Mast Cell TLR2 Signaling Is Crucial for Effective Killing of *Francisella tularensis*


*J Immunol* 2012; 188:5604-5611; Prepublished online 23 April 2012;
doi: 10.4049/jimmunol.1200039

http://www.jimmunol.org/content/188/11/5604

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Supplementary Material [http://www.jimmunol.org/content/suppl/2012/04/24/jimmunol.1200039.DC1](http://www.jimmunol.org/content/suppl/2012/04/24/jimmunol.1200039.DC1)

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Mast Cell TLR2 Signaling Is Crucial for Effective Killing of Francisella tularensis

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TLR signaling is critical for early host defense against pathogens, but the contributions of mast cell TLR-mediated mechanisms and subsequent effector functions during pulmonary infection are largely unknown. We have previously demonstrated that mast cells, through the production of IL-4, effectively control Francisella tularensis replication. In this study, the highly virulent strain of F. tularensis SCHU S4 and the live vaccine strain were used to investigate the contribution of mast cell/TLR regulation of Francisella. Mast cells required TLR2 for effective bacterial killing, regulation of the hydrolytic enzyme cathepsin L, and for coordination and trafficking of MHC class II and lysosomal-associated membrane protein 2. Infected TLR2−/− mast cells, in contrast to wild-type and TLR4−/− cells, lacked detectable IL-4 and displayed increased cell death with a 2–3 log increase of F. tularensis replication, but could be rescued with rIL-4 treatment. Importantly, MHC class II and lysosomal-associated membrane protein 2 localization with labeled F. tularensis in the lungs was greater in wild-type than in TLR2−/− mice. These results provide evidence for the important effector contribution of mast cells and TLR2-mediated signaling on early innate processes in the lung following pulmonary F. tularensis infection and provide additional insight into possible mechanisms by which intracellular pathogens modulate respiratory immune defenses. The Journal of Immunology, 2012, 188: 5604–5611.

Mast cells act as sentinels that sense bacterial products via host surface pattern recognition receptors such as TLR2 and TLR4 (1, 2). Although mast cell cytokine production following TLR recognition has been well documented (3–7), much is still unknown about mast cell TLR signaling and function during bacterial infection. In dendritic cells, TLR signaling triggers bacterial phagocytosis and promotes phagosome maturation, which entails nascent phagosome fusion with endosomes and phagolysosomal formation (8). Phagocytosis, phagosome maturation (9), and phagosome movement along microtubules to perinuclear regions require optimal pH regulation (10, 11). Although the maturation process has been studied in dendritic cells (8, 12, 13), the contribution of TLR function in mast cell phagocytosis is yet to be elucidated.

Phagosome maturation in myeloid lineage cells is marked by protein accumulation, including CD63 (14), lysosomal-associated membrane protein (LAMP)1/2 (15), and Rab7 (16, 17). Additional proteins in this process include the V-type ATPase, MHC class II (MHCII), and lysosomal hydrolases. To this end, lysosomal hydrolases are comprised of several cathepsins such as serine, aspartate, and cysteine proteases (18–20). Among the cathepsins, cathepsin L is critical for cellular homeostasis, autophagy, apoptosis, and Ag processing and presentation (21, 22). Importantly, this protease also has been implicated in various diseases, including diabetes (23), cancer (24), and lung inflammation (25), and therefore must be expediently regulated to maintain homeostasis.

Under ideal conditions, phagosome/lysosome fusion leads to a decrease in pH and protease activation, which culminates in killing of pathogens within the phagolysosome. However, a number of pathogens have developed evasion mechanisms by modifying the phagosome or maturation events. For example, Mycobacterium spp. block the Rab5-positive stage (26), Leishmania spp. inhibit phagosome maturation (27), and Coxiella burnetii converts the phagosome into a hybrid autophagosome (28). Francisella tularensis, the causative agent of pneumonic tularemia, has been shown to escape the phagosome to evade lysosomal degradation and undergoes extensive replication within the macrophage cytosol (29).

TLR2 signaling and ensuing immune responses are important for the control of pulmonary F. tularensis (30). Whereas TLR2 has been shown to be involved in recognition of Francisella lipoproteins (30–32), wild-type (WT), TLR2−/−, and TLR4−/− macrophages have been shown to exhibit similar susceptibility to F. tularensis infection. We have recently shown that mast cells are essential for host survival during pulmonary F. tularensis challenge, and that mast cells cocultured with macrophages promote killing of the organism via IL-4 secretion (33) and increased macrophage cellular ATP production with subsequent acidification (34).

Although cellular acidic conditions have been shown to control trafficking along microtubules (10, 35), and Yates and Russell (36) have shown that macrophage TLR2/4 signaling is not required for phagosome maturation, there is a paucity of information on mast

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Received for publication January 6, 2012. Accepted for publication March 28, 2012.

This work was supported in part by National Institutes of Health Grant P01 AI057986, National Institutes of Health/National Institute of General Medical Sciences Grant GM60655 for Minority Biomedical Research Support/Research Initiative for Scientific Enhancement, the Alfred P. Sloan Foundation Graduate Scholarship Program, and Army Research Office of the Department of Defense Contract W911NF-11-1-01346.

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The online version of this article contains supplemental material.

Abbreviations used in this article: LAMP, lysosomal-associated membrane protein; LVS, live vaccine strain; MHCII, MHC class II; MOI, multiplicity of infection; WT, wild-type.

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www.jimmunol.org/cgi/doi/10.4049/jimmunol.1200039

The Journal of Immunology
cell TLR function and control of cellular trafficking and lysosomal function. Accordingly, using the highly human virulent strain of *F. tularensis* (SCHU S4) and *F. tularensis* live vaccine strain (LVS), we investigated the contribution of mast cell TLR2/4 and IL-4 production to killing by mast cells. Our findings show that mast cell TLR2 is critical for optimal trafficking of cellular proteins and effective host responses during Franciscella infection.

**Materials and Methods**

**Mice**
Specific pathogen-free 4- to 8-wk-old mice were used for all procedures. C57BL/6 mice were purchased from the National Cancer Institute. TLR2 (37) and TLR4 (38) mice were provided by Dr. M.T. Berton. All experimental procedures and animal care were performed in accordance with the Institutional Animal Care and Use Committee guidelines.

**Bacteria**

*F. tularensis* LVS (obtained from Dr. R. Lyons at the University of New Mexico), *F. tularensis* SCHU S4 (obtained from the Centers for Disease Control), and mCherry LVS (KFK314) were grown in tryptic soy broth supplemented with t-cysteine (33). Experiments using SCHU S4 were conducted in a licensed animal biosafety level 3 facility.

**Generation of primary cells and in vitro infection**
Mas cells were derived from mouse bone marrow and injected in vitro as described previously (35). Briefly, cells were counted, plated, and incubated at 37°C for 5 h, then washed with Hank's balanced salt solution (HBSS) and resuspended in 2% paraformaldehyde for flow cytometric analysis. Analyses were performed using CellQuest Pro software.

**In vivo challenge and flow cytometry**
Six- to 8-wk-old C57BL/6 and TLR2/–/– mice were challenged intranasally with either 1 or 10 LD50 LVS or with PBS (mock challenge). At days 2 and 4 postchallenge, mice were euthanized. The lungs were perfused with 1 × PBS and stained with a 70-μm cell strainer or formalin was injected into the trachea and the lungs were fixed in neutral formalin overnight, dehydrated, and embedded in paraffin. Serial sections of 5-μm thickness were prepared. Tissue sections were permeabilized with 1% Permea/Wash buffer and blocked with BSA or rat serum. Tissues were stained for 45 min at room temperature with fluorochrome-conjugated Abs, including anti-CD11b, anti-LAMP2, anti-CD49b, anti-CD11c, or anti-F4/80 (BD Biosciences), and DAPI. Lung cells were washed with 2% FBS in 1 × PBS with 0.09% sodium azide (PBS wash), then blocked with 10% rat serum in 1 × PBS at 4°C for 30 min. Samples were washed and surface stained with appropriate fluorochrome-conjugated Ab or isotype control, incubated at 4°C for 30 min, and washed. Samples were then fixed in CytoFix/Cytoperm for 20 min, washed with PBS, and treated with 1 × Perm/Wash for 10 min, and the wash was removed. Cells were then stained with fluorochrome-conjugated Ab or isotype control diluted in 100 μl 1 × Perm/Wash. Appropriate samples were then incubated for 60 min with intermittently agitated, washed in 1 × Perm/Wash, and resuspended in 2% (w/v) paraformaldehyde for flow cytometric analysis (FACSCalibur; BD Biosciences). Analyses were performed using CellQuest software (BD Biosciences). Lung tissues also were examined using a 510 Meta confocal microscope (Zeiss). LSM 510 confocal microscopy. Lasers and settings for detection of fluorophores included: 405 for detection of DAPI (BP 420–480), 488 for detection of Alexa 488 (NFT 490 and BP 505–530), 594 for detection of mCherry (NFT 545 and BP 560–615), and 633 for detection of allophycocyanin (NFT 545 and LP 650).

**Results**

WT and TLR4**/–** mast cells effectively control Franciscella replication, whereas TLR2**/–** mast cells are permissive to Franciscella replication

TLR2-deficient (TLR2**/–**) mice have been shown to exhibit higher mortality than do WT mice following *F. tularensis* pulmonary challenge (30, 32). Given that WT, TLR2+/–, and TLR4+/– macrophages exhibit similar susceptibility to *Francisella*, and that we have previously shown that mast cells are important for bacterial control and survival during pulmonary *F. tularensis* infection (33), we compared replication in WT and TLR-deficient mast cells during *Francisella* infection. Bacterial replication was examined at 3 and 24 h in WT mast cells infected with LVS and the highly human virulent type A strain, SCHU S4, and compared with infected TLR2+/– and TLR4+/– mast cells. These analyses revealed that whereas bacterial replication of both LVS and SCHU S4 was restricted in WT and TLR4+/– mast cells, TLR2+/– mast cells exhibited significantly (*p* < 0.002) increased (2–3 log) replication compared with WT mast cells at 24 h (Fig. 1). Macrophages were used for comparison, and as previously observed (33, 34), LVS and SCHU S4 replicated robustly within primary WT macrophages. These results suggest that TLR2 recognition of bacteria and/or TLR2 signaling is critical for the control of *F. tularensis* replication within mast cells.

TLR2**/–** mast cells lack detectable IL-4 production and exhibit reduced cathepsin L activation immediately following infection

Given that we have previously demonstrated that IL-4 is critical for mast cell inhibition of *F. tularensis* replication (33), and that TNF-α has been suggested to be an important innate immune component in host defense (40, 41), supernatants collected from WT, TLR2+/–, and TLR4+/– mast cells during mock or LVS infection...
were analyzed for IL-4 and TNF-α production. Both IL-4 and TNF-α were produced by LVS-infected WT and TLR4−/− mast cells, whereas TLR2−/− mast cells lacked detectable IL-4 production, but produced similar levels of TNF-α to WT mast cells (Fig. 2A). LVS-infected TLR4−/− mast cells exhibited marked reduction of TNF-α production compared with WT and TLR2−/− mast cells (Fig. 2A). Given that LVS- or SCHU S4-infected TLR2−/− mast cells exhibited marked susceptibility to the organisms, but impaired IL-4 production in contrast to WT or TLR4−/− mast cells, additional studies were focused on the contribution of TLR2.

Because IL-4 treatment increases phagosome acidification during Francisella infection (34), and the hydrolytic protein cathepsin L has been shown to be enhanced by IL-4 (42), we subsequently analyzed the expression of cathepsin L by flow cytometry. These analyses revealed reduced active cathepsin L expression in TLR2−/− LVS-infected mast cells (Fig. 2B; 55.0%, representative histogram shown) at early time points (30 min shown; filled lines) compared with WT (86.0%, representative histogram shown). However, by 1 h after LVS challenge (Fig. 2B; hatched lines), cathepsin L decreased in WT (12.5%, representative histogram shown) and increased in TLR2−/− mast cells (95%, representative histogram shown). Taken together, these results suggest that TLR2 recognition of Francisella and downstream signaling are important for IL-4 production and subsequent regulation of cathepsin L synthesis in mast cells.

TLR2−/− mast cells express altered LAMP and MHCII trafficking and acidification during F. tularensis infection

Because IL-4 has been shown to upregulate MHCII expression and acidification in macrophages (34, 43), and given that accumulation of LAMP2 is important during phagosome maturation and ensuing phagosome/lysosome fusion (21, 44), MHCII and LAMP2 were examined in Lucifer yellow-labeled LVS-infected WT, TLR2−/−, and TLR4−/− mast cells. Flow cytometry analysis of MHCII surface expression during early (30 min) LVS infection revealed that WT mast cells express reduced MHCII surface proteins (22%) compared with TLR2−/− mast cells, which expressed almost twice (41%) the level and were comparable to TLR4−/− mast cells (37%) (Fig. 3A). We then used confocal microscopy to examine the relationship between MHCII and colocalization with labeled LVS. Interestingly, confocal microscopy analysis revealed that...
whereas TLR2−/− mast cells showed high levels of MHCII expression at the cell surface, intracellular MHCII expression was markedly reduced and greater numbers of LVS were present in the TLR2−/− mast cells, in contrast to WT mast cells, which showed high levels of intracellular MHCII along with reduced Lucifer yellow-labeled bacteria (Fig. 3B). For further examination, we used three-dimensional confocal microscopy analysis at a higher magnification (×1000) and visualized notable aggregation of MHCII molecules with LAMP2-positive proteins in WT mast cells early (30 min) during LVS infection, in contrast to TLR2−/− mast cells with dispersed MHCII and LAMP2-positive proteins throughout the cells (Fig. 4A). However, by 3 h LAMP2 and MHCII trafficking to the cell surface increased, along with decreased c-Kit–positive fluorescent proteins in TLR2−/− mast cells, when compared with WT mast cells (Fig. 4B). Moreover, increased levels of Lucifer yellow-labeled bacteria in TLR2−/− mast cells also were noted compared with WT mast cells (Fig. 4B), which was in agreement with bacterial plate counts (Fig. 4C). Because TLR2−/− mast cells exhibited increased LVS replication and reduced colocalization with MHCII and acidification marker, we analyzed cell death by examining active caspase-3 induction as well as the effect of IL-4 on these processes. Importantly, LVS- and SCHU S4-infected TLR2−/− mast cells exhibited a significant increase in active caspase-3 expression when compared with similarly infected WT or TLR4−/− mast cells (Fig. 4C, only SCHU S4 shown), whereas addition of IL-4 to the TLR2−/− mast cells reduced active caspase-3 induction and conferred control of SCHU S4 bacterial replication similar to WT mast cells. These results suggest that mast cell TLR2 recognition of F. tularensis and consequential downstream signaling promote phagosome maturation, trafficking of LAMP2 and MHCII molecules, and killing of Francisella, which is likely related to IL-4 signaling. Addition of IL-4 rescued SCHU S4-infected TLR2−/− mast cells from apoptosis and promoted effective killing of the organism.

**The infiltration and localization of mast cells with mCherry-labeled LVS following pulmonary infection**

Given the striking in vitro differences between WT and TLR2-deficient mast cells during F. tularensis infection, we analyzed the early cellular events as well as mast cell migration to the lungs following pulmonary challenge. C57BL/6 mice were challenged (10 LD50) intranasally with mCherry-labeled LVS and lungs were collected at days 2 and 4 postchallenge. At day 2 postchallenge, minimal c-Kit–positive cells were noted (data not shown), in agreement with our previous study (33). However, cellular infiltration to the lungs was evident by day 4 postchallenge. Concurrently, analysis of lung tissue collected from LVS-challenged WT mice revealed notable aggregated LAMP2 molecules that encompassed mCherry-labeled LVS when compared with lung tissue from TLR2−/−-challenged mice by day 4 (Fig. 5A). Moreover, the intensity of LAMP staining within these regions was greater in the WT compared with TLR2−/− lung sections (Fig. 5B).

Because subpopulations of dendritic cells and NK cells have been reported to express c-Kit, lung cells were analyzed for c-Kit expression in addition to CD49b (a pan-NK cell marker) and CD11c (dendritic cell marker). Flow cytometry analyses (Fig. 6) of WT lung cells collected from mock and mCherry-labeled LVS-challenged mice revealed that cells expressing c-Kit during pulmonary F. tularensis challenge primarily consisted of mast cells and NK cells. At day 4 postchallenge, ~26% of lung cells were c-Kit–positive; 65% of this c-Kit population also were FcεRI–positive, indicative of mast cells, and 8.5% were CD49b–positive, indicative of cytotoxic NK cells (Fig. 6A, 6B). Importantly, lung cell analyses also indicated that c-Kit–positive cells interact with Francisella during pulmonary infection (Fig. 6C), although minimal mCherry–c-Kit–positive cells were noted (Fig. 6C, insert, higher magnification). Because WT mast cells are not permissive to F. tularensis replication, live cell imaging was used to further analyze the WT and TLR2−/− mast cell/Francisella interaction. Live cell imaging (Supplemental Video 1) demonstrated that WT

**FIGURE 3.** MHCII and LAMP2 expression are altered in TLR2−/−-deficient mast cells. (A) WT, TLR2−/−, and TLR4−/− mast cell MHCII surface analysis by flow cytometry. Representative histograms are shown. (B) WT, TLR2−/−, and TLR4−/− mast cells infected with Lucifer yellow-labeled LVS (green) and labeled with c-Kit (purple) surface fluorescent-conjugated Ab with intracellular MHCII (light blue), LAMP2 (orange), and acidification probe (red). Representative images are shown at 30 min. Original magnification ×400.
mast cells were polarized, highly mobile, and efficient in killing of Lucifer yellow-labeled LVS in contrast to TLR2−/− mast cells. Furthermore, live cell imaging of TLR2−/− mast cells (Supplemental Video 2) demonstrated that the cells efficiently engulf Lucifer yellow-labeled LVS (green); however, bacteria did not localize within acidified regions (red), and instead persisted (green) within the mast cells. Sequential images of WT mast cells (Supplemental Fig. 1A–D) show polarized acidification regions (white arrows) during phagocytosis of bacteria, in contrast to TLR2−/− mast cells with acidification regions around the mast cell periphery (Supplemental Fig. 1E–H, lower panel, white arrows). Collectively, the live cell imaging of WT mast cells (Supplemental Video 1) revealed rapid phagocytosis, acidification (red), and degradation of LVS (green), suggestive of a more efficient phagosome and phagolysosomal maturation process. Importantly, these results are in agreement with bacterial plate count information showing that TLR2−/− mast cells efficiently engulf Francisella, but permit survival and growth of the organisms, whereas WT mast cells demonstrate effective killing of the pathogen.

**Discussion**

Mast cells provide a dynamic defense against mucosal pathogens. Control of certain bacterial pulmonary pathogens, including *F. tularensis*.
TLR2 signaling has been reported to link phagocytosis and discrimination of self to autophagy and requires acidification (57, 58). To this end, the increase in IL-4 secretion, optimal cathepsin L activation, and phagosome maturation, resulting in localization of LAMP2 (47, 48), requires TLR2 receptor signaling. This study provides further mechanistic insight into mast cell bacterial recognition and control of infection.

Importantly, mast cell TLR2-mediated innate immune responses included optimal trafficking and early accumulation of LAMP2, MHCII, and cathepsin L, which resulted in effective killing of F. tularensis. TLR signaling and subsequent lysosomal function have been primarily characterized in dendritic cells (49). Trombetta et al. (13) used LPS to demonstrate that mature dendritic cells accumulate vacuolar pumps, resulting in enhanced acidified lysosomes and Ag proteolysis. These dendritic cells also were shown to accumulate MHCII molecules. Blander and Medzhitov (50) later demonstrated that dendritic cell TLR4 recognition of LPS is critical for phagosome maturation and discrimination of self (apoptotic cells) and nonself for Ag presentation. In contrast, Yates and Russell (36) have proposed that phagosome maturation occurs independently of TLR2 or TLR4 stimulation, using bone marrow derived macrophages stimulated with Pam3Cys-Ser-Pro-Gly (green) and spot (red) LVS regions of >0.5 μm following bacterial challenge analysis. Original magnification ×400.

Our findings suggest that TLR2 signaling was required for induction of IL-4 secretion, optimal cathepsin L activation, and phagosome maturation, resulting in localization of LAMP2 and MHCII molecules with F. tularensis. It has previously been demonstrated that bone marrow-derived macrophages are highly permissive to Francisella infection; however, coculture with mast cells or the addition of rIL-4 markedly reduced bacterial replication (33, 34). Importantly, we noted that bone marrow-derived macrophages did not produce IL-4 during infection, in contrast to bone marrow-derived mast cells. We also have provided evidence that in mast cell/macrophage coculture, the production of IL-4 by mast cells promotes macrophage ATP production and subsequent phagosome acidification (34). Therefore, the differences noted by Yates and Russell (36) may have been partially due to the lack of IL-4 production by macrophages.

Previous studies (51–53) have shown that IFN-γ and TNF-α are primary control mechanisms during Francisella infection. In contrast, addition of IFN-γ also improved killing of LVS in mast cells and increased IL-4 production (data not shown), TNF-α was not sufficient to induce Francisella killing in mast cells as demonstrated by TLR2−/− mast cells, which produced comparable levels to WT mast cells. Although reactive oxygen species induced by TNF-α have been shown to promote signaling via the IL-4 receptor pathway, TNF-α is not required for initiation of IL-4 signaling (54). IL-4 enhances ATP production (55), cathepsin L activation (42), and phagosome acidification (34). Additionally, our findings suggest that TLR2 signaling was required for initiation of mast cell IL-4 secretion, optimal cathepsin L activation, and phagosome maturation, resulting in localization of LAMP2 and MHCII molecules with F. tularensis. The seminal work of Heuser (35) demonstrated that cellular acidic and alkaline conditions influence lysosome trafficking along microtubules. Our findings suggest that cathepsin L activity, which targets multiple cellular proteins implicated in trafficking such as dynamin (59) and actin (60), must also be regulated in a manner for efficient movement and maturation of the phagosome/lysosome to ensure destruction of Francisella, as overexpression is associated with induction of apoptosis and tissue damage (24, 61).
Furthermore, TLR2 signaling has been reported to be important for control of *M. tuberculosis* (46). Effective control of this pathogen also may be dependent on ensuing acidification required for TLR9 conformational changes and subsequent function (62). Results from work reported in this study support a probable link for TLR2 signaling and ensuing acidification for TLR9 function. Given that early TLR2 signaling and phagosome maturation were key to efficient mast cell bacterial killing, this study provides additional avenues to exploit for control of intracellular bacterial pathogens. TLR2 signaling and IL-4 production resulted in early enhanced levels of active cathepsin L, which is important for cleavage of the invariant chain (20, 63). Additionally, IL-4 is associated with the production of cathepsins B and S, which may be involved in development of cancers (64), whereas extracellular cathepsin L has been implicated in apoptosis (61), tissue damage, and tumorigenesis (24, 65). TLR2<sup>–/–</sup> mast cells demonstrated an excess of active cathepsin L compared with WT cells after 1 h LVS infection as demonstrated by confocal microscopy and flow cytometry. Confocal microscopy analyses also showed disintegration of the mast cell plasma membrane and release of LVS. Intense LAMP-2 and cathepsin L-positive regions at the membrane of TLR2<sup>–/–</sup> mast cells, along with numerous Lucifer yellow-labeled LVS and diminished or undetectable c-Kit proteins, suggest that lysosomes were localized at the plasma membrane, contributing to the destruction of membrane integrity. To this end, it has previously been shown that lysosomes localize at the plasma membrane for repair processes following initial translocation of phosphatidylyserine to the outer surface of the membrane (66). This repair process requires an increase of calcium and actin polymerization, and bacteria may take advantage of the process (67, 68) for escape into the extracellular region, demonstrating the critical requirement for stringent mast cell TLR2 regulation. In summary, this study provides additional mechanistic insight into mast cell-mediated innate immune mechanisms and the positive contribution of this cell type during pulmonary infection with Gram-negative bacteria.

Acknowledgments

We thank the University of Texas at San Antonio Research Centers in Minority Institutions Research Core staff and Dr. Colleen Witt for confocal microscopy analyses.

Disclosures

The authors have no financial conflicts of interest.

References


Figure S1, Supplemental Data. Wild-type mast cell acidification regions surround bacteria in contrast to TLR2/- mast cells. Imaging frames of wild-type (A-D) and TLR2/- (E-H) mast cells. (A-B) Imaging frames of LVS infected WT mast cells show polarized regions with fluorescent clusters that project for bacteria uptake; acidification fluorescent probe shown in frames projecting (arrows) toward bacteria.; (C-D) localization of acidification probe to clustered regions (arrows) following uptake of bacteria. (E-H) Imaging frames of LVS infected TLR2/- mast cells show that acidification regions lack polarization and are at the dispersed at the cell periphery (arrows).
SUPPLEMENTAL INFORMATION

Video 1, Supplemental Data. Wild-type mast cells effectively phagocytose and kill Francisella tularensis. Live imaging of Lucifer labeled LVS (green, FITC EX filter 10%) and WT mast cells with acidification probe (red, TRITC EX filter 1%) at 37°C, Olympus 100X objective, Z sectioning, and time-lapse of 60 seconds for 2 h. Imaging shows that WT mast cells migrate rapidly toward bacteria with polarized regions (red) and projections that encompass bacteria. Acidification probe shows increased intensity and surrounds bacteria; the green fluorescence of bacteria is diminished.

Video 2, Supplementary Data. TLR2−/− mast cells effectively phagocytose Francisella tularensis, but lack effective killing potential. Live imaging of Lucifer labeled LVS (green, FITC EX filter 10%) and TLR2−/− mast cells with acidification probe (red, TRITC EX filter 1%) at 37°C, Olympus 100X objective, Z sectioning, and time-lapse of 60 seconds for 2h. Imaging shows that TLR2−/− mast cells engulf bacteria which are dispersed within the mast cells, but with minimal red acidification regions and high Lucifer-LVS green regions.