

Francisella tularensis LVS grown in macrophages has reduced ability to stimulate the secretion of inflammatory cytokines by macrophages in vitro

Daniel J. Loegering^{a,*}, James R. Drake^b, Jeffrey A. Banas^b, Tamara L. McNealy^b,
Debbie G. Mc Arthur^b, Laura M. Webster^c, Michelle R. Lennartz^c

^aCenter for Cardiovascular Sciences, Albany Medical College, 47 New Scotland Avenue, Albany, NY 12208, USA

^bCenter for Immunology and Microbial Disease, Albany Medical College, 47 New Scotland Avenue, Albany, NY 12208, USA

^cCenter for Cell Biology and Cancer Research, Albany Medical College, 47 New Scotland Avenue, Albany, NY 12208, USA

Received 18 April 2006; received in revised form 25 July 2006; accepted 27 July 2006
Available online 25 September 2006

Abstract

The virulence of *Francisella tularensis* LVS is determined in part by its ability to invade and replicate within macrophages and stimulate the production of inflammatory cytokines. The present study determined the effects of growing *F. tularensis* in macrophages on its ability to stimulate cytokine secretion by macrophages. *F. tularensis* grown in Mueller–Hinton broth (FtB) stimulated the secretion of large amounts of TNF- α , IL-12p40, IL-6 and MCP-1/CCL2 when incubated with macrophages overnight. In contrast, *F. tularensis* released from infected macrophages (FtMac) stimulated very little secretion of these cytokines by primary cultures of murine peritoneal macrophages, human monocytes or macrophage cell lines. Stimulation of nitric oxide production by FtMac was also less than that elicited by FtB. FtMac killed with gentamicin or paraformaldehyde also stimulated low levels of cytokine secretion. FtMac recovered the ability to stimulate cytokine secretion after overnight culture in broth. Infection of macrophages with FtMac inhibited the cytokine response to subsequent stimulation with LPS from *Escherichia coli* but did not affect Fc γ receptor-mediated phagocytosis. FtMac were ingested by macrophages at about half the rate of FtB, however, this did not account for the lower cytokine secretion. FtMac and FtB replicated at similar rates within macrophages. Finally, Mice infected with FtMac had a higher mortality rate than those infected with FtB. These results reveal that growth in macrophages causes a reversible phenotypic change in *F. tularensis* that is associated with decreased stimulation of cytokine secretion, inhibition of LPS-stimulated secretion of inflammatory cytokines by macrophages and increased lethality in mice.

© 2006 Elsevier Ltd. All rights reserved.

Keywords: *Francisella tularensis*; Phagocytosis; TNF- α ; IL-12; IL-6; MCP-1

1. Introduction

Francisella tularensis is an intracellular pathogen that causes the clinical entity known as tularemia. While not a particularly important clinical pathogen in the United States, interest in this organism has increased since it was placed on the A list of bioterror agents. Its extremely high infectivity, ease of aerosol dispersal and high pathogenicity makes *F. tularensis* a fearsome bioweapon.

F. tularensis invades and proliferates within tissue macrophages. Macrophages are also one of the first lines of defense against *F. tularensis* and activated macrophages can kill this organism [1–3]. Thus, secretion of inflammatory cytokines during the early stages of an infection may limit bacterial proliferation by increasing the activation state of macrophages. Consistent with this hypothesis, it has been shown that administration of neutralizing antibodies against tumor necrosis factor- α (TNF- α) or interferon- γ (IFN- γ) results in early death or a decrease in LD50 of mice infected with *F. tularensis* [4–8]. IFN- γ null mice are also more susceptible to *F. tularensis* infection

*Corresponding author. Tel.: +1 518 262 5662; fax: +1 518 262 8101.
E-mail address: loegerd@mail.amc.edu (D.J. Loegering).

than their wild-type counterparts [9]. Treatment of macrophages with IFN- γ allows these cells to limit the growth of *F. tularensis* [10–13]. The ability of IFN- γ and TNF- α to limit the growth of intracellular pathogens in macrophages is often mediated by nitric oxide [14]. IFN- γ and TNF- α stimulate the induction of nitric oxide synthase that generates nitric oxide in macrophages. Interleukin-12 (IL-12), which is primarily produced by macrophages, stimulates the secretion of IFN- γ by T cells. Infection of naive macrophages with *F. tularensis* stimulates low levels of IL-12 and TNF- α secretion, however, exposure to IFN- γ greatly increases the production of these cytokines and nitric oxide [13,15,16]. Thus, the acute production of inflammatory cytokines such as TNF- α and IL-12 by macrophages may be important in controlling *F. tularensis* infections.

Here, we show that *F. tularensis* grown in macrophages has substantially different characteristics from those grown in broth. *F. tularensis* that has broken out of macrophages shows greatly reduced ability to stimulate the secretion of inflammatory cytokines by macrophages and inhibits the ability of LPS from *Escherichia coli* to stimulate cytokine secretion. As expected from these findings, *F. tularensis* grown in macrophages was found to cause a higher mortality rate in mice than bacteria grown in broth.

2. Results

Secretion of inflammatory cytokines by macrophages is an important mechanism for controlling the proliferation of *F. tularensis* [13,15–17]. Since *F. tularensis* that has grown in macrophages infects bystander cells, we compared *F. tularensis* grown in macrophages (FtMac) with bacteria grown in broth (FtB) for the ability to stimulate the secretion of inflammatory cytokines by macrophages. FtB stimulated the secretion of large amounts of TNF- α , IL-12p40, IL-6 and MCP-1/CCL2 by elicited peritoneal murine macrophages (Fig. 1). Similar results were obtained when human monocytes were used (Fig. 2). LPS from *E. coli* was used as a positive control. Bacteria grown to late exponential phase in broth or on CA agar stimulated similar levels of cytokine secretion as FtB grown to early exponential phase (data not shown). The levels of cytokine secretion stimulated by FtB for 18 h were nearly as great as those induced by LPS from *E. coli* (Fig. 1). In contrast, FtMac stimulated about 10% of the level of cytokines stimulated by FtB (Fig. 1). Similar results were obtained with RAW 264.7, MHS or U937 cells (data not shown). Likewise, the species of macrophage used to generate FtMac was irrelevant as FtMac recovered from either murine RAW 264.7 cells or human U937 cells had similar effects. Cytokine levels detected in media of unstimulated

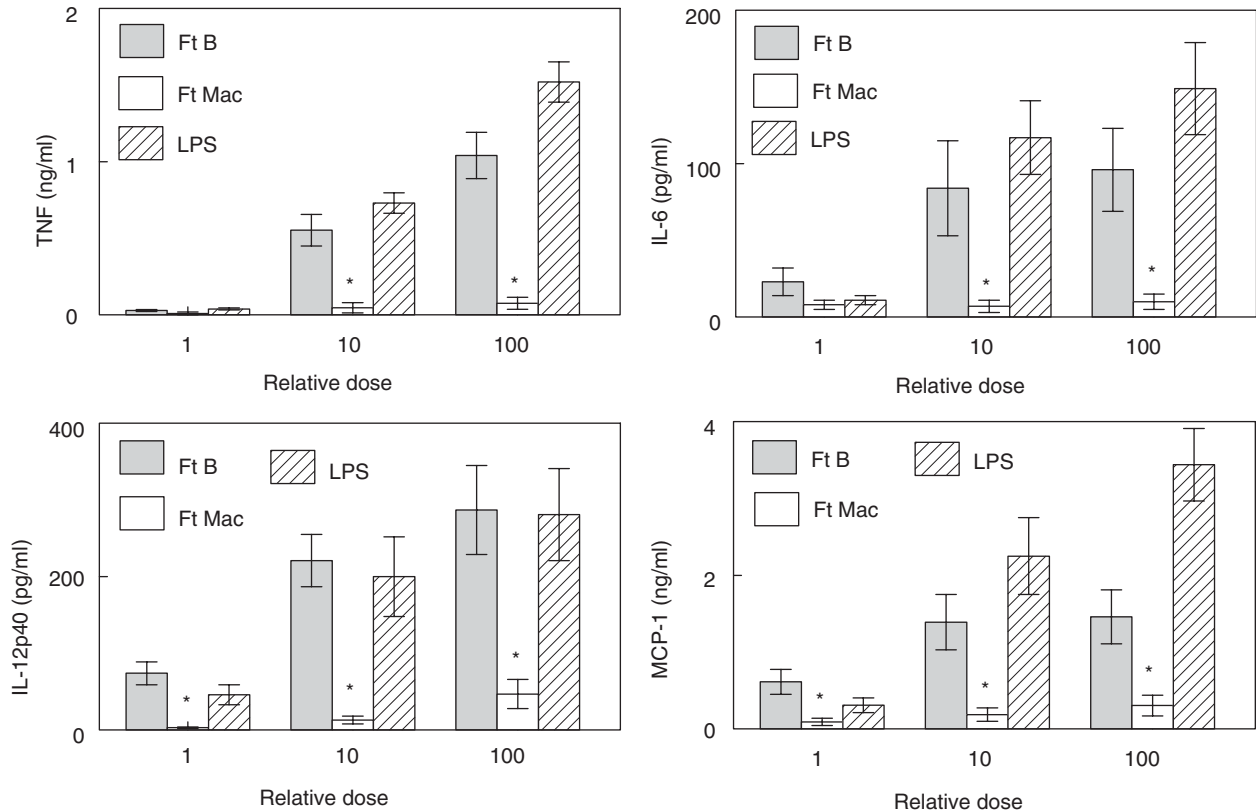


Fig. 1. Secretion of cytokines by murine elicited peritoneal macrophages stimulated with *F. tularensis* grown in Mueller-Hinton broth (FtB) or in macrophages (FtMac), or LPS from *E. coli*. Macrophages were incubated for 18 h with bacteria at an MOI of 1, 10 or 100 or with LPS at 1, 10 or 100 ng/ml. Cytokines were determined by bead array (TNF- α , IL-6, IL-1 β and MCP-1) or ELISA (IL-12p40) and values are the mean \pm SEM for 5 experiments. * p < 0.05 compared with the respective FtB value.

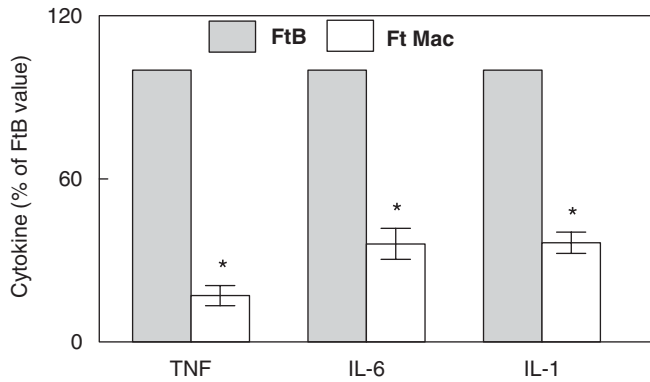


Fig. 2. Secretion of cytokines by human monocytes stimulated with *F. tularensis* grown in Mueller–Hinton broth (FtB) or in macrophages (FtMac). Monocytes were incubated with FtB or FtMac at an MOI of 100 for 18 h. Control levels of cytokine stimulated by FtB in monocytes were: TNF- α : 11.9 ± 0.9 , IL-6: 19.4 ± 0.6 , IL-1 β : 8.2 ± 0.8 ng/ml. Cytokines were determined by bead array (TNF- α , IL-6, IL-1 β and MCP-1) or ELISA (IL-12p40) and values are the mean \pm SEM for 5 experiments. * $p < 0.05$ compared with the respective FtB value.

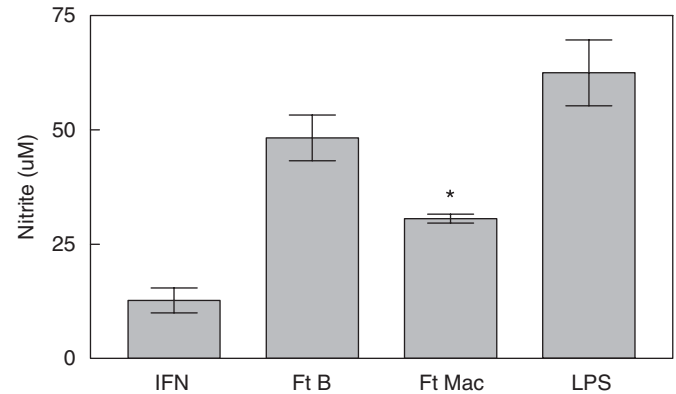


Fig. 3. Nitric oxide production by RAW 264.7 cells stimulated with *F. tularensis* grown in broth (FtB) or in macrophages (FtMac), or LPS from *E. coli*. Macrophages were incubated 18 h with bacteria at an MOI of 100 or with LPS at 100 ng/ml. All cultures contained interferon- γ (100 units/ml). Nitrite concentrations are expressed as the mean \pm SEM for 5 experiments. * $p < 0.05$ compared with the FtB value.

macrophages were always less than 10% of the LPS-stimulated levels. To determine if soluble material was contributing to cytokine secretion, the suspension of bacteria (5×10^9 /ml) used to inoculate macrophages was centrifuged at 8000g for 10 min and a volume of supernatant equal to that used for an MOI of 100 (10 μ l) was added to the macrophages. This supernatant did not consistently stimulate detectable cytokine secretion after 18 h (data not shown).

The production of nitric oxide by macrophages is an important defense mechanism for intracellular pathogens [14]. Neither elicited peritoneal macrophages nor RAW 264.7 cells consistently produced detectable nitrite when stimulated with FtB or FtMac overnight. However, combining either of the stimuli with IFN- γ (100 units/ml) resulted in nitric oxide production (Fig. 3). FtB stimulated the production of 46% more nitrate than FtMac when the amount stimulated by IFN- γ alone is excluded. It is possible that the presence of IFN- γ in these experiments accounted for FtMac stimulating relatively higher levels of nitrite than cytokines. Indeed, when FtMac-stimulated TNF- α secretion was determined in the presence of IFN- γ , FtMac stimulated 30.7% of the level of TNF- α stimulated with FtB compared with 10.3% in the absence of IFN- γ (data not shown). Thus, as with cytokines, FtMac stimulates lower levels of nitric oxide production than FtB but IFN- γ enhances the cytokine and nitric oxide response to FtMac.

FtMac were grown in broth to determine how long it would take to regain the ability to stimulate cytokine secretion (Fig. 4). Incubation of FtMac in Mueller–Hinton broth resulted in a progressive recovery of the ability to stimulate TNF- α secretion by RAW 264.7 cells. Full recovery required 18 h. In addition, killing FtMac with gentamicin or fixed in paraformaldehyde did not increase the ability to stimulate cytokine secretion (Fig. 5). These

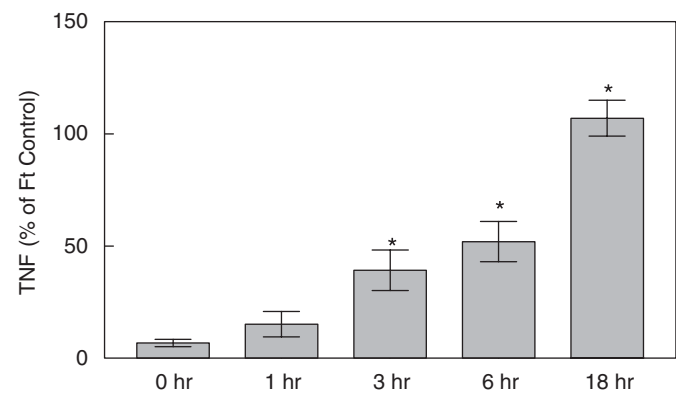


Fig. 4. FtMac regains the ability to stimulate TNF- α secretion by macrophages following incubation in broth. FtMac were incubated in broth for the indicated time, washed and incubated with RAW 264.7 cells for 18 h at an MOI of 100. TNF- α levels were determined by ELISA and results are expressed as the percent or the level stimulated by FtB and are the mean \pm SEM for 4 experiments. The control level of TNF- α stimulated by FtB was 3.27 ± 1.17 ng/ml. * $p < 0.05$ compared with the zero time value.

results show that FtMac can revert to the FtB phenotype and that reduced cytokine secretion does not require live bacteria.

To determine if inhibition of cytokine secretion is the mechanism for the reduced cytokine response to FtMac, macrophages were infected with *F. tularensis* for 2 h and then stimulated with *E. coli* LPS. Infection of macrophages with FtB did not inhibit subsequent LPS-stimulated cytokine secretion. In contrast, cytokine secretion stimulated with *E. coli* LPS was reduced in macrophages previously infected with FtMac (Fig. 6). The percent of LPS-stimulated cytokine secretion by macrophages infected with FtMac compared with FtB was $47.2 \pm 8.4\%$ for TNF- α , $56.0 \pm 8.5\%$ for IL-12p40, $43.2 \pm 10.3\%$ for IL-6

and $43.8 \pm 13.8\%$ for MCP-1 ($n = 6$, mean \pm SEM). These results suggest that FtMac may inhibit signaling pathways for cytokine secretion.

Determination of bacterial phagocytosis revealed that fewer FtMac were ingested than FtB (Fig. 7). The phagocytic

index of FtMac at an MOI of 100 was 51% of that for FtB and at an MOI of 10 it was 58%. The percentage of macrophages that had ingested at least one bacterium was 19.3 ± 0.33 for FtMac and 27.6 ± 2.67 for FtB ($n = 3$) (data not shown). The number of FtB and FtMac that were bound but not ingested was about half of the number ingested for either MOI (data not shown). A lower level of ingestion by FtMac compared with FtB was also seen when internalization was determined by CFU (Fig. 8). However, the lower ingestion of FtMac does not account for the minimal stimulation of cytokine secretion, because FtB stimulated greater cytokine secretion at a MOI of 10 than FtMac at an MOI of 100 (Fig. 1) even though more FtMac were ingested at an MOI of 100 than FtB were at an MOI of 10 (Fig. 7).

To determine if the lower phagocytosis of FtMac was due to inhibition of phagocytic function in general, the ability of infected macrophages to phagocytose IgG-coated beads was determined. Fc γ R phagocytic function was unaffected by infection of macrophages with FtB or FtMac (Fig. 9). Thus, in contrast to the effect on LPS-stimulated cytokine secretion, infection of macrophages with FtMac does not inhibit Fc γ R phagocytic function.

The ability of intracellular pathogens such as *F. tularensis* to replicate within macrophages is critical

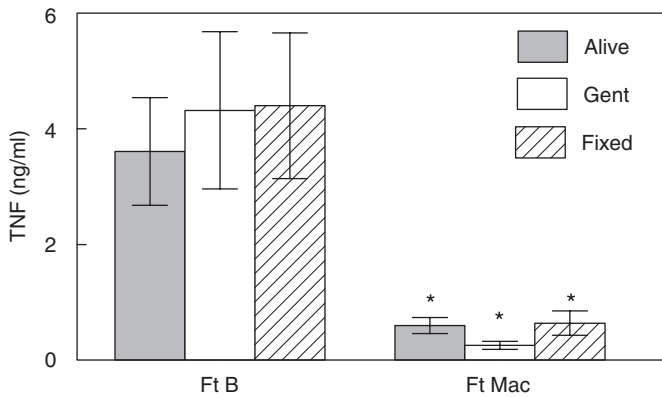


Fig. 5. Killing FtB or FtMac has no effect on the ability to stimulate TNF- α secretion by macrophages. Elicited peritoneal macrophages were incubated with FtB or FtMac for 18 h at an MOI of 100. Bacteria were killed with gentamicin (Gent) or with paraformaldehyde (Fixed). TNF- α was determined by ELISA and the values are the mean \pm SEM of 3 separate experiments. * $p < 0.05$ compared with the respective FtB value.

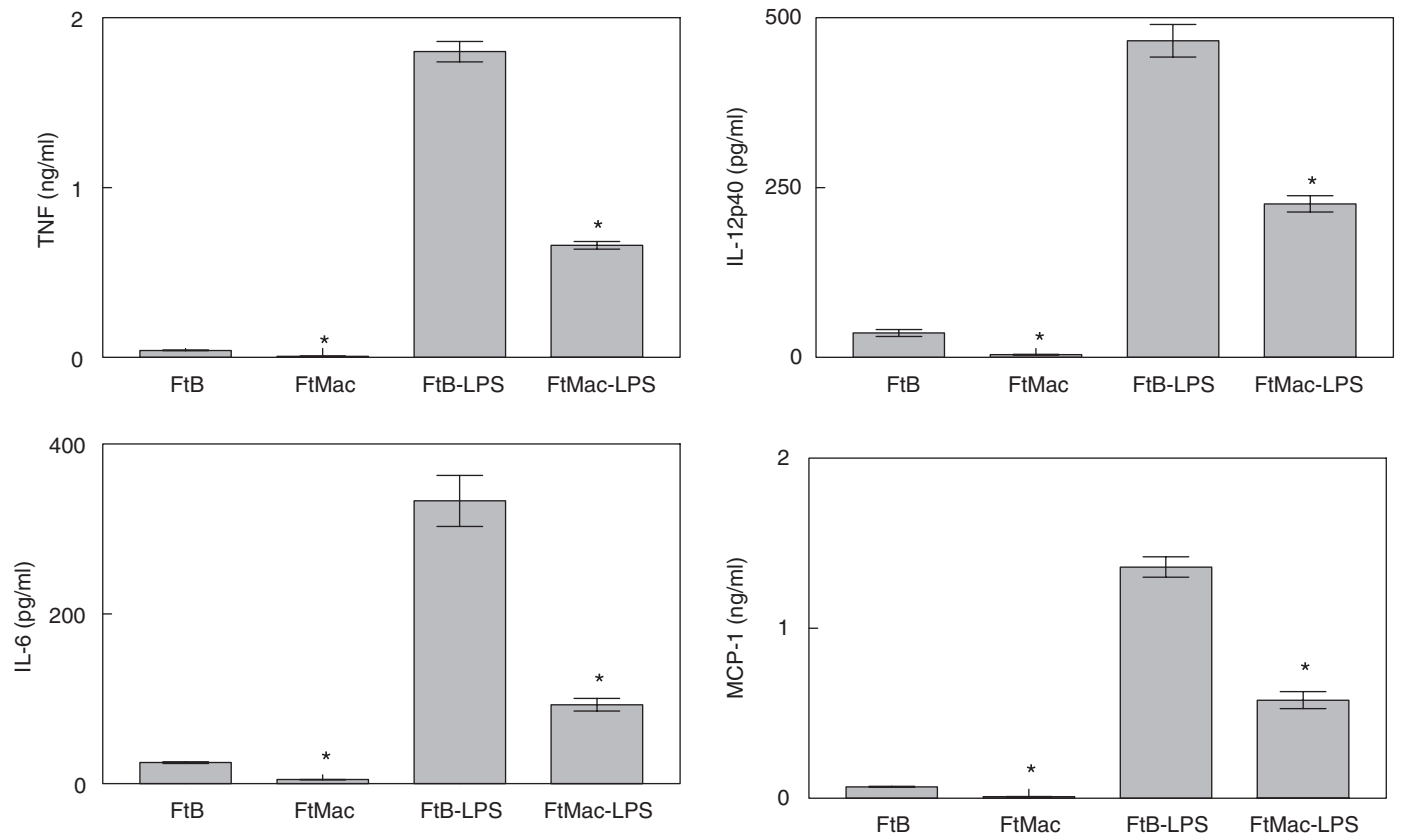


Fig. 6. Effect FtB or FtMac on LPS-stimulated TNF- α , IL-12p40, IL-6 and MCP-1 secretion. Elicited peritoneal macrophages were incubated with Ft or Ft M (FtMac) (MOI of 100) for 2 h, washed, external bacteria killed with gentamicin (50 μ g/ml), and then incubated for 18 h. In other experiments, macrophages were infected in the same way except that after infection they were stimulated with *E. coli* LPS (100 ng/ml). Values are representative of 6 separate experiments. * $p < 0.05$ compared with the respective FtB value.

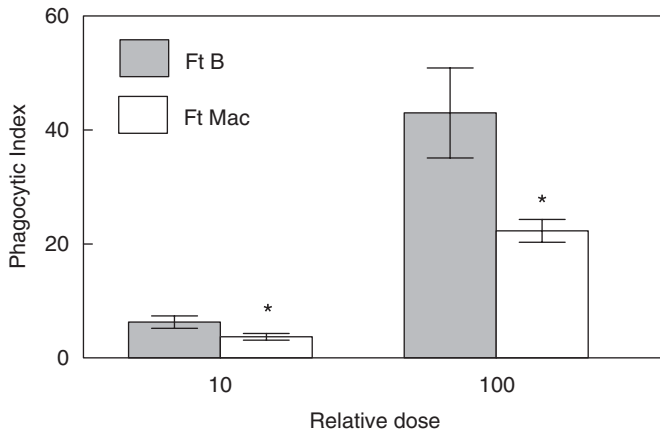


Fig. 7. Phagocytosis of FtB or FtMac by macrophages. RAW 264.7 cells were incubated with FtB or FtMac at an MOI of 10 or 100 for 2 h. Cells were fixed and stained with a fluorescently labeled anti-*F. tularensis* antibody and phagocytosis was quantified by immunofluorescence microscopy. Phagocytic index (number of ingested bacteria per 100 macrophages) values are the mean \pm SEM for 3 separate experiments. * $p < 0.05$ compared with the respective FtB value.

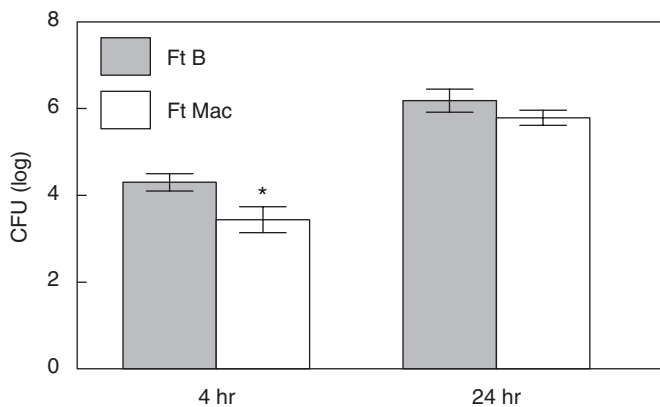


Fig. 8. Replication of FtB or FtMac in macrophages. RAW 264.7 cells were incubated with FtB or FtMac at an MOI of 100 for 2 h and washed. External bacteria were killed and CFU determined at 4 and 24 h. Values are the mean \pm SEM for 4 separate experiments. * $p < 0.05$ compared with the FtB value.

for their pathogenicity. Following ingestion, *F. tularensis* requires 4 h to break out of the phagosome of macrophages and begin to replicate within the cytoplasm [2]. It was found that FtB and FtMac replicated within macrophages at similar rates between 4 and 24 h after ingestion (Fig. 8). Similar results were obtained with U937 and MHS cells over 48 h. In U937 cells, FtMac replicated 3.63 ± 0.37 log and FtB replicated 3.49 ± 0.44 log; and for MHS cells, FtMac replicated 4.24 ± 0.96 log and FtB replicated 6.06 ± 0.38 log ($n = 4$, mean \pm SEM, not significantly different). Thus, FtMac replicates at a rate similar to that of FtB in macrophages.

Survival studies demonstrated that FtMac is more lethal than FtB. Significantly more mice died when infected with FtMac than with FtB (Fig. 10). The increased mortality caused by FtMac may be due to failure of this phenotype

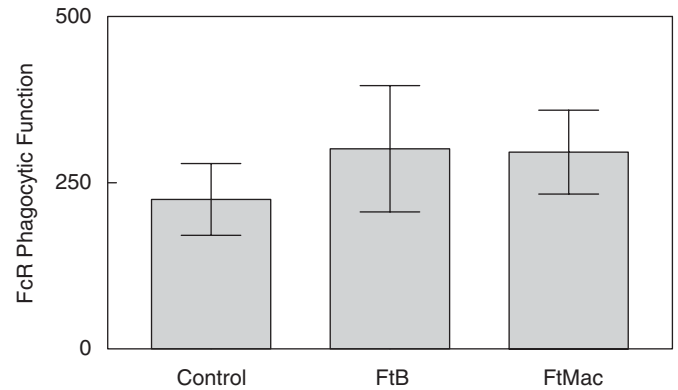


Fig. 9. Fc γ R phagocytic function, as assessed from the ingestion of IgG coated beads, was determined after infection of macrophages with FtB or FtMac. RAW264.7 cells were incubated with FtB or FtMac (MOI of 100) for 2 h, washed and then allowed to phagocytose IgG-coated beads (MOI of 10) for 30 min. Macrophages were fixed and the number of ingested beads counted by immunofluorescence. Results are expressed as the number of ingested beads per 100 macrophages and the values are the mean \pm SEM for 3 separate experiments.

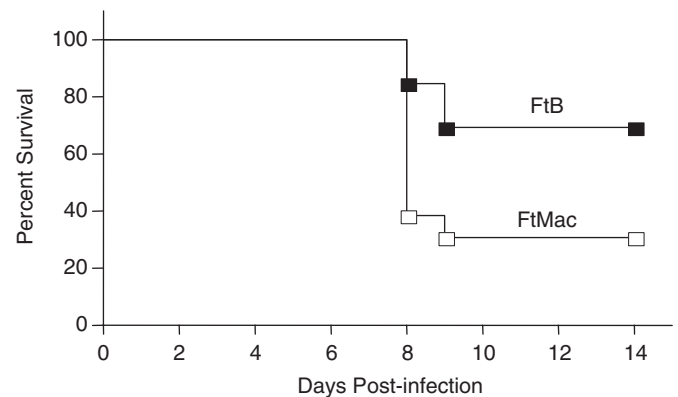


Fig. 10. Survival of Balb/C mice following infection with FtB or FtMac. Mice were anesthetized and infected by the intranasal route with 5000 CFU of bacteria. Survival was recorded twice a day for 21 days. There were no additional deaths beyond day 14. There were 13 mice per group. There was a significant difference in survival rate ($p < 0.05$).

to stimulate bactericidal mechanisms due to the secretion of low levels of inflammatory cytokines.

3. Discussion

An important factor in the spread of an *F. tularensis* infection is the ability of this organism to infect macrophages, replicate within them, break out and infect other macrophages [1–3]. One of the defenses against this strategy is for the macrophage to limit replication of the bacteria by producing inflammatory cytokines that increase the bactericidal capacity of the macrophages. The results reported here show that *F. tularensis* grown in broth (FtB) stimulate the secretion of inflammatory cytokines by macrophages, however, bacteria that have broken out of macrophages (FtMac) stimulate a limited cytokine response. This may allow an infection with *F. tularensis* to

progress without activating the bactericidal functions of the host. Such a paradigm may explain the high infectivity of *F. tularensis* in that a small inoculum would stimulate a limited innate response and subsequent amplification of the infection with bacteria released from macrophages would cause a minimal inflammatory cytokine response. Indeed, it was found that FtMac causes a greater mortality rate than FtB.

Consistent with previous reports, the present study shows that levels of inflammatory cytokines stimulated by LPS from *E. coli* were in the same range as those caused by FtB when the macrophages were incubated continuously with the bacteria [4,18]. FtB stimulated the secretion of TNF- α and IL-12p40 with a time course similar to that caused by LPS from *E. coli* (data not shown). We also found that when FtB were allowed to infect the macrophages for 2 h very low levels of cytokines were produced which is also in agreement with previous work [19,20]. Other studies have shown that *F. tularensis*, LVS can stimulate the secretion of cytokines after 3 days in culture [15,21,22].

F. tularensis that has undergone a growth cycle through macrophages (FtMac) stimulated the secretion of inflammatory cytokines 10-fold less than FtB. This difference was seen with different MOI, macrophages or human monocytes and when macrophages were incubated with the bacteria overnight (Figs. 1 and 2) or infected for 2 h (Fig. 6). Low levels of cytokine secretion induced by FtMac was unaltered when the organism was killed suggesting that the reduced cytokine secretion was not due to a factor secreted by the bacteria but rather due to a change in the surface of the bacteria. In addition, colonies of FtB and FtMac grown on agar have an identical appearance, arguing against the possibility that FtMac is a phase variant.

As with inflammatory cytokines, FtMac also stimulated lower levels of nitric oxide than FtB. The reduced production of this bactericidal agent could allow increased survival of the FtMac phenotype. FtMac did not reduce the production of nitric oxide to the same extent as inflammatory cytokines. However, it is possible that FtMac would stimulate little nitric oxide in a host because the production of nitric oxide requires the presence of IFN- γ . Since IFN- γ is stimulated by IL-12 and FtMac stimulates low levels of IL-12, it is unlikely that FtMac would cause the production of high levels of nitric oxide.

Infection of macrophages with FtMac was found to reduce subsequent cytokine secretion stimulated with *E. coli* LPS. However, LPS-stimulated cytokine secretion was not fully blocked by infection with FtMac, which may reflect the fact that not all of the macrophages were infected. A likely mechanism for the inhibition of cytokine secretion by FtMac is interference with the MAPK to NF- κ B signaling pathways. Telepnev et al. [20,23] have shown that the inhibition of cytokine secretion due to infection of macrophages with *F. tularensis* was associated with a reduction in the activation of MAPK and NF- κ B [20,23].

Inhibition of these signaling pathways is common among intracellular pathogens but the mechanism by which the inhibition occurs differs between organisms [24–26]. In contrast to the effect on cytokine secretion, FtMac did not inhibit Fc γ R-mediated phagocytosis suggesting that specific signaling pathways are inhibited by FtMac. We are currently investigating the signaling pathways involved in the effects of FtMac.

Previous work by Telepnev et al. [20] has shown that infection of macrophages with *F. tularensis* LVS grown on Thayer–Martin agar, inhibited cytokine secretion stimulated by LPS and bacterial lipoproteins. They found that the degree of inhibition of LPS-stimulated TNF- α secretion was dependent on the MOI and that when each macrophage had ingested at least one bacteria cytokine secretion was fully blocked. We did not detect an inhibition of LPS-stimulated cytokine secretion when macrophages were infected with FtB. However, there are several differences in experimental protocol between the two studies that could explain the disparate results including the conditions for growing the bacteria and the macrophages used.

FtMac was ingested at a lower rate than FtB by macrophages. This was not due to a general depression of phagocytic function because macrophages infected with FtMac had unchanged Fc γ R-mediated phagocytosis. Changes in the bacteria caused by growth in macrophages appear to alter its recognition by macrophages. Interpretation of these results is hampered by incomplete knowledge of the receptors involved in the recognition of *F. tularensis*. A recent paper by Clemens et al. [27] found that complement receptors could mediate the phagocytosis of serum-opsonized *F. tularensis*. Since we used heat-inactivated serum, it is unlikely that complement components are involved in our phagocytosis measurements. Further work is required to determine the role of receptors such as mannose, dectin and complement receptor 3 in the phagocytosis of *F. tularensis*. Even though the phagocytosis of FtMac was reduced, its rate of replication within macrophages is similar to that of FtB.

The results of this study expand our understanding of factors that can influence the ability of *F. tularensis* to stimulate or suppress cytokine secretion. In addition, this study emphasizes the importance of the growth conditions when studying the effect of *F. tularensis* on macrophages. Our results suggest that initial encounters with macrophages results in recognition of *F. tularensis* that causes the stimulation of inflammatory cytokines. However, when subsequent generations of bacteria break out of the macrophages they are changed in a way that allows them to invade new cells and replicate within them without stimulating cytokine secretion. The reduced cytokine response limits the ability of the host to control the infection resulting in increased mortality. An alternative interpretation of these results is that since *F. tularensis* may reside within cells in nature, natural infections may be by bacteria that have grown in cells and may cause little innate response by host macrophages.

4. Methods and materials

F. tularensis live vaccine strain (LVS) was a gift from Karen L. Elkins. Bacteria were grown in Mueller–Hinton broth to early exponential phase. For growth in macrophages, RAW 264.7 cells (2×10^6 per well) were adhered to 6 well plates and *F. tularensis* was added at an MOI of 100, incubated for 2 h, washed and extracellular bacteria killed with Gentamicin (50 µg/ml for 1 h). The wells were washed twice with PBS and incubated with fresh media for 72 h. Extracellular bacteria were collected by removing the media, washing the adherent macrophages and then centrifuging the media and washes at 200g to remove any macrophages. A total of $1\text{--}5 \times 10^9$ FtMac were recovered from three 6 well plates of RAW 264.7 cells. FtMac could not be obtained from elicited peritoneal macrophages probably due to their partial activation. No FtMac were obtained after 24 h and 10% of the number collected at 72 h could be collected at 48 h. *F. tularensis* did not multiply in the cell culture media used. For all experimental conditions, *F. tularensis* were washed (3000g for 20 min), suspended in cell culture media without antibiotics and counted by dark field microscopy. Similar numbers were obtained from colony-forming units determined by growth on chocolate agar plates. FtMac were never stored more than 24 h at 4 °C before use. Bacteria were more than 95% viable by BacLight™ analysis (Molecular Probes).

Peritoneal macrophages were elicited with thioglycolate in Balb/C mice and were obtained by lavage of the peritoneal cavity with 2×8 ml of PBS. The peritoneal exudate cells were washed, suspended in DMEM media and enriched for macrophages by adhesion for 2 h (48 well tissue culture plates at 5×10^5 /well). RAW 264.7 cells and MHS were cultured in DMEM. U937 cells were grown in RPMI media and treated with PMA (1 µg/ml) for 3 days prior to use. Elutriated human monocytes were purchased from the University of Nebraska Medical Center, Omaha, NE and incubated in RPMI media. Cell culture media contained 10% heat inactivated newborn calf serum supplemented with iron. All macrophage cultures containing *F. tularensis* were antibiotic free except during gentamicin treatment (1 h) to kill extracellular bacteria.

For cytokine studies, *F. tularensis* grown in broth (FtB) or macrophages (FtMac) were added to macrophages in antibiotic-free media at an MOI of 1 (5×10^5 /well), 10 (5×10^6 /well) or 100 (5×10^7 /well). LPS (*E. coli* 0111:B4) (Sigma) was used as a positive control for cytokine secretion. Macrophages were incubated with each stimulus for 18 h and cytokine levels in the media were determined. TNF- α and IL-12p40 levels were determined using ELISA kits from R&D Systems and BD Biosciences, respectively. TNF- α , IL-6 and MCP-1 were determined using the mouse inflammation cytokine cytometric bead array kit from BD Biosciences. Similar results for TNF- α levels were obtained from ELISA and cytometric bead array assays. Nitric oxide production by macrophages was assessed from nitrite levels in the media as determined using the Griess reaction.

At 24 h after addition of FtB or FtMac at an MOI of 100, more than 95% of the macrophages were viable as determined by exclusion of trypan blue and there was no change in the number of attached macrophages.

Phagocytosis of *F. tularensis* was determined using a fluorescent phagocytosis assay. FtB or FtMac were added to macrophages at an MOI of 10 or 100 and centrifuged on to the cells at 800g for 10 min to increase contact between bacteria and macrophages. Phagocytosis was allowed to proceed for 2 h, after which the macrophages were washed and fixed with 3.7% paraformaldehyde. External bacteria were stained with an antibody against *F. tularensis* (FB11, Research Diagnostics) and visualized with an Alexa 488 (green) secondary antibody. The macrophages were then permeabilized, stained with FB11 again and visualized with an Alexa 568 (red) secondary antibody. In this way the internal bacteria (red) were distinguished from the bound/external organisms (red + green = orange/yellow). Results were quantified visually and reported as phagocytic index (number of ingested bacteria per 100 macrophages).

The method described above was also used to determine Fc γ receptor (Fc γ R) phagocytic function as assessed by on the ability of macrophages to phagocytose IgG opsonized glass beads (2 µm). In this case, macrophages were incubated with FtB or FtMac at a MOI of 100 for 2 h, washed and then incubated with IgG-coated beads (MOI of 10) labeled with Alexa 568 for 30 min. The cells were washed and fixed and external beads labeled with Alexa 488. Phagocytic index for the beads was determined. Glass beads not coated with IgG were not phagocytosed by the macrophages.

Phagocytosis and proliferation of *F. tularensis* in macrophages was determined using macrophages seeded onto 6 well plates at a million per well. *F. tularensis*, grown in Mueller–Hinton broth or in macrophages, were added to the macrophages at an MOI of 100 and centrifuged on to the cells (800g, 10 min). The macrophages were infected for 2 h, washed and extracellular bacteria were killed with gentamicin (50 µg/ml, 1 h). The cells were then washed twice in PBS with rocking for 5 min. Culture media was added and incubated for 1, 24 or 48 h. The media was removed and the macrophages lysed with sodium deoxycholate (700 µl, 0.1%) for 5 min at 37 °C. The lysate was removed and the plate washed 5 times with media. The media, lysate and wash media were combined and the bacteria were recovered by centrifugation. Serial dilutions were incubated on CA plates for 72 h at 37 °C. CFU at 1 h indicated the number of *F. tularensis* that were taken up by the macrophages and the 24 and 48 h time points indicated subsequent proliferation.

Survival studies were carried out using intranasal infection of mice as described by Duckett et al. [9]. Briefly, mice were anesthetized with ketamine and xylazine and were infected with FtB or FtMac in 40 µl of Ringer's solution containing 5000 CFU. The number of CFU inoculated was confirmed at the time of the experiment.

Data are expressed as the mean \pm SE. Survival data were analyzed using the Kaplan–Meir survival analysis and all other comparisons were analyzed using the Students' *t*-test. The level of confidence was placed at 95% for all experiments.

Acknowledgements

The authors thank Dr. Karen L. Elkins for the *F. tularensis* LVS that was used for this study. The authors acknowledge the assistance of the Immunology Core and Microbiology Core facilities in the Center for Immunology and Microbial Disease, the technical assistance of Scott Granger and secretarial assistance of Wendy M. Hobb. This work was supported by PO1 AI056321, Subproject 1 and GM50821.

References

- [1] Elkins KL, Cowley SC, Bosio CM. Innate and adaptive immune responses to an intracellular bacterium, *Francisella tularensis* live vaccine strain. *Microbes Infect* 2003;5:135–42.
- [2] Oyston PC, Sjostedt A, Titball RW. Tularaemia: bioterrorism defence renews interest in *Francisella tularensis*. *Nat Rev Microbiol* 2004;2:967–78.
- [3] Sjostedt A. Intracellular survival mechanisms of *Francisella tularensis*, a stealth pathogen. *Microbes Infect* 2006;8:561–7.
- [4] Conlan JW, KuoLee R, Shen H, Webb A. Different host defences are required to protect mice from primary systemic vs pulmonary infection with the facultative intracellular bacterial pathogen, *Francisella tularensis* LVS. *Microb Pathogenesis* 2002;32:127–34.
- [5] Elkins KL, Rhinehart-Jones TR, Culkun SJ, Yee D, Winegar RK. Minimal requirements for murine resistance to infection with *Francisella tularensis* LVS. *Infect Immun* 1996;64:3288–93.
- [6] Green SJ, Nacy CA, Schreiber RD, Granger DL, Crawford RM, Meltzer MS, et al. Neutralization of gamma interferon and tumor necrosis factor alpha blocks in vivo synthesis of nitrogen oxides from L-arginine and protection against *Francisella tularensis* infection in *Mycobacterium bovis* BCG-treated mice. *Infect Immun* 1993;61:689–98.
- [7] Leiby DA, Fortier AH, Crawford RM, Schreiber RD, Nacy CA. In vivo modulation of the murine immune response to *Francisella tularensis* LVS by administration of anticytokine antibodies. *Infect Immun* 1992;60:84–9.
- [8] Sjostedt A, North RJ, Conlan JW. The requirement of tumour necrosis factor-alpha and interferon-gamma for the expression of protective immunity to secondary murine tularaemia depends on the size of the challenge inoculum. *Microbiology* 1996;142(Part 6):1369–74.
- [9] Duckett NS, Olmos S, Durrant DM, Metzger DW. Intranasal interleukin-12 treatment for protection against respiratory infection with the *Francisella tularensis* live vaccine strain. *Infect Immun* 2005;73:2306–11.
- [10] Anthony LS, Ghadirian E, Nestel FP, Kongshavn PA. The requirement for gamma interferon in resistance of mice to experimental tularaemia. *Microb Pathogenesis* 1989;7:421–8.
- [11] Anthony LS, Morrissey PJ, Nano FE. Growth inhibition of *Francisella tularensis* live vaccine strain by IFN-gamma-activated macrophages is mediated by reactive nitrogen intermediates derived from L-arginine metabolism. *J Immunol* 1992;148:1829–34.
- [12] Fortier AH, Slayter MV, Ziemba R, Meltzer MS, Nacy CA. Live vaccine strain of *Francisella tularensis*: infection and immunity in mice. *Infect Immun* 1991;59:2922–8.
- [13] Polsinelli T, Meltzer MS, Fortier AH. Nitric oxide-independent killing of *Francisella tularensis* by IFN-gamma-stimulated murine alveolar macrophages. *J Immunol* 1994;153:1238–45.
- [14] Chakravorty D, Hensel M. Inducible nitric oxide synthase and control of intracellular bacterial pathogens. *Microbes Infect* 2003;5:621–7.
- [15] Bosio CM, Elkins KL. Susceptibility to secondary *Francisella tularensis* live vaccine strain infection in B-cell-deficient mice is associated with neutrophilia but not with defects in specific T-cell-mediated immunity. *Infect Immun* 2001;69:194–203.
- [16] Stenmark S, Sunnemark D, Bucht A, Sjostedt A. Rapid local expression of interleukin-12, tumor necrosis factor alpha, and gamma interferon after cutaneous *Francisella tularensis* infection in tularaemia-immune mice. *Infect Immun* 1999;67:1789–97.
- [17] Bolger CE, Forestal CA, Italo JK, Benach JL, Furie MB. The live vaccine strain of *Francisella tularensis* replicates in human and murine macrophages but induces only the human cells to secrete proinflammatory cytokines. *J Leukoc Biol* 2005;77:893–7.
- [18] Gavrilin MA, Bouakl IJ, Knatz NL, Duncan MD, Hall MW, Gunn JS, et al. Internalization and phagosome escape required for *Francisella* to induce human monocyte IL-1beta processing and release. *Proc Natl Acad Sci USA* 2006;103:141–6.
- [19] Bosio CM, Dow SW. *Francisella tularensis* induces aberrant activation of pulmonary dendritic cells. *J Immunol* 2005;175:6792–801.
- [20] Telepnev M, Golovliov I, Grundstrom T, Tarnvik A, Sjostedt A. *Francisella tularensis* inhibits Toll-like receptor-mediated activation of intracellular signalling and secretion of TNF-alpha and IL-1 from murine macrophages. *Cell Microbiol* 2003;5:41–51.
- [21] Cowley SC, Elkins KL. Multiple T cell subsets control *Francisella tularensis* LVS intracellular growth without stimulation through macrophage interferon gamma receptors. *J Exp Med* 2003;198:379–89.
- [22] Elkins KL, Cooper A, Colombini SM, Cowley SC, Kieffer TL. In vivo clearance of an intracellular bacterium, *Francisella tularensis* LVS, is dependent on the p40 subunit of interleukin-12 (IL-12) but not on IL-12 p70. *Infect Immun* 2002;70:1936–48.
- [23] Telepnev M, Golovliov I, Sjostedt A. *Francisella tularensis* LVS initially activates but subsequently down-regulates intracellular signaling and cytokine secretion in mouse monocyte and human peripheral blood mononuclear cells. *Microb Pathogenesis* 2005;38:239–47.
- [24] Denkers EY, Butcher BA. Sabotage and exploitation in macrophages parasitized by intracellular protozoans. *Trends Parasitol* 2005;21:35–41.
- [25] Koul A, Herget T, Klebl B, Ullrich A. Interplay between mycobacteria and host signalling pathways. *Nat Rev Microbiol* 2004;2:189–202.
- [26] Olivier M, Gregory DJ, Forget G. Subversion mechanisms by which *Leishmania* parasites can escape the host immune response: a signaling point of view. *Clin Microbiol Rev* 2005;18:293–305.
- [27] Clemens DL, Lee BY, Horwitz MA. *Francisella tularensis* enters macrophages via a novel process involving pseudopod loops. *Infect Immun* 2005;73:5892–902.