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Features of sepsis caused by pulmonary infection with *Francisella tularensis* Type A strain

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Abstract

The virulence mechanisms of *Francisella tularensis*, the causative agent of severe pneumonia in humans and a CDC category A bioterrorism agent, are not fully defined. As sepsis is the leading cause of mortality associated with respiratory infections, we determined whether, in the absence of any known bacterial toxins, a deregulated host response resulting in sepsis syndrome is associated with lethality of respiratory infection with the virulent human Type A strain SchuS4 of *F. tularensis*. The C57BL/6 mice infected intranasally with a lethal dose of SchuS4 exhibited high bacterial burden in systemic organs and blood indicative of bacteremia. In correlation, infected mice displayed severe tissue pathology and associated cell death in lungs, liver and spleen. Consistent with our studies with murine model strain *F. novicida*, infection with SchuS4 caused an initial delay in upregulation of inflammatory mediators followed by development of severe sepsis characterized by exaggerated cytokine release, upregulation of cardiovascular injury markers and sepsis mediator alarmins S100A9 and HMGB-1. This study shows that pulmonary tularemia caused by the Type A strain of *F. tularensis* results in a deregulated host response leading to severe sepsis and likely represents the major cause of mortality associated with this virulent pathogen.

Keywords

tularemia; SchuS4; sepsis; hypercytokinemia; bacteremia; alarmin

1. Introduction

Francisella tularensis, the causative agent of tularemia, is a Gram negative intracellular coccobacillus. Although clinical manifestations of the disease vary with the route of infection and the organs affected, respiratory infections with *Francisella tularensis* Type A strains cause the deadliest form of disease resulting in 30-40 % mortality with an infectious dose of 1-10 organisms [1]. The ease of an aerosol spread of virulent type A strains coupled

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with the low infectious dose range led to its CDC classification as a Class A bioterrorism agent [1]. The mechanisms involved in the extreme virulence of *Francisella* infections remain to be fully understood. The high degree of lethality is particularly intriguing in light of the lack of known bacterial factor/s acting as toxin/s. A pathogenicity island comprising many virulence factors has been defined but most of these factors are required for growth of the organism inside the host cell [2]. We hypothesized that host determinants are involved in the disease severity associated with this infection as deregulated host responses are shown to play an important role in determining the lethality associated with several pulmonary infections [3]. The current study is aimed at identifying host factors/ immune responses that may be contributing to the lethal outcome of respiratory tularemia caused by Type A strain of *Francisella*.

Sepsis is the leading cause of mortality associated with respiratory infections [4]. In fact, infections occurring via the respiratory route are more likely to cause sepsis as compared to other routes [5]. During sepsis, excessive activation of inflammatory and coagulation cascades along with activated innate immune cells leads to vasodilatation, microvascular clotting and tissue edema [6, 7]. In tandem, wide-spread cell death depletes immune cells necessary for fighting the infection as well as releases endogenous host proteins which act as “alarmins” or danger signals that exacerbate the ongoing inflammation, thus forming a positive feed-back loop [8-10]. The excessive tissue destruction coupled with coagulopathy results in loss of blood flow to vital organs causing multiple organ failure and ultimately death.

Early studies on tularemia pathogenesis in the 1940s showed the presence of bacteria in spleen, liver and blood following intraperitoneal infection, suggesting a bacteremia resulting from the infection [11, 12]. However, a wealth of more recent experimental and epidemiological studies of several respiratory diseases has now established that sepsis development is a complex interplay of several immune networks as indicated above. Indeed, recent studies from our laboratory showed that respiratory infection with the murine model strain *F. novicida* results in inflammatory cell infiltration and profound cytokine and chemokine expression consistent with severe sepsis [13-15]. In addition, bacteremia and dissemination of bacteria to systemic organs followed by extensive cell death in these *F. novicida* infected mice correlated with upregulation and release of alarmins [14-16]. Furthermore, a depletion of CD4⁺ T cells by apoptosis in lungs of *F. novicida* infected mice was observed consistent with previous studies showing extensive lymphocyte apoptosis during sepsis [17, 18].

As *F. tularensis* Type A strains are the fully virulent human pathogens, it is important to determine whether severe sepsis is evident during pneumonic infection with Type A strains which could lead to new strategies for effective therapeutics. To this end, mice infected intranasally with the Type A strain SchuS4 were monitored for several characteristic indicators of severe sepsis. Consistent with our previous studies with *F. novicida* [13-15], our data indicate that pulmonary infection of mice with Type A strain SchuS4 causes severe sepsis resulting in a rapid death before adaptive immunity can be established. The results of this study indicate several targets for therapeutic intervention of this debilitating infection.

2. Results

2.1. Intranasal infection with SchuS4 results in bacteremia and severe pathology in systemic organs

Mice intranasally infected with various doses of Type A strain SchuS4 started displaying signs of disease (lethargy, hunched gait and piloerection) by 72 h p.i. All the mice infected with a dose of 3×10^2 CFUs became moribund by 96 h p.i. and succumbed to infection the

same day (Fig. 1A). For all the experiments described henceforth, mice were infected with this dose of SchuS4. An analysis of bacterial burden in systemic organs and blood of the SchuS4 infected mice showed that while the bacteria were detected only in lungs at 24 hp.i., high bacterial loads were recovered from lungs, liver, spleen as well as blood at 72 and 96 hp.i., indicative of bacteremia (Fig. 1B). This was also reflected in severe tissue pathologies in lungs, liver and spleen of infected mice at 72 h p.i., a time when the mice started displaying external signs of disease. The H & E stained tissue sections of mock control mice displayed normal lung architecture with clear air spaces (Fig. 2A), normal histological appearance of liver (Fig. 2B) and defined red and white pulps in spleen (Fig. 2C). The infected mice, on the other hand, exhibited large perivascular as well as peribronchial granuloma-like areas of cellular infiltration in lungs (Fig. 2D). Histopathological changes in liver were marked by increased sinusoidal spaces, accumulation of fat bodies in hepatocytic cytoplasm as well as appearance of hepatic granulomas (Fig. 2E, arrows). The spleen displayed extensive destruction of lymphoid follicles as well as the cells in red pulp (Fig. 2F). Bacteremia, extensive immune cell infiltration and tissue destruction in these organs following pulmonary infection with SchuS4 is indicative of a systemic inflammatory response typical of sepsis syndrome.

2.2. Pulmonary infection with SchuS4 results in extensive cell death in systemic organs

Extensive tissue destruction during SchuS4 infection prompted an analysis of the extent of cell death in lungs, liver and spleen of infected mice. A TUNEL assay at 96 h p.i. showed that the most extensive cell death was visible in lungs and spleen (Fig. 3). In lungs, most of the TUNEL positive cells were localized to the lesions, while the spleen showed massive cell death occurring in red pulp as well as white pulp (Fig. 3D and 3F). The liver, on the other hand, exhibited TUNEL positive cells concentrated in small foci of infiltrating immune cells, possibly depicting necrotic granulomas (Fig. 3E). While all the hepatic granulomatous regions stained positive for bacteria (Fig. 3H and 3K), cell death was not detected in all the granulomas. The lungs and spleen, on the other hand showed the presence of bacteria in most areas of cell death analyzed (Fig. 3G, 3J, 3I and 3L).

2.3. Pulmonary infection with SchuS4 causes hypercytokinemia following an initial delay in immune response

Hypercytokinemia or “cytokine-storm” refers to uncontrolled upregulation of pro- as well as anti-inflammatory host mediators and is indicative of sepsis syndrome. Quantitative analyses of several cytokines, chemokines and host biomarkers were performed at various times post-infection in lungs of SchuS4 infected mice. Similar to our observations with the murine model strain *F. novicida* [13, 15], no significant change in the levels of these host mediators was observed at 24 h p.i. (Fig. 4 and Fig. 5). However, at 72 h and 96 h p.i., extensive upregulation in levels of both pro- and anti-inflammatory cytokines was observed (Fig. 4). The prototypic inflammatory cytokines TNF- α , IL-6, CXCL-1 and IL-1 as well as anti-inflammatory cytokine IL-10, deduced as markers of sepsis [19], were upregulated 10-2000 fold over mock control levels (Fig. 4). In addition, the results showed a profound upregulation in levels of several chemokines involved in activation and chemoattraction of innate immune cells in these mice at 72 and 96 h p.i. (Fig. 4). The highly elevated concentrations of CXCL1 (KC), CCL2 (MCP-1), and CXCL10 (IP10) in SchuS4 infected mice compared with mock controls (Fig. 4 and data not shown) are of particular interest as these cytokines at excessive levels correlate with disease severity, sepsis and mortality [20].

2.4. Mediators of coagulopathy and cardio-vascular injury are upregulated during pulmonary SchuS4 infection

Coagulopathy or deregulation of the coagulation cascade is a major clinical complication of sepsis [21]. Upregulation and activation of coagulation factors causes disseminated

intravascular coagulation leading to multi-organ failure due to disrupted blood circulation [21]. The levels of several of these mediators were examined in lung homogenates of SchuS4 infected animals at various times p.i. As shown in figure 5, SchuS4 infection resulted in upregulation in levels of tissue factor, Factor VII and fibrinogen, all of which are involved in coagulant activities. Pro-inflammatory cytokines such as IL-6 and TNF- α as well as acute phase proteins are known to accentuate the coagulation cascade [22]. Indeed, SchuS4 infection resulted in upregulation of classical acute phase proteins C-Reactive protein (CRP) and serum amyloid protein (SAP) as well as the scavenger protein haptoglobin by several fold over the normal mock control levels (Fig. 5). In addition to mediators of coagulopathy, the markers of cardiac and liver dysfunction such as the vasoconstrictor protein endothelin-1 and serum glutamic oxaloacetic transaminase (SGOT) were elevated in a time dependent fashion which is indicative of progressive hemodynamic and cardiovascular instability during pulmonary SchuS4 infection (Fig. 5).

2.5. Sepsis mediators S100A9 and HMGB1 are upregulated during SchuS4 infection

During sepsis, host immune responses causing tissue and cell damage can be triggered by pathogen associated molecular patterns (PAMPs) during an infection or by intracellular host molecules (alarmins) released by dead cells during trauma. We tested the levels of two prototypic alarmins S100A9 and HMGB-1 in systemic organs and sera of mice infected with SchuS4. As depicted in Fig. 6A and B, mock control animals showed baseline levels of expression of S100A9 in lungs, liver as well as in spleen. The SchuS4 infected mice, on the other hand, exhibited extensive upregulation and relocalization of this alarmin in cells of these organs, as observed by IF staining (Fig. 6A) and western blot analysis (Fig. 6B). In lungs, the S100A9 expression was mainly associated with lesion areas which are the main sites of localization of infiltrating immune cells (Fig. 2D), but not in the tissues away from foci of immune cell infiltration. Importantly, this alarmin was also observed to be extracellularly localized, particularly in lungs (Fig. 6A G'-I') where the extent of cell death was the highest and the S100A9 staining could be observed in cell-free areas depicted by absence of nuclear staining (Fig. 6A G'-I' asterisks). As the systemic increase in levels of alarmins is also determined by their detection in body fluids during sepsis [23], we looked at the levels of HMGB-1 in sera of mice infected with SchuS4. As shown in Fig. 6C, HMGB-1 was undetected in sera of mock control mice and the infected animals at 6 hp.i.. In contrast, HMGB-1 was substantially increased in sera of SchuS4 infected mice at 24 hp.i. onwards (Fig. 6C).

3. Discussion

The mechanisms associated with the extreme virulence of human Type A strains of *Francisella tularensis* are not well understood. The respiratory infections present the deadliest form of the disease caused by this pathogen. As sepsis develops most commonly with respiratory infections and with significant mortality [5], the current study was carried out to determine if severe sepsis is associated with the lethality of Type A *F. tularensis* infections in mice. Our results indicate that mortality of the mice infected intranasally with Type A strain SchuS4 is associated with the development of severe sepsis. Additionally, this study highlights the utility of *F. novicida* as a surrogate organism to understand mechanisms of sepsis development since our previous studies have shown a similar sepsis syndrome occurring during respiratory infection with this murine model strain [13, 15].

Hematogenous spread of respiratory pathogens is a major risk factor for development of sepsis with lung infections resulting in sepsis at twice the frequency as infections at other sites [24]. In the current study, bacteremia and systemic dissemination occurred during respiratory infection with the Type A strain SchuS4. Further, histopathological observations shown here indicated that pathophysiological progression of pulmonary tularemia following

infection with SchuS4 is coupled with immune cell death resulting in development of tissue pathologies in systemic organs. This is consistent with other reports showing severe pathology in systemic organs during pulmonary infection by Type A and Live Vaccine strain (LVS) of *Francisella* [25-27]. These observations indicate that although lungs are the initial site of bacterial replication, bacteremia results in systemic dissemination of the pathogen to liver and spleen causing severe tissue pathologies in these organs. In sepsis, a high bacterial load can overwhelm the host defense system triggering a systemic inflammatory reaction [28]. The exaggerated inflammatory response culminates in extensive cell death which results in tissue pathology and depletion of immune cells necessary for fighting the infection [29]. Our previous studies with *F. novicida* have shown extensive cell death as well as apoptosis induced loss of immune cells during respiratory infection [14, 17], and our results with SchuS4 infected mice parallel these observations. We have also observed a depletion of T cells in the lungs of SchuS4 infected mice (data not shown). These results are consistent with studies where sepsis patients exhibited apoptosis induced loss of lymphocytes [30]. This wide-spread cell death in organs of SchuS4 infected mice observed in the current study is indicative of multi-organ dysfunction correlating with sepsis syndrome.

Similar to previous studies by our laboratory [13, 15] and Bosio et al [31], we observed a delay in the host immune response during the initial 24 h of infection with SchuS4 where no upregulation of host immune mediators was observed despite substantial bacterial burden in the lungs (Fig. 1, 4 and 5). Following this, the onset of hypercytokinemia observed corroborated with the appearance of tissue pathology and cell death in systemic organs only at later times of infection i.e. 72h onwards (Fig. 2 and 3 and data not shown). The extensive cell death observed in the infected organs by TUNEL staining in the current study and by increased levels of activated caspase-1 in our previous study with *F. novicida* [15] is in contrast with the findings of Bosio et al where no apoptosis by way of caspase-3 activation was observed at 72 hp.i. [31]. The differences observed in two studies are most likely due to the infection dose, where Bosio et al used 50 CFUs, while this dose delayed the death of mice by 24h in our study as shown in Figure 1A.

A hypercytokinemia or unbridled upregulation of inflammatory cytokines and chemokines was observed during SchuS4 infection in this study. Several of these mediators act as chemoattractants and activators of innate immune cells leading to production of secondary mediators such as reactive oxygen and nitrogen species [32]. These innate immune mediators non-specifically destroy host cells contributing to tissue pathology [32]. The upregulation of these immune mediators in SchuS4 infected animals correlates with the pathological analyses of systemic organs from these mice which show extensive cellular infiltration as well as profound cell death. During sepsis, to counter this acute phase inflammation, an anti-inflammatory response develops which leads to immune depression and increased susceptibility to secondary infections [33]. Consistent with this in the current study, 1600 fold increase in inflammatory cytokine IL-6 and 26 fold increase in TNF- α was coupled with a 7 fold increase in anti-inflammatory cytokine IL-10 levels. To the best of our knowledge, systematic examination of such wide array of cytokines and chemokines in the same experimental setup as performed in this study has not been done with other murine models of sepsis. Nonetheless, the increase in levels of sepsis marker cytokines and chemokines TNF- α , IL-10, IL-6, CCL2 (MCP-1), CXCL2 (MIP-2) and IL-1 β in the current study were at par with that observed in other murine model of lethal sepsis developed by LPS injection or by cecal ligation and puncture [34-37]. Although the kinetics of hypercytokinemia development 72h p.i. onwards in SchuS4 induced sepsis were similar to those observed in whole lung homogenates of mice infected with similar doses of *F. novicida* strain U112 [13, 14], subtle differences were observed in the levels of some cytokines (Table 1). For example, while similar levels of IFN- γ , TNF- α , IL-10, CXCL10

and CCL2 were observed in both cases, infection with SchuS4 induced a higher increase in the levels of IL-6, IL-18, IL-1 β and CXCL1. On the other hand, *F. novicida* infection elicited higher levels of IL-1 α , CCL4, CCL9 and CXCL6 as compared to SchuS4 infection.

A dysregulated production of inflammatory cytokines and chemokines can result in persistent activation of secondary cascades such as acute phase proteins, complement and coagulopathy leading to dysfunction of systemic organs. In this study respiratory SchuS4 infection resulted in upregulation of several such host mediators. Endothelin-1 has been suggested to play a role in development of sepsis induced myocardial depression [38]. SGOT, an enzyme normally present in liver is released during sepsis indicating liver damage [39]. Additionally cytokines such as TNF- α , IL-1 β and IL-6 are also causative agents of sepsis induced cardiac injury [40]. These cytokines were highly upregulated in SchuS4 infected mice. The sepsis induced upregulation in markers of cardiac injury during SchuS4 infection may account for the development of endocarditis observed during this infection [41]. Acute phase proteins such as CRP (C-reactive protein), SAP (serum amyloid protein) and haptoglobin are elevated during sepsis in response to up-regulated IL-6 levels [42]. During SchuS4 infection upregulation of IL-6, IL-1 and TNF- α , cytokines known to induce the acute phase response, correlated well with the elevation in levels of these acute phase proteins [20]. The acute phase reaction, in turn, leads to increased production of complement and coagulation proteins, which generally have a protective role in containment of infection. However, the same protective mediators can be deleterious and lead to tissue damage if the intricate balance between these complex immune pathways is lost. Excessive upregulation in mediators of inflammatory, acute phase as well as coagulation pathways during SchuS4 infection indicates that this balance is indeed compromised providing further evidence of sepsis syndrome developing during respiratory SchuS4 infection.

Vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (FGF-basic) are required for growth and survival of endothelial cells and angiogenesis, respectively. Curiously, SchuS4 infection resulted in significant reduction in levels of FGF-basic (Fig. 5). This suggests a lack of vascular remodeling during infection. The levels of VEGF were also slightly reduced at 72 hp.i. although this change was not statistically significant. While the role of basic FGF in sepsis is undefined, a decrease in lung levels of VEGF associated with endothelial cell apoptosis has been observed during experimental sepsis [43] as well in septic patients [44].

Under various pathological conditions, the dead or dying cells release mediators which act as “danger signals” to exacerbate the inflammatory response resulting in a positive feedback loop for inflammation and tissue destruction [45]. Alarmins are endogenous proteins lacking signal sequences for active secretion but are rapidly released into the extracellular milieu in response to tissue injury or infection. Once released, they act as chemoattractants and immune activators to “overstimulate” immune cells thus converting the otherwise beneficial immune responses into excessive, damaging inflammation [8]. S100A9 and HMGB1 are two such prototypic alarmins. S100A9 belong to the S100 family of calcium binding cytosolic proteins, known to interact with cell cytoskeleton and as markers of phagocyte differentiation, have recently been recognized as alarmins which, upon extracellular release can activate innate immune receptors [46]. Upregulation in levels of S100A8 and S100A9 has been identified as a marker for inflammatory processes in diseases such as rheumatoid arthritis, juvenile idiopathic arthritis, and inflammatory bowel disease [47, 48]. In the current study, although we could not detect S100A9 in sera of infected animals, its upregulation in systemic organs with the progression of disease and the extracellular localization of S100A9 coincided with extensive cell death observed by TUNEL staining in SchuS4 infected animals. Under normal conditions, HMGB-1, another prototypic alarmin, remains associated with the nucleus and acts as a transcriptional regulator. However,

extracellular HMGB-1, secreted by activated immune cells or released passively from dead cells, acts as an alarmin and has been identified as a late mediator of sepsis [8]. The current study showed an increase in HMGB1 at systemic level in sera of SchuS4 infected animals. The upregulation and release of S100A9 and HMGB1 observed with SchuS4 infection was similar to that in our previous studies with *F. novicida* [14, 15]. These and possibly other alarmins during respiratory SchuS4 infection likely contribute to the excessive inflammatory response culminating in severe sepsis.

Our previous studies have shown that a sepsis syndrome occurs during respiratory *F. novicida* infection. In the absence of any endotoxin activity of LPS of Type A strains of Francisella or the murine model strain *F. novicida* [49], the similarity of disease progression and sepsis development during infection with both these strains highlights the fact that a deregulated host response with extensive cell death releasing immune activating mediators is central to disease development in tularemia.

Taken together, the results of the present study indicate that following an initial delay in the innate immune response, a severe sepsis elicited by bacteremia, hypercytokinemia and alarmin release occurs at the later stages of infection which is likely responsible for the mortality associated with respiratory Type A Francisella infections. Although the role of these mediators in pulmonary Francisella induced sepsis is speculative at this stage, this study reports, for the first time, a modulation in the levels of several host immune mediators acting in various immune pathways intricately associated with the development of sepsis syndrome. Due to the complexity of sepsis, where several immune pathways converge to elicit the observed response, a combinatorial approach to concomitantly target several immune mediators is likely the most effective approach for the treatment of this immune disorder, which is still lacking. This is underscored by our observations where methods of targeting individual mediators/pathway have met with little success in our hands (unpublished results). Nonetheless, the murine model of Francisella induced sepsis represents an excellent model for pulmonary infection induced sepsis which may help delineate the underlying mechanisms and thus define targets of therapeutic interventions. We are currently using inhibitors to concomitantly block inflammatory pathways, activate anti-inflammatory mediators and control cell death, in various combinations in order to block sepsis and improve host survival in this infection.

4. Materials and methods

4.1. Bacterial strains and mice

Francisella tularensis Type A strain SchuS4 was kindly provided by Dr. Karl E. Klose (University of Texas at San Antonio). The bacteria were cultivated on chocolate II agar plates (BD Diagnostic Systems, NJ) at 37 °C. All the *in-vivo* experiments were carried out in a biosafety level 3 facility at University of Texas at San Antonio certified by the Centers for Disease Control and Prevention, USA. Six to 8 weeks old female C57BL/6 (Charles River Laboratories, Wilmington, MA) were used for the experiments. The animal usage protocols were approved by the Institutional Animal Care and Usage Committee at University of Texas at San Antonio and followed federal guidelines.

4.2. Infection of mice

Mice were anaesthetized with a mixture of ketamine HCL and xylazine (30mg/ml ketamine, 4 mg/ml xylazine in phosphate buffered saline, PBS) and were infected intranasally with $0.6-3 \times 10^2$ CFUs of Type A strain SchuS4 in 20 µl of PBS. In our hands, this dose range of bacteria resulted in 100% mortality rate within 4-5 days post-infection (p.i.). The mock inoculated mice received 20 µl of PBS alone intranasally.

4.3. Bacterial burden analysis

Mice infected with SchuS4 were euthanized at 24, 72 and 96 h p.i. and the bacterial burden in blood, lungs, liver and spleen was analysed as described previously [13, 14].

4.4. Multi-analyte profile analysis

The biomarker levels in lung homogenates harvested at 24, 72 and 96 h p.i. were determined commercially by Rules-based Medicine (Austin, TX, USA) as previously described [13, 14]. The lung homogenates were passed through 0.2 µm filters (Nalgene) and streaked on chocolate II agar plates. The plates were incubated at 37°C for 1 week to ensure the sterility of the homogenates before sending them for biomarker analysis.

4.5. Histological and immunofluorescence staining

For histological and immunofluorescence staining, frozen tissues embedded in Optimal Cutting Temperature (O.C.T.) compound (Tissue-Tek, Sakura Finetek, CA) were processed as previously described [15]. For detection of cell death, the terminal deoxynucleotidyl transferase-mediated triphosphate (dUTP)-biotin nick end labeling (TUNEL) method was used according to manufacturer's instructions (Chemicon International, CA). A rabbit anti-*Francisella tularensis* antiserum (Becton Dickinson Co., MD) followed by goat anti-rabbit antibody conjugated with Rhodamine-Red (Jackson ImmunoResearch, PA) was used to detect bacteria in the organs. A goat anti-S100A9 antibody (R&D Systems, Minneapolis, MN) followed by Cy3-conjugated rabbit anti-goat antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA.) was used for S100A9 detection in frozen tissue sections. The images were acquired using a Leica DMR epifluorescent microscope (Leica Microsystems, Wetzlar, Germany) with an attached cooled CCD SPOT RT camera (Diagnostic Instruments Inc., Sterling Heights, MI). The images were processed and analyzed using Adobe Photoshop 7.0 software (Adobe, Mountain view, CA).

4.6. Immunoblotting of S100A9 and HMGB-1

The lungs, liver and spleen of mock control and SchuS4 infected animals harvested at indicated times post-infection were homogenized as described before in Section 2.4. Thirty microgram total protein of each sample was loaded on SDS polyacrylamide gels and transferred onto polyvinylidene difluoride (PVDF) membrane. S100A9 was detected by enhanced chemiluminescence (Amersham Biosciences) using a polyclonal goat anti-mouse S100A9 antibody (R&D Systems, MN) followed by a donkey anti-goat IgG conjugated with HRP (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA.). The levels of HMGB1 in the sera from mock or SchuS4 infected mice were determined by Western blotting analysis as described previously [50]. Briefly, 6 µl of sera were run on SDS polyacrylamide gels and transferred onto polyvinylidene difluoride (PVDF) membrane. HMGB-1 was detected by enhanced chemiluminescence (Amersham Biosciences) using a polyclonal rabbit anti-mouse HMGB1 antibody (Abcam, MA) followed by a rabbit Trueblot^R HRP conjugated rabbit IgG (eBiosciences, CA).

4.7. Statistical Analysis

Statistical comparison of multianalyte data of different experimental groups with mock control was performed by ANOVA with Dunnett's multiple comparison post-test.

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Fig. 1.

(A). Disease progression in the mice infected intranasally with indicated doses of SchuS4 was recorded daily. The infected mice showed disease symptoms by 3 days p.i., became moribund and succumbed to infection by 4-5 days p.i.. (B). Pulmonary infection with SchuS4 resulted in bacteremia and dissemination of bacteria to systemic organs. C57BL/6 mice were infected intranasally with 3×10^2 CFU of Type A strain SchuS4. At indicated times post-infection, blood, liver, spleen and lung tissues were recovered and total bacterial burdens in each organ were determined by dilution plating onto chocolate II agar. The data shown is average of 3-5 mice in two independent experiments.

Fig. 2.

Histopathological analysis of SchuS4 infected tissues. C57BL/6 mice were infected intranasally with 3×10^2 CFU of Type A strain SchuS4. At 96 h p.i., liver, spleen and lungs were harvested, sectioned, and stained with Hematoxylin & Eosin. The figure shows representative sections of lung, liver and spleen from mock control (A, B and C respectively) and SchuS4 infected (D, E and F respectively) mice. Data are from one representative experiment of three performed ($n=3$ for each group per experiment). H&E staining, Bar = 100 μ m.

Fig. 3.

Mice infected with SchuS4 display extensive apoptosis in lungs, liver and spleen. Organs from mice infected intranasally with 3×10^2 CFU of Type A strain SchuS4 were harvested 96h p.i., embedded in OCT and sectioned as described in Materials and Methods. In-situ terminal deoxyribonucleotidyl transferase-mediated triphosphate (dUTP)-biotin nick end labeling (TUNEL) was used for detection of DNA fragmentation of nuclei (green) in mock control (A, B and C) or in SchuS4 infected mice (D, E and F). The same sections were co-stained for detection of bacteria (red) using a rabbit anti-*F. tularensis* serum as primary antibody followed by goat anti-rabbit antibody conjugated with Rhodamine-red (G, H and I). Images J, K and L show merged TUNEL and bacterial staining. Nuclei (blue) were stained with 4'6' diamidino-2-phenylindol-dilactate (DAPI). Bar = 100 μ m.

Fig. 4.

Lethal infection with SchuS4 results in hypercytokinemia. The lungs from mock control and SchuS4 infected mice were harvested at 24 h, 72 h and 96 h p.i., homogenized in PBS with protease inhibitors and analyzed for rodent multi-analyte profile (Rules-Based Medicine, Austin, TX). Cytokines and chemokines described as markers of sepsis are depicted. Results shown are average of three infected and mock control mice from 2-3 independent experiments. Statistical analysis of the data comparing mock control with infected samples was performed by ANOVA with Dunnett's multiple comparison post-test, * $p < 0.05$; ** $p < 0.005$; *** $p < 0.0005$.

Fig. 5.

Lethal infection with SchuS4 results in coagulopathy, cardiovascular injury and acute phase response associated with severe sepsis. The lungs from mock control and SchuS4 infected mice were harvested at 24 h, 72 h and 96 h p.i., homogenized in PBS with protease inhibitors and analyzed for rodent multi-analyte profile (Rules-Based Medicine, Austin, TX). Results shown are average of three infected and mock control mice from 3 independent experiments. FGF-6, fibroblast growth factor-9; FGF-basic, basic fibroblast growth factor ; SAP, Serum Amyloid Protein; SGOT, Serum glutamic oxaloacetic transaminase; TPO, Thrombopoietin; VCAM-1, vascular cell adhesion molecule-1; VEGF, vascular endothelial growth factor. Statistical analysis of the data comparing mock control with infected samples was performed by ANOVA with Dunnett's multiple comparison post-test, * $p < 0.05$; ** $p < 0.005$; *** $p < 0.0005$.

Fig. 6.

Lethal pulmonary infection with SchuS4 causes upregulation and release of alarmins in systemic organs. (A) Immunofluorescence staining of S100A9, an alarmin, in the lung, liver and spleen of mock, and SchuS4 infected mice. The organs from mock control and SchuS4 infected mice were harvested at 96 h p.i., sectioned and stained for S100A9 (red) using a goat anti-S100A9 antibody followed by Cy3-conjugated rabbit anti-goat antibody. Nuclei (blue) were stained with 4'6' diamidino-2-phenylindol-dilactate (DAPI). Bar = 100 μm. Images G'-I' represent higher magnification of S100A9 positive lesion areas in lungs (bar = 50 μm). Asterisks in these images show S100A9 staining in cell free areas depicted by absence of nuclear staining. Images are from one representative experiment of two performed ($n=3$ mice per group). (B). Kinetics of S100A9 upregulation in lungs, liver and spleen of mock control and SchuS4 infected mice determined by Western blotting. Thirty

micrograms tissue homogenates from mock control and SchuS4 infected mice harvested at indicated times p.i. were run on SDS-polyacrylamide gels, transferred to PVDF membranes and probed with anti-S100A9 antibody as described in Materials and Methods. The data shown is representative of two independent experiments with 3 animals for each experimental group. (C). Detection of HMGB1 by Western blot in sera of mock control and SchuS4 infected mice harvested at indicated times p.i.. Equal volumes of sera from mock control and SchuS4 infected mice were run on SDS-polyacrylamide gels, transferred to PVDF membranes and probed with anti-HMGB1 antibody as described in Materials and Methods. The data shown is representative of two independent experiments with 3 animals for each experimental group.

Table 1

Amounts of cytokines and chemokines in lungs of mice infected with *F. tularensis* strain SchuS4 in comparison with those infected with similar dose of *F. novicida* strain U112.

Immune mediator	Amount in SchuS4 infected lungs	Amount in U112 infected lungs ^a
IFN- γ	569 $\pm$ 54 pg	712 $\pm$ 380 pg
IL-10	1059 $\pm$ 4 pg	983 $\pm$ 67 pg
IL-18	30 $\pm$ 2.3 ng	7 $\pm$ 0.3 ng
IL-6	5167 $\pm$ 168 pg	2470 $\pm$ 617 pg
IL-1 α	6 $\pm$ 0.6 ng	8 $\pm$ 1.8 ng
IL-1 β	17 $\pm$ 0.6 ng	10 $\pm$ 0.9 ng
CCL2	24 $\pm$ 0.4 ng	17 $\pm$ 5 ng
CCL4	3.3 $\pm$ 0.12 ng	6.4 $\pm$ 1.7 ng
CCL9	8.5 $\pm$ 0.4 ng	13.5 $\pm$ 1.6 ng
CXCL1	12 $\pm$ 0.8 ng	5 $\pm$ 0.6 ng
CXCL10	35 $\pm$ 3.4 ng	29 $\pm$ 7 ng
CXCL6	1.6 $\pm$ 0.06 ng	19 $\pm$ 2.7 ng
TNF- α	538 $\pm$ 35 pg	862 $\pm$ 122 pg

^aReference [13]