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Mast Cell/IL-4 Control of *Francisella tularensis* Replication and Host Cell Death is Associated with Increased ATP Production and Phagosomal Acidification

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Abstract

Mast cells are now recognized as effective modulators of innate immunity. We recently reported that mast cells and secreted interleukin-4 (IL-4) effectively control intramacrophage replication of *Francisella tularensis* Live Vaccine Strain (LVS), and that mice deficient in mast cells or IL-4 receptor (IL-4R$^{-/-}$) exhibited greater susceptibility to pulmonary challenge. In this study, we further evaluated the mechanism(s) by which mast cells/IL-4 control intramacrophage bacterial replication and host cell death, and found that IL-4R$^{-/-}$ mice exhibited significantly greater induction of active caspase-3 within lung macrophages than wild-type animals following intranasal challenge with either LVS or the human virulent type A strain SCHU S4. Treatment of LVS infected bone marrow-derived macrophages with a pancaspase inhibitor (zVAD) did not alter bacterial replication, but minimized active caspase-3 and other markers (Annexin V and propidium iodide) of cell death, while treatment with both rIL-4 and zVAD resulted in concomitant reduction of both parameters, suggesting that inhibition of bacterial replication by IL-4 was independent of caspase activation. Interestingly, IL-4-treated infected macrophages exhibited significantly increased ATP production and phagolysosomal acidification, as well as enhanced mannose receptor up-regulation and increased internalization with acidification, which correlated with observations in mast cell-macrophage co-cultures, with resultant decreases in *F. tularensis* replication.

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Disclosure

The authors declared no conflict of interest.

Introduction

Francisella tularensis is a Gram-negative bacterial pathogen and a potential biological weapon due to ease of dissemination and high mortality following pulmonary infection [1,2]. *F. tularensis* subspecies *tularensis* Type A is the most significant and virulent agent of tularemia and may infect humans with as few as 10 organisms resulting in pneumonic disease[3]. In contrast, *F. tularensis* subsp. *holarctica*, also known as Type B, is less virulent and causes a milder form of disease that is rarely fatal[1]. Most studies have utilized a murine model with the Live Vaccine Strain (LVS) derived from subsp. *holarctica* to elucidate innate and adaptive immunity following respiratory exposure. *F. tularensis* LVS primarily infects macrophages and evades lysosomal degradation resulting in high bacterial replication within the cytosol[4–7].

The respiratory compartment and specifically the lungs are primary sites that encounter respiratory pathogens and have developed dynamic immune defenses for clearance of microorganisms. We recently reported that mast cells, in addition to conventional phagocytic cells, infiltrate the cervical lymph nodes and lungs after intranasal LVS challenge[8]. Mast cells have the capacity to produce a broad range of secreted factors and cytokines, including interferon-gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), interleukin-4 (IL-4), and interleukin-15 (IL-15)[9–12], upon antigenic stimulation. The high degree of plasticity associated with this cell type allows mast cells to (a) directly phagocytose and kill microorganisms[13–15], (b) influence the activity of other cell types in the vicinity by enhancing cellular recruitment and subsequent activation[16–18], and (c) promote survival by production and induction of cytokines[19].

Our previous data demonstrated that mast cells significantly inhibit LVS uptake and growth within macrophages via contact dependent events and secreted products, including IL-4. Additionally, mice deficient in mast cells or the IL-4 receptor were found to be more susceptible to pulmonary LVS challenge with resultant higher burdens in lungs and spleens than in wild-type animals[8]. These findings and the pleiotropic nature of IL-4 have led us to further define the mechanisms of mast cell/IL-4 inhibition of *F. tularensis* replication and cell death.

In this study, lung cells from mice deficient in the IL-4 receptor showed increased active caspase-3 expression in $CD11b^+$ macrophages, compared to similarly challenged wild-type animals, following LVS or SCHU S4 challenge. Additionally, bone marrow-derived mast cells effectively reduced intramacrophage replication, as well as the induction of active caspase-3, following *in vitro* LVS or SCHU S4 challenge. Furthermore, macrophages treated with recombinant IL-4 (rIL-4) during *Francisella* infection displayed decreased expression of the cell death markers active caspase-3 and PARP (poly-ADP-ribosyl protein), and exhibited reduced propidium iodide uptake. Notably, IL-4 inhibition of bacterial replication was associated with increased ATP production, mannose receptor recycling and localization of bacteria within acidified organelles. These results suggest that mast cell and IL-4 reduction of *Francisella* replication and host cell death are linked via ATP and the resulting enhanced acidification of invading bacterial pathogens.

Results

IL-4 signaling regulates active caspase-3 expression in lung macrophages during F. tularensis pulmonary infection

We previously reported that mice deficient in mast cells or IL-4 receptor (R) expression have greater susceptibility to intranasal (i.n.) LVS challenge[8]. Given that IL-4 has been reported to reduce induction of active caspase-3 and progression to cell death and/or necrosis[20], we evaluated the lungs of BALB/c IL-4R$^{+/+}$ and IL-4R$^{-/-}$ mice for expression of active caspase-3 by flow cytometry following i.n. LVS or SCHU S4 challenge. BALB/c mice were challenged i.n. with 1600 CFU of LVS, a dose used in our previous studies, or with 125 CFU of SCHU S4. Lung tissues were collected at day 4 post-challenge, and single cell suspensions were prepared, stained with fluorescent conjugated CD11b and active caspase-3 antibodies, and evaluated by flow cytometry. Flow cytometry scatter plots (side scatter versus active caspase-3, representative SCHU S4 plots shown in Fig. 1A) used for analysis of total lung cells, revealed that active caspase-3 expression was greater in lung cells obtained from IL-4R$^{-/-}$ KO compared to IL-4R$^{+/+}$ mice. Given that macrophages are highly permissive to *Francisella* infection[1,2,4,6,21], we specifically analyzed the CD11b^{+} macrophage population (Fig. 1B). As shown in Figure 1C, 29.4% of macrophages (CD11b^{hi} gate, black arrow, Fig. 1B) were positive for active caspase-3 in LVS-challenged IL-4R$^{-/-}$ mice compared to 15.5 % in IL-4R$^{+/+}$ animals at day 4 post-challenge (n=4, p<0.005). Cells within the CD11b^{lo} gate (Fig. 1B, open arrow), identified as natural killer cells, were negligible for active caspase-3 (data not shown). As expected, there was minimal infiltration of CD11b^{+} cells or caspase-3 positive cells within the lungs of mock challenged animals (Fig. 1C). Given the degree of active caspase-3 expression following LVS infection, similar analyses were performed in lung cells collected at day 4 from animals challenged i.n. with the human virulent strain SCHU S4. Active caspase-3 expression was significantly increased in IL-4R$^{-/-}$mice (19.2%) compared to IL-4R$^{+/+}$ mice (10.5%) after challenge (Fig. 1D). Moreover, active caspase-3 expression also was increased within IL-4R$^{-/-}$ CD11b^{hi} lung macrophages (11.1%) compared to IL-4R$^{+/+}$ macrophages (7.1%). These results suggest that IL-4R-mediated signaling modulates active caspase-3 expression within the pulmonary compartment following LVS and SCHU S4 challenge.

Mast cells and IL-4 inhibit F. tularensis-induced host cell death

Given that mice deficient in IL-4R signaling exhibited greater caspase-3 activation following pulmonary *F. tularensis* challenge, we utilized an established bone marrow derived primary macrophage-mast cell co-culture system[8] to directly assess the role of IL-4 inhibition of host cell death. These analyses revealed that macrophages (CD11b^{+} cells) cultured alone during LVS or SCHU S4 infection (100 MOI) exhibited greater than 40% active caspase-3 expression while infected macrophages co-cultured in the presence of mast cells exhibited a significant ($p<0.05$) reduction in caspase-3 activity (22%) [Fig. 2]. A significant ($p<0.05$) reduction in active caspase-3 also was evident in macrophages treated with rIL-4 (25 ng/ml) and infected with LVS (18%) and SCHU S4 (27%). Given the effect of mast cells and IL-4 in reducing *F. tularensis*-induced caspase-3 activation, we analyzed additional indicators of host cell death including Annexin V[22], PARP (poly-ADP ribose polymerase) and propidium iodide[23,24] staining by flow cytometry over a time course.

While propidium iodide stains dead and necrotic host cells, Annexin V binds membrane phospholipid phosphatidylserine (PS) molecules that are exposed during the early stages of cell death or apoptosis[22]. Induction of active caspase-3 and PARP are downstream events following mitochondrial damage and release of cytochrome c[23],[24]. These analyses (Fig. 3) revealed that induction of active caspase (11%) and PARP (9%) as well as Annexin V (12%) staining were detectable as early as 12 h. At 24 h, LVS-infected macrophages exhibited 62.4%, 42.2%, and 59.3% of Annexin V, active caspase-3, and PARP positive cells, respectively. Approximately, 12.0% of LVS-infected macrophages were double positive for propidium iodide and Annexin V (data not shown). In comparison, LVS-infected macrophages treated with rIL4 exhibited a significant ($p < 0.05$) reduction of Annexin V (18.2%), active caspase-3 (19.0%) and PARP (17.9%) expression at 24 h, compared to untreated infected macrophages. We further examined mast cell secreted IL-4 and influence on cell death by using co-culture systems consisting of wild-type macrophages and STAT6$^{-/-}$ mast cells that have negligible IL-4 production, but normal TNF-α production[25]. Macrophages co-cultured with STAT6$^{-/-}$ mast cells and infected with LVS resulted in a significant loss of mast cell inhibitory effects on intramacrophage bacterial replication as well as active caspase-3 expression compared to macrophages cultured with wild-type mast cells (Supplementary Fig. S1). These results provide additional evidence for the role of mast-cell secreted products, including IL-4, in modulating host cell death in infected macrophages.

Mast cells promote the maintenance of morphological integrity in macrophages during LVS infection

To further analyze the pattern of host cell death following *F. tularensis* infection, we observed changes in cellular morphology by 3-dimensional confocal microscopy in individual cell cultures or co-cultures after 24 h of infection with Lucifer yellow (green fluorescence)-labeled LVS (MOI 100) [Fig. 4A]. These analyses demonstrated that macrophages, but not mast cells, undergo marked morphological changes after LVS infection. Infected macrophages were seen to encompass greater numbers of Lucifer labeled LVS within the cytoplasm, compared to infected mast cells. In the co-cultures, macrophages exhibited CD11b^{hi}, while mast cells expressed negligible to minimal CD11b^{lo} and also were discernible by morphology. Importantly, LVS-infected macrophages (white arrow) cultured in the presence of mast cells (red arrow) largely retained normal cellular morphology when compared to infected macrophages cultured alone. The structural alteration of cells was further quantitatively evaluated using IMARIS software (Fig 4B). Measurements of surface area (LVS=1572 μ^3, mock=425 μ^3), volume (LVS=1777 μ^3, mock=2408 μ^3) and sphericity (LVS=0.28, mock=0.73; a value of 1.00 represents a perfect sphere) revealed that macrophages undergo significant quantifiable morphological changes following LVS infection. Macrophages co-cultured with mast cells exhibited more normal morphology (e.g., sphericity: 0.61) compared to macrophages cultured alone (sphericity: 0.28). Mast cells exhibited minimal alterations and comparable quantitative morphological measurements after LVS or mock infection. Given that we have previously shown that mast cell secreted IL-4 inhibits intramacrophage LVS replication, we next examined if this cytokine is also responsible for maintenance of macrophage morphology in the co-culture system. Uninfected and LVS infected macrophages were treated with rIL-4 (5 or 25 ng/ml)

and also evaluated for surface area, volume and sphericity. These data revealed that treatment with rIL-4 rescues macrophage morphology (Fig. 4C and Supplementary Fig. 2S) with similar fashion to mast cells during mast cell-macrophage co-culture. These results further support that mast cell secreted IL-4 exerts an effect on LVS-infected macrophages that allows these cells to maintain cellular integrity and reduce bacterial replication and cell death.

Inhibition of active caspase-3 does not alter bacterial replication

To determine if IL-4 inhibition of bacterial replication was dependent on inhibition of active caspase-3 and host cell death, we used a pancaspase inhibitor, zVAD[26,27] during the course of LVS infection. The pancaspase inhibitor was selected given that active caspase-3 has been suggested to be an important factor during LVS or SCHU S4 infection[28] and the possibility of involvement of other caspases. In this regard, and in contrast to SCHU S4 and LVS, induction of active caspase-1, which cleaves proIL1β, has been shown to be an important factor during *Francisella novicida* infection and ensuing inflammation[29]. In the mast cell-macrophage co-culture system used for this study, minimal IL-1β was detected in supernatants during LVS infection compared to a much higher quantity produced during *F. novicida* infection (data not shown), in agreement with previous studies. Thus, macrophages infected with LVS were compared to LVS infected macrophages that were pre-treated with zVAD, zVAD plus rIL4, or rIL4 (Fig. 5). Interestingly, macrophages treated with zVAD and infected with LVS exhibited a significant decrease of active caspase-3 expression (38% to 5%); however, bacterial replication was minimally altered. Macrophages treated with zVAD plus rIL4 or with rIL4 alone during infection exhibited reductions in both bacterial replication and active caspase-3. These results suggest that although IL-4 limits active caspase-3 and host cell death, the key mechanism for inhibition of bacterial replication by mast cells/IL4 may be unrelated to host cell death, but rather to an IL-4 mediated function that is essential for inhibition of both processes upstream of active caspase-3.

Interleukin-4 increases cellular ATP, acidification, and mannose receptor expression

Interleukin-4 has recently been shown to inhibit host cell death by an increase of cellular ATP via increased uptake of glucose[30]. Furthermore, ATP has been shown to promote phagosome-lysosome fusion and acidification with inhibition of bacterial replication[31,32]. Therefore, we examined IL-4 modulation of cellular ATP and acidification of phago-lysosomes in conjunction with mannose receptor expression during *Francisella* infection. The mannose receptor (MR) is important for *Francisella* recognition and is up-regulated by IL-4[33]. Macrophages were treated with rIL-4 (5 or 25 ng/ml) for 2 h prior to infection; cell lysates (normalized by Bradford assay) were analyzed for ATP after 1 h of infection with LVS; and acidification and MR expression were examined by confocal microscopy and flow cytometry. Macrophages treated with 5 ng/ml of rIL4 exhibited an increase of cellular ATP (15 to 18 mM), and a significant ($p<0.001$) increase (25 mM) with 25 ng/ml of rIL4 compared to untreated macrophages during LVS infection (Fig. 6A). Additionally, ATP production was significantly increased in LVS infected macrophages treated with 25 ng/mL compared to uninfected macrophages treated with the same concentration, suggesting a possible synergistic induction of ATP in LVS infected macrophages with addition of rIL-4. Since ATP enhances phagosome acidification, an acidification probe was added during the

last 30 minutes of infection for evaluation of phagosome acidity. The acidification probe is a fluorophore containing a weakly basic amine that selectively accumulates in acidic organelles and can be used in labeling live cells[34]. Cells were surface stained with anti-CD206 (mannose receptor, MR) and anti-CD11b at room temperature, and DAPI was added after final wash of antibodies. Surprisingly, confocal microscopy imaging, which allows 3-D analysis, showed that while the cells were only surface stained with anti-CD206 and anti-CD11b (cell permeabilizing agents were not used), the MR fluorescent conjugated antibody was taken into the macrophages and co-localized (white arrows) with the acidification probe which labels acidic organelles (Fig. 6B). This colocalization of MR with acidification regions was notably higher in rIL-4 treated compared to untreated macrophages. To further quantify MR expression, untreated macrophages were compared to rIL-4 treated macrophages, and analyzed by flow cytometry (Fig. 6C). Analyses revealed that rIL-4 treated macrophages exhibited a significant increase of MR expression compared to untreated macrophages during LVS infection. Furthermore, infected macrophages in co-culture with mast cells significantly increased MR expression from approximately 40% to over 80% (Fig. 6C). To further examine the significance of MR up-regulation and acidification, we generated mannose receptor deficient macrophages from C57BL/6 $MR^{-/-}$ mice[35]. Wild-type and $MR^{-/-}$ macrophages were treated with rIL4 and infected with LVS, and compared to untreated LVS infected macrophages for bacterial replication, MR expression, ATP (Fig. 7) and acidification (Supplementary Fig. S3). Given that additional mechanisms for bacteria uptake have been previously suggested[36] , it was not surprising that $MR^{-/-}$ macrophages also phagocytosed LVS. Addition of rIL-4 increased ATP in $MR^{-/-}$ macrophages as expected, however bacterial reduction was not significant, and a high percentage of bacteria did not co-localize with acidified regions (Supplementary Fig.S3). In separate experiments, addition of rIL-4 to IL-4 receptor deficient macrophages did not alter bacterial replication, MR expression, or ATP production (Fig. 8). Together, these data demonstrate that mast cells and IL-4 increase MR expression, and importantly, suggest that mast cell secreted IL-4 promotes uptake and acidification of MR in organelles during *F. tularensis* infection.

Interleukin-4 promotes co-localization of Francisella within acidified organelles

Given our findings that mast cells and IL-4 enhance MR expression and acidification of recycling receptors, we next determined if intramacrophage *Francisella* organisms were contained within acidified organelles or regions using Lucifer labeled LVS. We analyzed untreated and rIL-4 treated macrophages with addition of an acidification probe during the last 30 minutes of LVS infection *in vitro* and analyzed cells by confocal microscopy (Fig. 9). At one hour post-challenge, untreated macrophages contained numerous Lucifer labeled LVS (green) visible with minimal co-localization (yellow) with acidification regions (red). However, in rIL-4 treated macrophages, the lower number of Lucifer labeled LVS (green) colocalized with the numerous sphere-shaped regions encompassed by the acidification probe (Fig. 9) with Lucifer labeled LVS co-localizing as yellow.

Interestingly, 3D enlargement and comparison of the acidified regions revealed that addition of rIL-4 results in notable encompassing of bacteria within the red acidified regions, in contrast to untreated LVS infected macrophages (Fig. 9A, 3D enlargement). This may

suggest that the bacteria in the untreated macrophages escape the phagosome, rather than efficiently killed as in the rIL-4 treated macrophages. Furthermore, statistical analysis of *Francisella* within the acidification regions using ImarisR software, demonstrated that greater than 80% of the Lucifer labeled bacteria in the rIL-4 treated macrophages localized within the acidified regions compared to less than 10% (Fig. 9B) in the untreated macrophages. We further examined IL-4 influence on phagosome acidification using IL-4 receptor deficient macrophages in co-culture with mast cells and compared resulting acidification to wild-type mast cell macrophage co-cultures. These experiments revealed a notable increase of acidification regions and decreased Lucifer labeled LVS in wild-type co-cultures, but reduced acidification in IL4R$^{-/-}$ macrophages (Supplementary Fig. S4). However, after 24 h LVS infection (MOI 100), IL4R$^{-/-}$macrophages in co-culture with mast cells exhibited high cell lysis, which may be directly related to lack of IL-4 uptake and resulting cell death. Thus, by use of the acidificationprobe, which is highly selective for acidic organelles, we demonstrated that mast cell secreted IL-4 improves acidification of *Francisella* within macrophages.

Discussion

Mast cells are efficient innate immune responders located at mucosal surfaces and within tissues[37,38]. We previously reported that mast cells migrate to the lungs during *F. tularensis* LVS pulmonary infection and that mast cell secreted IL-4 significantly reduces intramacrophage replication[8]. In this study, we further analyzed mast cell/IL-4 mediated inhibition of bacterial replication and host cell death using mice deficient in IL-4 signaling. These mice (IL4R$^{-/-}$) exhibited significantly higher levels of active caspase-3, a known cysteine protease associated with cell death[39], compared to wild-type mice, following LVS or human virulent SCHU S4 pulmonary challenge. However, there was higher macrophage recruitment to the lungs and increased cell death within this population following LVS infection compared to SCHU S4. This increase in active caspase-3 expression within lung macrophages of LVS or SCHU S4-infected IL-4R$^{-/-}$mice suggests a physiological contribution of IL-4 in modulating host cell death following pulmonary infection. In this regard, Wickstrum and colleagues[28] have recently reported that mice infected via aerosolization of SCHU S4 exhibit active caspase-3 and cell death, but minimal cleaved caspase-1 associated with the inflammasome, within the lungs.

Notably, our study demonstrates a plausible mechanism by which IL-4-mediated inhibition of *F. tularensis* replication is associated with increased ATP production and improved acidification of organelles containing bacteria. Specifically, IL-4 increased ATP production, and MR expression and uptake with increased co-localization of MR within acidified regions in macrophages, as well as localization of Lucifer labeled *F. tularensis* to the acidified organelles. Interestingly, addition of rIL-4 resulted in bacteria well encompassed by acidified regions in contrast to untreated macrophages. Furthermore, when IL4R$^{-/-}$ macrophages were co-cultured with mast cells, there was reduced acidification and increased Lucifer labeled bacteria compared to wild-type macrophages in co-culture with mast cells (Supplementary Fig. S4). Also, at 24 h, IL4R$^{-/-}$ macrophages in co-culture with mast cells exhibited greater cell lysis than wild-type macrophages, which may be directly related to lack of IL4 uptake and resulting cell death. These results provide a foundation by

which mast cells and IL-4 production mediate reduction of bacterial replication and host cell death associated with the modulation of ATP expression.

To analyze parameters associated with cell death following bacterial infection, we evaluated the effect of mast cells on LVS and SCHU S4 intramacrophage replication and induction of apoptosis. Under similar co-culture conditions, mast cells were shown to inhibit the intramacrophage replication of SCHU S4 and LVS, and to diminish active caspase-3 induction. Additionally, infected macrophages treated with rIL-4 demonstrated decreased Annexin V and propidium iodide staining. To determine if the inhibition of bacterial burden was dependent upon the reduction of host cell death, we utilized a pancaspase inhibitor, zVAD. Although active caspase-3 was significantly diminished in the presence of the inhibitor, bacterial replication was not altered, suggesting that the effect of IL-4 was upstream and independent of active caspase-3 inhibition. Interleukin-4 up-regulation of ATP by enhanced glucose uptake has been shown to inhibit cell death[30].

Additionally, IL-4 up-regulates Bcl-xL, which functions in maintenance of mitochondrial integrity[30],[40], and prevention of cell death upstream of cytochrome c release, thereby reducing the progression to necrosis[20]. Interestingly, ATP also has been shown to promote phagosome-lysosome fusion and intracellular killing of bacterial pathogens[31],[32]. Our recent findings[8] and current published reports[30]–[32], suggest that the association between IL-4 inhibition of *Francisella* bacterial replication and host cell death may result from increased ATP production. Enhancement of ATP, not only supports cell survival, but also promotes acidification and killing of invading pathogens.

Additionally, to determine if the inhibitory effects observed with mast cells are mast cells specific, primary hepatocytes were co-cultured with macrophages, and then infected with LVS as conducted in previous experiments of co-culture with mast cells. Bacterial replication and macrophage active caspase-3 expression were not altered (data not shown), supporting the findings that inhibition is dependent upon mast cell secreted IL-4. While mast cell spent supernatant or rIL-4 treatment reduced LVS intramacrophage replication, the levels of nitric oxide were not increased (Supplemental Fig. 5S). Therefore, nitric oxide is ruled out as a possible factor influencing bacterial reduction. Additionally, $STAT6^{-/-}$ deficient mast cells which do not product detectable levels of IL-4, did not reduce bacterial replication or active caspase-3 expression in macrophages during co-culture. Considering that TNF-α has been shown to amplify IL-4 receptor signaling via inactivation of protein tyrosine phosphatase 1B[41], we also examined the effects of neutralizing antibody against TNF-α. This neutralizing antibody reduced the levels of IL4 (data not shown) in the system and the inhibitory effects of mast cells in co-culture. The highest level of abrogation of mast cell effects resulted with addition of anti-IL-4 and anti-TNF-α to the co-culture system during LVS infection (Supplemental Fig. S6). In view of the fact that *Francisella* infected macrophages produce TNF-α, this cytokine alone is not responsible for the decrease of bacterial replication or macrophage cell death observed with mast cells/IL4. The IL-4–TNF-α feedback mechanism during *Francisella* infection is currently under further investigation.

Interleukin-4 has been shown to enhance MR expression and increase recycling of these receptors into the lysosomes[33],[42]. Additionally, up-regulation of MR has been reported to be

essential to control of pulmonary pathogens such as *Pseudomonas*[43] and *Mycobacterium*[44]. To this end, Schulert and Allen[33] demonstrated that IL-4 treatment promotes phagocytosis of LVS and up-regulation of MR expression within infected macrophages. However, the degree of expression of IL-4 and the origin (e.g., peritoneal or bone marrow derived) of macrophages may lead to phenotypic differences in cellular activity. MR expression induced by extended (48 h) rIL-4 pretreatment compared to the short term (2 h) pretreatment used in this study was associated with decreased phagocytosis and inefficient bacterial killing[45]. The inefficient bacterial killing during extended rIL-4 treatment may promote excessive stimulation and reduced recycling of the MR. This is supported by a recent finding that sustained receptor stimulation results in sequestration of recycling endosomes[46]. Given the plasticity of mononuclear cells and the stimuli of the microenvironment (i.e., peritoneal, lung, systemic or tissue), there may be several facets to the response of macrophages to cytokines such as IL-4 *in vivo*. Collectively, our recent findings provide new insights to the contribution of mast cells and secreted IL-4 in mediating anti-bacterial innate immunity.

Methods

Mice

Specific pathogen-free 4–8 week old mice were utilized for all procedures. C57BL/6 and BALB/c mice were purchased from the National Cancer Institute. BALB/c IL-4R$^{-/-}$mice[47] were purchased from Taconic Farms. C57BL/6 MR$^{-/-}$ mice were a generous gift from Dr. Gary Cole. All experimental procedures and animal care were performed in accordance with the Institutional Animal Care and Use Committee (IACUC) guidelines.

Bacteria

Francisella tularensis LVS (obtained from Dr. R. Lyons at University of New Mexico) and *F. tularensis* SCHU S4 (obtained from the Centers for Disease Control) were grown in tryptic soy broth supplemented with L-cysteine[8]. Experiments using SCHU S4 were conducted in a licensed ABSL-3 facility.

Generation of primary cells and in vitro infection

Macrophages and mast cells were derived from mouse bone marrow and infected *in vitro* as described previously[8]. Briefly, cells were counted, plated and incubated for a 4-hour rest period without cytokines prior to infection. Cells were infected for 2 h, treated with gentamicin for 1 h, washed, incubated at 37 °C, and analyzed at various time-points. At 3, 12, or 24 h post-infection with LVS (MOI 100) or *F. tularensis* SCHU S4 (MOI 100), bacteria were enumerated using cellular lysates. In separate experiments, murine recombinant IL-4 (rIL-4, 5 or 25 ng/ml, eBioscience) was added to cultures 2 h prior to infection and rIL4 was replaced after each wash step. Cells were used for determination of bacterial replication, or active caspase-3 expression. For caspase inhibition experiments, z-VAD FMK (Fluromethylketone, BD Pharmingen), a pancaspase inhibitor, was dissolved in DMSO, and added to cultures 30 min prior to addition of rIL-4. Similar concentrations of DMSO were used as controls.

In vivo challenge and flow cytometry

Six to eight week old BALB/c (IL4R$^{+/+}$ and IL-4R$^{-/-}$) mice were challenged intranasally (i.n.) with 1600 CFU of LVS (a dose used in our previous studies) or PBS (mock challenge). At day 4 post-challenge, mice were euthanized. Additionally, BALB/c (IL4R$^{+/+}$ and IL-4R$^{-/-}$) mice were challenged i.n. with 125 CFU of SCHU S4 or PBS and mice were euthanized in a similar manner at day 4 post-challenge. The lungs were perfused with 1X PBS, then excised and placed in a sterile dish. Lungs were rinsed with cold 10 % RPMI and transferred to fresh media. The lung tissue was minced, and cells were filtered through a 70 μm strainer. The cells were then treated with collogenase (0.7 mg/ml) plus DNAse and incubated at 37 °C for 15 minutes. Next, the lung cells were washed in cold 10 % RPMI, and RBC lysis buffer was added to the cells. The cells were incubated for 5 minutes at 37°C, washed and aliquoted into polystyrene tubes at 1 X 10^6 for staining with fluorescent conjugated antibodies. Next, the cells were washed with 2 % FBS in 1X PBS with 0.09 % sodium azide (PBS wash), then blocked with 10 % rat sera 1X PBS at 4 °C for 30 minutes. Samples were washed and surfaced stained with CD11b allophycocyanin (APC) or isotype control (IgG2a κ APC), incubated at 4 °C for 30 minutes, and washed. The samples were then fixed in Cytofix/Cytoperm™ for 20 min, washed with PBS wash, and treated with 1X PermWash™ for 10 min and wash was removed. Active caspase-3 PE (Clone C92-605, BD Biosciences), or isotype control (IgG1 PE) was diluted in 100 μL 1X PermWash™, and added to appropriate samples. Samples were incubated for 60 min with intermittent vortexing, washed in 1X PermWash™, and resuspended in 2% (w/v) paraformaldehyde for flow cytometer (FACSCalibur, BD Biosciences) analyses. Analyses were performed using CellQuest Pro software (BD Biosciences).

For *in vitro* analysis of macrophages, mast cells and co-cultures, cells were collected at 3, 12 or 24 h and washed in PBS wash. Samples were subsequently blocked with anti-mouse CD16/CD32 (BD Biosciences), or 10 % rat sera at 4°C, followed by addition of fluorescent conjugated antibodies for surface and/or intracellular staining as noted above. Fluorescent antibodies included FcεRI (phycoerythrin, PE), c-Kit fluorescein isothiocyanate (FITC) (Clone 2B8, eBioscience), CD11b (FITC or APC, allophycocyanin), CD206 (Clone MR5D3, BioLegend), active caspase-3 PE (Clone C92-605, BD Biosciences), or cleaved PARP PE (Clone F21-852, BD Biosciences) or isotype controls (IgG1 PE, IgG2a κ FITC, IgG2aκ APC, IgG2a κ Alexa 488). Gating for analyses included side scatter versus c-Kit for primary mast cells, or CD11b for macrophages. In separate experiments, samples were washed with 1X Binding Buffer and stained for 15 minutes with cell death markers Annexin V-FITC and propidium iodide for 15 minutes; unstained samples were used as controls. The samples were then washed with 1X Binding buffer (BD Biosciences), resuspended in 400 uL 1X Binding buffer, and acquired (FACSCalibur) within 30 minutes. Analyses were performed using CellQuest Pro software (BD Biosciences).

Microscopy

LVS bacteria were labeled with Lucifer yellow (Sigma)[48] at 37°C for 2 h, washed and placed on ice. Lucifer labeled bacteria were used for *in vitro* infection as described in the previous section, however mast cells and macrophages were cultured on coverslips in 24-well plates. For analysis of acidification[34], a specific acidification probe (LysoTracker Red

DND-99, Invitrogen, Eugene, OR) was added for 30 min to 1 h in culture at 37°C, followed by several washings. CD206 Alexa 488 was added to cells at room temperature for 30 min. and washed. Additional staining with fluorescent conjugated antibodies to FcεRI (PE) or CD11b (PE) was conducted at 4°C for 30 min followed by washing. The cells were fixed with 2 % Paraformaldehyde for 20 minutes and washed. Mounting media with DAPI (Calibiochem, Canada) was added, and coverslips were placed on slides. Samples were examined using a confocal microscope (Zeiss 510 Meta, Carl Zeiss Microimaging Inc., Thornwood, NY) and analyzed with Imaris software (Bitplane Inc., St. Paul MN).

Statistics

Data were analyzed by Student's t-test or ANOVA using the statistical software program SigmaStat (Chicago, IL). ANOVA was used for comparison of more than two groups. A *P* value of 0.05 or less was considered statistically significant. Data are representative of experiments repeated two to three times.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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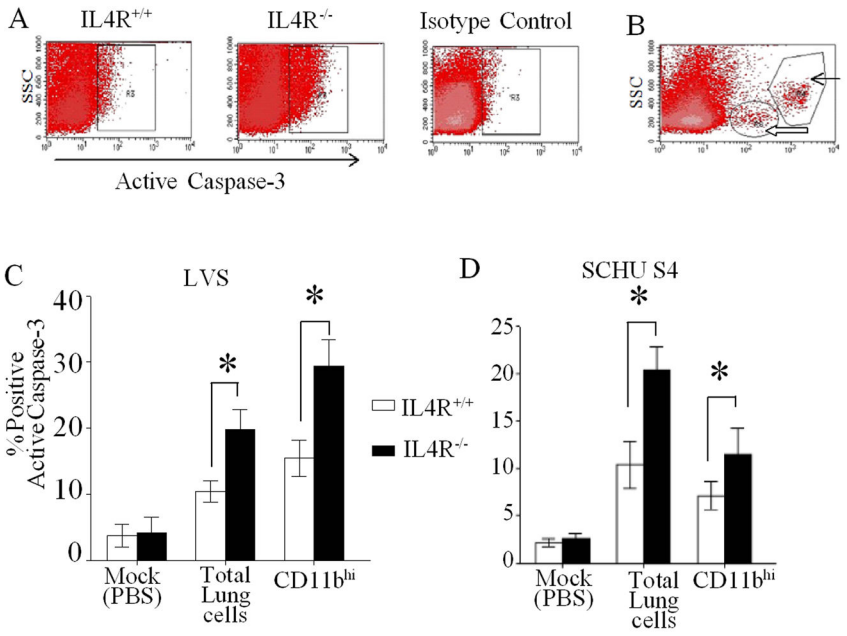


Fig. 1. IL-4 signaling regulates active caspase-3 expression in lung macrophages during *F. tularensis* LVS and SCHU S4 pulmonary infection

Lung cells from WT and IL-4R$^{-/-}$ mice were analyzed by flow cytometry for active caspase-3 expression on day 4 after i.n. LVS challenge (1600 CFU; n= 4 /group); or day 4 after SCHU S4 challenge (125 CFU; n=4/group); (A) Total lung cells and cells within the CD11b^{hi} gate were analyzed; 100,000 events collected. Representative scatter plots (SCHU S4 i.n. infection) are shown for total active caspase-3 positive lung cells for WT and IL-4R$^{-/-}$ mice, isotype controls, and (B) Side scatter versus CD11b; CD11b^{hi} gate (arrow) used for macrophage analysis (open arrow, CD11b^{lo}, NK cell population). Expression of active caspase-3 positive total lung cells and CD11b positive active caspase-3 positive lung cells are compared from WT and IL-4R$^{-/-}$ mice following LVS (C) or SCHU S4 (D) i.n. challenge. Unparied *t* test, $*p < 0.05$. Results are representative of three independent experiments.

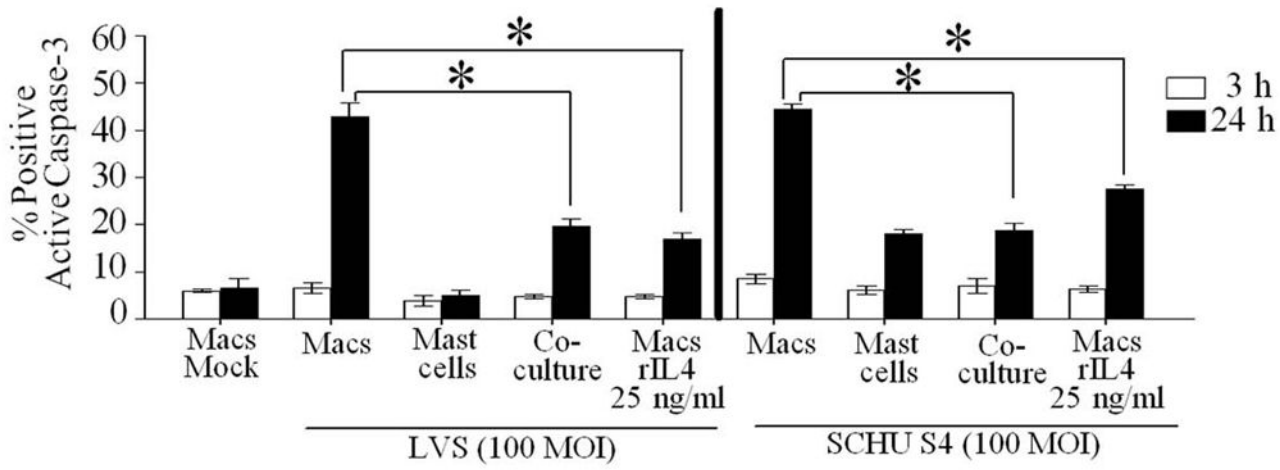


Fig. 2. Mast cells inhibit *F. tularensis* LVS and SCHU S4 induction of active caspase-3

Macrophages, mast cells, co-cultures, or macrophages plus 25 ng/ml of rIL-4 were infected with LVS (MOI=100) or SCHU S4 (MOI=100). Active caspase-3 was evaluated by flow cytometry (mast cells, macrophages, or co-cultures) at 3 and 24 h. Expression of active caspase-3 in LVS or SCHU S4-infected macrophages was compared to infected macrophages in co-culture with mast cells or with addition of rIL-4 (25 ng/ml) [Unpaired *t* test, $*p < 0.05$]. Within co-cultures, cells were gated on $CD11b^+$ for macrophage analysis; 10,000 events were acquired. Results are representative of three independent experiments.

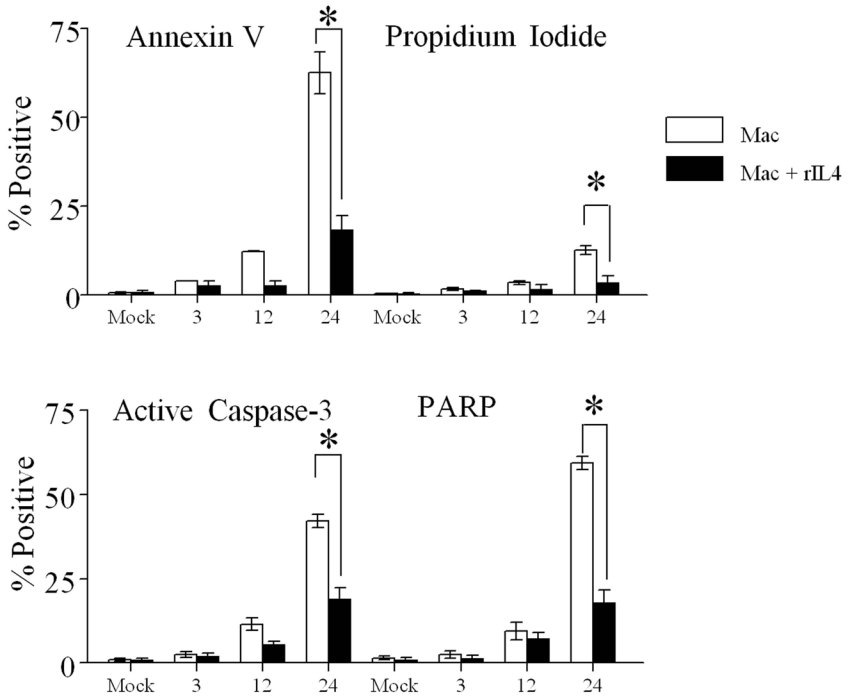


Fig. 3. IL-4 inhibits *Francisella* induced cell death in macrophages

Macrophages + rIL-4 (25 ng/ml) were infected with LVS (MOI=100) and compared to LVS infected untreated macrophages at 3, 12 and 24 h for Annexin V and propidium iodide staining. Uninfected macrophages were used as controls. A separate group of similar treated cells were permeabilized and stained for active caspase-3, and PARP, and analyzed by flow cytometry. Untreated macrophages were compared to rIL4 (25 ng/ml) treated macrophages [Unpaired *t* test, $*p < 0.05$]. 10,000 events were acquired. Results are representative of three independent experiments.

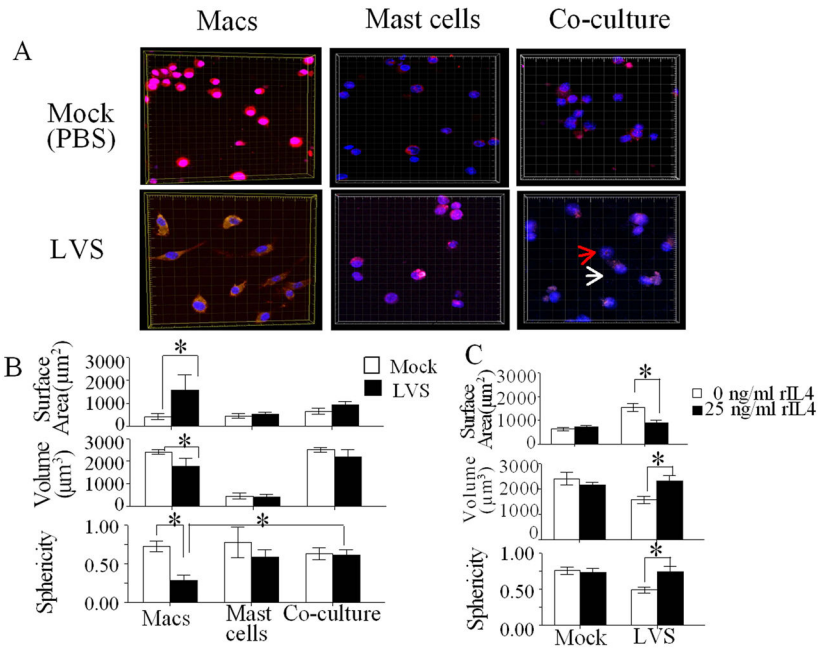


Fig. 4. Macrophages exhibit reduced morphological alterations in co-culture with mast cells during *F. tularensis* LVS infection

Mast cells, macrophages, or co-cultures were infected (MOI=100) with Lucifer yellow-labeled LVS. (A) Cells were analyzed by confocal microscopy after 24 h in culture. Macrophages were stained with anti-CD11b PE (red), while mast cells were stained with anti-FcεRI PE (red) and nuclear stain (blue). Macrophages (white arrow), mast cells (red arrow). Magnification 400X. (B) Cells from culture were quantitatively analyzed by IMARIS software for area, volume, and sphericity (1.00 represents a perfect sphere) after LVS infection. (*$p < 0.05$) LVS-infected macrophages compared to mock-infected macrophages. Comparison of sphericity of LVS-infected macrophages compared to mast cell macrophage co-cultures (C) Uninfected or LVS infected macrophages were treated with rIL-4 (5 or 25 ng/ml) or untreated and cellular morphology was compared for surface area, volume and sphericity. (Unpaired *t* test, *$p < 0.05$). Results are representative of three independent experiments.

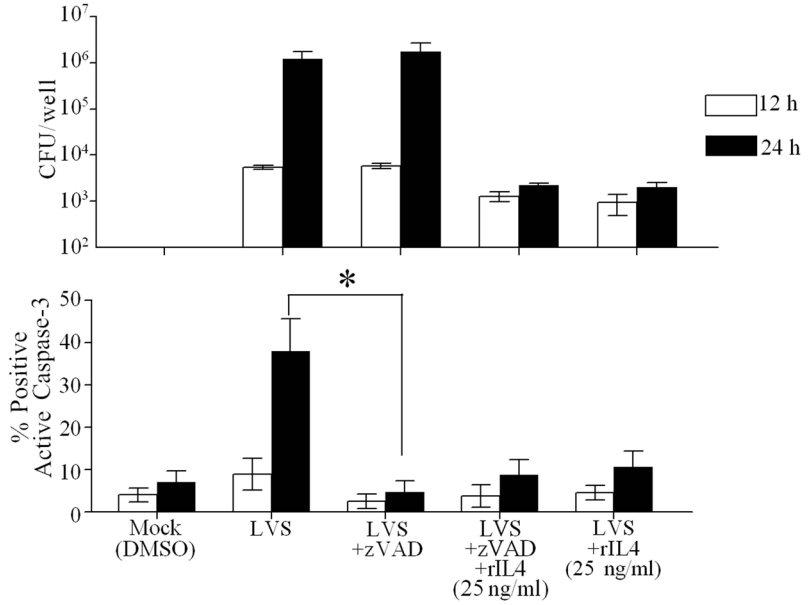
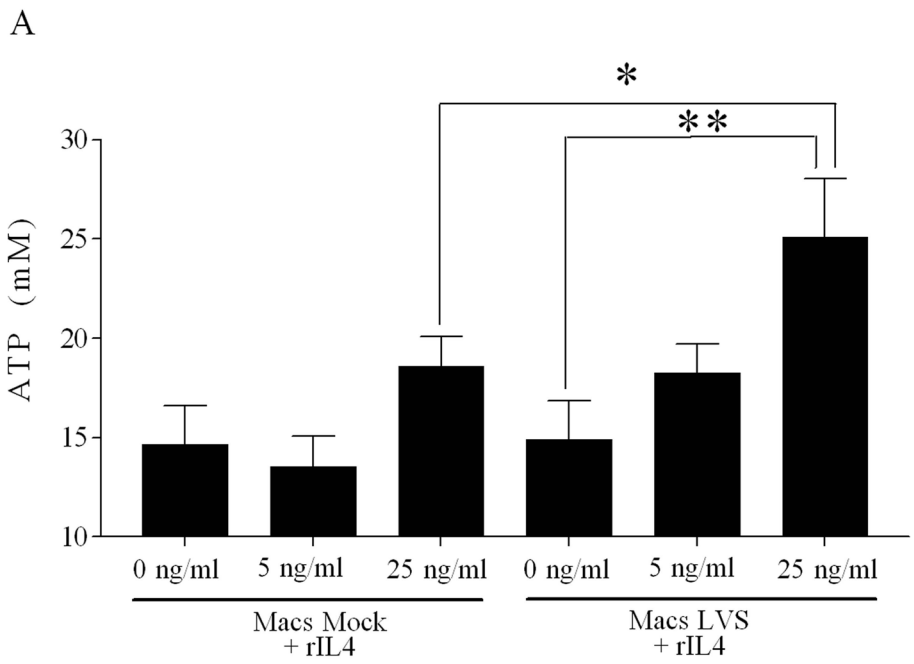


Fig. 5. Inhibition of active caspase-3 does not alter bacterial replication

Macrophages, macrophages + zVAD (20μM), macrophages + zVAD (20μM) and rIL-4 (25 ng/ml), or macrophages + rIL4 (25 ng/ml) were infected (MOI=100) with LVS and compared for bacterial replication and active caspase-3 induction at 12 and 24 h. (Unpaired *t* test, $*p < 0.05$). Results are representative of three independent experiments.



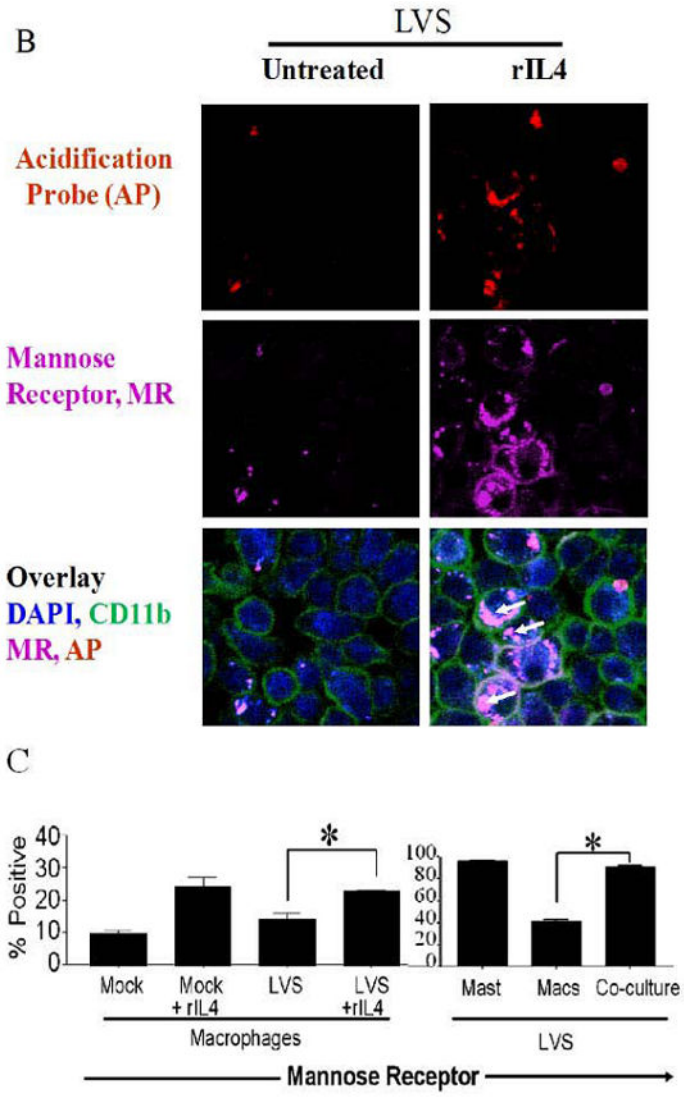


Fig. 6. IL-4 promotes ATP production and mannose receptor expression with recycling

(A) Uninfected macrophages and macrophages + rIL-4 (5 or 25 ng/ml) were compared to LVS infected (MOI=100) macrophages and LVS infected macrophages + rIL-4 (5 or 25 ng/ml) for induction of ATP production by bioluminescence assay, (Unpaired *t* test, $*p < 0.001$). (B) LVS infected (MOI=100) macrophages were compared to LVS infected macrophages + rIL-4 (25 ng/ml) and analyzed by confocal microscopy (400X). Macrophages were treated with the acidification probe during incubation and surfaced stained with anti-CD206 (MR, purple), anti-CD11b (green), and DAPI (nuclear stain, blue). Representative images are compared for untreated and rIL-4 treated macrophages: acidification probe (red), and MR (purple) alone and co-localization of internalized MR (white arrow) and organelle acidification probe (pink). (C) Macrophages (± rIL-4 treatment), mast cells or co-cultures were infected (MOI=100) with LVS and analyzed for MR expression; macrophages in co-culture were analyzed within the CD11b gate; 10,000 events were acquired. (Unpaired *t* test, $*p < 0.05$). Results are representative of three independent experiments.

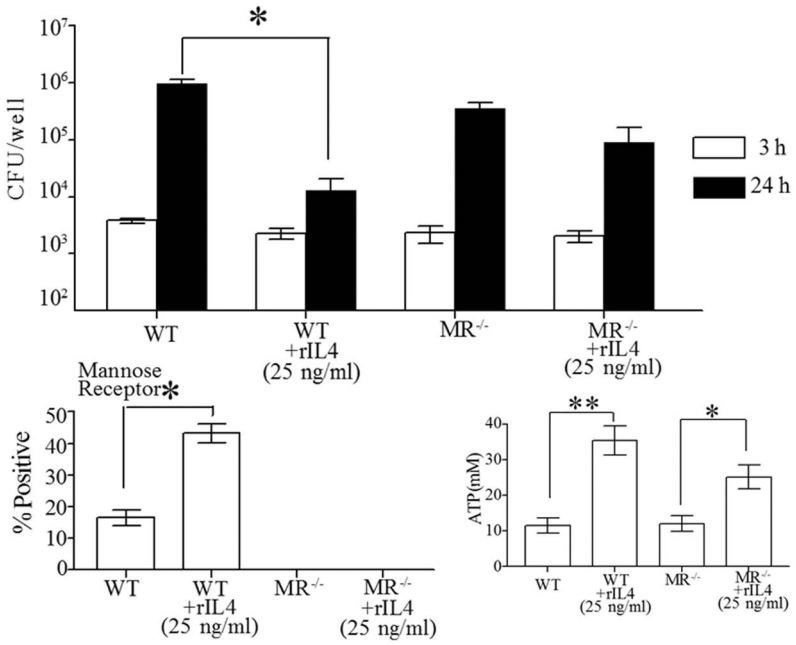


Fig. 7. Mannose receptor deficient macrophages exhibit reduced bacterial killing compared to WT macrophages

WT and $MR^{-/-}$ macrophages were treated rIL-4 (25 ng/ml), infected with LVS (MOI=100), and compared to corresponding untreated LVS infected (MOI=100) macrophages. Cells were analyzed for bacterial replication (A) MR expression (B) and ATP production (C) as previously described (Unpaired *t* test, **p* <0.003).

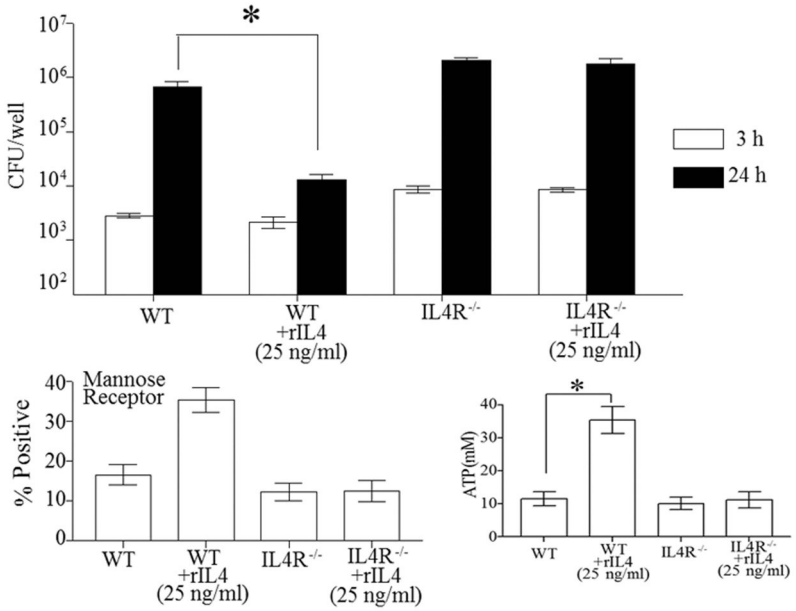


Figure 8. IL-4 receptor deficient macrophages do not exhibit increased ATP production or mannose receptor expression with rIL-4 treatment

WT and IL4R$^{-/-}$ macrophages were treated with rIL-4 (25 ng/ml), infected with LVS (MOI=100) and compared to corresponding untreated LVS infected (MOI=100) macrophages. Cells were analyzed for bacterial replication (A), MR expression (B) and ATP production (C) as previously described (Unpaired *t* test, *$p < 0.003$).

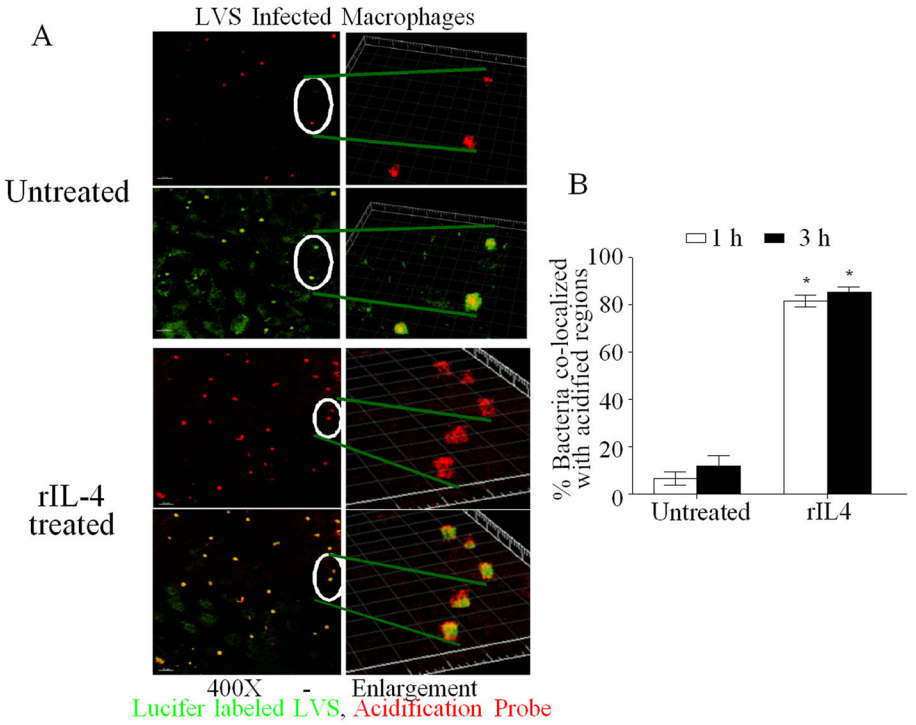


Fig. 9. Interleukin-4 promotes co-localization of *Francisella* within acidified organelles

Lucifer labeled LVS (green) infected (MOI=100) macrophages were compared to Lucifer labeled LVS (green) infected macrophages + rIL-4 (25 ng/ml) and analyzed by confocal microscopy (400X) at 1 h post-challenge. Macrophages were treated with the organelle acidification probe (red) during incubation. (A) Representative images are compared for untreated and rIL-4 treated macrophages: acidification probe (red) alone, and co-localization of Lucifer labeled LVS (green) with acidification probe (yellow). 3-D enlargement of the acidified organelles: Lucifer labeled LVS, and co-localization shown (white arrows). (B) Lucifer labeled LVS infected (MOI=100) macrophages were compared to Lucifer labeled LVS infected macrophages + rIL-4 (25 ng/ml) and analyzed using IMARIS^{R} software at 1 and 3 h post-challenge. 3-D spot localization analysis of Lucifer yellow (488nm channel, greater than 0.8um) and acidification probe (594nm channel, greater than 2.0 um) were compared to co-localization (Lucifer yellow with red) and calculated as a percent of the total for the two groups. At least 100 cells were analyzed from each group, *$p<0.002$.