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

Jessica A. Edwards,^{1†‡} Dedee Rockx-Brouwer,^{1†} Vinod Nair² and Jean Celli¹

Correspondence

Jean Celli

jcelli@niaid.nih.gov

¹Tularemia Pathogenesis Section, Laboratory of Intracellular Parasites, Research Technologies Branch, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Hamilton, MT 59840, USA

²Electron Microscopy Unit, Research Technologies Branch, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Hamilton, MT 59840, USA

The intracellular bacterium *Francisella tularensis* ensures its survival and proliferation within phagocytes of the infected host through phagosomal escape and cytosolic replication, to cause the disease tularemia. The cytokine interferon- γ (IFN- γ) is important in controlling primary infections *in vivo*, and *in vitro* intracellular proliferation of *Francisella* in macrophages, but its actual effects on the intracellular cycle of the bacterium are ambiguous. Here, we have performed an extensive analysis of the intracellular fate of the virulent *F. tularensis* subsp. *tularensis* strain Schu S4 in primary IFN- γ -activated murine and human macrophages to understand how this cytokine controls *Francisella* proliferation. In both murine bone marrow-derived macrophages (muBMMs) and human blood monocyte-derived macrophages (MDMs), IFN- γ controlled bacterial proliferation. Schu S4 growth inhibition was not due to a defect in phagosomal escape, since bacteria disrupted their phagosomes with indistinguishable kinetics in both muBMMs and MDMs, regardless of their activation state. Rather, IFN- γ activation restricted cytosolic replication of Schu S4 in a manner independent of reactive oxygen or nitrogen species. Hence, IFN- γ induces phagocyte NADPH oxidase Phox- and inducible nitric oxide synthase (iNOS)-independent cytosolic effector mechanisms that restrict growth of virulent *Francisella* in macrophages.

Received 16 June 2009

Revised 16 November 2009

Accepted 17 November 2009

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-

†These authors contributed equally to this work.

‡Present address: Department of Microbiology, Ohio State University, Columbus, OH 43210, USA.

Abbreviations: FAC, ferric ammonium citrate; IFN- γ , interferon- γ ; iNOS, inducible nitric oxide synthase; LVS, live vaccine strain; MDM, human blood monocyte-derived macrophage; muBMM, murine bone marrow-derived macrophage; NAC, *N*-acetyl-L-cysteine; NMMLA, *N*5-[imino(methylamino)methyl]-L-ornithine; PEC, peritoneal exudate cells; pi, post-infection; RNS, reactive nitrogen species; ROS, reactive oxygen species; TEM, transmission electron microscopy; TNF- α , tumour necrosis factor α .

Three supplementary figures, showing reassociation of intracellular Schu S4 with endosomal membranes following phagosomal escape, ROS production in *gp91^{Phox}*^{-/-} *NOS2*^{-/-} knockout muBMMs, and the effect of excess L-tryptophan on IFN- γ -induced growth restriction of Schu S4 in muBMMs, are available with the online version of this paper.

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 inhibits 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 μ g 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 *gp91^{Phox}-/-* *NOS2^{-/-}* knockout mice [a gift from Dr Carl Nathan, Weill Medical College of Cornell University; (Shiloh *et al.*, 1999)], differentiated into macrophages as described previously (Chong *et al.*, 2008), and replated in 6-, 24- or 96-well cell culture-treated plates at a density of 1 $\times$ 10⁶ (6-well plates) or 1 $\times$ 10⁵ (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 $\times$ 10⁵ ml⁻¹ (24-well plate) or 8 $\times$ 10⁵ 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 *N*5-[imino-(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, Alexa Fluor 568–donkey anti-rat, Alexa Fluor 568–donkey anti-rabbit or Alexa Fluor 568–donkey anti-goat antibodies (Invitrogen). Because both anti-*Francisella* LPS and anti-human EEA-1 antibodies were raised in mice, bacteria were detected using 4'-6-diamidino-2-phenylindole (DAPI) (100 ng ml⁻¹ in H₂O) labelling 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 $\times 63/1.4$ objective for quantitative analysis, or Carl Zeiss LSM 510 or LSM 710 confocal laser scanning microscopes for image acquisition. Confocal images of 1024 $\times$ 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 $\times$ 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 μ l 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 μ l 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 μ l 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 μ M 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, phorbol-12-myristate-13-acetate (PMA; Sigma) in KRG buffer was added at a final concentration of 1 μ M 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 /TNFSF1A kit according to the manufacturer's instructions (R&D Systems). In these assays, uninfected muBMMs treated with 1 μ g 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

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 $\pm$ 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 μ m (insets). (c) Representative growth curves of Schu S4 in control and IFN- γ -activated MDMs. Values are means $\pm$ 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 does not affect *Francisella* phagosomal 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

Fig. 2. IFN- γ does not affect maturation of Schu S4-containing phagosomes 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 5, 10, 20, 30 or 60 min and processed for immunolabelling of *Francisella* LPS and either EEA-1 or LAMP-1. (a) Colocalization of intracellular bacteria with either EEA-1 or LAMP-1 in untreated or IFN- γ -treated muBMMs. (b) Colocalization of intracellular bacteria with either EEA-1 or LAMP-1 in untreated or IFN- γ -treated MDMs. Values are means $\pm$ SD of three independent experiments, where at least 100 bacteria were analysed per condition in each experiment.

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

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

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 $\pm$ 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 μ m. (c) Percentages of cytoplasmic Schu S4 in control and IFN- γ -treated MDMs. Values are means $\pm$ 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 μ m.

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 GFP^{low} to a GFP^{high} phenotype (Fig. 4a–c), indicating extensive bacterial replication. In contrast, in IFN- γ -activated muBMMs, a smaller population of GFP⁺CD11b⁺ cells (a maximum of $32 \pm 17\%$ at 6 h pi; Fig. 4b) was detected from 4 h pi and onwards, which did not evolve into a GFP^{high}CD11b⁺ population (Figs 1b and 4c), demonstrating a lack of extensive cytosolic growth. 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 $11.5 \pm 13.1\%$ 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 GFP^{low} 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 ($4.6 \pm 3.7\%$ at 12 h pi) exhibited extensive replication in IFN- γ -activated cells, compared with control macrophages ($29.3 \pm 8.9\%$ 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 (Polsinelli *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 $\pm$ 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 $\pm$ SD from three or four independent experiments and correspond to percentages from gated quadrants 2. 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 $\pm$ SD from three independent experiments.

Fig. 5. IFN- γ restricts cytosolic proliferation of Schu S4 in MDMs. MDMs were left untreated or were 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, b) or fluorescence microscopy (c, d), as described in Methods. (a) Representative flow cytometry plots of infected MDMs at 4 and 16 h pi from three independent experiments. Uninfected MDMs were used as negative controls for GFP fluorescence. Percentages in the top-right quadrant indicate the fraction of CD11b⁺ GFP⁺ cells. (b) Flow cytometry-based quantification of Schu S4 infection in either control or IFN- γ -activated MDMs. Cells with Schu S4 replication refer to infected MDMs that contained more than five bacteria. Values are means $\pm$ 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 ($P < 0.05$). (c, d) Percentages of either control (c) or IFN- γ -activated (d) infected MDMs 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. Values are means $\pm$ 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.

Fig. 6. IFN- γ -mediated restriction of Schu S4 cytosolic growth does not depend upon either Phox or iNOS. (a) ROS production by either control or IFN- γ -activated muBMMs that were pretreated with *E. coli* LPS for 24 h and stimulated or not stimulated with PMA. Cells were incubated in luminol-containing KRG buffer and luminescence (counts per second, cps) was measured over a 60 min period. Data are means $\pm$ SD from triplicate samples from a representative experiment out of three. (b–d) ROS production by either control or IFN- γ -activated muBMMs infected with Schu S4 for 1 (b), 8 (c) or 20 h (d) and stimulated or not stimulated with PMA. Data are means $\pm$ SD from triplicate samples of a representative experiment of two. (e) NO production by either control or IFN- γ -activated C57BL/6 or *gp91^{Phox}-/-* NOS2^{-/-} muBMMs that were treated with *E. coli* LPS for 24 h (left panel), or either control or IFN- γ -activated C57BL/6 muBMMs infected with Schu S4 for 1, 8 or 20 h (right panel). Data are means $\pm$ SD from triplicate samples of a representative experiment out of three. (f) TNF- α secretion by either control or IFN- γ -activated Schu S4-infected muBMMs at 1, 8 and 20 h pi. Uninfected, *E. coli* LPS-treated, control or IFN- γ -activated muBMMs were used as positive controls. Data are means $\pm$ SD from triplicate samples of a representative experiment of two. (g) Representative growth curves of Schu S4 in either control or IFN- γ -activated *Phox*- and iNOS-deficient muBMMs. *gp91^{Phox}-/-* NOS2^{-/-} muBMMs 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 1, 4, 8, 12 and 24 h and processed for c.f.u. enumeration. (h, i) Representative growth curves of Schu S4 in either control (h) or IFN- γ -activated (i) MDMs that were additionally treated with NAC, NMMLA, or NMMLA and NAC for 1 h prior to and throughout the infection. At 1, 4, 8 and 12 h pi, macrophages were processed for c.f.u. enumeration. Plots are representative of three independent experiments.

We then monitored bacterial growth in either untreated or IFN- γ -treated muBMMs derived from *gp91^{Phox}-/-* 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) (Zaslhoff, 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 *Rickettsia 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 .

ACKNOWLEDGEMENTS

We are grateful to Anders Sjöstedt (Umeå University, Umeå, Sweden) for providing the Schu S4 strain, to Leigh Knodler and laboratory members for critical reading of the manuscript, and to Catharine Bosio, Jennifer Chase, Robin Ireland and Rebecca Anderson for helpful advice and assistance with flow cytometry and preparation of human macrophages. We also thank Steve Holland, Eugene Howerton and the Department of Transfusion Medicine at the National Institutes of Health (NIH) for their assistance in providing human monocytes. This work was supported by the Intramural Research Program of the NIH, National Institute of Allergy and Infectious Diseases.

REFERENCES

- Anthony, L. S., Morrissey, P. J. & Nano, F. E. (1992). 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* **148**, 1829–1834.
- Beatty, W. L., Belanger, T. A., Desai, A. A., Morrison, R. P. & Byrne, G. I. (1994). Tryptophan depletion as a mechanism of gamma interferon-mediated chlamydial persistence. *Infect Immun* **62**, 3705–3711.
- Bonquist, L., Lindgren, H., Golovliov, I., Guina, T. & Sjöstedt, A. (2008). MglA and Igl proteins contribute to the modulation of *Francisella tularensis* live vaccine strain-containing phagosomes in murine macrophages. *Infect Immun* **76**, 3502–3510.
- Bosio, C. M. & Dow, S. W. (2005). *Francisella tularensis* induces aberrant activation of pulmonary dendritic cells. *J Immunol* **175**, 6792–6801.
- Chase, J. C., Celli, J. & Bosio, C. M. (2009). Direct and indirect impairment of human dendritic cell function by virulent *Francisella tularensis* Schu S4. *Infect Immun* **77**, 180–195.
- Checroun, C., Wehrly, T. D., Fischer, E. R., Hayes, S. F. & Celli, J. (2006). Autophagy-mediated reentry of *Francisella tularensis* into the endocytic compartment after cytoplasmic replication. *Proc Natl Acad Sci U S A* **103**, 14578–14583.
- Chong, A., Wehrly, T. D., Nair, V., Fischer, E. R., Barker, J. R., Klose, K. E. & Celli, J. (2008). The early phagosomal stage of *Francisella tularensis* determines optimal phagosomal escape and *Francisella* pathogenicity island protein expression. *Infect Immun* **76**, 5488–5499.
- Clemens, D. L., Lee, B. Y. & Horwitz, M. A. (2004). Virulent and avirulent strains of *Francisella tularensis* prevent acidification and maturation of their phagosomes and escape into the cytoplasm in human macrophages. *Infect Immun* **72**, 3204–3217.
- Dahlgren, C. & Karlsson, A. (1999). Respiratory burst in human neutrophils. *J Immunol Methods* **232**, 3–14.

- Elkins, K. L., Rhinehart-Jones, T. R., Culkin, S. J., Yee, D. & Winegar, R. K. (1996). Minimal requirements for murine resistance to infection with *Francisella tularensis* LVS. *Infect Immun* **64**, 3288–3293.
- Ellis, J., Oyston, P. C., Green, M. & Titball, R. W. (2002). Tularemia. *Clin Microbiol Rev* **15**, 631–646.
- Fortier, A. H., Polsinelli, T., Green, S. J. & Nacy, C. A. (1992). Activation of macrophages for destruction of *Francisella tularensis*: identification of cytokines, effector cells, and effector molecules. *Infect Immun* **60**, 817–825.
- Golovliov, I., Baranov, V., Krocova, Z., Kovarova, H. & Sjostedt, A. (2003). An attenuated strain of the facultative intracellular bacterium *Francisella tularensis* can escape the phagosome of monocytic cells. *Infect Immun* **71**, 5940–5950.
- Hall, J. D., Woolard, M. D., Gunn, B. M., Craven, R. R., Taft-Benz, S., Frelinger, J. A. & Kawula, T. H. (2008). Infected-host-cell repertoire and cellular response in the lung following inhalation of *Francisella tularensis* Schu S4, LVS, or U112. *Infect Immun* **76**, 5843–5852.
- Hiemstra, P. S., van den Barselaar, M. T., Roest, M., Nibbering, P. H. & van Furth, R. (1999). Ubiquitin, a novel murine microbicidal protein present in the cytosolic fraction of macrophages. *J Leukoc Biol* **66**, 423–428.
- Leiby, D. A., Fortier, A. H., Crawford, R. M., Schreiber, R. D. & Nacy, C. A. (1992). In vivo modulation of the murine immune response to *Francisella tularensis* LVS by administration of anticytokine antibodies. *Infect Immun* **60**, 84–89.
- Levine, B. & Deretic, V. (2007). Unveiling the roles of autophagy in innate and adaptive immunity. *Nat Rev Immunol* **7**, 767–777.
- Lindgren, H., Golovliov, I., Baranov, V., Ernst, R. K., Telepnev, M. & Sjostedt, A. (2004). Factors affecting the escape of *Francisella tularensis* from the phagolysosome. *J Med Microbiol* **53**, 953–958.
- Lindgren, H., Stenman, L., Tarnvik, A. & Sjostedt, A. (2005). The contribution of reactive nitrogen and oxygen species to the killing of *Francisella tularensis* LVS by murine macrophages. *Microbes Infect* **7**, 467–475.
- Lindgren, H., Shen, H., Zingmark, C., Golovliov, I., Conlan, W. & Sjostedt, A. (2007). Resistance of *Francisella tularensis* strains against reactive nitrogen and oxygen species with special reference to the role of KatG. *Infect Immun* **75**, 1303–1309.
- Mariathasan, S., Weiss, D. S., Dixit, V. M. & Monack, D. M. (2005). Innate immunity against *Francisella tularensis* is dependent on the ASC/caspase-1 axis. *J Exp Med* **202**, 1043–1049.
- McCaffrey, R. L. & Allen, L. A. (2006). *Francisella tularensis* LVS evades killing by human neutrophils via inhibition of the respiratory burst and phagosome escape. *J Leukoc Biol* **80**, 1224–1230.
- Oyston, P. C., Sjostedt, A. & Titball, R. W. (2004). Tularemia: bioterrorism defence renews interest in *Francisella tularensis*. *Nat Rev Microbiol* **2**, 967–978.
- Paradkar, P. N., De Domenico, I., Durchfort, N., Zohn, I., Kaplan, J. & Ward, D. M. (2008). Iron depletion limits intracellular bacterial growth in macrophages. *Blood* **112**, 866–874.
- Polsinelli, T., Meltzer, M. S. & Fortier, A. H. (1994). Nitric oxide-independent killing of *Francisella tularensis* by IFN- γ -stimulated murine alveolar macrophages. *J Immunol* **153**, 1238–1245.
- Portnoy, D. A., Schreiber, R. D., Connelly, P. & Tilney, L. G. (1989). γ Interferon limits access of *Listeria monocytogenes* to the macrophage cytoplasm. *J Exp Med* **170**, 2141–2146.
- Radtke, A. L. & O’Riordan, M. X. (2006). Intracellular innate resistance to bacterial pathogens. *Cell Microbiol* **8**, 1720–1729.
- Santic, M., Molmeret, M. & Abu Kwaik, Y. (2005a). Modulation of biogenesis of the *Francisella tularensis* subsp. *novicida*-containing phagosome in quiescent human macrophages and its maturation into a phagolysosome upon activation by IFN- γ . *Cell Microbiol* **7**, 957–967.
- Santic, M., Molmeret, M., Klose, K. E., Jones, S. & Abu Kwaik, Y. (2005b). The *Francisella tularensis* pathogenicity island protein IgIC and its regulator MglA are essential for modulating phagosome biogenesis and subsequent bacterial escape into the cytoplasm. *Cell Microbiol* **7**, 969–979.
- Santic, M., Asare, R., Skrobbonja, I., Jones, S. & Abu Kwaik, Y. (2008). Acquisition of the vATPase proton pump and phagosome acidification is essential for escape of *Francisella tularensis* into the macrophage cytosol. *Infect Immun* **76**, 2671–2677.
- Schaible, U. E. & Kaufmann, S. H. (2004). Iron and microbial infection. *Nat Rev Microbiol* **2**, 946–953.
- Shiloh, M. U., MacMicking, J. D., Nicholson, S., Brause, J. E., Potter, S., Marino, M., Fang, F., Dinuer, M. & Nathan, C. (1999). Phenotype of mice and macrophages deficient in both phagocyte oxidase and inducible nitric oxide synthase. *Immunity* **10**, 29–38.
- Turco, J. & Winkler, H. H. (1983). Cloned mouse interferon- γ inhibits the growth of *Rickettsia prowazekii* in cultured mouse fibroblasts. *J Exp Med* **158**, 2159–2164.
- Way, S. S., Borczuk, A. C., Dominitz, R. & Goldberg, M. B. (1998). An essential role for gamma interferon in innate resistance to *Shigella flexneri* infection. *Infect Immun* **66**, 1342–1348.
- Wehrly, T. D., Chong, A., Virtaneva, K., Sturdevant, D. E., Child, R., Edwards, J. A., Brouwer, D., Nair, V., Fischer, E. R. & other authors (2009). Intracellular biology and virulence determinants of *Francisella tularensis* revealed by transcriptional profiling inside macrophages. *Cell Microbiol* **11**, 1128–1150.
- Zaslloff, M. (2002). Antimicrobial peptides of multicellular organisms. *Nature* **415**, 389–395.

Edited by: P. C. F. Oyston