effector molecules at the host's disposal during the early stages of infection when the bacterial number is low could enable the host to better handle the infection (153). At this stage of infection, the infection has not progressed to full-blown bacteremia. This may also explain the loss of protection in our passive immunization model when the challenge dose was increased (Table II and Figure 12).

While our data suggests the value of LVS-immune Abs in the early phase of infection (~48 h) with SCHU S4, it did not manifest as decreased bacterial burden in the sampled tissues in this time frame (Figure 9 and Chapter IV Discussion). This result appears to conflict with the direct effect of Abs on intracellular SCHU S4 growth in PBMCs *in vitro.* While opsonization with LVS-specific Abs significantly enhanced uptake of SCHU S4 and curbed its fold-increase in 24h, the SCHU S4 numbers remained

significantly higher than the controls at 24h p.i. (Figure 15). This implies that Abs may contribute towards increased infection of cells. However, these *in vitro* findings may need to be viewed with caution until further investigations are carried *in vivo* in the context of whole animal as well as developing *in vitro* and *ex vivo* assays with purified cell populations to dissect Abs' role on SCHU S4 growth. As demonstrated by Geier *et al*. Abs can indeed restrict phagosomal escape and cytosolic proliferation of *Francisella* when phagocytosed through FcγRs on macrophages (189). The resulting escalated superoxide production ultimately led to significantly decreased intracellular growth (189). Altered intracellular fate of the pathogen through IgG (190) and FcγRs have similarly been reported for *Mycobacterium* spps. and *Legionella pneumophila* (191, 192) where the IgG/FcγR-mediated endocytic pathways culminated in phago-lysosome fusion and bacterial degradation.

The reduced tissue damage in the IRS-treated rats may be due to the prevention of the development of sepsis-like condition reported recently in mice challenged with SCHU S4 (53). By delaying the hematogenous spread of SCHU S4, IRS might have averted a dysregulated immune response which results from uncontrolled production of inflammatory mediators leading to increased vascular permeability, tissue injury, and organ failure (193, 194). Restricting the tissue destruction may be critical as end-stage tularemia in humans and non-human primates associated with extensively damaged cells in the microabcesses and pyogranulomatous necrotic foci of the spleen, liver, and lymph nodes (182, 195). Similarly, in mice with respiratory tularemia caused by type A strains, a steep drop in the leukocyte count in the peripheral blood and a significant increase in the apoptosis of $F4/80^{+}$ and $Ly\text{-}6G^{+}$ cells in the necrotic areas of liver and spleen has

been reported (52, 88, 173, 196). Type A strains of *Francisella* may induce apoptosis of host cells to evade the host inflammatory responses and disseminate successfully, similar to several other intracellular pathogens like *Salmonella*, *Yersinia*, and *Streptococcus* (197-199). Thus, the death of the macrophages in a caspase-3 dependent manner during pneumonic tularemia may subvert the host innate immune responses and decrease the survival (51, 52). On the other hand, the host itself may induce apoptosis of the infected cells in a non-inflammatory manner in the early stages of infection to result in decreased pathogen viability without injuring the bystander cells. Thus, the direct influence of LVS-immune Abs on the viability of immune cells and its contribution towards survival of SCHU S4-infected animals needs to be studied.

The sustained viability of DCs, B, and T cells in the LALNs of IRS-treated rats may contribute towards rapid and adequate development of adaptive immunity, which is otherwise dramatically compromised in the NRS-treated rats (Figure 24). Since direct quantitation of cell death in LNs has not been done, it could be argued that the disappearance of cells from the LALNs at day 7 p.i. is perhaps due to failure of the aforementioned cells to home to the LNs or due to their increased egress from LNs to other anatomic locations where active SCHU S4 multiplication is occurring. However, this scenario may be unlikely since IRS-treated rats that consistently survive lethal SCHU S4 challenges had significantly higher viable cell counts than NRS-treated rats, which usually succumb to SCHU S4 infection. The *Ft*-specific adaptive T cell responses measured indirectly through IFNγ ELISPOT in our study may not be an accurate representation of T cell immunity development in IRS-treated rats, since the T cell counts are significantly disproportionate between the treatment groups at day 7 p.i. Moreover,

IFNγ production by non-T and –NK cells (DCs, NK-DCs, macrophages, neutrophils) further dilutes the clarity of our interpretations (200). Intracellular cytokine staining would identify specific cell types secreting IFNγ and thereby precisely measure the adaptive immune response development. Several pathogens exploit the inherent properties of DCs to phagocytose the invading pathogens. Similar to *Mycobacterium*, (201, 202) *Ft* disseminates and multiplies in the LDLNs mainly by infecting the lung DCs (188). This ability has been attributed to its virulence during pulmonary infection. Immune Ab treatment may be preventing the manipulation of lung DC characteristics in our model since the bacterial burden in the LALNs of IRS-treated rats was significantly higher than negative controls at 3 days p.i. It must be noted that at the same time point, SCHU S4 dissemination to liver and spleen was significantly lower than NRS-treated rats in all the experiments (Figure 9). However, it needs to be determined if SCHU S4 reached the LNs of infected F344 rats alone or in association with DCs as shown by Bar-Haim et al. with LVS (188). The gradual and significant decline of SCHU S4 in the LALN of IRS-treated rats by day 7 p.i. may indicate adaptive immune response development in the LNs.

S. typhimurium (203) and *M. tuberculosis* (204, 205) have been shown to undermine the Ag presenting capability of DCs. However, upon targeting *Salmonella* to the FcγRs on DCs with specific IgG, the impaired Ag presenting ability was reversed (203). Although there is no proof yet of type A *Ft* strains employing the same immune evasion strategy, Bosio et al. have reported significantly lower MHC II expression on murine DCs during pulmonary infection with SCHU S4 (55). Further studies on Ab-DC interactions and kinetics of *Ft* and immune cell trafficking in the context of our passive

immunization model are therefore warranted. Overall, our data suggests the possible role of Abs in rapidly shepherding SCHU S4 to the draining LNs in a form favorable to eliciting a rapid adaptive immune response, which ultimately confers immunological advantage to the passively immunized rat.

With regard to the mechanism of Ab-mediated protection against pulmonary tularemia by type A *Ft* strains in F344 rats, our data suggests LVS-immune Abs exert their effect during the first 2 days after infection by acting on the components of the innate immune system. Since IRS-treatment prevented the development of a hyper-inflammatory state associated with immune cell death and tissue damage, the end result of these as yet unidentified interactions was immunomodulatory in enabling the development of adaptive immunity and the survival of the infected animals. Indeed, dysregulated immune response to pulmonary type A *Ft* infections has been reported to be associated with poor outcome in mice. Hypercytokinemia observed in various SCHU S4-infected tissues (Figures 18 to 21) of NRS-treated rats is an important attribute of sepsis syndrome. Hyperactive immune response has been shown to ultimately lead to the development of disseminated intravascular coagulopathy, increased vascular permeability, and organ injury and failure. IRS treatment may have controlled the development of unrestrained inflammatory response and thereby prevented the progression of SCHU S4 infection to a sepsis-like condition. Thus, IRS-treated rats may have escaped death resulting from type A *Ft* infections despite high bacterial burden. In summary, the current study provides a better understanding of SCHU S4 virulence mechanisms and the role of immune Abs in limiting the pathogenesis and death associated with lung infection.

CHAPTER 6: DISCUSSION AND CONCLUSIONS

Summary and Future directions

The research study presented herein provides evidence for the protective role of Abs against pulmonary tularemia caused by type A strains of *Ft.* The data shows that while Abs alone significantly prolonged the survival of infected animals in the absence of T cells, their ability to provide long-term survival critically depended on CD8 T cells. In addition, immune Ab treatment prevented dissemination of SCHU S4 from the lungs and was associated with decreased inflammation and tissue damage. By limiting the loss of immune cell viability, IRS treatment may have allowed for the development of adaptive immunity, which ultimately helped clear the infection. This concept adds to our understanding of Abs' role in pulmonary tularemia caused by SCHU S4 and provides insight into further studies.

Type A strains of *Ft* are notorious for the wide array of virulence mechanisms they employ to successfully evade the host. By being able to be successfully vaccinated and passively immunized, the F344 rat strain has certain advantages over other rodent models in studying respiratory tularemia., eExperiments examining the virulence attributes of SCHU S4 in rats with pneumonic tularemia and how Abs aid in overcoming these pathogenic mechanisms would further characterize the F344 rat passive immunization model., In addition, the role of complement in the inflammatory process has not been tested in this system. With the recent advances in rat gene knockout technologies (206, 207), delineation of IgG-mediated protective mechanisms is possible.

Signals transduced by cross-linking FcγRs through IgG have several downstream effects on inflammatory processes. The indispensability of IgG for protection against

SCHU S4 in this study therefore warrants further experimentation on the role of FcγRs. First, signaling cascades and resulting SCHU S4 intracellular fate during FcγR-mediated phagocytosis through IgG opsonization must be compared with those events resulting from when FcγRs alone are engaged. This would help tease out the relative importance of IgG and FcγRs. Second, the synergistic effects of phagocytosis by FcγRs and other phagocytic receptors need to be studied. In the study by Celli & coworkers, although SCHU S4 uptake by both FcγRs andcomplement receptors (CRs) resulted in decreased phagosomal escape while only FcγR-mediated phagocytosis caused respiratory burst, which enhanced bacterial killing (189). Hence the net result of the combined actions of these different receptors would be interesting when probed using different opsonins. Third, since our study clearly points to Ab-mediated effects during the first three days of SCHU S4 infection, the role of Ab-FcγR interactions on chemokine expression in different cell types must be elucidated. FcγR engagement by IgG was shown to induce MIP-1α and other chemokines in human and mice myeloid leukocytes (208, 209). And finally, infecting rats with Ab-opsonized SCHU S4 could give us further insight into the potential outcomes resulting from Ab- FcγR interactions.

While macrophages are preferentially infected at a high rate by type A *Ft*, several research groups have recently found other cells also to be infected. SCHU S4 mutants, which cannot replicate in macrophages, were shown to infect and multiply in non-macrophage cells and cause lethal disease in mice (210). In addition, the invasion of RBCs reported by the Nau lab has profound implications on the role of Abs in altering the SCHU S4-infected outcomes (59) since it is therefore possible that Ab opsonization

of SCHU S4 *in vivo* could skew towards increased uptake through FcγRs and subsequent killing. This effect of Abs on the repertoire of infected cells could also have consequences on cell recruitment through Ab-mediated chemokine production. Hence the percentages of different cell populations and the kinetics of their infection in addition to their recruitment to the lungs must be tested with fluorochrome-tagged SCHU S4 to better understand the actions of LVS-specific Abs in the F344 rats.

SCHU S4-infected animals succumb to infection before developing T cell immunity, the critical importance of which has been exhaustively documented (118-121). Hence it is possible that the type A strains obstruct multiple steps involved in the development of T and B cell responses. For example, different pathogens have been shown to inhibit peptide presentation on MHC molecules (203-205). It needs to be determined whether SCHU S4 interferes with these pathways and if Abs prevent this interference and enable primary immune responses in the lymphoid tissues, which is normally seen during vaccination with live immunogen. Also, it would be interesting to see if Abs hasten the development of primary immune responses in the regional LNs.

The increased SCHU S4 levels in the LALNs of IRS-treated rats early in the infection did not correlate with the dissemination to liver and spleen (Figures 9 and 27). Performing *in vivo* vital staining experiments would provide information if the pathogen travelled to the LALNs on its own through capillary leakage or through migrating phagocytes subsequent to their infection. In addition, the dissemination of SCHU S4 to LNs and other tissues could be compared at the same time in the same rats to better

understand the role of Abs in rapid dissemination to the LNs to give a head start in initiating rapid immune responses.

Our findings point to the critical role of CD8 T cells in protecting the passively immunized rats (Figure 13). Upon recognition of peptides in context with MHC I molecules, CD8 T cells primarily mediate their effects against intracellular infections by causing apoptosis of infected cells induced by perforin and granzyme B or by FasL. They also secrete IFNγ and TNFα, which have been shown to be critical against *Ft* infections. The exact nature of CD8 T cells actions in the context of our passive immunization model and the influential role(s) of Abs on CD8 T cells must be studied. A simple and effective way to study the actions of these components is by developing an *in vitro* assay similar to the macrophage T cell assay developed by Elkins and coworkers (122, 123). The effect of Abs and immune T cells alone, and in combination, can then be evaluated by measuring the SCHU S4 intramacrophage growth and cytokines and other effector molecules. The assay can be translated *in vivo* by adoptive transfer of T cells and Abs and the resulting mechanisms and net effects can be studied and compared. Thus a clear understanding of how Abs and T cells coordinate during pulmonary *Ft* infection could help in designing vaccines with greater efficacy. Besides the timely development of T cell immunity, immune Abs' role in controlling the cell death must be elucidated. The relative levels of different cell death pathways *viz.* necrosis, apoptosis, and pyroptosis during pulmonary SCHU S4 infection should be studied with the availability of appropriate tools for studying rats. Since the bacterial burden the tissues of both NRS- and IRS-treated rats is more or less similar, limiting the inflammation and

cell death could be crucial for the positive outcome in IRS-treated rats. Although the levels of different DAMPs were not measured in the current study, their relevance in pathogenesis during an infection is gaining ground (211, 212). Elevated levels of high-mobility group box-1 (HMGB-1) and other DAMPs have been reported recently in mice infected i.n. with SCHU S4 (53). While it is possible that a complex interplay of host- and pathogen-derived factors may raise the levels of DAMPs and lead to the development of sepsis-like condition and multiple organ failure, the direct effect of Abs on the release of DAMPs must be studied. The LPS levels in the plasma of infected animals could provide further clues regarding the reduced inflammation in the IRS-treated rats. Perhaps, Ab binding to *Ft* surface and subsequent phagocytosis could limit LPS shedding. The increased LPS levels could trigger an aberrant and chronic activated inflammatory state and this could be easily tested both *in vitro* and *in vivo.* Finally, a number of *in vitro* experiments suggested here could be performed with human cell samples and sera to see if the findings in rats and humans are similar. This would ultimately aid in designing better vaccines and therapeutics by incorporating Ab functions in to these designs.

Conclusions

In summary, the results and interpretations described in this manuscript detail the protective role of immune IgG Abs in protection against respiratory tularemia caused by the virulent type A strains of *Ft.* Overall, the data shows that Ab treatment is associated with decreased inflammation and immune cell death prior to the development of T cell

immunity. Moreover, the experiments provide the groundwork and clues for understanding the several other mechanisms through which Abs enhance survival during respiratory infections with bacterial pathogens. The model ultimately adds significantly to our understanding of virulence mechanisms of pathogens in general and how Abs modulate the host-pathogen interactions to have a positive influence on the overall health of the infected host.

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