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Francisella gains a survival advantage within mononuclear phagocytes by suppressing the host IFNγ response

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Abstract

Tularemia is a zoonotic disease caused by the Gram-negative intracellular pathogen *Francisella tularensis*. These bacteria evade phagolysosomal fusion, escape from the phagosome and replicate in the host cell cytoplasm. IFNγ has been shown to suppress the intra-macrophage growth of *Francisella* through both nitric oxide-dependent and -independent pathways. Since *Francisella* is known to subvert host immune responses, we hypothesized that this pathogen could interfere with IFNγ signaling. Here, we report that infection with *Francisella* suppresses IFNγ-induced STAT1 expression and phosphorylation in both human and murine mononuclear phagocytes. This suppressive effect of *Francisella* is independent of phagosomal escape or replication and is mediated by a heat-stable and constitutively-expressed bacterial factor. An analysis of the molecular mechanism of STAT1 inhibition indicated that expression of SOCS3, an established negative regulator of IFNγ signaling, is highly up-regulated during infection and suppresses STAT1 phosphorylation. Functional analyses revealed that this interference with IFNγ signaling is accompanied by the suppression of IP-10 production and iNOS induction resulting in increased intracellular bacterial survival. Importantly, the suppressive effect on IFNγ-mediated host cell protection is most effective when IFNγ is added post infection, suggesting that the bacteria establish a permissive environment within the host cell.

Keywords

Francisella; IFNγ; SOCS3; immune evasion

Introduction

Francisella tularensis is a Gram-negative, intracellular, zoonotic pathogen that causes the disease tularemia. *F. tularensis* subspecies *tularensis* (Type A) and subspecies *holarctica*

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(Type B) cause disease in humans. *F. tularensis* has been categorized by the CDC as a Category A Select agent and is perceived as a potential bio-weapon. However little is known about its pathogenesis[1]. Other subspecies include *F. tularensis novicida* and *mediasiatica. F. novicida* is attenuated in humans but highly infectious in mice. Thus it is a widely used experimental model for tularemia.

F. tularensis predominantly infects monocytes and macrophages. The phagosomes containing *F. tularensis* do not fuse with lysosomes and the bacteria escape into the cytosol where they replicate and subsequently trigger apoptosis of the host cell[2,3]. Escape of this pathogen into the cytosol requires the expression of bacterial proteins such as IglC, MglA, PmrA and AcpA[4–8]. Priming of macrophages with IFNγ, however, inhibits this escape[9]

IFNγ is a cytokine mainly produced by natural killer and T cells[10], although we have found that infected monocytes also secrete very low levels of IFNγ. The signaling cascade initiated by engagement of the IFNγ receptor (IFNγR) has been comprehensively examined[10]. Recruitment and activation of the JAK kinases and STAT1 are a prerequisite for the transcription of IFNγ response genes. IFNγ signaling is negatively regulated by several mechanisms including the internalization of IFNγR, dephosphorylation of JAKs by the tyrosine phosphatase SHP-1 and the induction of the SOCS (Suppressors Of Cytokine Signaling) proteins. SOCS proteins function by binding to JAKs and inhibiting their phosphorylation, and thereby the JAK-dependent phosphorylation of the receptor and STAT1[12].

IFNγ plays a crucial role in modulating host immune responses. In particular, IFNγ is important for the activation of anti-microbial events such as the production of nitric oxide and reactive oxygen species and the up-regulation of FcγRI, complement receptor CR3 and NRAMP1[10]. Several pathogens such as *Leishmania donovani*[13], *Mycobacterium avium*[14], *Mycobacterim tuberculosis*[15,16], and *Listeria monocytogenes*[17] have developed mechanisms to evade IFNγ-mediated host responses. Since *Francisella* is often referred to as a 'stealth pathogen' that effectively evades host immune responses, it is reasonable to postulate that this organism may also interfere with the host IFNγ response.

In this study we report that *Francisella* suppresses IFNγ-induced STAT1 expression and tyrosine phosphorylation in both human and murine mononuclear phagocytes. Examination of downstream events shows that IFNγ-induced iNOS expression is reduced, suggesting a mechanism for increased bacterial survival. This signaling interference is independent of phagosomal escape, replication and viability of the pathogen. Further, we demonstrate that the negative regulator SOCS3 is highly up-regulated and accounts, at least in part, to the suppression of STAT1 phosphorylation. Finally, we show that administration of recombinant IFNγ leads to reduced intracellular bacterial survival as previously reported[9], but that administration after infection offers little benefit. This suggests that *Francisella* subverts IFNγ signaling upon infection.

Materials and Methods

Cells, antibodies and reagents

Raw 264.7 and THP-1 cells were obtained from ATCC and maintained in RPMI 1640 with 5% heat-inactivated fetal bovine serum (FBS). Recombinant IFNγ (mouse and human) and mouse IFNβ were purchased from R& D Systems (Minneapolis, MN). Antibodies specific for phospho-STAT1, phospho-JAK2, phospho-JAK1, were purchased from Cell Signaling Technology (Beverly, MA). Actin and SOCS3 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against STAT1 and iNOS were obtained from BD Biosciences (San Jose, CA). *F. novicida* U112 (JSG1819), an m*glA* mutant of *F. novicida* (JSG2250) and *F. tularensis* LVS were used in all experiments. The *iglD* mutant of *F. novicida* bacteria were

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a generous gift from Dr. Yousef Abu Kwaik (U. of Louisville, KY). Control siRNA and SOCS3-specific siRNA (On-Target*plus* SMARTpool) were obtained from Dharmacon (Lafayette, CO). *Francisella* strains were streaked and grown overnight on Chocolate II agar plates (Becton Dickinson and company, MD) at 37°C.

Isolation of peripheral blood monocytes

Peripheral blood monocytes (PBMs) were isolated as previously described[11]. Briefly, PBMCs were first isolated by density gradient centrifugation over Histopaque (Sigma-Aldrich, St Louis, MO) and monocytes were then purified from PBMCs by negative selection using MACS Monocyte Isolation kit (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. The purity of the monocytes was more than 97% as determined by flow cytometry using CD14 antibodies.

Cell stimulation, lysis, and Western blotting

Macrophages were infected with plate-grown (grown on Chocolate II agar plates for 16–18 h at 37°C) *F. novicida* as previously described[18] at an MOI of 10 unless described otherwise. Briefly, RAW 264.7 cells were plated overnight in 12-well or 6 well plates and allowed to adhere. *F. novicida* resuspended in RPMI medium containing 5% heat inactivated FBS was added to the adherent macrophages and then incubated at 37°C and 5% CO_2 for the indicated time points. In parallel, the viability of bacteria was tested by plating the inoculum on chocolate II agar plates and bacterial numbers in the inoculum were quantified using the Petroff-Hauser chamber. These data indicated that >98% of the bacteria in the inoculum were viable in all cases (wild type and mutants). During the infection in the experiments shown the cells were not washed at any point. However, similar responses were seen when cells were extensively washed 1 hour post infection, treated with gentamicin and allowed to incubate (data not shown). Where indicated, cells were stimulated with 25 ng/ml of IFNγ. In various experiments IFNγ was added at different points -either 8 hours prior to infection (pre), at the time of infection (co) or 8 hours post infection (post). These details are provided in the corresponding Figure legends. In some experiments, bacteria were killed by either heating the bacterial suspension at 98°C for 10 min or by treating the bacteria with 50 μg/ml of gentamicin for 60 min. In some experiments macrophages were treated with vehicle control (DMSO) or 5 μg/ml of cytochalasin D for 30 minutes before infection.

Uninfected and infected cells were lysed in TN1 buffer (50mM Tris pH 8.0, 10mM EDTA, 10mM $Na_4P_2O_7$, 10mM NaF, 1% Triton-X 100, 125mM NaCl, 10mM Na_3VO_4, 10μg/ml each aprotinin and leupeptin). Post-nuclear lysates were boiled in Laemmli Sample Buffer and were separated by SDS/PAGE, transferred to nitrocellulose filters, probed with the antibody of interest and developed by enhanced chemiluminescence.

Microarray analysis

PBMs were isolated from 4 separate donors and resuspended in RPMI 1640 with 5% heat-inactivated FBS at 5 million cells per ml. The monocytes were then infected with *F. novicida* at 100 MOI for 24 hours. Bone marrow-derived macrophages were isolated and differentiated from 3 C57Bl/6 mice as described previously[18]. Seven days after differentiation, macrophages from each mouse were plated into 2 wells of a 6-well dish at 2.5 million per well in RPMI containing 5% heat-inactivated FBS. *F. novicida* was added to each well at an MOI of 100. Cells were gently mixed and incubated at 37°C in 5% CO_2 for 24 h.

Twenty four hours post-infection, RNA was extracted from both PBMs and BMMs using TRIzol® Reagent (Invitrogen Life Technologies, Carlsbad, CA), column-purified using RNeasy columns (Qiagen, Valencia, CA) and hybridized to Affymetrix GeneChip® Human Genome 133 plus 2.0 and Mouse Genome 430 2.0 Arrays (Affymetrix, Santa Clara, CA).

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Expression values were calculated using the "gcrma" package in BioConductor (www.bioconductor.org) and the "limma" package (Smyth, 2004) was used to find genes significantly different ($p \leq 0.05$) between infected and uninfected samples.

siRNA transfection

RAW 264.7 cells were transfected with either control siRNA or SOCS3-specific siRNA using the Amaxa Nucleofector (Amaxa biosystems, Germany) as previously described[18]. Briefly, 7×10^6 cells were resuspended in 100 µl Nucelofector Solution V and were nucelofected with 10 µl of 100µM siRNA. Immediately post- nucleofection, 500 µl of pre-warmed RPMI was added to the transfection mix before transferring to 12-well plates containing 1.5 ml pre-warmed RPMI per well. Plates were incubated for 16 hours at 37°C before infections were performed.

ELISA measurement of cytokine production

Raw 264.7 cells were infected with *F. novicida* for varying time points. Cell supernatants were harvested, centrifuged to remove dead cells and analyzed by ELISA using an IP-10 specific kit from R & D Systems (Minneapolis, MN). Data were analyzed using an unpaired Student's *t-test*. A p value ≤ 0.05 was considered significant.

Intracellular survival assay

This assay was performed as previously reported with a few modifications[18]. Briefly, 90 min post-infection with 100 MOI of *F. novicida*, cells were washed twice with RPMI and further incubated for 22 h. The cell cultures were treated with 50 µg/ml of gentamicin for 60 min at 37°C and 5 % CO_2, washed twice with RPMI and subsequently lysed in 0.1 % SDS for 5 minutes. Immediately, 10 fold serial dilutions were made and appropriate dilutions were plated on Chocolate II agar plates. Assays were performed in triplicate for each test group. To maintain constant infection and IFNγ exposure periods in "pre-, co- and post-groups" the following protocol was followed. Briefly, within each group two parallel sets of macrophage cultures were infected for a constant infection period. One set of the samples in each group was exposed to 25 ng/ml of IFNγ for 24 hours to maintain constant IFNγ exposure across the groups. Subsequently, both sets in a group were processed to measure the intra-cellular number of the bacteria, and the bacterial count in IFNγ-treated samples was expressed as a percentage of bacterial number obtained in untreated samples of that group.

Measurement of nitrite by Griess Reagent

The levels of NO were measured by assaying the culture supernatants for NO_2^-, a stable product of NO with molecular oxygen. The assay was performed as previously described[19]. Macrophages were plated in RPMI containing no phenol red and no serum. Cells were then infected with 10 MOI of *F. novicida* for 24 hours and where indicated cells were simultaneously exposed to bacteria and 25 ng/ml of IFNγ. Harvested cell supernatants were centrifuged to clear any dead cells and the concentration of nitrite in the cell supernatants was estimated by Griess reaction. For nitrite assay, 100 µl of supernatant was mixed with 50 µl of Griess Reagent and incubated at room temperature in dark for 30 minutes. The absorbance was measured at 520 nm and nitrite concentrations were calculated from a standard curve obtained using standards containing increasing concentration of $NaNO_2$.

Results

Francisella suppresses IFNγ-induced STAT1 phosphorylation

IFNγ orchestrates diverse immune processes that are critical for efficient pathogen clearance. Recently Santic et al. demonstrated that phagosomes containing *Francisella* are able to fuse

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with lysosomes in IFNγ-activated macrophages[9]. Thus, we hypothesized that *Francisella* may hinder the IFNγ-mediated host response. To test this hypothesis, we examined tyrosine phosphorylation of the downstream STAT1 (pY STAT1). For this, RAW 264.7 cells were infected with *F. novicida* in the presence or absence of IFNγ. Unless indicated otherwise both bacteria and IFNγ were added at the same time in all the experiments. Protein-matched lysates were analyzed by Western blotting with antibodies specific for tyrosine phosphorylated STAT1 (Figure 1A, upper panel). IFNγ treatment of the RAW 264.7 cells resulted in a robust induction of STAT1 phosphorylation which was dampened by infection with *F. novicida* (lane 4). To ensure equal loading of protein in all lanes the same membranes were reprobed with actin antibody. STAT-1 protein expression is upregulated by IFNγ treatment. Interestingly, we also observed that *Francisella* partially prevented the IFNγ-mediated induction of STAT1 protein itself (Figure 1B). The decrease in the induction of IFNγ-mediated STAT1 protein during *Francisella* infection is likely a consequence of the suppressed activation of STAT1, which can feed back to up-regulate itself. Thus, hereafter we have assessed the tyrosine phosphorylation levels of STAT1 which reflect the activation status of IFNγ signaling cascade. The reduction in IFNγ-induced STAT1 phosphorylation when challenged with *Francisella* was also observed in primary bone marrow-derived macrophages (BMM; Figure 1C).

To examine whether the suppressive effect of *F. novicida* on IFNγ-induced pY STAT1 levels is dose-dependent, RAW 264.7 cells were infected with increasing MOI in the presence or absence of IFNγ. The results indicated that there is a decrease in IFNγ-induced STAT1 phosphorylation at all of the multiplicities of infection tested (Figure 1D). Further characterization revealed that this suppressive effect of *F. novicida* on IFNγ-induced STAT1 phosphorylation was seen as early as 1 hour (data not shown).

To examine whether *F. tularensis* LVS (Type B, vaccine strain) could likewise suppress IFNγ-induced STAT1 phosphorylation, similar infection experiments were performed with this latter strain. The results shown in Figure 1E demonstrate that *F. tularensis* LVS is also capable of suppressing IFNγ-induced STAT1 phosphorylation. A recent study reported that Type 1 IFN is produced and is important for the inflammasome activation during *Francisella* infection [20]. In addition to STAT1-STAT2 heterodimers, STAT1 homodimers are also formed during Type 1 interferon signaling, although to a lesser extent. So we have examined the influence of *Francisella* on Type 1 interferon signaling as well and observed that *Francisella* is capable of suppressing STAT1 phosphorylation induced by IFNβ (Figure 1F). Thus, it is possible that *Francisella* may attempt to interfere with the signaling induced by IFNs in general.

Phagosomal escape, replication and viability of *Francisella* are dispensable for its suppressive effect on IFNγ-induced STAT1 phosphorylation

We next examined the bacterial factors that may be responsible for *Francisella*-mediated down-regulation of IFNγ-induced STAT1 phosphorylation. MglA is a global transcriptional regulator of *Francisella* essential for the expression of many virulence genes. In addition, MglA is critical for the bacterial escape from the phagosome[4]. To test the possible involvement of any MglA-dependent protein/event in the *Francisella*-mediated suppression of IFNγ signaling, RAW 264.7 cells were infected with *F. novicida* or an *mglA* mutant of *F. novicida* (*FN mglA*), in the presence or absence of IFNγ. In parallel, we examined whether bacterial replication is required for the suppression of IFNγ-induced STAT1 phosphorylation. For this, we used an *iglD* mutant of *F. novicida*. IglD is an MglA-dependent protein that was recently reported to be critical for the replication of the bacteria within host cells[21]. Protein-matched lysates were resolved by SDS/PAGE and analyzed by Western blotting with pY STAT1 antibody (Figure 2A, upper panel). Results indicated that both *FN mglA* and *FN iglD* effectively suppressed IFNγ-induced STAT1 phosphorylation, indicating that the suppressive effect is not

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dependent on phagosomal escape nor intracellular replication of *Francisella*. Of note, we also tested the number of viable bacteria in the inoculum immediately after infection to confirm that a comparable MOI was achieved in these experiments.

To examine the possible involvement of an inducible bacterial factor in the down-regulation of IFNγ-induced STAT1 phosphorylation, macrophages were infected with live or killed bacteria in the presence or absence of IFNγ, and pY STAT1 levels were analyzed by Western blotting (Figure 2B). Bacteria were killed by either heat treatment (HKFN) or exposure to gentamicin (GKFN). Both live and dead bacteria effectively suppressed IFNγ-induced pY STAT1. These results suggest that a constitutively-expressed and heat-stable bacterial factor is involved in the suppression of IFNγ-induced STAT1 phosphorylation.

Francisella-induced SOCS3 expression dampens STAT1 phosphorylation

We next examined the molecular mechanism of *Francisella*-mediated suppression of IFNγ-induced STAT1 phosphorylation. Here, we considered two possibilities: a) that *Francisella* down-regulates positive regulators upstream of STAT1 phosphorylation, and/or b) that *Francisella* up-regulates negative regulators of STAT1 phosphorylation. To address whether positive regulators are down-regulated, Raw 264.7 cells were infected with *F. novicida* in the presence or absence of IFNγ and the expression levels of JAK1 and JAK2 were assessed by Western blot analysis. In parallel, phosphorylation (indicative of activation) of JAK1 and JAK2 were assessed by Western blotting with phospho-specific antibodies to JAK1 and JAK2. Results indicated that there is no suppressive effect on the expression nor activation of JAK1 and JAK2 during infection (data not shown).

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Since the tyrosine phosphorylation level of the JAKs was not suppressed by *F. novicida* infection, we did not examine the activation of SHP-1, the tyrosine phosphatase that dephosphorylates JAK kinases. However, we examined whether other negative regulators of IFNγ signaling, the SOCS proteins, were up-regulated during infection. SOCS1 and SOCS3 are established negative regulators of the IFNγ signaling pathway. Down-regulation of IFNγ-induced signaling by several pathogens is associated with the up-regulation of SOCS3 protein[17,22–24]. To this end, we began by examining microarray results from an experiment comparing infected and uninfected BMMs and analyzed the expression of various SOCS proteins. Results shown in Figure 3A indicate that during *F. novicida* infection SOCS3 but not SOCS1 mRNA was highly up-regulated in murine BMMs. The expression of SOCS3 was confirmed at the level of protein by Western blotting in RAW 264.7 cells (Figure 3B). Infection alone induced the expression of SOCS3 as early as 6h post-infection and the protein levels persisted even at 24h post-infection (Figures 3 and data not shown). In addition, co-stimulation of macrophages with *F. novicida* and IFNγ resulted in a further increase in the SOCS3 protein levels. Similar results were obtained with *F. tularensis* LVS (data not shown). Consistently, phagosomal escape, replication and viability of the bacteria, which were not necessary for the suppression of IFNγ-induced STAT1 phosphorylation, were also found to be dispensable for the *Francisella*-induced up-regulation of SOCS3 protein (data not shown).

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We next tested whether *Francisella*-induced SOCS3 may contribute to the suppression of IFNγ-induced STAT1 phosphorylation. For this, RAW 264.7 cells were transfected with either control or SOCS3-specific siRNA. Approximately 16 hours after transfection, cells were infected and the tyrosine phosphorylation of STAT1 was assessed by Western blotting (Figure 3C). When exposed to both IFNγ and *F. novicida*, SOCS3-specific siRNA transfectants displayed higher STAT1 phosphorylation levels than control transfectants. This suggests that SOCS3 may at least in part contribute to the *Francisella*-mediated suppression of IFNγ-induced STAT1 phosphorylation. In parallel, we also examined the levels of SOCS3 protein in the transfectants by Western blotting with SOCS3 antibody and found that cells transfected with

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SOCS3-specific siRNA contained less SOCS3 protein than the control transfectants (Figure 3D).

Francisella suppresses IFNγ-induced STAT1 phosphorylation in human monocytes

To test whether *Francisella* can also down-regulate IFNγ-mediated STAT1 phosphorylation in human cells, we infected human peripheral blood monocytes (PBM) or THP-1 (human monocytic cell line) with *F. novicida* in the presence or absence of IFNγ. In all these experiments cells were exposed to IFNγ before infection with *Francisella*. Similar to the results obtained with murine macrophages (RAW 264.7 and BMMs), we observed that *Francisella* infection suppressed IFNγ-induced STAT1 phosphorylation in both PBMs and THP-1 cells (Figure 4A and 4B). Similar results were obtained when THP-1 cells were infected with *F. tularensis* LVS (Figure 4B). Moreover, the reduction in the IFNγ-induced STAT1 phosphorylation was evident as early as 2h post-infection of THP-1 cells (data not shown). Also consistent with the results obtained in RAW 264.7 cells, *FN mglA* or *FN iglD* effectively suppressed IFNγ-induced STAT1 phosphorylation (Figure 4B). Finally, SOCS3 gene expression was also found to be up-regulated during *F. novicida* infection of PBMs (Figure 4C).

Francisella suppresses STAT1-dependent nitric oxide production

IFNγ treatment of macrophages is reported to induce the expression of iNOS leading to the production of NO, which is essential for killing pathogens[25]. In agreement with previous reports, we observed that co-stimulation of macrophages with IFNγ and *F. novicida* resulted in NO production[19,26] (Figure 5A). Likewise, co-stimulation of cells with IFNγ and *F. novicida* resulted in the up-regulation of iNOS (Figure 5B). The observation that *Francisella* down-regulates IFNγ-induced STAT1 phosphorylation but does not suppress STAT1-dependent iNOS induction appeared paradoxical. We reasoned that *Francisella* may require some time to establish a permissive environment and be able to counter IFNγ-induced iNOS expression. To test this notion, we compared the induction levels of iNOS under priming, co-stimulatory and post-infection conditions. For this, IFNγ was added to RAW 264.7 cells 8h prior to infection (priming), at the time of infection (co-stimulation) or 8h after infection (post-infection) and the expression of iNOS under these conditions was analyzed by Western blotting (Figure 6A, upper panel). Infection alone or treatment with IFNγ alone induced very low levels of iNOS expression. The induction of iNOS was highly enhanced when IFNγ-primed macrophages were infected with *F. novicida*. However, when IFNγ was administered at the time of infection (co-stimulation) the induction of iNOS was dramatically reduced, and almost completely abrogated if IFNγ was administered post infection. Likewise, compared to the IFNγ priming condition, co-stimulation of macrophages with IFNγ and *F. novicida* resulted in lower levels of STAT1 phosphorylation, which is further reduced when cells were exposed to IFNγ after infection (Figure 6A, middle panel). A similar trend was seen with IFNγ-induced IP-10, an IFNγ-dependent chemokine (Figure 6B). Collectively, these data indicate that *F. novicida* establishes a permissive environment in the host cell over time to counteract the host protective effects of IFNγ.

Establishment of *Francisella* infection in macrophages leads to resistance to IFNγ-induced bacterial death

Earlier studies demonstrated that prior exposure of infected cells to IFNγ drastically reduces the intra-macrophage survival of bacteria[9]. This is consistent with the induction of iNOS in our model. However, our data indicate that if IFNγ is administered either at the time of infection or after infection *Francisella* can suppress IFNγ-induced iNOS expression. Thus, to test if this reduction in iNOS levels correlates with an increase in bacterial survival, CFUs from infected cell lysates under various conditions of IFNγ exposure (priming, co-stimulatory and post-

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infection) were compared. The results are shown in Figure 6C. When IFNγ was added 8 hours prior to or at the time of infection, there was ~ 90% decrease in intracellular bacterial survival, compared to the survival in the absence of IFNγ administration. However, when IFNγ was added 8 hours after infection there was only a 30–40% reduction in intracellular bacterial survival. The reduced efficiency of IFNγ to induce bacterial death when added 8 hours post-infection correlates with the decrease in the levels of iNOS under these conditions as shown in Figure 6A.

Together, our results provide evidence that in both mouse and human cells *Francisella* suppresses IFNγ-induced STAT1 phosphorylation and this *Francisella*-mediated interference of IFNγ signaling is associated with an induction of SOCS3 (Figure 7). The results also show that SOCS3, at least in part, contributes to the *Francisella*-mediated suppression of IFNγ-induced STAT1 phosphorylation. Further, *Francisella*-mediated suppression of IFNγ-induced STAT1 phosphorylation results in a lower induction of STAT1-dependent proteins such as iNOS, leading to enhanced intra-macrophage survival of the bacteria.

Discussion

Phagocytic cells such as monocytes and macrophages ingest and subsequently destroy pathogens through the phago-lysosomal pathway and the production of inflammatory mediators such as cytokines. These microbicidal activities are greatly enhanced by exposure of the cells to IFNγ. Such IFNγ-activated macrophages are often referred to as classically activated or Type I activated macrophages. Highly successful pathogens have developed various strategies to subvert microbicidal responses of the host cell and, instead, create a favorable intracellular environment to suit their needs. Our knowledge regarding the immune evasion mechanisms utilized by *Francisella* remains limited. These mechanisms have been reported to include evasion of phago-lysosomal fusion[2,3], dampening of the inflammatory response upon escape into the cytosol[27,28] and abrogation of T cell responses[29]. One strategy employed by pathogens such as *Leishmania donovani*[13], *Mycobacterium avium*[14], *Mycobacterium tuberculosis*[15,16], *and Listeria monocytogenes*[17] is to interfere with the IFNγ-mediated activation of macrophages. In this process, pathogens such as *Leishmania donovani* inhibit IFNγ-induced STAT1 activation[13] whereas *Mycobacterium tuberculosis* does not influence STAT1 activation but exerts a gene-selective inhibition of IFNγ-induced transcriptional responses by disrupting its interaction with transcriptional co-activators[15]. Results from the present study provide evidence that *Francisella* suppresses the IFNγ-mediated signaling response and gene expression which results in decreased microbicidal activity of the host cell.

The inhibitory effect of *Francisella* on IFNγ-mediated signaling does not require the bacteria to be metabolically active. Our finding that killed *Francisella* also downregulates IFNγ signaling is consistent with the findings obtained with other pathogens such as *L. donovani*[24] and *Mycobacterium bovis*[23]. Together, these data provide evidence that a heat-stable bacterial component is responsible for the down-regulation of IFNγ-induced STAT1 tyrosine phosphorylation. This observation is in accord with the results obtained in other studies which demonstrate that cells treated with heat-stable bacterial components such as LPS[30] or Mycobacterial TDM[23] efficiently suppressed IFNγ-induced STAT1 phosphorylation. In addition, we have observed that prevention of bacterial uptake by treating the cells with cytochalasin D did not reverse the *Francisella*-mediated inhibition of IFNγ signaling, suggesting that bacterial contact itself is sufficient to cause this effect (data not shown). Perhaps the ligation of a host cell receptor by a surface bacterial component such as LPS may contribute to the interference of IFNγ signaling. For example the inhibition of IFNγ-mediated responses by *Mycobacterium tuberculosis*[31–33] and *Mycobacterium avium*[32] is at least partly dependent upon TLR2 engagement. TLR2 is one of the major macrophage receptors involved in the

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recognition of *Francisella*[34–36]. This tempts us to speculate that TLR2 may play a critical role in *Francisella*-mediated interference of IFNγ signaling as well.

In our murine model, *Francisella* infection did not reduce the IFNγ-induced phosphorylation of JAK tyrosine kinases, which is in contrast with findings obtained with other intracellular pathogens[13,23]. Instead, we found that *Francisella*-mediated suppression of IFNγ signaling is associated with the up-regulation of SOCS3 and that knock-down of SOCS3 partially restored the IFNγ-induced STAT1 phosphorylation during *Francisella* infection. The interference of IFNγ signaling by several pathogens has been correlated with expression of either SOCS1 or SOCS3 or both. For example, *Salmonella enterica* serovar Typhi induces both SOCS1 and SOCS3 expression while *Burkholderia pseudomallei* induces only SOCS3 expression[22]. SOCS proteins are suppressors of cytokine signaling that form a key part of the negative feedback of cytokine signal transduction. Our microarray data obtained with infected murine BMMs and human PBMs indicate that SOCS3 but not SOCS1 is highly up-regulated during *Francisella* infection. SOCS1 can directly interact with JAK1 and JAK2 and inhibit their phosphorylation and kinase activity [37]. Therefore, the unchanged levels of IFNγ-induced JAK phosphorylation during *Francisella* infection correlate with the lack of induction of SOCS1 in our experiments.

The mechanism of SOCS3 action is not clear. One study showed that although SOCS3 associated with the JAK kinases, overexpression of SOCS3 did not inhibit the *in vitro* kinase activity of JAK1 or JAK2[38]. Other studies showed that SOCS3 binds to phosphorylated receptors including the leptin receptor[39] and IL-2 receptor β chain[40]. When bound to the activated receptors, SOCS3 may inhibit JAK2 activation. However, high levels of activated growth hormone receptor were found to be essential for SOCS3-mediated inhibition of growth hormone induced JAK2 activation[41].

Our data demonstrate that akin to the suppression of IFNγ-induced STAT1 phosphorylation, a heat-stable bacterial factor(s), but not live bacteria, is essential for the induction of SOCS3. These results are in agreement with results from other studies showing purified LPS[30] of other bacteria or mycobacterial TDM[23] could activate SOCS3 expression. Ongoing experiments are aimed at understanding the molecular mechanism(s) of *Francisella*-induced SOCS3 expression. One formal possibility is that the induction of SOCS3 is mediated by feedback signaling initiated by cytokines secreted by infected cells. Since cytokine induction during *Francisella* infection is dependent on host cell contact with the bacteria via TLR2 but independent of bacterial uptake or viability, this is consistent with the induction of SOCS3 expression.

The observation that iNOS induction is suppressed during *Francisella* infection is consistent with previous findings that over-expression of SOCS3 results in the suppression of the mouse iNOS gene promoter[30]. Interestingly, we observed that *Francisella* requires some time (in the absence of IFNγ exposure) for the establishment of maximal inhibition of host-protective events. Such a requirement was also observed in the case of *Burkholderia pseudomallei* infection[22]. This suggests that *Francisella* regulates the host cell response to protective inflammatory mediators over time, the net result of which is increased intracellular survival. Thus, there continues to be a dynamic interplay between the pathogen and host cell during the course of infection.

Data obtained from various mouse models strongly support the protective role of IFNγ during tularemia[42,43]. Leiby et al. have shown that intra-peritoneal injections of IFNγ neutralizing antibody at the time of infection resulted in the death of mice even at a very low dose of infection[44]. On the other hand, administration of the IFNγ neutralizing antibody two days post infection did not significantly influence the progress of the disease, suggesting that the

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protective effect of IFNγ is most effective at early time points after infection. This is consistent with our infection model where IFNγ is ineffective at protecting host cells if administered after the infection has occurred. Such strict time dependence for IFNγ therapy is also seen with *Listeria monocytogenes,* a pathogen previously reported to suppress the IFNγ response[45]. Further, Conlan et al observed that neutralizing antibodies against IFNγ did not alter the progress of primary murine tularemia initiated through intra-nasal administration of *Francisella*[46]. Thus, based on our studies it may be speculated that alveolar macrophages may be more effective in suppressing IFNγ responses and may thus contribute to the rapid lethality observed during the pneumonic form of tularemia.

As described above, in mouse models of tularemia, IFNγ is produced but mice still succumb. This could be partly explained by the ability of *Francisella* to induce SOCS3 and thereby potentially circumvent the IFNγ-mediated survival benefit. To our knowledge, this is the first study to demonstrate *Francisella*-mediated suppression of IFNγ signaling and provide evidence for the functional consequence of this signaling interference.

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Abbreviations used in this paper

IFNγ
: Interferon gamma

STAT1
: signal transducer and activator of transcription 1

SOCS
: suppressor of cytokine signaling

iNOS
: inducible nitric oxide synthase

BMM
: bone marrow-derived macrophages

PBM
: peripheral blood monocytes

MOI
: multiplicity of infection

HKFN
: heat killed *Francisella novicida*

GKFN
: gentamicin killed *Francisella novicida*

CFU
: colony forming unit

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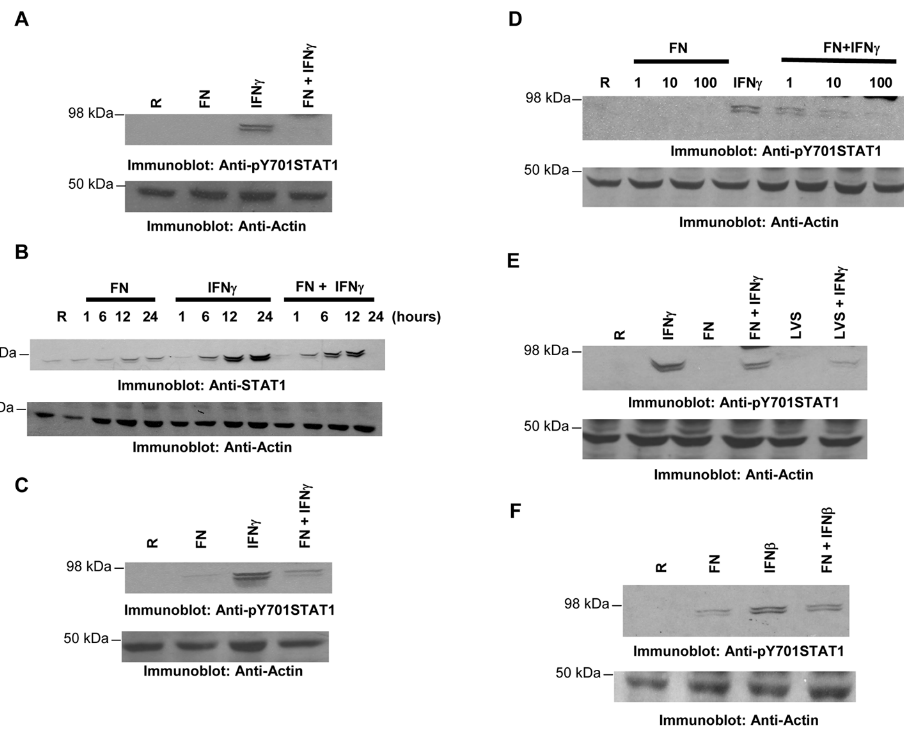

Figure 1. *Francisella* suppresses IFNγ-induced STAT1 phosphorylation

A. RAW 264.7 were infected with 10 MOI of *F. novicida* (FN) in the presence or absence of IFNγ (25 ng/ml) for 24 h. Protein-matched lysates were resolved by SDS PAGE and analyzed by Western blotting with phosphotyrosine STAT1 (pY STAT1) antibody (upper panel). The same membranes were re-probed with actin antibody (lower panel). The results are representative of six independent experiments. **B.** RAW 264.7 cells were infected with 10 MOI of *F. novicida* (FN) in the presence or absence of IFNγ (25 ng/ml) for the indicated time points. Protein-matched lysates were resolved by SDS PAGE and analyzed by Western blotting with STAT1 antibody (upper panel) and later with actin antibody (lower panel). These results are representative of three similar experiments. **C.** BMMs were infected with 10 MOI of *F. novicida* (FN) in the presence or absence of IFNγ (25 ng/ml) for 24 h and protein-matched lysates were resolved by SDS PAGE and analyzed by Western blotting with pY STAT1 antibody (upper panel). The same membrane was re-probed with actin antibody (lower panel). These results are representative of two independent experiments. **D.** Raw 264.7 cells were infected with 1, 10 or 100 MOI of FN for 24 hours in the presence or absence of IFNγ and the levels of pY STAT1 were analyzed by Western blotting (upper panel). The lower panel is a reprobe of the same membrane with actin antibody. These results are representative of two independent experiments. **E**. Raw 264.7 were infected with 10 MOI of *F. novicida* (FN) or *F. tularensis* LVS in the presence or absence of IFNγ (25 ng/ml) for 24 h. Protein-matched lysates were analyzed by Western blotting with pY STAT1 antibody (upper panel). The same membranes were reprobed with actin antibody (lower panel). These results are representative of three independent experiments. **F.** RAW 264.7 cells were infected in the presence or absence of IFNβ (500 IU/ml). 24 hours after the infection, the phosphorylation of STAT1 was assessed by Western blotting. Results are representative of three independent experiments. In all experiments described above, bacteria and IFNγ were added to the macrophages at the same time (co-stimulation). R, resting.

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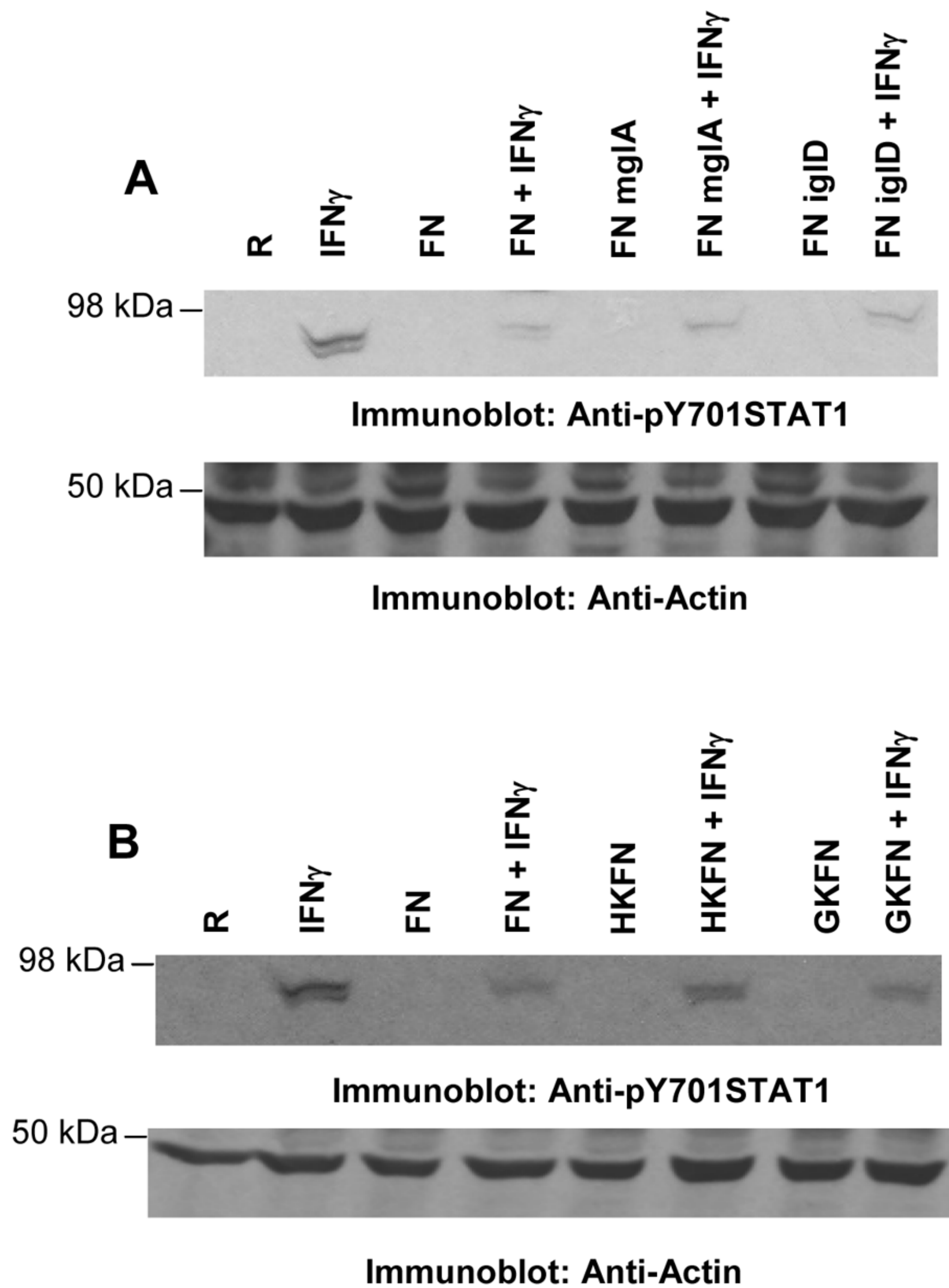

Figure 2. Phagosomal escape, replication and viability of *Francisella* are dispensable for the inhibition of IFNγ signaling

A & B RAW 264.7 cells were infected with wild type (FN), *FN mglA* or *FN iglD* **(A)** and with live (FN), heat-killed (HKFN) or gentamicin-killed (GKFN) bacteria **(B)** in the presence or absence of IFNγ for 24 h. Protein-matched lysates were analyzed by immunoblotting with pY STAT1 antibody (upper panels). The same membranes were reprobed with actin antibody to ensure equal loading (lower panels). In all experiments described above bacteria and IFNγ were added to the macrophages at the same time (co-stimulation). R, resting. These results are representative of four independent experiments.

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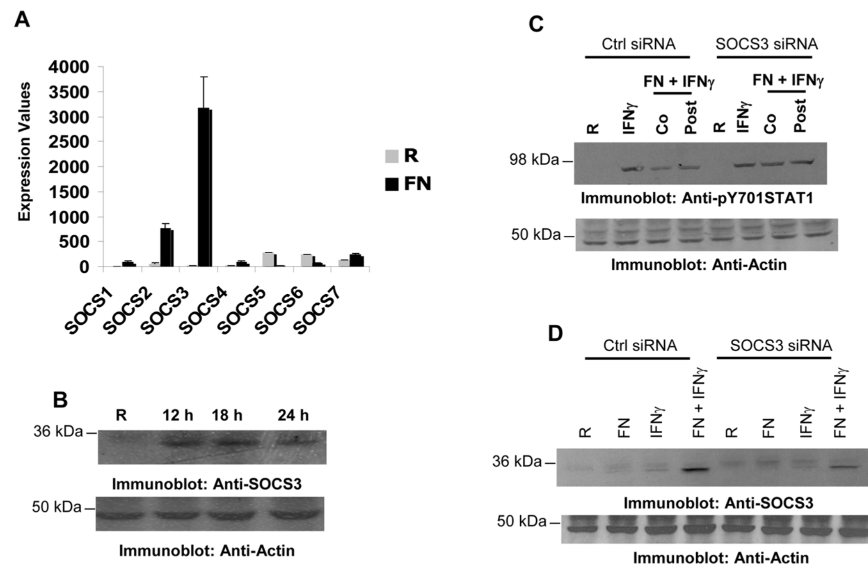

Figure 3. *Francisella* induces the expression of SOCS3

A. Bone marrow-derived macrophages (BMM) were infected with 100 MOI of *F. novicida* for 24 h. RNA was extracted, purified and microarray analysis was performed. Expression values for SOCS mRNA from three independent infections are shown. **B.** RAW 264.7 cells were infected with 10 MOI of *F. novicida* and the expression of SOCS3 protein was analyzed by Western blotting (upper panel). The same membrane was re-probed with actin antibody to ensure equal loading (lower panel). **C.** RAW 264.7 cells were nucleofected with either control or SOCS3-specific siRNA. 24 hours after transfection, cells were infected with *F. novicida* in the presence or absence of IFNγ (25 ng/ml) for 16 hours and the tyrosine phosphorylation of STAT1 was assessed by Western blotting. Co, IFNγ was added to the transfectants at the time of infection; Post, IFNγ was added 6 hours after infection; R, resting. These results are representative of three independent experiments. **D.** RAW 264.7 cells were transfected with control or SOCS3-specific siRNA and 16 hours after transfection, cells were infected with *F. novicida* in the presence or absence of IFNγ (25ng/ml) for 16 hours and expression of SOCS3 was analyzed by Western blotting (upper panel). The same membrane was reprobed with actin antibody to ensure equal loading. (lower panel). These results are representative of 4 similar and independent experiments.

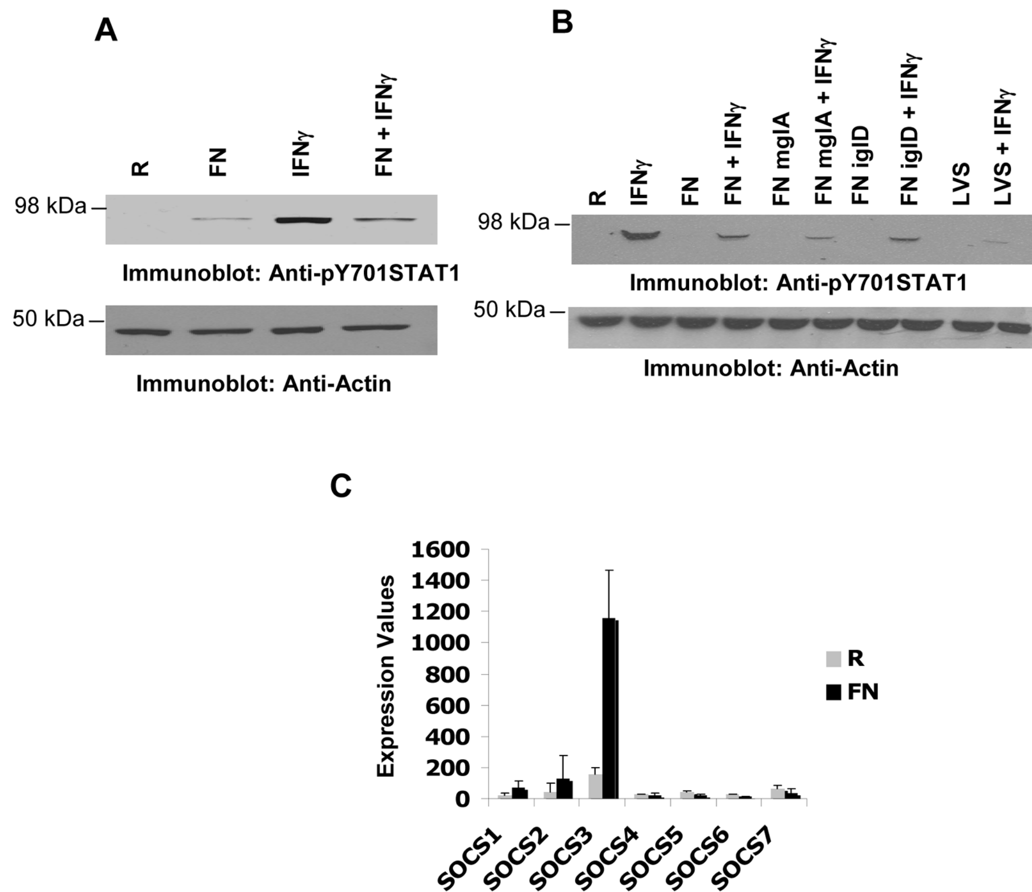

Figure 4. *Francisella* suppresses IFNγ-induced STAT1 phosphorylation in human cells

A. PBMs were pre-treated with IFNγ for 8 hours and then infected with 10 MOI of *F. novicida* in the presence or absence of IFNγ for 24 h. Protein-matched lysates were analyzed by Western blotting with pY STAT1 antibody (upper panel). The same membranes were re-probed with actin antibody (lower panel). **B.** THP-1 cells were pre-treated with IFNγ for 8 hours and then infected with 10 MOI of wild type (FN), *FN mglA*, *FN iglD* or *F. tularensis* LVS bacteria in the presence or absence of IFNγ. Tyrosine phosphorylation of STAT1 was assessed by Western blotting (upper panel). The same membranes were reprobed with actin antibody to ensure equal loading (lower panel). These data are representative of 3 independent experimenta. **C.** PBMs were infected with 100 MOI of *F. novicida* for 24 h. RNA was extracted, purified and microarray analysis was performed. Expression values for SOCS mRNA from four monocyte donors are shown. R, resting.

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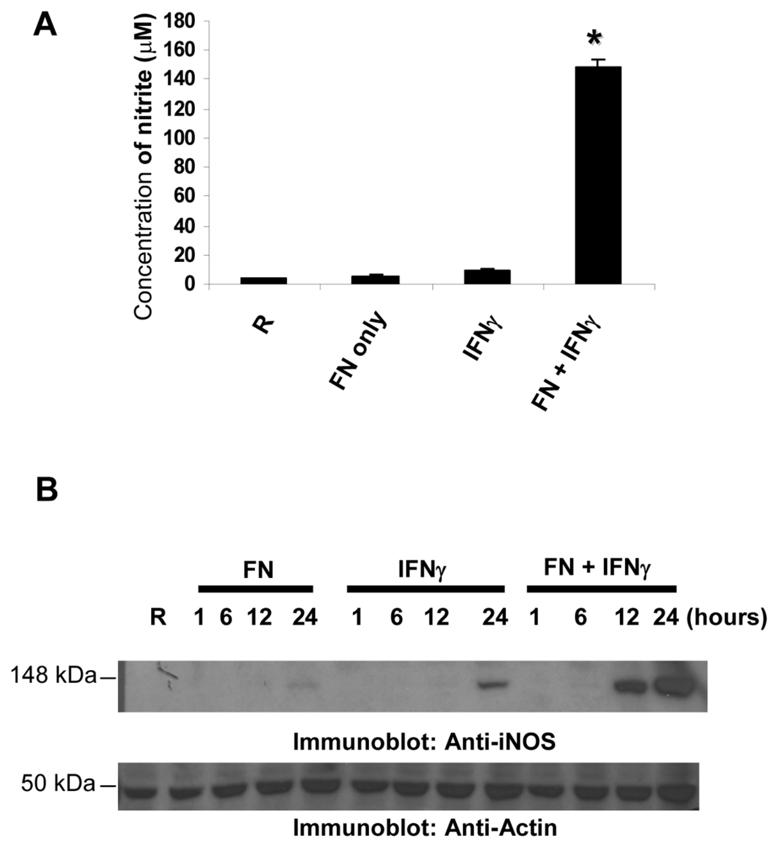

Figure 5. Co-stimulation of macrophages with *Francisella* and IFNγ induces iNOS/nitric oxide
A. RAW 264.7 cells were infected with 10 MOI of *F. novicida* in the presence or absence of IFNγ (25 ng/ml; co-stimulation) for 24 h and the amount of NO in the cell supernatants was measured by the Griess reagent. The graphs show mean and SD of values obtained from 3 independent experiments. * $p<0.05$ compared with IFNγ value. **B.** RAW 264.7 cells were infected in the presence or absence of IFNγ for the indicated time points (co-stimulation) and protein-matched cell lysates were analyzed for the expression of iNOS by Western blotting. R, resting. These results are representative of three independent experiments.

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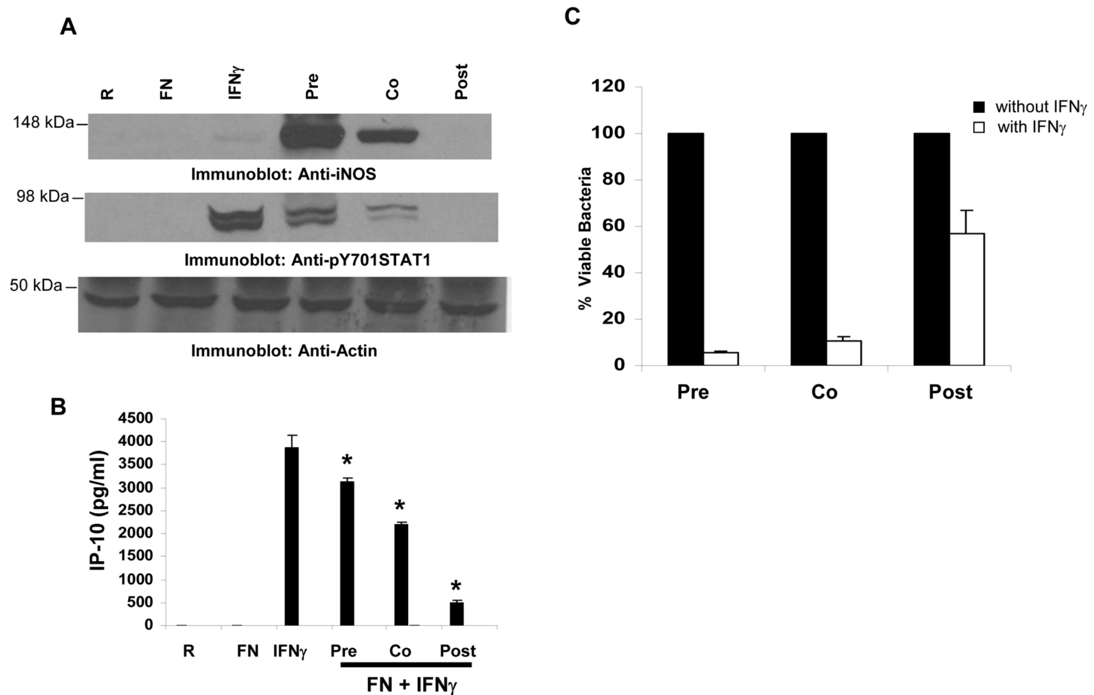

Figure 6. *Francisella* suppresses IFNγ-induced iNOS leading to enhanced bacterial survival

A. RAW 264.7 cells were exposed to IFNγ 8 h prior to infection (pre), at the time of infection (co) or 8 h post-infection (post) for a constant IFNγ-exposure period of 24 hours and the cellular levels of iNOS and pY STAT1 were analyzed by Western blotting. The same membranes were re-probed with actin antibody. These data are representative of three independent experiments. **B.** RAW 264.7 cells were treated as described in **A** and the amount of IP-10 produced was measured by ELISA. The graph shows mean and SD of values obtained from triplicate samples. These data are representative of 3 independent experiments. * $p<0.05$ compared with the IFNγ value. R, resting. **C.** RAW 264.7 cells were treated as described in the Materials and Methods section to maintain a constant infection period within each of the three groups (pre, co and post) and a constant IFNγ exposure period across the three groups. CFUs in samples that were not treated with IFNγ were set as 100%. The CFUs obtained from the samples treated with IFNγ were expressed as a percent of the corresponding non-IFNγ treated sample. The graph shows the mean and SD of values obtained from 3 independent experiments.

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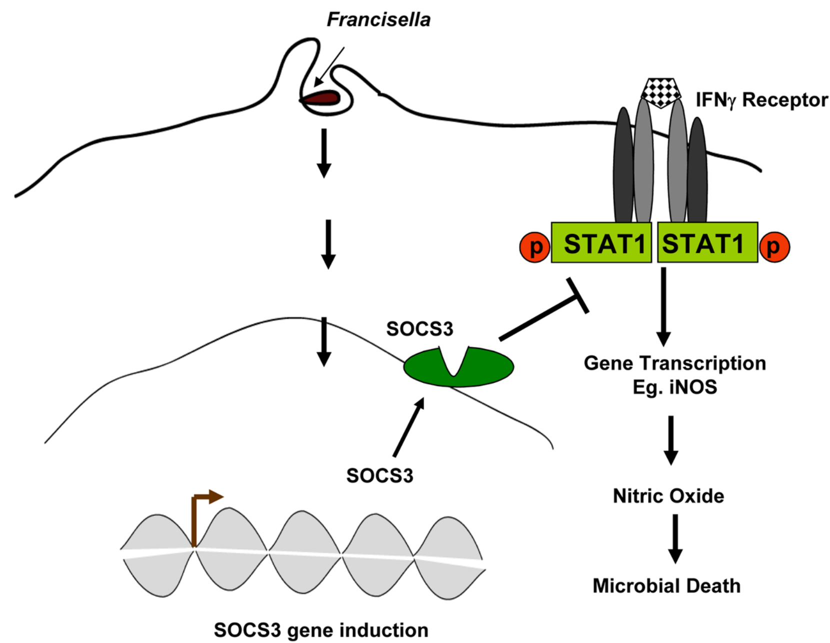

Figure 7. Proposed model of *Francisella*-mediated interference of IFNγ signaling response
Francisella infection up-regulates SOCS3 expression, which suppresses STAT1 phosphorylation potentially by binding to the IFNγ receptor and dampening subsequent recruitment and activation of STAT1. Ultimately, *Francisella*-mediated suppression of the IFNγ response leads to the inhibition of IFNγ-induced iNOS and other anti-microbial events, resulting in enhanced bacterial survival in host cells.