Galectin-3 Functions as an Alarmin: Pathogenic Role for Sepsis Development in Murine Respiratory Tularemia

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Abstract

Sepsis is a complex immune disorder with a mortality rate of 20–50% and currently has no therapeutic interventions. It is thus critical to identify and characterize molecules/factors responsible for its development. We have recently shown that pulmonary infection with Francisella results in sepsis development. As extensive cell death is a prominent feature of sepsis, we hypothesized that host endogenous molecules called alarmins released from dead or dying host cells cause a hyperinflammatory response culminating in sepsis development. In the current study we investigated the role of galectin-3, a mammalian β-galactoside binding lectin, as an alarmin in sepsis development during F. novicida infection. We observed an upregulated expression and extracellular release of galectin-3 in the lungs of mice undergoing lethal pulmonary infection with virulent strain of F. novicida but not in those infected with a non-lethal, attenuated strain of the bacteria. In comparison with their wild-type C57Bl/6 counterparts, F. novicida infected galectin-3 deficient (galectin-3−/−) mice demonstrated significantly reduced leukocyte infiltration, particularly neutrophils in their lungs. They also exhibited a marked decrease in inflammatory cytokines, vascular injury markers, and neutrophil-associated inflammatory mediators. Concomitantly, in-vitro pre-treatment of primary neutrophils and macrophages with recombinant galectin-3 significantly reduced pro-inflammatory cytokine release and leukocyte infiltration. Moreover, the lipid A of Francisella LPS does not stimulate TLR4 and is hypo-inflammatory [6]. Studies from our and other laboratories have shown that extensive tissue damage and widespread cell death is a hallmark of Francisella infection, regardless of the bacterial strain [4,7–10]. Additionally, our studies show that Francisella infected macrophages are defective in clearance of dead cell debris, a process termed efferocytosis, leading to accumulation of these dead cells and their contents [11]. We thus hypothesized that in the absence of any bacterial toxins, host endogenous molecules released from these dead or dying cells contribute to the inflammatory response culminating in sepsis development during respiratory infection with Francisella.

Alarmins are host endogenous factors which perform homeostatic functions when contained within cellular compartments [12]. However, under pathological conditions, these molecules can be released either passively from dead cells or actively via non-classical secretion pathways [13]. Once in the extracellular milieu, they exhibit immune modulatory properties such as induction of pro-inflammatory cytokines, immune cell chemotaxis, and regulation of cell death [12–14]. The overt inflammation during sepsis is primarily a result of the interaction between innate immune receptors with pathogen derived molecules (Pathogen Associated Molecular Patterns [PAMPs]) and alarmins. PAMPs and alarmins together constitute Danger-Associated Molecular Patterns.
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(DAMPs). The interaction of Toll-Like receptors (TLRs) as well as NOD-Like receptors (NLRs) with pathogen derived PAMPs during sepsis has been studied extensively (reviewed in [15,16]). However, the recognition of self-molecules (alarmins) by signaling receptors and the concomitant inflammatory response is an area of research which is still in its infancy. Moreover, in a complex immune disorder like sepsis which is an interplay of several host immune pathways such as the coagulation system, complement cascade and even the autonomic nervous system [15], it is likely that several alarmins are involved at the intersections of these pathways. Thus, identification of novel alarmins may aide in understanding this complex disorder and may present additional targets for effective therapeutics. As sepsis developed during pulmonary infection with Francisella is associated with extensive cell death in lungs and other systemic organs, we sought to identify novel alarmins that might be released during this infection and may contribute to disease development.

Galectins constitute a soluble mammalian β-galactoside binding lectin family which play homeostatic roles in regulation of cell cycle and apoptosis, as well as display inflammatory and immune modulatory activities in various pathological conditions [17–20]. Previous studies have implicated galectin-3 in regulation of various inflammatory conditions including endotoxemia and airway inflammation [21–23]. In this study we show that galectin-3, a mammalian galactoside binding soluble lectin is upregulated and released in lungs of mice undergoing lethal respiratory infection with F.n. but not in mice vaccinated with an attenuated mutant strain of the bacteria that protects these mice from an otherwise lethal challenge. We thus hypothesized that galectin-3 exacerbates the inflammatory response during lethal infection. The outcome of this study, with use of galectin-3 deficient mice, shows that galectin-3 plays the role of an alarmin in Francisella infection induced sepsis development.

Materials and Methods

Ethics Statement

The animal usage protocols were approved by the Institutional Animal Care and Usage Committee at the University of North Dakota (protocol no. 1108-3) and the University of Texas at San Antonio (protocol no. MU066). All the procedures strictly followed the institutional and federal guidelines and all efforts were made to minimize animal suffering.

Bacterial Strains and Mice

The F.n. strain U112 and an attenuated transposon mutant lacking a 58 kDa protein of hypothetical function (kindly provided by Dr. Larry Gallagher, University of Washington) were grown on Trypticase Soy Agar (TSA) medium supplemented with L-cysteine at 37°C. After overnight growth, the bacteria were harvested and suspended in a freezing medium (250 mM sucrose, 10 mM sodium phosphate pH 7.2 and 5 mM glutamic acid). Stocks were aliquoted and frozen at −80°C for further use.

All in-vivo experiments were performed using 6–8 wk old female C57Bl/6 wild-type and galectin-3−/− mice. Galectin-3−/− mice were purchased from Jackson Laboratories (Bar Harbor, ME). Sex- and age-matched galectin-3−/− mice with the same genetic background were used as control.

Antibodies and Reagents

All reagents were purchased from Sigma-Aldrich unless otherwise indicated. For detection of galectin-3 by immunofluorescence (IF) staining, a purified rat anti-mouse galectin-3 antibody (eBioscience, San Diego, CA) followed by Alexa-546 conjugated chicken anti-rat antibody (Molecular Probes, OR) was used. A rat anti-mouse CD11b antibody conjugated to PE (BD Pharmingen) and a purified rat anti-mouse Gr1 monoclonal antibody, clone Ly-6G (Clone Accurate Chemical, Westbury, NY, USA), followed by the secondary antibody RRX-conjugated Affipure goat anti-rat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) were used for double staining of activated neutrophils. The terminal deoxynucleotidyl transferase-mediated triphosphate (dUTP)-biotin nick end labeling (TUNEL) staining kit was purchased from Chemicon International, CA. Purified recombinant galectin-3 was purchased from R&D Systems, MN. The endotoxin level was <1.0 EU per μg of protein. For detection of reactive oxygen species, Fc OxyBURST assay reagent was purchased from Molecular Probes, Eugene, OR. Mouse IL-6 and TNF-α ELISA kits (BD OptEIA) were from BD Biosciences, San Diego, CA.

Infection of Mice, Survival and Bacterial Burden

Mice were anaesthetized with a mixture of ketamine HCL and xylazine (30 mg/ml ketamine, 4 mg/ml xylazine in PBS) and were infected intranasally with 50–70 CFUs of the wild-type F.n. strain U112 in 20 μl of PBS or with 20 μl of PBS alone. Mice were monitored daily for signs of disease, which typically included piloerection, hunched gait, lethargy and eye discharge. The survival of infected mice was recorded for up to 2 weeks post-infection (p.i.). Mice displaying severe signs of distress (labored breathing, non-responsiveness to cage tapping, failure of grooming and severe eye discharge) were humanely sacrificed by injecting a mixture of ketamine (90–120 mg/kg) and xylazine (10 mg/kg) followed by cervical dislocation. The death was recorded as tularemia induced mortality. For non-lethal infection, the mice were similarly inoculated with the mutant bacteria followed 3 weeks later by challenge with similar dose of the wild-type organisms. In some experiments, the mice were euthanized at 3 days p.i. and blood, lungs and liver were aseptically harvested. The organs were homogenized aseptically in cold PBS with Complete™ protease inhibitor cocktail (Roche Diagnostics, Germany). For the bacterial burden analyses, the homogenates and blood were serially diluted in PBS and plated on TSA. CFU counts per mouse were calculated after incubating the plates at 37°C overnight.

Quantitative Real-time PCR

Lungs from infected and mock control mice at various times post-infection were immediately removed after perfusion and total RNA was extracted using Trizol reagent (Invitrogen) according to the manufacturer’s instructions. Real-time PCR analysis of the samples was performed using SYBR green (Applied Biosystems, CA, USA) as the detection dye to measure the expression levels of Galectin-3-specific mRNAs. Briefly, one microgram of total RNA from either infected or mock infected mice was reverse transcribed into cDNA by using a high capacity cDNA reverse transcription kit according to the manufacturer’s instructions (Applied Biosystems, CA, USA). Transcripts of the housekeeping ribosomal 18S and galectin-3 were PCR amplified in each sample by using specific primers [Advanced Nucleic Acids Core Facility, UTHSCSA, TX]: 18S (sense) 5′-CAGTGGTTGTTGTAGAGAAAGCG-3′ and (anti sense) 5′-GTCCCGTGGTTCTCTCGATG-3′; Gal-3 (sense) 5′-CAGTGCAGAGGCGTCGG-3′ and (anti-sense) 5′-CTGCCAGACGCGCTGGGTTT-3′. The target gene expression levels were normalized to levels of the house keeping 18S gene in the same sample. Expression of galectin-3 in infected samples...
stimulated as described above for the neutrophils. Culture pretreatment of the cells with 10⁻⁸ M of purified recombinant galectin-3 served as controls. One hour after stimulation, the cells were counted by trypan blue exclusion staining.

Enumeration of Cellular Infiltration in Lungs

Lungs were harvested from infected and mock control mice at 3 days p.i. after perfusion with PBS and were treated with collagenase to obtain single cell suspensions as previously described [3,4,25]. Total numbers of viable immune cells in lungs of infected and mock control galectin-3⁻/⁻ or WT mice were counted 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).

Multi-analyte Profile Analysis

The lung homogenates were prepared as described for the bacterial burden analysis above and were centrifuged at 2000×g for 15 min to clear cellular debris. The supernatants were immediately frozen at −80°C. The biomarker levels in lung homogenates were determined commercially by Myriad Rules-based Medicine (Austin, TX, USA) utilizing a multiplexed flow-based system: Mouse MAP™ (Multi-Analyte Profiles) analysis technology.

Neutrophil and Macrophage Activation

Cells were isolated from the peritoneal cavities of naïve C57BL/6 mice 12–14 h after intraperitoneal injection with sterile 4% thioglycollate. Neutrophil percentage was determined by flow cytometry using neutrophil specific anti-mouse Gr-1 (anti-Ly-6G and Ly6C). Additionally, the lavage cells were cytocoentrifuged on glass slides and were stained with H&E as described above. The cells were plated at the density of 1×10⁶ cells and were infected with wild-type F.n. strain U112 at MOI 50 with or without pretreatment of the cells with 10 µg/ml of purified recombinant galectin-3. Cells stimulated with galectin-3 alone or with 10 ng/ml of phorbol myristate acetate (PMA) served as controls. One hour after stimulation, production of reactive oxygen species (ROS) was measured in the cells by flow cytometry using Fc OxyBURST reagent according to the manufacturer’s instructions. A minimum of 10,000 events was read for each sample and all the cells measuring fluorescence positive in FITC channel (excitation and emission maxima of ∼490 nm and ∼520 nm, respectively) were gated to get the percentage of ROS producing cells.

Bone marrow was isolated from wild-type and galectin-3⁻/⁻ mice and the cells were differentiated to macrophages as previously described [26]. On day 6 of culture 90–95% cells were macrophages as determined by flow cytometry using macrophage specific markers CD11b and F4/80. The cells were plated at 8×10⁶ cells per well in 96-well flat-bottom plates and were stimulated as described above for the neutrophils. Culture supernatants were collected 24 h after stimulation and measurement of IL-6 and TNF-α was performed by ELISA according to the manufacturer’s instructions (BD OptEIA, BD Biosciences).

Histological and Immunofluorescence Staining

For histological and immunofluorescence staining, frozen lung sections thus obtained were stained with hematoxylin and eosin for pathological analysis, or for detection of galectin-3 and activated neutrophils (CD11b⁻/⁻Gr1⁻) by immunofluorescence staining, as previously described [24]. For detection of cell death, TUNEL method was used according to manufacturer’s instructions (Chemicon International, CA). 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).

Results

Galectin-3 is Highly Expressed and is Localized Extracellularly in Lungs during the Septic Phase of F.n. Infection

Alarmins are characterized as intracellular host factors which display extracellular release under pathological conditions. To examine if galectin-3 exhibits this alarmin property in pulmonary tularemia, the expression and distribution of this lectin was analyzed. We compared the transcript and protein level expression of galectin-3 at various times post-infection (p.i.) in lungs of mice undergoing lethal pulmonary infection with the wild-type strain of F.n. versus the mice vaccinated with an attenuated mutant of F.n. (Mut/WT mice), which protects the mice from sepsis. This mutant has been characterized extensively in our previous studies [3]. As shown in Figure 1A, galectin-3 transcript levels showed maximal increase at 3 days p.i. (dp.i.) in the lungs of mice infected with the wild-type bacteria as well as in the Mut/WT mice. However, this increase was significantly higher in mice undergoing lethal infection as compared to the protected Mut/WT mice. This increase in galectin-3 expression at 3 dp.i. is consistent with the appearance of other sepsis features (extensive cell death, hyperinflammatory response, increased vascular injury) at this time, as shown in our previous studies with F.n. as well as the fully virulent F. tularensis [3,4]. We thus termed this as septic phase of Francisella infection and carried out the rest of our analysis at this time point. Immunofluorescence (IF) analysis of galectin-3 protein expression in frozen sections of lungs harvested at 3dp.i. showed a low basal level expression in mock infected mice inoculated with PBS alone (Fig. 1.B1). Consistent with the transcript data, lungs of mice undergoing septic infection with the wild-type F.n. exhibited upregulated expression of this lectin at 3d p.i. (Fig. 1. B2). This increase in expression was substantially higher than that in Mut/WT mice (Fig. 1.B4). The mice infected with mutant alone for 3 weeks and without challenge with WT bacteria (Mut-3 wk) served as control for the Mut/WT mice. In these mice, galectin-3 was observed to be expressed at low basal level similar to mock control animals (Fig. 1B3). Importantly, most of galectin-3 expressed in septic mice was localized extracellularly in large granuloma-like areas of cellular infiltration, undergoing extensive cell death (Fig. 1.B2). The non-septic Mut/WT mice, on the other hand, showed intracellular galectin-3 associated with live cells (Fig. 1.B4). Western blot analysis of bronchoalveolar lavage (BAL) from mice infected with the WT F. novicida also showed a significantly high extracellular release of galectin-3 (Figure S1). Taken together, these data clearly showed that galectin-3 exhibits a characteristic alarmin property of extracellular release during septic phase of pulmonary F.n. infection.

F.n. Infected Galectin-3⁻/⁻ Mice Display Reduced Inflammatory Response and Neutrophil Accumulation

We hypothesized that, similar to the function of alarmins, increased expression and extracellular localization of galectin-3 may be contributing to the hyperinflammatory response culmi-
nating in sepsis during lethal Francisella infection. In order to analyze this, lungs were harvested at 3d.p.i. from F.n. infected galectin-3^{−/−} and wild-type mice and the levels of multiple cytokines, chemokines as well as vascular injury markers were measured using a multiplex assay. Galectin-3^{−/−} mice displayed significant reduction in levels of several vascular injury markers in comparison with their wild-type counterparts (Fig. 2A). In addition, levels of several inflammatory cytokines (TNF-α, IL-10, IL-1β), described as markers of sepsis, were reduced in galectin-3^{−/−} mice (Fig. 2B). These observations strongly suggested an

Figure 1. Upregulated expression and extracellular release of Galectin-3 in lungs during respiratory F. novicida infection. (A) Total RNA was extracted by Trizol method from lungs harvested at the indicated times after infection with the Wild-type bacteria (WT) or from mice vaccinated with an attenuated mutant strain followed by challenge with WT bacteria (Mut/WT mice). The mRNA levels of Galectin-3 were analyzed by real-time PCR as described in Materials and Methods and are expressed as fold changes over the levels in mock control mice. Data shown are the averages of 3–4 mice per group. Statistically significant differences are denoted by asterisks (**p<0.005). (B) In-situ IF staining of frozen lung sections from mock infected and WT U112 infected or Mut/WT mice harvested at 3 d. p.i. Lung harvested 3 weeks after vaccination with the mutant alone (Mut-3 wk) served as controls for Mut/WT mice. The sections were stained for galectin-3 (red) using a purified rat anti-mouse galectin-3 antibody followed by Alexa-546 conjugated chicken anti-rat antibody. Nuclei (blue) were stained with 4′,6-diamidino-2-phenylindol-dilactate (DAPI). Magnification x200. Insets depict extracellular galectin-3 in WT F. novicida infected mouse lungs (B2′) and cytosolic galectin-3 in Mut/WT (B4′) mouse lungs.

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immune-stimulatory role of galectin-3 during pulmonary Francisella infection. Interestingly, in comparison with wild-type mice, infected galectin-3\(^{2/2}\) mice displayed a reduction in several chemokines involved in neutrophil recruitment (Fig. 2C). Furthermore, levels of myeloperoxidase (MPO), a neutrophil associated protease and marker of neutrophil activation, was also reduced in infected galectin-3\(^{2/2}\) mice. In order to correlate these observations with cellular infiltration in-vivo, IF staining for co-expression of CD11b and Gr1, markers for activated neutrophils [27], was performed on lung sections from galectin-3\(^{2/2}\) and wild-type mice. Consistent with the chemokine data, cells infiltrating the lungs of infected wild-type mice showed high co-expression of CD11b and Gr1, suggesting an activated neutrophil phenotype. These cells were mostly accumulated in large lesion like areas in the lungs of these mice. The cells infected galectin-3\(^{2/2}\) mice, on the other hand, expressed CD11b, but low or no Gr1 (Fig. 3). These results suggested a role of galectin-3 in regulation of myeloid cell accumulation, particularly neutrophils, in the lungs of mice during pulmonary F.n. infection.

**Galectin-3 Regulates F.n. Infection Induced Inflammatory Response In-vitro**

In order to further investigate the immune stimulatory properties of galectin-3, we examined the role of this lectin in in-vitro activation of myeloid cells, particularly neutrophils and macrophages. These are the major cell types that infiltrate the lungs of Francisella infected mice [3,28]. In-vitro infection of WT bone marrow derived macrophages (BMDMs) with wild-type F.n. U112 resulted in an inflammatory response in terms of increased TNF-\(\alpha\) and IL-6 production (Fig. 4A). Galectin-3\(^{2/2}\) macrophages on the other hand, produced significantly lower amounts of these cytokines in response to infection (Fig. 4A). As the extracellularly released galectin-3 may be playing a role in activation of myeloid cells in-vivo, we examined if pretreatment of these cells with galectin-3 has any effect on Francisella infection induced cytokine production. Stimulation of macrophages with purified galectin-3 induced minimal amount of TNF-\(\alpha\) and IL-6 production (Fig. 4B). The optimal concentration of galectin-3 was experimentally determined by using 1–20 \(\mu\)g/ml of the recombinant protein (data not shown). Infection with wild-type F.n. strain

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**Figure 2. Galectin-3\(^{2/2}\) mice display reduced levels of inflammatory mediators in lungs after pulmonary infection with F.n.** The lungs from WT mock infected (WT-M), galectin-3\(^{2/2}\) mock infected (Gal-3\(^{2/2}\)M), WT F. novicida infected (WT-Inf) or galectin-3\(^{2/2}\) F. novicida infected mice (Gal3\(^{2/2}\)Inf) were harvested at 3 d.p.i., homogenized with protease inhibitors in PBS and analyzed commercially for rodent multi-analyte profiles (Rules-Based Medicine, Austin, TX). (A), levels of vascular injury markers; (B), levels of inflammatory cytokines; and (C), levels of neutrophil attractant chemokines and activation markers in lung homogenates. Results shown are from 3–4 mice per group from 3 different experiments. CRP; C-reactive protein, MMP-9; matrix metalloproteinase 9, MPO; myeloperoxidase. *\(p<0.05\); **\(p<0.005\). doi:10.1371/journal.pone.0059616.g002
U112 infection, on the other hand, induced substantial amounts of these cytokines in macrophages. Interestingly, pre-treatment of macrophages with purified galectin-3 exacerbated this Francisella-induced inflammatory cytokine production (Fig. 4B). Immune stimulatory effect of galectin-3 was also examined on peritoneal neutrophils. Cells collected by peritoneal lavage following intraperitoneal injection of thioglycollate were 80–85% neutrophils as determined by flow cytometry and morphological analysis with characteristic multilobed nuclei (Figure S2). Unlike macrophages, treatment of neutrophils with purified galectin-3 alone activated these cells to produce substantial levels of ROS as determined by oxidation of Fc OxyBURST dye (Fig. 4C). Importantly, pre-treatment of neutrophils with this lectin primed these cells to produce further increased amounts of ROS in response to F. novicida infection, which was significantly higher than that elicited by F. novicida infection alone (Fig. 4C). This cell-type specific response of galectin-3 indicates involvement of distinct receptors and/or signaling pathways, which is currently being investigated in our laboratory. Nonetheless, this augmentation of Francisella infection-induced myeloid cell activation by galectin-3 likely has implications in exacerbation of inflammation culminating in sepsis development during this infection.

Galectin-3−/− Mice Exhibit Reduced Lung Pathology after F. novicida Infection

Lung cryosections from wild-type and galectin-3−/− mice infected with a lethal dose of F. novicida were stained with H&E and processed for histopathological analyses as described in Materials and Methods. Mock infected wild-type and galectin-3−/− mice exhibited similar normal lung architecture with minimal cellular infiltration and clear air spaces (Fig. 5A). As expected, a massive increase in cellular infiltration and extensive pathology, along with severe bronchopneumonia and massive cell death occurring in the center of large granuloma-like areas of infiltration, was evident in the lungs of wild-type mice at 3 dp.i. (Fig. 5A). The lungs of galectin-3−/− mice, on the other hand, showed moderate peribronchial and perivascular infiltration (Fig. 5A). The infiltrating cells in these areas appeared to be viable and the areas of infiltration lacked cellular debris that is typical of extensive apoptosis and necrosis in the wild-type mice. This was consistent with reduced numbers of leukocytes enumerated after collagenase treatment of the lungs harvested from galectin-3−/− mice (Fig. 5B). Galectin-3 deficiency did not affect the basal number of cells as mock infected wild-type and galectin-3−/− animals showed similar low number of cells in the lungs. To further analyze the extent of cell death in the lungs of infected wild-type and galectin-3−/− mice, TUNEL assay was performed on frozen sections of lungs harvested at 3 dp.i. As shown in Fig. 5C, mock infected wild-type and galectin-3−/− mice showed minimal numbers of TUNEL

Figure 3. Galectin-3−/− mice display reduced accumulation of neutrophils in lungs during F. novicida infection. Frozen sections of lungs harvested at 3 d. p.i. from mock infected and F. novicida infected WT or galectin-3−/− mice were co-stained with antibodies against myeloid cell markers CD11b (red) and Gr1 (green). A high co-expression of both markers is depicted by yellow color in infected WT lungs while cells infiltrating lungs of galectin-3−/− mice exhibited expression of only CD11b. Nuclei (blue) were stained with 4′,6′-diamidino-2-phenylindol-dilactate (DAPI). Magnification x200. Asterisks depict lesions in the lungs.
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positive cells in their lungs. On the other hand, septic lungs of F.n. infected wild-type mice showed extensive cell death within perivascular and peribronchial lesions which are the main sites of immune cell infiltration during infection (Fig. 5C). In contrast, the numbers of apoptotic TUNEL positive cells in infected galectin-3−/− mice were much less as compared to their wild-type counterparts following infection with F.n. The improved lung architecture and reduced cell death in the absence of galectin-3 indicates a pathological role of this lectin during pulmonary Francisella infection.

Galectin-3−/− Mice Show Improved Survival Following F.n. Infection
In order to see the effect of improved lung pathology and reduced inflammatory responses in the absence of galectin-3, overall disease severity and survival was compared in C57BL/6 wild-type and galectin-3−/− mice infected with a lethal dose of F.n. In the infected wild-type mice, visible signs of disease started to appear by day 3 p.i. which typically included piloerection, hunched gait, lethargy, and eye discharge. All of these mice succumbed to infection by day 5 p.i. (Fig. 6A). By contrast galectin-3−/− mice exhibited delayed appearance of disease symptoms and showed significantly improved survival as compared to the infected wild-type mice (Fig. 6A). Intriguingly, enumeration of bacterial burden in the organs of these mice at the peak of infection, i.e., 3 d.p.i. showed that both galectin-3−/− and the wild-type animals exhibited similar bacterial burdens in their systemic organs as well as in blood (Fig. 6B).
Figure 5. Galectin-3 deficiency leads to improved lung pathology, reduced leukocyte accumulation and reduced cell death upon pulmonary F.n. infection. (A) Lungs from mock infected and F.n. infected wild-type (WT) or galectin-3⁻/⁻ mice were harvested at the septic phase (3 d. p.i.), embedded in optimal-cutting-temperature (OCT) compound, and sectioned as described in Materials and Methods.
Discussion

Sepsis is the 2nd leading cause of death in ICU patients and pulmonary infections in turn are a major source of sepsis [29]. It is a complex immune disorder resulting from deregulation of multiple host defense pathways. Accumulating evidence suggests that host endogenous molecules termed alarmins, likely play an important role in pathophysiology of sepsis [30]. In this study we show that galectin-3, a mammalian β-galactoside binding lectin acts as a novel alarmin in development of sepsis during pulmonary infection with *F. novicida*. Consistent with characteristic properties of alarmins, galectin-3 was upregulated and extracellularly released during the septic phase of infection and could amplify the Francisella infection-induced inflammatory response of neutrophils and macrophages. Furthermore, galectin-3−/− mice showed improved pathology, reduced inflammation and improved survival during pulmonary Francisella infection. These results suggest that galectin-3 functions as an alarmin and plays a pathogenic role in development of sepsis in pulmonary bacterial infection.

Alarmins are structurally diverse multifunctional host proteins with some common properties [12–15]. These are endogenous proteins performing homeostatic functions that lack any signal sequence for active secretion and have chemoattractant and immune activating properties, once released in extracellular milieu. With advances in our understanding of host responses to pathogenic events, the list of alarmins has continued to grow over the past decade. Several well-characterized alarmins such as High Mobility Group Box1 (HMGB-1), S100 family of proteins, and heat shock proteins have been shown to perform dual functions as factors controlling homeostatic processes like transcriptional regulation when localized to intracellular compartments and as pro-inflammatory factors upon their release from necrotic cells during a pathogenic insult [31–33]. Similarly, galectin-3 when localized in the nucleus, has been shown to act as an RNA splicing factor [34] and performs homeostatic functions such as embryogenesis and cell cycle regulation [19]. The results of current study showed that galectin-3 can be released extracellularly in lungs under pathogenic conditions such as an infection. Curiously, galectin-3 does not contain any signal sequence for golgi mediated classical secretion. Thus active secretion of this lectin is likely via a yet unclear non-classical secretion pathway, a property shared by most alarmins characterized to date [20]. However, in F.n. infection, this lectin is likely released passively from dead/dying cells since the extracellular galectin-3 is detected only during lethal infection with F.n., which typically results in extensive cell death [3,4,7]. On the other hand, the mice vaccinated with an attenuated F.n. mutant causing little or no cell death showed this lectin to be intracellular and largely associated with live cells, with minimal levels in BAL. Notwithstanding the mechanism involved, release of galectin-3 in the septic phase of F.n. infection indicates a role for this molecule in pathogenesis of this infection.

Consistent with alarmin properties, galectin-3 exhibited immune activating properties such as stimulation of oxidative burst in neutrophils and inflammatory cytokine production in macrophages. Importantly, this lectin was able to augment F.n. infection induced inflammatory response from neutrophils as well as macrophages, which can have important implications under in-vivo conditions. Previous studies from our laboratory have shown that pulmonary infection with F.n., as well as *F. novicida*...
In this regard a recent study showed a defect in neutrophil turnover and thus potentiating its interaction with immune activating receptor/ s, as has been shown in case of HMGB1 [33]. Studies regarding the identity of Francisella factors possibly engaged by galectin-3 and immunological consequences of these interactions are currently on-going in our laboratory. The survival advantage of *F. novicida* infected galectin-3−/− mice was dependent on the infection dose of bacteria. The galectin-3−/− mice succumbed to the infection at a similar rate as WT mice when infected with 300–500 CFUs of bacteria (data not shown). It is possible that at that dose, a higher bacterial burden leads to a further increase in cell death leading to an increased accumulation of other alarmins which mask the advantageous effect of the absence of galectin-3. This observation highlights the complex nature of sepsis syndrome where multiple host and pathogen derived factors cross talk and regulate various immune pathways. It is also consistent with previous studies showing partial or no protection upon blocking single alarmin such as HMGB1 [52–54]. Nonetheless, as the bacteria can be cleared by successful antibiotic therapy, the complications often arise from tissue damage during sepsis. Thus, a combinatorial approach using blockage of galectin-3 along with antibiotics could prove to be a successful therapy for treating Francisella infection induced sepsis.

In Teto, our findings indicate that galectin-3 plays a pathogenic role as an alarmin to exacerbate the inflammatory response during pulmonary infection with Francisella and contributes to sepsis development. Galectin-3 thus may represent a potential target for treatment of sepsis during this infection.
Supporting Information

Figure S1 Upregulated expression and extracellular release of Galectin-3 in lungs during respiratory F. novicida infection. Bronchoalveolar lavage (BAL) was obtained from lungs of mice infected with the wild-type F. novicida strain U112 or PBS alone as previously described (9). Galectin-3 was immunoprecipitated from BAL using a purified rat anti-mouse galectin-3 antibody (eBioscience, San Diego, CA) by previously described method [55] with modifications. Briefly 1 mg of total BAL proteins were incubated with 10 μg anti-galectin-3 antibody at 4°C overnight. Immune complexes were pulled down with using 30 μl of 30% Protein A Plus agarose beads (Pierce) for 2 h at 4°C. The beads were washed, solubilized in 1 x SDS gel loading buffer and resolved on 12% acrylamide gels (BioRad). The gels were processed for western blotting as described previously [3] for detection of galectin-3 using anti-mouse galectin-3 antibody. Densitometric analysis of bands was performed using the Lumi-Imager software (Roche Applied Science). Bar graph depicts densitometry analysis of galectin-3 bands represented in arbitrary units. Statistically significant differences are denoted by asterisks (∗∗∗p<0.001).

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Critical reviewed the manuscript: DWJ. Conceived and designed the experiments: JS BBM. Performed the experiments: BBM QJ. ALS RB. Analyzed the data: BBM JM JF. Contributed reagents/materials/analysis tools: JS JM JF. Wrote the paper: BBM JS.
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