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Non FcεR-bearing Mast Cells Secrete Sufficient Interleukin-4 to Control *Francisella tularensis* Replication within Macrophages

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Abstract

Mast cells have classically been implicated in the triggering of allergic and anaphylactic reactions. However, recent findings have elucidated the ability of these cells to selectively release a variety of cytokines leading to bacterial clearance through neutrophil and dendritic cell mobilization, and suggest an important role in innate host defenses. Our laboratory has established a primary bone marrow derived mast cell-macrophage co-culture system and found that mast cells mediated a significant inhibition of *Francisella tularensis* LVS uptake and replication within macrophages through contact and the secreted product interleukin-4 (IL-4). In this study, we utilized P815 mast cells and J774 macrophages to further investigate whether mast cell activation by non-FcεR driven signals could produce IL-4 and control intramacrophage LVS replication. P815 supernatants collected upon activation by the mast cell activating peptide MP7, as well as P815 cells co-cultured with J774 macrophages, exhibited marked inhibition of bacterial uptake and replication, which correlated with the production of IL-4. The inhibition noted in vitro was titratable and preserved at ratios relevant to cellular infiltration events following pulmonary challenge. Collectively, our data suggest that both primary mast cell and P815 mast cell (lacking FcεR) secreted IL-4 can control intramacrophage *Francisella* replication.

Keywords

mast cells; Gram negative bacteria; IL-4; respiratory infection; *Francisella tularensis*; innate immunity

1. Introduction

Although the conventional view of mast cells is associated with IgE-mediated allergic disease [1; 2], there is accumulating evidence indicating that mast cells are a crucial component of the innate immune system [3; 4]. Specifically, several studies [3; 4; 5] have reported that mast cells play a vital role in microbial recognition and modulation of host

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defense mechanisms in response to Gram-negative bacteria. This may be attributed to the preferential location of mast cells at the mucosal interface, as well as the ability of these cells to release a wide array of preformed proinflammatory mediators, such as tumor necrosis factor alpha (TNF- α), which mediate the interaction of CD48, a mast cell surface receptor, with the bacterial adhesion protein FimH of type 1 pili present on Gram-negative bacteria [6; 7].

Our laboratory has recently adapted a mast cell-macrophage co-culture system based on the in vitro co-culture system developed by Cowley et al [8] in order to examine the interaction of these cell types with the facultative intracellular, Gram negative bacterium *Francisella tularensis*, the causative agent of tularemia [9; 10]. We reported that bone-marrow derived mast cells cultured with *F. tularensis* Live Vaccine Strain (LVS), an attenuated organism [11], exhibited minimal bacterial uptake and replication. Importantly, mast cells co-cultured (1 to 1 ratio) with bone-marrow derived macrophages, which are highly permissive to *F. tularensis*, were shown to exhibit a significant inhibition of intramacrophage bacterial replication [12]. This effect of mast cells on intramacrophage replication was mediated by contact-mediated mechanisms and by the production of mast cell-derived IL-4.

In this study, we utilized P815 mast cells (that do not express the Fc ϵ R) and J774 macrophages to determine the role of non-Fc ϵ R mediated activation of mast cells in production of secretory products, including IL-4, and regulation of intramacrophage *F. tularensis* replication. Our results indicate that Fc ϵ R deficient mast cells produce secretory products through non-classical pathways to control intramacrophage *Francisella* replication and, moreover, that the ratios required to achieve this inhibition may be physiologically relevant.

2. Materials and Methods

2.1. Mice

Pathogen-free mice (5–8 weeks) were used in all experiments. C57BL/6 mice were purchased from the National Cancer Institute and housed at the University of Texas at San Antonio Animal Facility. Institutional Animal Care and Use Committee (IACUC) guidelines were followed with regard to animal housing, care, and experimental procedures.

2.2. Bacteria

Francisella tularensis LVS (lot 703-0303-016) was obtained from Rick Lyons at the University of New Mexico and was grown in trypticase soy broth (TSB) supplemented with 0.1% cysteine. Bacteria were grown into late log phase, pelleted by centrifugation, and resuspended in freezing medium containing 70% TSB and 30% glycerol. Each bacterial stock was held at –80 degrees until thawed and used for experiments. The bacterial concentration was determined by serial dilution plating on supplemented trypticase soy agar (TSA) plates.

2.3. Cell lines

The J774 murine macrophage cell line (ATCC Number TIB-67) was grown to 90–95% confluence in Dulbecco's Modified Eagles Medium (DMEM) (Mediatech, Inc., Manassas, VA) supplemented with 10% fetal bovine serum (FBS) (HyClone Laboratories, Logan, UT), and was passaged every 2 to 3 days in T75 tissue culture flasks (BD Biosciences, Bedford, MA). The P815 mast cell line (ATCC Number TIB-64) was suspended in DMEM supplemented with 10% FBS and 1.5 g/L of sodium bicarbonate. Fresh medium was added every 2 to 3 days, and the cells were washed and resuspended in new medium every 6 to 8 days.

2.4. Generation of primary mast cells

Bone marrow derived mast cells (BMMC) were obtained using aseptic procedures described previously (Ketavarupu et. al [12]). Briefly, mice were euthanized and the femurs removed and flushed with ice cold RPMI 1640 (Mediatech, Inc., Manassas, VA) medium. Cells were resuspended in RPMI medium supplemented with 10% FBS, recombinant IL-3 (5 ng/ml) and stem cell factor (5 ng/ml) (PeproTech, Rocky Hill, NJ) for differentiation into mast cells. The cells were then seeded into culture flasks for 24 h and nonadherent mast cells transferred into new flasks. The mast cells were maintained by the addition of cytokine enriched RPMI medium with 10% FBS every 3 days and harvested for all experiments at approximately 4 weeks.

2.5. Mast cell granule staining

The P815 mast cells and BMMCs were stained using Wright's stain (Sigma-Aldrich, St. Louis, MO) (manufacturer's instructions) to visualize the granules. Briefly, 1×10^5 cells were adhered to Cytopro poly-L-lysine coated slides using a Cytopro 7620 Cyto centrifuge (Wescor, Logan, UT) and treated with Wright's stain for 2 min followed by Wright's buffer for 4 min until a green metallic sheen was formed. The slides were rinsed with deionized water, and allowed to dry before microscopic analyses.

2.6. Flow cytometry

P815 and bone marrow derived mast cells were seeded into polystyrene tubes at a density of 2.5×10^5 cells in phosphate buffered saline (PBS) and then blocked with purified rat anti-mouse CD16/CD32 Fc block. Cells were stained with PE (phycoerythrin) conjugated anti-mouse FcεRI antibody (Clone: MAR1, eBioscience, San Diego, CA) and FITC (fluorescein isothiocyanate) conjugated anti-mouse cKit antibody (eBioscience, San Diego, CA). The samples were acquired using the BD LSR II flow cytometer (BD Biosciences, San Jose, CA) and analyzed by FACSDiva software. Lung cells were stained with FITC conjugated anti-mouse CD11b antibody (eBioscience, San Diego, CA), APC (allophycocyanin) conjugated anti-mouse F4/80 antibody (eBioscience, San Diego, CA), FITC conjugated cKit or PE conjugated FcεRI. Appropriate isotype controls were used for analyses. Cells were then washed twice with 2% fetal bovine serum in 1X PBS and the samples were acquired using FACSCalibur (BD Biosciences) and analyzed with CellQuest Pro software (BD Biosciences).

2.7. Preparation of cell suspensions from lungs

C57/BL6 mice (n = 3/group) were challenged intranasally with 1600 CFU of LVS (LD₅₀ approximately 5000 CFU) or mock infected (PBS). On day 3 post-challenge, mice were euthanized. Lung tissues were collected, rinsed and transferred to fresh medium. Tissues were minced and passed through 70 μm cell strainers. Cells were treated with collagenase (0.7 mg/ml, Sigma C7657) plus Type IV Bovine DNase (Sigma D5025) at 37°C for 20 min, washed in DMEM and added to RBC lysis buffer (NH₄CL + KH₂PO₄) for 5 minutes at 37°C. Cells were then washed, counted, and aliquoted into polystyrene tubes for flow cytometry analysis. 50K to 100K events were collected using FACSCalibur and analyzed with BD CellQuest Pro software.

2.8. In vitro infection of cells

J774 macrophages, P815 mast cells, and BMMCs were seeded into sterile 96 well tissue culture plates at a total cell density of 5×10^5 cells/well. For 1:1 ratio co-cultures, the wells contained an equal amount of mast cells and macrophages with a total of 5×10^5 cells/well. For all other ratios, mast cells were decreased while maintaining the total number of cells/well at a constant 5×10^5 . The seeded cells were incubated with *F. tularensis* LVS at a

multiplicity of infection (MOI) of 10 or 100 for 2 h, and then treated with gentamicin (MP Biomedicals, Solon, OH) for an additional 2 h to kill extracellular bacteria. Following gentamicin treatment, the 4 h time point cells were lysed with a 0.2% sodium deoxycholate solution and serial dilutions were prepared and plated on supplemented TSA. For 24 h time points, cells were washed, replaced with fresh medium and incubated at 37°C in 5% CO₂ for the next 20 h. After incubation, cells were lysed and dilution plated on supplemented TSA. The plates from both time points were incubated at 37°C for 48 to 72 h and colonies counted for enumeration. Uptake and replication assays were performed as above, but supernatants in addition to cell lysates were collected at 4 and 24 h time points and dilution plated. For some experiments, supernatants were collected at the 2 and 24 h time points, centrifuged to remove bacteria, and stored at -80°C for cytokine analysis. For experiments using recombinant IL-4 (rIL-4) (eBioscience, San Diego, CA), macrophages were pretreated with various concentrations of rIL-4 for 2 h before infection, and throughout the assay. In separate experiments, assays were conducted as stated above, and supernatants were collected 24 h post LVS or mock infection, centrifuged, filtered through 0.22 µm filters (Millipore Corporation, Bedford, MA), and held on ice for use as spent supernatants. J774 macrophages were pretreated for 2 h with mock or LVS infected cell spent supernatant and throughout the infection period and assayed for intramacrophage replication as previously described. In some experiments, P815 mast cells were infected with LVS (MOI 10) and cultured in media alone, or with neutralizing anti-IL-4 antibody (50 µg/ml; clone 11B11) during the 24 h incubation period. Bacterial replication was determined by dilution plating of lysed cells. Supernatants also were obtained following treatment of P815 cells with the small-molecule mast cell activator MP7 or the inactive analog MP17 (Peptide 2.0 Inc, Herndon, VA) [13; 14]. The P815 mast cells were seeded into sterile 96-well tissue culture plates at a total cell density of 5×10^5 cells/well and treated with either MP7 (50 µg/ml), MP17 (50 µg/ml), or PBS and incubated at 37°C for 30 min, and supernatants collected and used for pretreatment of J774 macrophages 2 h before LVS infection and throughout the uptake and replication assay. To further investigate the mast-macrophage interactions during *F. tularensis* infection, J774 macrophages were infected with LVS (10 MOI) for 24 h, treated with gentamicin for additional 24 h and supernatants collected after 24 h. The supernatants were filtered and utilized as a spent-culture medium and to activate P815 mast cells. Moreover, filtered supernatants obtained from the activated P815 mast cells were utilized to treat LVS-infected J774 macrophages.

2.9. ELISA

Cell supernatants were collected at 2 and 24 h and stored at -80°C until assayed. Cell supernatants also were collected 30 min after incubation of P815 mast cells with the mast cell activators. IL-4 and TNF-α enzyme linked immunosorbent assays (BD Pharmingen, San Diego, CA) were used to detect cytokines according to the manufacturer's procedure. Plates were analyzed on a microplate reader (Bio-Tek Instruments, Winooski, VT) and the test sample concentrations calculated against the standards.

2.10. Statistical analysis

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.

3. Results

Characteristics of bone marrow derived mast cells and the P815 mast cell line

We previously have shown that mast cells infiltrate the lungs following LVS intranasal challenge, and that BMMC significantly reduce LVS intramacrophage replication in vitro [12]. In this study, we investigated the use of P815 mast cells as an alternative to BMMC. The P815 mastocytoma cells have been shown to contain high levels of histamine and serotonin [15], but lack an abundance of metachromatic granules which are characteristic of normal mast cells. Additionally, P815 mast cells do not express FcεRI, the receptor involved in the cross linking and aggregation of IgE which leads to degranulation and the release of various inflammatory mediators from the granules [16; 17].

Using our established system, we compared the two mast cell types during LVS infection. First, the BMMC and P815 mast cells (P815s) were qualitatively characterized by staining with Wright's stain to show the presence or absence of granules (Fig. 1A, 1B). Both mast cell types displayed blue nuclei and lighter cytoplasm, while BMMC showed many purple stained granules (arrows) (Fig. 1A) in contrast to P815 mast cells which, as expected, did not display these granules (Fig. 1B). Subsequently, the BMMC and P815s were stained with fluorescent conjugated antibodies against the mast cell surface receptors cKit and FcεRI, and analyzed by flow cytometry (Fig. 1C–F). The BMMC displayed a heterogeneous population of surface receptor expression with 80% of the population staining double positive for cKit and FcεRI expression, 10% staining single positive with 8% staining positive only for FcεRI and 2% positive only for cKit (Fig. 1E). In contrast, P815 mast cells did not display a shift on the FcεRI axis due to lack of the complete and functional receptors but 100% of P815s stained positive for cKit (Fig. 1F). Moreover, to determine whether bacterial infection induced FcεR expression on P815 cell surface, these cells were infected with LVS (MOI 10 and 100) and stained for FcεR expression. These analyses revealed that LVS-infected P815 mast cells exhibited a lack of Fcε receptor staining (0.8% positive cells; data not shown). Taken together, these results confirm that P815 cells exhibit mast cell properties, although, the cells cannot be activated by IgE dependent mechanisms due to the lack of FcεR.

P815 mast cell lines inhibit *F. tularensis* LVS replication in J774 macrophages

To validate the use of the cell lines for studying *F. tularensis* LVS infection in the macrophage-mast cell co-culture model, J774 macrophages, P815 mast cells and BMMC, alone and in 1:1 co-cultures of J774s with P815s or BMMC, were challenged with LVS (MOI 10 or 100). After 2 h of infection, macrophages, mast cells, and co-cultures were treated with gentamicin for 2 h to kill extracellular bacteria. Cells were then lysed, and dilution plated at the 4 and 24 h time points to determine bacterial replication. *F. tularensis* LVS replication increased by 3 logs within macrophages from 4 to 24 h (Fig. 2) at both MOIs (10 and 100). However, there was significantly ($P = 0.01$) less replication in both the P815 mast cells and BMMC at 24 h compared to replication in the J774 macrophages. Additionally, there was significantly less ($P = 0.05$) LVS replication in the P815s when compared to the BMMC, suggesting that the P815 cell line controlled intracellular LVS replication better than BMMC. Inhibition of intramacrophage LVS replication also was demonstrated in co-cultures of J774s with either mast cell type ($P = 0.01$). However, there was significantly greater ($P = 0.05$) inhibition of bacterial replication in the P815 co-cultures than in the BMMC co-cultures at 100 MOI, further suggesting that the inhibitory effect is greater with the P815 mast cells (Fig. 2B).

Mast cells and macrophages infiltrate the lungs following intranasal challenge

In view of the fact that a 1:1 ratio of mast cells to macrophages was used for the in vitro system, we sought to establish the in vivo physiological relevance. C57/BL6 mice were

challenged intranasally with LVS (1600 CFU), and lungs were collected at day 3 post-challenge. The lungs were processed as described, and stained with fluorochrome conjugated antibodies to cKit, FcεRI, CD11b, and F4/80. Flow cytometry analyses were performed using side scatter versus cKit or side scatter versus CD11b for total lung cell evaluation (Fig. 3A). The lungs cells were further analyzed within the cKit gate for expression of FcεRI to identify mast cells, and within the CD11b^{hi} gate for expression of F4/80 to identify macrophages. The percentage of double positive cells (FcεRI⁺cKit⁺) increased from 2.1% (mock) to 6.5% (LVS) which revealed infiltrating mast cells following pulmonary LVS challenge (Fig. 3B). Similar analyses within the CD11b^{hi} gate revealed an increase of infiltrating macrophages (CD11b^{hi}+F4/80⁺) from 2.9% to 16.8% (Fig. 3C). Overall, mast cells (cKit⁺FcεRI⁺) infiltrated the lungs and increased from approximately 1.0 % (mock) to 5 % (LVS challenged) of total lung cells, while macrophages (CD11b^{hi}+F4/80⁺) increased from approximately 2.0 % (mock) to 15 % (LVS challenged), demonstrating an approximate 1:3 mast cell: macrophage ratio in the lungs following LVS challenge (Fig. 3D). We also have determined by confocal microscopy analyses that mast cells are localized within the vicinity of mCherry-labeled LVS in the lungs, as early as day 2 following bacterial challenge (data not shown).

Reduction of mast cells in co-cultures leads to decreased inhibition of intramacrophage *F. tularensis* LVS replication

To determine if the mast cell inhibition of intramacrophage LVS replication is maintained at ratios relevant to infiltration events following pulmonary challenge, J774s were cultured alone or co-cultured with P815s or BMMC at 1:1, 5:1, 10:1, 100:1, and 1000:1 ratios (Fig. 4B and C). The total number of cells per well was 5×10^5 at all ratios. The J774 macrophages cultured alone supported a high level of LVS replication within 24 h (Fig. 4A), while the P815 mast cells alone (Fig. 4B) and BMMC alone (Fig. 5C) demonstrated a markedly reduced LVS replication from 4 to 24 h. Interestingly, the cultures containing 1:1 and 5:1 ratios of J774 to P815 or BMMC showed similar levels of inhibition ($P = 0.007$, Fig. 4B & C). However, the results for the 10:1 ratio of macrophage: mast cell co-cultures showed an increase in bacterial replication when compared to the 5:1 and 1:1 ratios ($P = 0.005$). The loss of inhibition progressively increased from the 10:1 to the 1000:1 ratio of J774: P815 (Fig 4B), whereas co-cultures of J774 with BMMC produced an abrupt loss of inhibition at the 10:1 ratio (Fig. 4C). For the more effective P815 mast cell, a reduction to 100 macrophages/mast cell still resulted in a significant degree of inhibition further substantiating that the P815s were better effectors of the inhibition. Additionally, the optimal inhibitory effect on LVS intramacrophage replication with both mast cell types at the 1:1 and 5:1 ratios, provides a physiologically relevant ratio for evaluation of macrophage-mast cell interactions during LVS infection, which was found to be approximately 3:1.

Production of TNF-α and IL-4 in *F. tularensis* LVS infected co-cultures of macrophages and mast cells

Previously, macrophage derived TNF-α has been shown to control survival and bacterial burden during LVS infection [18; 19; 20] and IL-4 from BMMC has been shown to mediate the inhibition of LVS replication in primary bone marrow-derived macrophages [12]. To determine the role of TNF-α and IL-4 in the P815 mast cell inhibition of LVS replication within J774 macrophages, the cytokine profiles of each test group were determined by ELISA. Supernatants from the 24 h cultures of J774 macrophages (Fig. 5A), P815 mast cells (Fig. 5B) and BMMC (Fig. 5C) cultured alone, and co-cultures of J774 macrophages with P815 or BMMC at ratios of 1:1, 5:1, 10:1, 100:1, and 1000:1 (macrophages: mast cells) were analyzed. TNF-α was recovered from all test conditions at the 24 h time point with the highest concentrations produced in J774 macrophages cultured alone and at the highest

ratios of macrophages to mast cells, peaking at over 500 pg/ml for macrophages alone. Both mast cell types produced TNF- α upon infection with LVS, but at much lower concentrations ($P = 0.003$) of approximately 100 pg/ml. The TNF- α production by J774 macrophages in co-culture with either mast cell type increased with decreasing number of mast cells, suggesting that this cytokine was not responsible for the observed inhibition of bacterial replication in co-cultures. As expected, mock infected cultures produced minimal TNF- α .

IL-4 at the same 24 h time period was not detected in the supernatant of mock or infected J774 macrophages cultured alone. However, there were high levels of IL-4 in the supernatants of infected P815 mast cells (>125 pg/ml) and BMMC (35 pg/ml) cultures at 10 MOI ($P = 0.007$) (Fig. 6B & C). Moreover, P815s in co-culture with J774s at 1:1 and 5:1 (macrophage: mast cell) produced similar levels of IL-4 (Fig. 6B), whereas BMMC in co-culture with J774s at the same ratios also produced similar levels of IL-4 (Fig. 6C), albeit less than in P815 mast cell co-cultures. IFN- γ (interferon- γ) also was measured along with IL-4 and TNF- α . However, minimal induction of IFN- γ was observed in any supernatant (data not shown). These data verify that the mast cells are the primary producers of IL-4 in the co-culture system, further suggesting that IL-4 mediates the inhibitory effect of mast cells upon intramacrophage LVS replication.

Effect of IL-4 or P815 spent supernatant on intramacrophage replication of *F. tularensis* LVS

To directly study the effects of IL-4 on intramacrophage LVS replication in J774 macrophages, the macrophages were treated with either 5 or 25 ng/ml of recombinant IL-4 (rIL-4), 2 h before and throughout the LVS infection and compared to untreated macrophages and macrophages in co-culture with P815 mast cells. While 5 ng/ml of rIL-4 did not produce a significant reduction, 25 ng/ml of rIL-4 significantly reduced ($P = 0.002$) bacterial replication compared to replication in J774 macrophages alone (Fig. 7A). The bacterial inhibition was similar to the reduction demonstrated in the 1:1 co-cultures of macrophages and mast cells. To investigate whether supernatants collected from LVS-activated P815 mast cells would confer inhibition of intramacrophage bacterial replication, filtered mock or spent P815 supernatants were used to treat LVS-challenged J774 macrophages. As shown in Fig. 7B, LVS-challenged J774 macrophages treated with spent P815 supernatants exhibited a significant reduction ($P = 0.01$) in bacterial replication compared with macrophages incubated with mock-treated supernatants. To further assess whether IL-4 within the spent-treated supernatants modulated the inhibitory affect on intramacrophage LVS replication, J774 cells were incubated with P815 spent supernatants treated with neutralizing anti-IL-4 antibody. These analyses revealed a significant loss ($P = 0.004$) of inhibition of intramacrophage LVS replication in neutralizing anti-IL-4 treated J774 macrophages when compared to mock neutralized P815 supernatants (Fig. 7B). These results along with those that direct treatment of J774 macrophages with rIL-4 results in inhibition of intramacrophage LVS replication suggest that IL-4 production by both mast cell types effectively controlled intramacrophage bacterial replication.

IL-4 released from activated P815 mast cells inhibits *F. tularensis* LVS replication in J774 macrophages

Given the ability of LVS to activate P815 mast cells to control bacterial replication within infected macrophages, we further evaluated the role of mast cell activation with the small molecule mast cell activator MP7 and the control peptide MP17 in secretion of IL-4 [13; 14]. MP7 (INLKALAALAKALL-NH₂) is a potent analog of mastoporan and is known to be a direct activator of G protein [13; 14]. MP17 (INLKAKAALAKKIL-OH) is an inactive analog of mastoporan [20], and hence was used as a control peptide. Following 30 min of treatment with MP7 or controls, the mast cells (P815s) were centrifuged to collect spent

supernatants. Then, J774 macrophages were treated with these respective supernatants for 2 h prior to LVS infection and throughout the incubation period, and intramacrophage LVS replication was compared to that of untreated macrophages. The macrophages treated with supernatant collected from MP7 activated P815 mast cells showed a statistically significant reduction of intramacrophage LVS replication ($P = 0.01$), when compared to untreated (mock) macrophages or macrophages treated with supernatants collected from MP17 treated P815 mast cells (Fig. 8A). Macrophage activated P815 cell supernatants obtained by treatment of P815 cells with filtered LVS infected J774 cell supernatants also exhibited marked inhibition ($P = 0.01$) of intramacrophage LVS replication which was comparable to that observed with activation of P815s with the small molecule activator (Fig. 8B). Moreover, the MP7 treated mast cells also produced increased levels of IL-4 (>110 pg/ml) when compared to MP17, or mock treated P815 mast cells ($P = 0.03$) (Fig. 8C). Minimal levels of TNF- α were detected under all conditions (data not shown). Together, these analyses reveal that mast cells activated by MP7, infection with LVS, or by filtered supernatants of infected macrophages may control LVS replication in the J774 macrophages by inducing the production of mast cell derived IL-4.

4. Discussion

In this study, we have shown that J774 macrophages are highly permissive [12; 21; 22; 23] to intracellular *F. tularensis* LVS replication, whereas P815 mast cells that do not express the Fc ϵ RI, and therefore cannot be activated by IgE dependent mechanisms, are much less permissive to intracellular LVS replication. This inhibitory effect of P815 mast cells on intramacrophage LVS replication was titratable; being dependent on the number of mast cells and correlating with levels of IL-4 produced. The optimal macrophage: mast cell ratio observed for inhibition in vitro (approximately 3:1) correlated with ratios demonstrated in the lungs (3:1) following pulmonary LVS challenge. The P815 mast cells, upon activation by mast cell activating peptide MP7, demonstrated a non-Fc ϵ R dependent release of secretory products which inhibited intramacrophage LVS replication. Moreover, the inhibitory effect on LVS replication of MP7 activated P815 cell spent supernatants correlated with increased levels of secreted IL-4.

Although infiltration of mast cells is typically associated with detrimental consequences such as mastocytosis and inflammatory disorders, these cells also aid in enhanced resolution of bacterial infections, chiefly by releasing a wide range of cytokines [24]. Mast cells located at the host-environmental interface are potent producers of the multifunctional lymphokine IL-4 and other crucial cytokines such as TNF- α [25; 26]. To this end, we have shown that mast cells infiltrate into the respiratory compartment following intranasal *F. tularensis* LVS challenge and produce IL-4 [12]. The predominate source of early IL-4 production in this pulmonary infection may be mast cells, since lymphocytes isolated from LVS-infected lungs produced minimal IL-4 [27]. Interleukin-4 is pre-synthesized and stored in mast cell granules for immediate release on cell activation as well as synthesized de novo within minutes after stimulation [28; 29]. Contrary to the established role of mast cell-derived IL-4 in granuloma and tumor progression, in vitro studies indicate that, unlike T lymphocytes which produce large amounts of IL-4 when fully activated [30], mast cells release much less IL-4 due to an intrinsic mechanism that regulates the IL-4 production [28]. Interleukin-4 transcription is positively regulated by STAT 6 in T cells, whereas a truncated STAT 6 isoform is utilized by mast cells to repress IL-4 production [28; 31; 32]. Hence, these findings suggest that mast cells produce IL-4 in a controlled fashion to exert anti-bacterial effects. To this end, IL-4 has been shown to regulate antimicrobial peptides and to target macrophages to induce tuberculostatic function [33], and is consistent with a recent report that has demonstrated that IL-4 promotes the transepithelial transport of the antimicrobial substrate thiocyanate in human airway epithelial cells [34]. In addition,

enhanced clearance of *Pseudomonas aeruginosa* was demonstrated in the lungs of transgenic mice expressing IL-4 [35]. In our co-culture system, IL-4 from mast cells may be suppressing TNF- α production from macrophages as indicated by the reduction in TNF- α levels within co-cultures (increased levels of IL-4) as compared to cultures with macrophages alone (minimal IL-4). In this regard, there are several reports demonstrating that IL-4 down-regulates TNF induced cellular responses [32–34]. Interleukin-4 also has been suggested to antagonize the effects of TNF in vitro [36; 37] and in vivo [38] by inducing shedding of both p60 and p80 forms of the TNF receptor. It has been demonstrated that IL-4 exhibits both stimulatory and down-regulatory effects on T cells and a variety of hematopoietic cell lineages and cytokines [25]. Our laboratory has provided substantial evidence for the role of mast cells and IL-4 in modulating bacterial replication and host cell death [21]. Specifically, we have found that mice deficient in IL-4R signaling exhibited significantly greater induction of active caspase-3 following pulmonary *F. tularensis* challenge. Moreover, mast cells and IL-4 mediated reduction of *Francisella* replication and host cell death may be associated with increased cellular ATP, improved acidification of invading pathogens and up-regulation of mannose receptor expression. Additionally, bone marrow-derived mast cells also were found to effectively reduce intramacrophage replication of the human virulent strain *F. tularensis* SCHU S4, as well as the induction of active caspase-3 [21]. These results are in agreement with other published findings [39] that indicate that IL-4 regulates active caspase-3 expression and progression to cell death and/or necrosis.

A review of the literature and our current and previous studies suggest that the in vitro and in vivo effects of IL-4 on LVS intramacrophage replication and virulence in mice may depend on the source of macrophages, the mode of IL-4 stimulation and the route of challenge. In accordance with our previous findings [12; 21], treatment of LVS-infected macrophages with rIL-4 significantly inhibits bacterial replication similar to that observed when these cells are co-cultured with P815 mast cells. Importantly, the inhibition of bacterial replication can be reversed by the addition of neutralizing anti-IL-4 treatment. In contrast, using a bone marrow derived macrophage-lymphocyte co-culture system, Elkins and colleagues [40; 41] observed that addition of neutralizing anti-IL-4 treatment exhibited negligible effect on control of LVS replication. This observation may be due to the fact that, within this particular co-culture system, there is minimal production of IL-4 in comparison to what is observed when macrophages are cultured with mast cells. Anthony et al [42] and Shirey et al [43] reported that resident peritoneal macrophages treated with rIL-4 also failed to inhibit intramacrophage bacterial growth. Given the phenotypic differences and plasticity between peritoneal and bone marrow derived macrophages, it may not be surprising that the responsiveness of these mononuclear cells to IL-4 may depend on the in vivo source and the mode of IL-4 stimulation. Moreover, Leiby et al [20] observed that selective removal of IL-4 in vivo with neutralizing antibody resulted in an increase in LVS LD₅₀ for mice challenged intradermally; but this increase was not statistically significant. We have reported [12] that adoptive transfer of IL-4 deficient or competent mast cells into mast cell-deficient mice reconstituted comparable levels of protection against pulmonary *Francisella* infection. In this situation, the effects of mast cell-derived IL-4 may have been masked by contact dependent events and/or non IL-4 secreted products of mast cells. Shirey et al [43] noted that LVS inoculated intraperitoneally into IL4R $\alpha^{-/-}$ mice exhibited increased survival. However, greater lung and spleen bacterial burdens and increased susceptibility to pulmonary infection in IL-4R $\alpha^{-/-}$ mice were observed in our study [12], suggesting that different routes of challenge may modulate outcomes in these infection models. Additionally, BMMC generated from IL-4 $^{-/-}$ mice were found to be unable to inhibit intracellular LVS replication when co-cultured with wild-type macrophages [12] lending additional support to our in vivo findings.

Mast cells may produce secretory products including IL-4 either through the classical IgE mediated pathway or by alternative activation of mast cells [44]. Specifically, mast cells may be induced by certain compounds in a non-FcεR mediated fashion to release secreted products that mediate mobilization of DCs and lymphocytes to draining lymph nodes, which is essential for priming adaptive immune responses [45]. In light of these findings, small-molecule mast cell activators, including compound 48/80, mastoporan and its active analogs (MP7 in our study), substance P and catestatin, have gained interest in the context of vaccine development [45]. These polycationic substances also may cause mast cells to undergo exocytosis by non-antigenic stimuli [44] with cellular responses based on direct [46; 47] or indirect [48] activation of G proteins. Our results, involving use of MP7 to activate P815 mast cells, provide further support in favor of these small molecule mast cell activators as inducers of secretory products.

Mast cells are assumed to be similarly activated in vivo due to the signaling mechanism involving *Francisella* infected macrophages. Based on several studies, it is known that *F. tularensis* infects primarily monocytes and macrophages [49; 50]. Macrophages respond to the infection by the production of several pro-inflammatory cytokines and chemokines [51; 52]. It has also been shown that mast cell-macrophage interactions play a crucial role in limiting LVS replication in macrophages in vitro as well as in vivo [10]. In this study, macrophage activated P815 filtered supernatants, obtained by treatment of P815s with LVS infected J774 filtered supernatants, exhibited inhibition of intramacrophage LVS replication comparable to that observed with infection of mast cells and activation of mast cells with the small molecule activator MP7. Together, this study suggests that mast cells may become activated through a variety of paths, and independent of FcεR, to serve a protective function by releasing secretory products, including IL-4, which effectively control the replication of Gram negative bacteria such as *Francisella tularensis*.

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Highlights

- We utilize P815 mast cells and J774 macrophages as surrogates for primary mast cells and macrophages respectively.
- We investigate whether mast cell activation by non-FcεR driven signals could produce IL-4 and control intramacrophage LVS replication.
- The inhibition noted in vitro is titratable and preserved at ratios relevant to cellular infiltration events following pulmonary challenge.

Bone Marrow Derived Mast Cells

Figure 1. Morphological examination and surface receptor expression of bone marrow derived mast cells and P815 mast cells

Mast cells were stained with Wright's stain to demonstrate the presence of granules. Black arrows show presence of granules in BMMC (A), but not in P815 mast cells (B). 1×10^5 of each type of mast cell stained with fluorescent conjugated antibodies against FcεRI (PE) and cKit (FITC) and analyzed by flow cytometry (C-F).

Figure 2. Inhibition of *F. tularensis* LVS replication in mast cells and co-cultures

5×10^5 cells/well alone or in 1:1 co-cultures of macrophages: mast cells infected with LVS for 2 h, treated with gentamicin for an additional 2 h, cultured in fresh media or lysed, and dilution plated for bacterial enumeration. **(A)** LVS at 10 MOI. **(B)** LVS at 100 MOI. Results are representative of independent experiments repeated twice. *, $P = 0.01$ (two-way ANOVA).

Figure 3. Infiltration of mast cells and macrophages into the lungs following intranasal LVS challenge

C57/BL6 mice were challenged intranasally with LVS (1600 CFU) and lungs collected at day 3 post-challenge, processed, and stained with fluorochrome conjugated antibodies to cKit, FcεRI, CD11b and F4/80. (A) Scatter plot analyses of total lung cells (mock or LVS infected) for mast cells and macrophages. Analysis of lung cells within the (B) cKit⁺ gate, (C) CD11b^{hi} gate. (D) Percentages of infiltrating macrophages and mast cells in the lungs following LVS challenge. Percentages shown are for total lung cells. *, $P < 0.05$ (Student's *t*-test).

Figure 4. Titration of inhibitory effect on LVS intramacrophage replication by P815 mast cells and bone marrow derived mast cells in co-culture with J774 macrophages

5×10^5 cells total/well infected (10 MOI) with LVS for 2 h, treated with gentamicin for an additional 2 h, cultured in fresh medium or lysed and dilution plated for bacterial enumeration. (A) J774 macrophages alone. (B) P815 mast cells or co-cultures of different ratios of macrophages:mast cells. (C) BMMC or co-cultures of different ratios of macrophages: mast cells. Results are representative of independent experiments repeated twice. (*, $P = 0.01$ (two-way ANOVA), P815 co-cultures and BMMC co-cultures compared with J774 macrophages alone at 24 h).

Figure 5. TNF- α production from macrophages alone and macrophages with decreasing ratios of P815 or bone marrow derived mast cells

TNF- α from 24 h cell culture supernatants from all conditions measured by ELISA. (A) J774 macrophages alone. (B) P815 mast cells alone and macrophages cultured with decreasing ratios of P815 mast cells. (C) BMMC alone and macrophages co-cultured with decreasing ratios of BMMC. Results are representative of independent experiments repeated twice. (*, $P = 0.01$ (two-way ANOVA), LVS infected P815 mast cells or co-cultures and BMMC or cocultures compared with mock infected cells at 24 h, *, $P = 0.003$ (two-way ANOVA), LVS infected P815 cells or BMMC compared with LVS infected J774 cells at 24 h).

Figure 6. IL-4 production from macrophages alone and macrophages with decreasing ratios of P815 or bone marrow derived mast cells

IL-4 from 24 h cell culture supernatants from all conditions measured by ELISA. (A) J774 macrophages alone. (B) P815 mast cells alone and macrophages co-cultured with decreasing ratios of P815 mast cells. (C) BMMC alone and macrophages co-cultured with decreasing ratios of BMMC. Results are representative of independent experiments repeated twice. (*, $P = 0.01$ (two-way ANOVA), LVS infected P815 mast cells or co-cultures and BMMC or co-cultures compared with mock infected cells at 24 h, *, $P = 0.007$ (two-way ANOVA), LVS infected P815 cells or BMMC compared with LVS infected J774 cells at 24 h).

Figure 7. Effect of IL-4/P815-spent supernatant on intramacrophage LVS replication (A) Untreated, recombinant (r) IL-4 (5 or 25 ng/ml) treated, and co-culture with P815 mast cells. *, P=0.002 (two-way ANOVA). (B) Neutralizing anti-IL-4 antibody (50 µg/ml) treated J774 macrophages. *, P=0.004 (two-way ANOVA). All seeded at 5×10^5 cells/well. All conditions following infection with LVS (10 MOI) for 2 h, gentamicin treatment for an additional 2 h, then lysed or cultured in fresh media until the 24 h time point, lysed and dilution plated for bacterial enumeration. Results are representative of independent experiments repeated twice.

Figure 8. Inhibition of *F. tularensis* LVS replication in J774 macrophages by IL-4 released from activated P815 mast cells

5×10^5 J774 cells/well infected (10 MOI) with LVS for 2 h, treated with gentamicin for an additional 2 h, cultured in fresh medium or lysed and dilution plated. **(A)** LVS replication in J774 macrophages treated with filtered spent supernatants obtained from P815 mast cells treated with PBS, MP7 or MP17. *, $P=0.01$ (two-way ANOVA). **(B)** LVS replication in J774 macrophages treated with mock or LVS infected P815 filtered spent supernatants. *, $P=0.006$ (one-way ANOVA). **(C)** IL-4 detected in respective P815 cell spent supernatants as measured by ELISA. *, $P=0.03$ (two-way ANOVA). Results are representative of independent experiments repeated twice.