Restricted cytosolic growth of *Francisella tularensis* subsp. *tularensis* by IFN-γ activation of macrophages

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INTRODUCTION

Macrophages are an essential component of innate host defences against microbial pathogens, and can recognize, phagocytose and destroy invading organisms through a repertoire of antimicrobial effectors. These include a degradative endosomal system that culminates in the delivery of degradative hydrolases to the maturing phagosome, the phagocyte NADPH oxidase Phox, an inducible nitric oxide synthase (iNOS) and cationic antimicrobial peptides, all of which act in conjunction to kill phagocytosed microbes (Radtke & O’Riordan, 2006). While many of these effectors are constitutively functional in macrophages, innate immune signals such as cytokines can further induce their expression and/or enhance their activities, therefore increasing the microbicidal potential of macrophages.

*Francisella tularensis* is a highly virulent, Gram-negative, facultative intracellular bacterium that causes tularemia in a wide variety of mammals, including humans (Oyston et al., 2004). *F. tularensis* subsp. *tularensis* (type A) and *F. tularensis* subsp. *holarctica* (type B) are the prominent cause of human tularemia, while *F. tularensis* subsp. *novicida* is considered non-pathogenic to humans (Ellis et al., 2002). Essential to *Francisella* pathogenesis is its ability to invade, survive and proliferate within mam-
malian cells, among which macrophages are considered to be an important target of this pathogen in the early stages of respiratory tularemia (Hall et al., 2008). Various murine models of Francisella infection using an attenuated live vaccine strain (LVS) of the subspecies holarctica have clearly established a key role for interferon-γ (IFN-γ) in controlling primary infections (Elkins et al., 1996; Leiby et al., 1992), therefore suggesting that IFN-γ priming of macrophages controls Francisella intracellular proliferation. Consistently, early in vitro studies using murine peritoneal exudate cells (PEC) have demonstrated that IFN-γ activation induces growth of LVS (Anthony et al., 1992; Fortier et al., 1992) in an iNOS-dependent manner (Anthony et al., 1992), while a comparable, yet iNOS-independent, inhibition of LVS growth was observed in murine alveolar macrophages (Polsinelli et al., 1994). More recently, Lindgren et al. (2005) have also documented a role for Phox and iNOS in growth inhibition of LVS in murine PEC. However, iNOS, but not Phox, was involved in killing of the highly virulent type A strain Schu S4 by IFN-γ activation in murine PEC (Lindgren et al., 2007), a difference attributed to the greater resistance of virulent strains to reactive oxygen (ROS) and nitrogen (RNS) species (Lindgren et al., 2007).

In quiescent murine and human macrophages, Francisella ensures its effective survival and proliferation via a rapid phagosomal escape followed by replication in the cytosol (Checroun et al., 2006; Clemens et al., 2004; Golovliov et al., 2003; Santic et al., 2005a), thereby circumventing the degradative endosomal system and its associated bactericidal activities. Phagosomal escape is a tightly regulated process and its efficiency depends upon conditions encountered within the early phagosome prior to disruption (Chong et al., 2008; Santic et al., 2008). An attractive hypothesis to explain the ability of IFN-γ to induce killing of intracellular Francisella is that this cytokine affects specific stages of the bacterium intracellular cycle to prevent otherwise efficient escape mechanisms from bactericidal activities. Indeed, IFN-γ activation of murine PEC has been proposed to affect phagosomal escape of the holarctica strain LVS (Lindgren et al., 2004), but does not in the murine macrophage-like cell line J774A.1 (Bonquist et al., 2008), where IFN-γ activation restricts intracellular growth, yet does not seem to affect the association of bacteria with endosomal membranes. By contrast, Santic et al. (2005a) have shown in human monocyte-derived macrophages (MDMs) that a strain of the human-avirulent subspecies novicida is unable to escape from its original phagosome upon IFN-γ activation and is killed by fusion with lysosomes.

Altogether, previous studies on the mode of action of IFN-γ towards intracellular growth of Francisella have led to discrepant conclusions. These can be attributed to the variety of infection models used, where potential functional differences in both the source of macrophages and the Francisella subspecies studied have generated ambiguous data on the mechanisms of control by IFN-γ and the effectors involved. In particular, only one study has addressed the effect of IFN-γ on virulent F. tularensis subsp. tularensis, indicating differences between virulent and attenuated strains (Lindgren et al., 2007). Additionally, several early studies have investigated the effect of IFN-γ on Francisella growth in both murine and human macrophages over several days (Anthony et al., 1992; Fortier et al., 1992; Polsinelli et al., 1994; Santic et al., 2005a). Such a time frame covers more than one intracellular cycle (Checroun et al., 2006; Wehrly et al., 2009), likely including macrophage death and reinfection events, and therefore cannot address how IFN-γ activation truly affects a single infection cycle.

Here we have performed the first, to our knowledge, comparative study of the effect of IFN-γ activation upon the intracellular survival and trafficking of the type A strain Schu S4 in both murine and human primary macrophages, to clarify the mode of action of IFN-γ activation on the intracellular cycle of virulent Francisella. We demonstrate that IFN-γ activation does not affect the kinetics or efficiency of Schu S4 phagosomal escape but instead restricts its cytosolic replication independently of known bactericidal mechanisms.

**METHODS**

**Bacterial strains and culture conditions.** The prototypic type A virulent strain F. tularensis subsp. tularensis Schu S4 was obtained from Anders Sjöstedt (Umeå University, Umeå, Sweden). Schu S4 expressing GFP was engineered as described previously (Chong et al., 2008). Bacteria were grown on modified Mueller–Hinton (MMH) agar plates [Mueller–Hinton medium supplemented with 0.1% glucose, 0.025% ferric pyrophosphate and 2% IsoVitalex (Becton Dickinson)], supplemented with 10 μg kanamycin ml⁻¹ for GFP-expressing Schu S4, for 3 days at 37 °C under 7% CO₂. Under these growth conditions, Schu S4 exhibits high virulence in mice upon either intranasal or intradermal inoculation (Wehrly et al., 2009). GFP expression did not affect Schu S4 intracellular behaviour. All manipulations of F. tularensis strain Schu S4 were performed in a Biosafety Level 3 facility according to standard operating procedures approved by the Rocky Mountain Laboratories Institutional Biosafety Committee.

**Macrophage culture and infection.** To generate murine bone marrow-derived macrophages (muBMMs), bone marrow cells were isolated from femurs of either 6–10-week-old C57BL/6 J female mice (Jackson Laboratories) or gg91kflox/⁻/NOS2⁻/⁻ knockout mice [a gift from Dr Carl Nathan, Weill Medical College of Cornell University; (Shiloh et al., 1999)], differentiated into macrophages as previously (Chong et al., 2008), and replated in 6-, 24- or 96-well cell culture-treated plates at a density of 1 x 10⁶ (6-well plates) or 1 x 10⁷ (24- or 96-well plates) macrophages per well. Human blood-derived macrophages (MDMs) were generated from peripheral blood monocytes subjected to apheresis and enriched by density centrifugation using Ficoll-Paque (GE Healthcare) and by negative selection using a Dynabeads Untouched Human Monocytes kit (Invitrogen) according to the manufacturer’s instructions. Mononuclear cells were seeded at a cell density of either 2 x 10⁶ ml⁻¹ (24-well plate) or 8 x 10⁶ ml⁻¹ (6-well plate) in RPMI medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, non-essential amino acids (NEAA; Invitrogen) and 50 ng ml⁻¹ recombinant...
human macrophage colony stimulating factor (M-CSF; PeproTech). Medium was replenished on day 3 and day 6 of culture, and MDMs were used for infections on day 7. Human blood cells were collected from anonymous volunteers under a protocol reviewed and approved by the NIH Institutional Biosafety Committee. Where indicated, the macrophage medium was supplemented with 50 U ml⁻¹ recombinant murine or human IFN-γ (Peprotech) 24 h prior to infection and maintained throughout the infection. Higher concentrations of IFN-γ did not enhance growth restriction of Francisella (data not shown). When required, N-acetyl-L-cysteine (NAC; Sigma-Aldrich) or N3-[mimino-(methylamino)methyl]-l-ornithine (NMMLA; Cayman Chemical) was added to the macrophage culture medium 1 h prior to infection at 10 or 1 mM, respectively, and maintained throughout the infection. Under these conditions, no significant cytotoxicity was observed.

Immediately prior to infection of macrophages, a few colonies from a freshly streaked plate were resuspended in MMH broth and OD₆₀₀ was measured to estimate bacterial numbers. Bacteria were diluted to the appropriate m.o.i. in either muBMM or MDM medium, and 0.5 ml (24-well plates) or 1.5 ml (6-well plates), respectively, was added to chilled macrophages. Macrophage infections were then performed in the absence of fresh serum as described previously (Chong et al., 2008) at an applied m.o.i. of 25–100, depending on the analysis performed. Under these conditions, 30–50% of macrophages were infected with one to two bacteria at the onset of infection.

Determination of bacterial intracellular growth. Intracellular growth of Schu S4 was monitored by determining the number of c.f.u. recovered from lysed macrophages, as described previously (Chong et al., 2008). The number of viable intracellular bacteria per well was determined in triplicate for each time point.

Immunofluorescence microscopy. Macrophages grown on 12 mm glass coverslips in 24-well plates were infected at an m.o.i. of 25 and processed for immunofluorescence labelling as described previously (Chong et al., 2008). Primary antibodies used were mouse anti-F. tularensis LPS (US Biological), rat anti-mouse LAMP-1 [clone 1D4B, developed by J. T. August and obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the National Institute of Child Health and Human Development (NICHD) and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA, USA], rabbit anti-human LAMP-1 (Novus Biologicals), goat anti-mouse EEA-1 N-19 (Santa Cruz Biotechnology) and mouse anti-human EEA-1 (BD Transduction Laboratories). Secondary antibodies were Alexa Fluor 488–donkey anti-mouse (Invitrogen) and mouse anti-human EEA-1 (BD Transduction Laboratories) or mouse anti-human LAMP-1 (Novus Biologicals) labelled in experiments involving colocalization with EEA-1 in MDMs. To quantify Francisella escape from its initial phagosome, phagosomal integrity assays were performed as described previously (Checroun et al., 2006) with minor modifications (Chong et al., 2008). Briefly, live infected cells were first permeabilized with digitonin to allow for cytosolic delivery of Alexa Fluor 488-conjugated anti-Francisella antibodies, which labelled bacteria located in the cytosol or within a compromised phagosome. Cells were then fixed and all bacteria were labelled after saponin permeabilization using Alexa Fluor 568-conjugated anti-Francisella antibodies, yielding samples with differentially labelled cytosolic and vacuolar bacteria (Checroun et al., 2006; Chong et al., 2008). Samples were observed on a Carl Zeiss Axio Imager epifluorescence microscope equipped with a Plan- APOCHROMAT x 63/1.4 objective for quantitative analysis, or Carl Zeiss LSM 510 or LSM 710 confocal laser scanning microscopes for image acquisition. Confocal images of 1024 x 1024 pixels were acquired and assembled using Adobe Photoshop CS3.

Transmission electron microscopy (TEM). Macrophages on 12 mm Aclar coverslips were infected at an m.o.i. of 100 and processed as described previously (Chong et al., 2008). Sections were viewed in a Hitachi H7500 transmission electron microscope at 80 kV. Images were acquired with a Hamamatsu 2K x 2K bottom mount AMT digital camera (Advanced Microscopy Techniques) and assembled in Adobe Photoshop CS3.

Flow cytometry. Flow cytometry was performed by infecting either muBMMs or MDMs cultured in 6-well plates with GFP-expressing Schu S4, as described above, at an m.o.i. of 50. At each time point, cells were washed five or six times with PBS in the absence of cations. PBS was replaced with 750 μl TrypLE Express (Invitrogen) and incubated at 37 °C and 7 % CO₂ for 15 min. Cells were then collected by gentle scraping followed by centrifugation at 200 g for 4 min at 4 °C. Microscopic observation of wells after cell detachment confirmed that most cells were harvested. The cell pellet was resuspended in PBS and distributed to a 96-well plate at 50 μl per well for immunostaining. An antibody panel composed of phycoerythrin (PE)-Cy7-conjugated anti-mouse CD11b and PE-conjugated anti-MHC class II, diluted 1:200 in FACS buffer (eBioscience), was used to identify IFN-γ activated muBMMs. Compensation controls using biotin-conjugated CD11b followed by either FITC-, PE-Cy7- or PE-conjugated streptavidin were performed in conjunction with each experiment. PE-Cy7-conjugated mouse anti-human CD11b/Mac-1 antibodies (BD Biosciences) were used to identify MDMs. Antibodies were incubated for 30 min at 4 °C. Cells were collected by centrifugation at 520 g and 4 °C for 1 min, and fixed in 1 % paraformaldehyde in PBS. After a 30 min incubation at 4 °C, cells were collected and resuspended in FACS buffer before analysis by flow cytometry in a Biosafety Level 3 facility using a CyFlow ML flow cytometer controlled by the Flowmax software (Partec). At least 10,000 cells were analysed in each sample. Flow cytometry data were analysed and plotted using FlowJo software (v7.2.5) (Tree Star). Cells were first gated on side (SSC) and forward (FSC) light scatter, then on either CD11b⁺/MHC class II⁺ or CD11b⁺/MHC class II⁺ populations for analysis of muBMM infections, or on CD11b⁺ cells for analysis of MDM infections. Typically, more than 98 % of IFN-γ-activated muBMMs expressed high levels of MHC class II molecules (data not shown). The minimal GFP fluorescence levels detectable by flow cytometry were estimated from fluorescence microscopy analysis to require at least five intracellular bacteria per cell.

Measurements of ROS, nitric oxide and tumour necrosis factor (TNF-α) production. Superoxide production by infected muBMMs was measured using a luminol-based chemiluminescence method, as described by Dahlgren & Karlsson (1999). MuBMMs in 96-well plates were infected at an m.o.i. of 25. At different time points, the macrophage medium was replaced with 100 μl Krebs-Ringer (KRG) buffer (120 mM NaCl, 5 mM KCl, 1.7 mM KH₂PO₄, 8.3 mM Na₂HPO₄, 10 mM glucose, 1 mM CaCl₂, and 1.5 mM MgCl₂, pH 7.3) containing 100 μM luminol (Sigma) and 12 U ml⁻¹ horseradish peroxidase (HRP; Sigma), and chemiluminescence was immediately measured at 25 °C over 60 min at 1 min intervals using an Infinite M1000 plate reader (Tecan US). Where required, phosphol-12-myristate-13-acetate (PMA; Sigma) in KRG buffer was added at a final concentration of 1 μM immediately before measurement. Nitrite production was measured in cell culture supernatants from Schu S4-infected muBMMs at various time points using a Griess Reagent System kit according to the manufacturer’s instructions (Promega). Secretion of TNF-α was measured in cell culture supernatants from Schu S4-infected muBMMs at various time points using a mouse TNF-α/TNFFSF1A kit according to the manufacturer’s instructions (R&D Systems). In these assays, uninfected muBMMs treated with 1 μg ml⁻¹ of Ultrapure Escherichia coli LPS (InvivoGen) for either 20 or 24 h were used as positive controls. All assays were run in triplicate.
RESULTS

Impaired growth of Schu S4 in IFN-γ-activated macrophages

To evaluate the effect of IFN-γ treatment on intracellular Francisella, we first monitored bacterial viability over an infection cycle in either untreated or IFN-γ-pretreated muBMMs or MDMs, by enumerating c.f.u. recovered from lysed infected cells. In control muBMMs, intracellular numbers of Schu S4 increased by nearly three orders of magnitude from 4 to 18 h post-infection (pi) after an initial 4 h lag phase (Fig. 1a), indicating efficient intracellular proliferation. In IFN-γ-pretreated muBMMs, numbers of intracellular viable bacteria were similar to those obtained in control muBMMs up to 8 h pi, then stalled and decreased afterwards (Fig. 1a), suggesting a defect in intracellular proliferation upon IFN-γ treatment. Consistent with a control of intracellular growth by IFN-γ, the number of intracellular bacteria in individual

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**Fig. 1.** IFN-γ impairs intracellular growth of Schu S4 in murine and human macrophages. muBMMs or MDMs were left untreated or were treated with 50 U recombinant IFN-γ ml⁻¹ 24 h prior to infection and infected with either Schu S4 (a, c) or GFP-expressing Schu S4 (b, d). Infected macrophages were then processed for either c.f.u. enumeration at 1, 4, 8, 12 and 18 h pi (muBMMs) or 1, 4, 10 and 16 h pi (MDMs) (a, c) or immunolabelling of LAMP-1 at 1 and 10 (MDMs) or 12 h pi (muBMMs). (a) Representative growth curves of Schu S4 in control and IFN-γ-activated muBMMs. Values are means ± SD of triplicate samples and are representative of three independent experiments. (b) Representative confocal micrographs of control and IFN-γ-activated muBMMs infected with GFP-Schu S4 at 1 and 12 h pi. Bacteria appear in green; LAMP-1 staining appears in red. Insets are magnifications of the boxed regions in the main images. Bars, 10 or 2 μm (insets). (c) Representative growth curves of Schu S4 in control and IFN-γ-activated MDMs. Values are means ± SD of triplicate samples and are representative of three independent experiments. (d) Representative confocal micrographs of control and IFN-γ-activated MDMs infected with GFP-Schu S4 at 1 and 10 h pi. Bacteria appear in green; LAMP-1 staining appears in red. Insets are magnifications of the boxed regions in the main images.
muBMMs increased more dramatically in untreated than in IFN-γ-treated cells between 1 and 12 h pi (Fig. 1b), where limited bacterial replication was observed in the latter case. Hence, IFN-γ treatment of muBMMs prior to infection restricts Schu S4 proliferation, but only at late stages of its intracellular cycle.

In MDMs, Schu S4 displayed exponential growth similar to that in muBMMs, as judged by the increase in intracellular c.f.u. recovered from infected cells over a 16 h time-course (Fig. 1c) and the increase in intracellular numbers of bacteria in individual cells between 1 and 10 h pi (Fig. 1d). In contrast, progressively decreasing numbers of intracellular c.f.u. were recovered from IFN-γ-activated MDMs (Fig. 1c), suggesting that IFN-γ activation of MDMs efficiently prevents proliferation and induces killing of Schu S4. Despite this net decrease in viable intracellular bacteria, some limited bacterial replication in a subset of infected MDMs was evident at 10 h pi by fluorescence microscopy of GFP-Schu S4 (Fig. 1d). Similar replication patterns were observed with Schu S4 (data not shown).

Hence, despite an overall decrease in c.f.u., bacteria underwent limited growth in a subset of IFN-γ-activated MDMs. Taken together, these results demonstrate a control of Schu S4 intracellular proliferation in human macrophages by IFN-γ that is the net result of both bacterial clearance and restricted multiplication.

**IFN-γ activation of macrophages escape**

*Francisella* phagosomal escape is a prerequisite for intracellular proliferation, since bacterial mutants that are affected in this process do not reach the cytosol or multiply in it (Bonquist *et al.*, 2008; Lindgren *et al.*, 2004; Santic *et al.*, 2005b), and delaying phagosomal escape delays the onset of bacterial growth (Chong *et al.*, 2008). While LVS apparently escapes from its phagosome in IFN-γ-activated J774A.1 macrophage-like cells (Bonquist *et al.*, 2008), control of *F. novicida* growth in IFN-γ-activated MDMs has been solely attributed to its inability to disrupt its original phagosome and avoid subsequent killing through fusion with lysosomes (Santic *et al.*, 2005b). Because of these discrepant results, we sought to determine whether the limited proliferation of Schu S4 in either muBMMs or MDMs could be accounted for by IFN-γ-induced defects in phagosomal escape. Since phagosomal escape of *Francisella* is in part determined by maturation of the early phagosome (Chong *et al.*, 2008; Santic *et al.*, 2008), we first examined the maturation events of Schu S4-containing phagosomes rapidly after phagocytosis by either untreated or IFN-γ-treated muBMMs or MDMs. With similar kinetics in both untreated and IFN-γ-treated muBMMs, bacterial phagosomes rapidly acquired then excluded the early endosomal marker EEA-1 (Fig. 2a), as previously shown (Chong *et al.*, 2008). Coincident with EEA-1 exclusion, LAMP-1 was progressively acquired, with a peak at 20 min pi in both untreated and IFN-γ-treated cells, before the percentages of LAMP-1-positive phagosomes decreased to less than 20 % by 60 min pi (Fig. 2a), suggesting phagosomal disruption. Similar to muBMMs, sequential kinetics of EEA-1 and LAMP-1 acquisitions and exclusions were observed in both untreated and IFN-γ-treated MDMs (Fig. 2a, b). Yet, phagosomal maturation events were slower and less extensive in MDMs than in muBMMs, since the peak of EEA-1 acquisitions (a maximum of 35.3 ± 13.3 % of positive phagosomes) occurred at 10 min pi and that of LAMP-1 acquisition never exceeded 42.5 ± 9.5 % of positive phagosomes before loss of colocalization (Fig. 2b). Altogether, these results indicate that although the maturation kinetics of Schu S4-containing phagosomes differ slightly between muBMMs

![Image](http://mic.sgmjournals.org)
and MDMs, IFN-γ activation of these cells prior to infection does not significantly affect these events.

Using a phagosomal integrity assay (Chong et al., 2008) to quantify phagosomal disruption and electron microscopy to analyse ultrastructures around intracellular Francisella, we found that the majority of bacteria had disrupted their phagosomal membrane by 1 h pi in both muBMMs (Fig. 3a, b) and MDMs (Fig. 3c, d), regardless of IFN-γ activation, and obvious membrane degradation was detected as early as 30 min pi under all conditions (Fig. 3b, d). By 8 h pi, all bacteria were found in the cytosol of

![Figure 3](https://www.microbiologyresearch.org/fig3.jpg)

**Fig. 3.** IFN-γ does not affect phagosomal escape of Schu S4 in macrophages. muBMMs or MDMs were left untreated or were treated with 50 U recombinant IFN-γ ml⁻¹ 24 h prior to infection and infected with Schu S4 for 0.5, 1, 2, 4 or 8 h and processed for phagosomal integrity assay (a, c) or TEM (b, d), as described in Methods. (a) Percentages of cytoplasmic Schu S4 in control and IFN-γ-treated muBMMs. Values are means ± sd from three independent experiments, in which 100 bacteria were analysed per condition in each experiment. (b) Representative TEM micrographs of either control (upper panels) or IFN-γ-treated (lower panels) muBMMs infected with Schu S4 for 30 min, 2 h or 8 h. Arrows indicate areas of phagosomal membrane degradation. Bar, 0.5 μm. (c) Percentages of cytoplasmic Schu S4 in control and IFN-γ-treated MDMs. Values are means ± sd from three independent experiments, in which 100 bacteria were analysed per condition in each experiment. (d) Representative TEM micrographs of either control (upper panels) or IFN-γ-treated (lower panels) MDMs infected with Schu S4 for 30 min, 2 h or 8 h. Arrows indicate areas of phagosomal membrane degradation. Bar, 0.5 μm.
both control and IFN-γ-activated macrophages (Fig. 3b, d). Overall, no difference in the nature and kinetics of phagosomal escape could be detected, which clearly demonstrates that IFN-γ activation does not affect the kinetics of phagosomal escape of Schu S4 in either murine or human macrophages.

**IFN-γ activation of macrophages restricts cytosolic growth of Francisella**

Since IFN-γ did not affect the ability of Schu S4 to disrupt its early phagosome and reach the cytosol, we postulated that its restrictive effect on bacterial proliferation acts upon the stage of cytosolic replication. To test this hypothesis, we analysed the extent of Schu S4 growth at times pi when all bacteria are cytosolic in muBMMs by flow cytometry and fluorescence microscopy of GFP-expressing bacteria. In control muBMMs, an increasing percentage of CD11b+ cells displayed detectable GFP fluorescence between 4 and 16 h pi (Fig. 4a), clearly revealing infected cells from the onset of bacterial replication in the cytosol (Wehrly et al., 2009). The increasing percentage of GFP+ cells observed between 4 and 6 h pi (Fig. 4a) was not due to additional infection of cells, but to bacterial replication in an increasing number of originally infected cells yielding fluorescence above the detection threshold (more than five bacteria per cell; Fig. 4d, e). At 12 and 16 h pi, a distinct and prominent population of infected cells with high GFP fluorescence was detected (gated quadrant 2; Fig. 4a), indicating extensive bacterial replication at these stages. This was confirmed by fluorescence microscopy, where such infected cells contained a high number of cytosolic bacteria (Fig. 1b) and were present in significant numbers from 12 h pi and onwards (cells containing more than 30 bacteria; Fig. 4d). Gating the GFP+ population for high fluorescence levels (Fig. 4a, c) revealed that the infected CD11b+ cells progressively evolved from a GFPlow to a GFPhigh phenotype (Fig. 4a–c), indicating extensive bacterial replication. In contrast, IFN-γ-activated muBMMs, a smaller population of GFP+CD11b+ cells (a maximum of 32 ± 17% at 6 h pi; Fig. 4b) was detected from 4 h pi and onwards, which did not evolve into a GFPhighCD11b+ population (Figs 1b and 4c), demonstrating a lack of extensive cytosolic replication. Microscopy analysis of individual macrophages confirmed that a very low percentage of IFN-γ-activated muBMMs were permissive for extensive bacterial replication (a maximum of 11.5 ± 13.1% of infected cells harboured >30 bacteria; Fig. 4e), and an increased percentage of cells with limited replication at 12 and 16 h pi, compared with untreated muBMMs (Fig. 4d), was observed. Furthermore, this GFPlow population decreased with time (Fig. 4a, b), suggesting either bacterial clearance or a decrease in intracellular numbers of bacteria below the GFP detection threshold by flow cytometry. Taken together, these analyses demonstrate that IFN-γ activation in muBMMs restricts bacterial proliferation in the cytosol.

To verify that restriction of cytosolic growth by IFN-γ also occurred in human macrophages, MDMs were infected with GFP-expressing Schu S4 and subjected to similar analyses. Although the percentages of GFP+CD11b+ cells were lower in MDMs than in muBMMs (compare Figs 4a and 5a), a clear population of infected cells with replicating bacteria was detected in untreated macrophages, but was severely restricted in IFN-γ-activated cells (Fig. 5a, b). Consistently, a very limited number of infected cells (4.6 ± 3.7% at 12 h pi) exhibited extensive replication in IFN-γ-activated cells, compared with control macrophages (29.3 ± 8.9% at 12 h pi; Fig. 5b). Similar to muBMMs, a detailed analysis of individual infected cells by fluorescence microscopy revealed an IFN-γ-induced shift of the infected population towards limited (6–30 bacteria per cell) replication (Fig. 5c, d), corroborating the flow cytometry data. Hence, IFN-γ-activated MDMs also restrict cytosolic proliferation of Schu S4, indicating that this cytokine exerts similar restrictive effects in both murine and human macrophages.

**Roles of IFN-γ-induced effector mechanisms in restriction of cytosolic replication**

IFN-γ-induced autophagy is an established mechanism of cytosolic pathogen killing, and delivers them to the degradative lysosomal compartment. We therefore examined whether intracellular Schu S4 reached the endosomal compartment concomitant with IFN-γ-dependent growth restriction in both muBMMs and MDMs. Except at 20 h pi in untreated muBMMs, where formation of late autophagic FCVs occurred (Checroun et al., 2006) (Supplementary Fig. S1a, b), bacteria did not show any significant colocalization with the late endosomal membrane marker LAMP-1 after reaching the cytosol in both untreated and IFN-γ-treated macrophages (Fig. 3a, c and Supplementary Fig. S1). This indicates that they were not delivered to the endosomal compartment at stages where growth restriction occurred. Additionally, the occurrence of FCVs was dramatically reduced in IFN-γ-treated muBMMs (Supplementary Fig. S1a and b), and FCVs did not form in either untreated or IFN-γ-treated MDMs (results not shown), ruling out any temporal or functional correlation between FCVs and Francisella growth restriction. Hence, autophagy is not involved in Francisella growth control by IFN-γ.

Previous reports have invoked ROS and RNS as effectors of intracellular control of Francisella growth in IFN-γ-activated murine macrophages, with RNS playing a prominent role in the control of Schu S4 growth in murine PECs (Lindgren et al., 2005). However, LVS killing by IFN-γ-activated murine alveolar macrophages does not depend upon iNOS (Polzinelli et al., 1994). To examine whether these antimicrobial products contribute to the limited growth of Schu S4 observed in IFN-γ-treated cells, we first examined whether infected muBMMs produce ROS and nitric oxide (NO). Uninfected cells treated with
Fig. 4. IFN-γ restricts cytosolic proliferation of Schu S4 in muBMMs. muBMMs were left untreated or treated with 50 U recombinant IFN-γ ml⁻¹ 24 h prior to infection and infected with GFP-Schu S4 for 0, 4, 6, 12 and 16 h and processed for CD11b staining and flow cytometry (a–c) or fluorescence microscopy (d, e), as described in Methods. (a) Representative flow cytometry plots of infected muBMMs from three independent experiments. Uninfected muBMMs were used as negative controls for GFP fluorescence. Percentages in either black or red gated quadrants indicate the fraction of CD11b⁺GFP⁺ (gated quadrant 1) cells and CD11b⁺GFP⁺high (gated quadrant 2) cells, respectively, revealing infected muBMMs with limited and extensive replication. Cells in gated quadrants 2 are a subset of cells in gated quadrants 1. (b) Flow cytometry-based quantification of Schu S4 infection in either control or IFN-γ-activated muBMMs. Values are means ± SD from three or four independent experiments and correspond to percentages from gated quadrants 1. GFP⁺ cells refer to infected muBMMs that contained more than five bacteria. (c) Flow cytometry-based quantification of Schu S4 extensive replication in either control or IFN-γ-activated muBMMs. Values are means ± SD from three independent experiments. Asterisks indicate statistically significant differences between control and IFN-γ-treated samples, as determined by unpaired Student’s t test (P < 0.05). (d, e) Percentages of either control (d) or IFN-γ-activated (e) infected muBMMs containing 1–5 (white bars), 6–30 (grey bars) or >30 (black bars) bacteria, as scored by fluorescence microscopy at 1, 4, 6, 12 and 16 h pi. At least 250 cells were analysed per condition in each experiment. Values are means ± SD from three independent experiments.
purified *E. coli* LPS for 24 h produced superoxide in response to phorbol 12-myristate 13-acetate (PMA) stimulation, with higher levels detectable for IFN-γ-activated cells (Fig. 6a). ROS production was dependent upon the NADPH oxidase, since muBMMs from *gp91^Phox^−/− NOS2^−/−* knockout mice failed to produce any ROS (Supplementary Fig. S2). Compared with uninfected, LPS-treated cells (Fig. 6a), no significant levels of ROS in Schu S4-infected muBMMs were detectable at 1, 8 and 20 h pi, whether cells were activated or not, even after PMA stimulation (Fig. 6b–d). Only IFN-γ-activated cells showed a very low response to PMA stimulation, which progressively decreased with the infection time (Fig. 6b–d). Hence, infected cells did not produce ROS and were inhibited in their capacity to produce ROS upon stimulation (Fig. 6a), as previously described in human neutrophils (McCaffrey & Allen, 2006). Similarly, no significant amounts of NO were produced by either untreated or IFN-γ-treated muBMMs at either 1, 8 or 20 h pi, compared with the iNOS-dependent, LPS-induced NO production in IFN-γ-activated cells (Fig. 6e).

Additionally, we examined proinflammatory responses of infected macrophages through secretion of TNF-α, and could not detect any significant secretion of this cytokine by Schu S4-infected muBMMs (Fig. 6f), consistent with recent findings in human dendritic cells (Chase et al., 2009). Hence, Schu S4 infection of muBMMs inhibits production of ROS, and fails to stimulate either NO production or secretion of the proinflammatory cytokine TNF-α in both quiescent and IFN-γ-activated muBMMs.
We then monitored bacterial growth in either untreated or IFN-γ-treated muBMMs derived from gp91Phox−/−NOS2−/− knockout mice (Shiloh et al., 1999), which are deficient in both NADPH oxidase and iNOS. The intracellular behaviour of Schu S4 in either untreated or IFN-γ-treated knockout muBMMs was similar to that observed in wild-type mouse
macrophages (Fig. 6g and Fig. 1a), with a significant growth in untreated muBMMs that was restricted after 8 h pi in IFN-γ-treated cells (Fig. 6g). Hence, ROS and RNS do not significantly contribute to the control of Schu S4 growth in IFN-γ-activated muBMMs. Supplementation of IFN-γ-activated muBMMs with either excess L-tryptophan (Supplementary Fig. S2) or ferric ammonium citrate (FAC; data not shown) did not restore normal growth of Schu S4, also excluding a significant role for the tryptophan-depleting enzyme indoleamine 2,3-dioxygenase (IDO) or iron sequestration in Francisella growth restriction by IFN-γ.

To extend our findings to human cells, we also examined the roles of ROS and RNS in IFN-γ-mediated restriction of Schu S4 cytosolic growth in MDMs. For this purpose, we monitored Schu S4 intracellular growth in either control or IFN-γ-activated MDMs that were additionally left untreated or treated with NAC and NMMLA, which respectively scavenge ROS and inhibit RNS production. Used individually or in combination, neither of these inhibitors affected the numbers of intracellular Schu S4 in either control or IFN-γ-activated MDMs (Fig. 6h, i).

Hence, neither ROS nor RNS can be invoked as an effector mechanism of Francisella cytosolic growth restriction in MDMs.

DISCUSSION

The virulence of F. tularensis relies on its ability to proliferate within phagocytes, a pathogenic trait that has been long known to be affected by IFN-γ (Anthony et al., 1992; Fortier et al., 1992), consistent with the established role of this cytokine in controlling primary infections (Elkins et al., 1996; Leiby et al., 1992). Yet, how IFN-γ activation allows macrophages to control Francisella proliferation has remained ambiguous and poorly documented for highly virulent strains. Here we have performed for the first time, to our knowledge, an extensive analysis of the intracellular cycle of the prototypical, highly virulent strain Schu S4 in either IFN-γ-activated primary murine or human macrophages, to characterize the effects that this cytokine exerts on intracellular bacteria. We showed that IFN-γ controls Schu S4 intracellular proliferation in both murine and human macrophages by restricting cytosolic replication but without affecting phagosomal escape. Our results are in line with the recent finding that LVS phagosomal escape in IFN-γ-activated J774A.1 cells is not affected (Bonquist et al., 2008) and extend these by demonstrating restriction of cytosolic growth of a highly virulent strain in primary macrophages of both murine and human origin. Although the concept of Francisella intracellular growth inhibition by IFN-γ has been proposed in the past as a default interpretation of c.f.u. numbers recovered from macrophages over several intracellular cycles (Anthony et al., 1992; Polsinelli et al., 1994; Santic et al., 2005a), our data clearly demonstrate growth restriction through a detailed analysis of a single infection cycle using an array of complementary techniques.

A caveat of intracellular c.f.u. enumeration is that it can be influenced by loss of infected cells due to cytotoxicity during the infection cycle, a phenomenon previously reported with F. novicida (Mariathasan et al., 2005). While we observed a loss of infected cells in both murine and human macrophages at the late stages of the Francisella infection cycle (results not shown), it did not significantly differ between untreated and IFN-γ-activated populations. Although one cannot exclude that the loss of infected cells could account for part of the decreased recovery of viable bacteria from IFN-γ-activated macrophages, our analysis of similar populations of infected macrophages by flow cytometry circumvented this problem and clearly demonstrated a restriction of bacterial replication within IFN-γ-activated cells, therefore confirming the restrictive effect of IFN-γ upon cytosolic Francisella at the cellular level.

Our results disagree with those of Santic et al. (2005a), who concluded that inhibition of F. novicida growth in IFN-γ-activated MDMs results from an impairment of phagosomal escape and eventual fusion of Francisella phagosomes with lysosomes. Under our experimental conditions, IFN-γ pretreatment of macrophages did not result in any phagosomal escape defects, as determined by fluorescence and electron microscopy. Since our data were similar in both murine and human macrophages, it is unlikely that the results of Santic and co-workers can be explained by their use of primary human cells. Instead, the use in human cells of a subspecies of low virulence in humans may have generated conclusions that are not applicable to the behaviour of virulent Francisella strains. Some earlier studies on the effect of IFN-γ on Francisella intracellular growth were also carried out with the attenuated LVS strain. In the only comparative study reported, Lindgren et al. (2007) have shown that LVS killing in IFN-γ-activated PEC involves ROS and RNS, while Schu S4 killing is RNS-dependent alone. Under our experimental conditions, cytosolic control of Schu S4 multiplication by IFN-γ did not require the activities of either Phox or iNOS, since normal intracellular growth of Schu S4 was not restored either in murine macrophages lacking Phox and iNOS activities or in human macrophages treated with the ROS scavenger NAC and the iNOS inhibitor NMMLA. Accordingly, Schu S4-infected muBMMs did not produce any detectable ROS or NO concomitant with cytosolic growth restriction. In murine PEC, inhibition of iNOS by NMMLA prevented Schu S4 killing (Lindgren et al., 2007), yet did not significantly restore intracellular growth. Although apparently contradictory, our results and those of Lindgren and co-workers may either simply reflect different experimental conditions or highlight functional differences between cell models. Indeed, PEC are isolated following an inflammatory elicitation process that may determine a different phenotype from that of in vitro-derived muBMMs. Consistently, inhibition of LVS growth by IFN-γ requires different effector mechanisms in PEC and alveolar macrophages (Anthony et al., 1992; Polsinelli et al., 1994), suggesting that tissue residency and harvesting
of macrophages phenotypically affect their response to IFN-γ when used ex vivo. Interestingly, under our experimental conditions, mubMMs and MDMs displayed an IFN-γ-induced response comparable with that of mouse alveolar macrophages (Polsinelli et al., 1994), which are target cells of Francisella during pulmonary infection (Bosio & Dow, 2005; Hall et al., 2008). This potentially validates these in vitro models in the context of pulmonary tularemia, in which IFN-γ-producing cells may be recruited at the site of infection during Francisella intracellular proliferation.

Although we have shown in this study that IFN-γ restricts cytosolic growth of Schu S4, the actual effector mechanisms remain elusive. Neither ROS nor RNS are involved in growth restriction of Schu S4, consistent with the inhibition of their production in infected mubMMs and the ability of this strain to resist such reactive species in vitro (Lindgren et al., 2007). Additionally, supplementation of the macrophage medium with either excess l-tryptophan or FAC did not restore cytosolic growth, indicating that growth restriction was not mediated by either IDO-mediated tryptophan deprivation or iron sequestration, which are both known IFN-γ-inducible mechanisms that restrict growth of intracellular parasites (Beatty et al., 1994; Paradkar et al., 2008; Schaible & Kaufmann, 2004). Autophagy is involved in IFN-γ-induced killing of several intracellular pathogens, by delivering them to the endocytic degradative pathway (Levine & Deretic, 2007). Although Schu S4 effectively escaped this intracellular compartment in IFN-γ-activated macrophages, there remained the possibility of delivery of cytosolic Francisella to the lysosomal compartment via autophagy, as reported in quiescent murine macrophages at late stages of the intracellular cycle (Checroun et al., 2006). However, endosomal membranes were not detected in association with individual bacteria during the restricted cytosolic growth in IFN-γ-activated cells (Supplementary Fig. S1), ruling out the autophagic pathway as a major effector mechanism of Francisella growth control. Additionally, the occurrence of post-replication FCVs (Checroun et al., 2006) was dramatically reduced in IFN-γ-activated macrophages. A likely explanation for this result is that FCV formation requires extensive cytosolic growth of Francisella (Checroun et al., 2006), to levels that are not attained in IFN-γ-activated macrophages due to the growth restriction effect of this cytokine. Another bactericidal mechanism induced by IFN-γ is the production of antimicrobial peptides (AMPs) (Zasloff, 2002). While most AMPs are either secreted or delivered to phagosomes, ubiquicidin has been isolated in the cytosol of IFN-γ-activated macrophages (Hiemstra et al., 1999), suggesting a cytosolic role for AMPs. It therefore remains possible that some cytosolic AMPs, including ubiquicidin, play an important role in controlling cytosolic growth of Francisella, a hypothesis that deserves further investigation.

In conclusion, we have characterized the cytosolic stage of the intracellular cycle of Francisella tularensis as the point of control of bacterial proliferation by IFN-γ activation. Although IFN-γ has long been known to control proliferation of many vacuolar and cytosolic pathogens, the actual mechanism of restriction has rarely been identified for cytosolic parasites. While IFN-γ activation prevents phagosomal escape of Listeria monocytogenes (Portnoy et al., 1989), how this cytokine mediates growth control of either Shigella flexneri or Ricetttsia prowazekii (Turco & Winkler, 1983; Way et al., 1998) is unclear. Our findings therefore make Francisella–macrophage infections an attractive pathogen–host cell interaction model to study cytosolic antimicrobial mechanisms induced by IFN-γ.

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Control of Francisella intracellular growth by IFN-γ


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