



Published in final edited form as:

*Mol Immunol.* 2008 May ; 45(10): 2962–2969. doi:10.1016/j.molimm.2008.01.022.

## Molecular immunology of experimental primary tularemia in mice infected by respiratory or intradermal routes with type A

### *Francisella tularensis*

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### Abstract

The type A subspecies of *Francisella tularensis* is a highly virulent facultative intracellular bacterial pathogen, and a potential biological weapon. Recently, there has been renewed interest in developing new vaccines and therapeutics against this bacterium. Natural cases of disease, tularemia, caused by the type A subspecies are very rare. Therefore, the United States Food and Drug Administration will rely on the so called Animal Rule for efficacy testing of anti-*Francisella* medicines. This requires the human disease to be modeled in one or more animal species in which the pathogenicity of the agent is reasonably well understood. Mice are natural hosts for *F. tularensis*, and might be able to satisfy this requirement. Tularemia pathogenesis appears to be primarily due to the host inflammatory response which is poorly understood at the molecular level. Additionally, the extent to which this response varies depending on host and pathogen genetic background, or by pathogen challenge route or dose is unknown. Therefore, the present study examined sera and infected tissues from C57BL/6 and BALB/c mice challenged by natural intradermal and respiratory routes with one of two distinct type A strains of the pathogen for cytokine and chemokine responses that might help explain the morbidity associated with tularemia. The results show that the molecular immune response was mostly similar regardless of the variables examined. For instance, mRNA for the proinflammatory cytokine IL-6, and chemokines KC, and IP-10 was consistently upregulated at all sites of infection. Upregulation of mRNA for several other cytokines and chemokines occurred in a more tissue restricted manner. For instance, IFN- $\gamma$  was highly upregulated in the skin of BALB/c, but not C57BL/6 mice after ID inoculation of the pathogen, whilst IL-10 mRNA upregulation was only consistently seen in the skin and lungs.

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## 1. Introduction

*Francisella tularensis* subspecies *tularensis* (type A subspecies) is a highly infectious and virulent facultative intracellular bacterial pathogen of many mammalian species including humans (reviewed in Sjostedt, 2007). It causes a spectrum of diseases collectively called tularemia and it was once developed on an industrial scale as a biological weapon (Dennis *et al.*, 2001; Ellis *et al.*, 2002). Nowadays, there are renewed concerns about the abuse of this pathogen which has led to the call for the development of new countermeasures (Dennis *et al.*, 2001). A major barrier to this aim is the natural clinical rarity of tularemia caused by type A *F. tularensis*. This makes it impossible to test the efficacy of anti-*Francisella* drugs and vaccines in human clinical trials. To circumvent this problem the United States Food and Drug Administration (FDA) developed regulations that allow the efficacy of countermeasures to be demonstrated in animal models considered to mimic the human disease (<http://www.fda.gov/cber/rules/humeffic.htm>). A major tenet of these regulations, referred to as the “Animal Rule,” is that the mechanism of pathogenicity of the disease agent is reasonably well-understood. Applying the Animal Rule to the development of *F. tularensis* countermeasures is not straightforward, since its pathogenicity is not well characterized (reviewed in Conlan and Oyston, 2007). *F. tularensis* does not elaborate any obvious exotoxins, and its lipopolysaccharide lacks endotoxicity. Instead, *F. tularensis* appears to cause disease by proliferating extensively in various host tissues and eliciting a cellular inflammatory response therein that disrupts normal physiology. Histopathological changes in the organs of humans or experimentally-infected model animals including mice support this view (Lillie and Francis, 1936; Baskerville and Hambleton, 1976; Baskerville *et al.*, 1978; Conlan *et al.*, 2003). Similarities in the histopathology of human and murine tularemia suggest that mice are a host species that may at least partially satisfy the Animal Rule for tularemia vaccines. However, it remains to be determined whether mouse and human tularemia pathogenesis is similar at the molecular level.

Inflammation is coordinated by multiple cytokines and chemokines operating in concert (Murdoch and Finn 2000; Borish and Steinke, 2003; Elenkov *et al.*, 2005) and understanding their production during tularemia will be pivotal for refining our understanding of *F. tularensis* pathogenicity and potentially provide data that would facilitate the application of the Animal Rule to medical countermeasures against it. Immune responses can be influenced by a multitude of parameters including host and pathogen genetic background, and route and dose of infection that can limit the utility of experimental models. Therefore, in the present study we undertook a detailed comparison of the serum and tissue cytokine and chemokine response of BALB/c and C57BL/6 mice infected by intradermal (ID) or respiratory routes with various doses of either of two distinct type A strains of *F. tularensis*. Overall, regardless of these variables, the results show that a broadly similar cytokine/chemokine response occurred during all cases of primary murine tularemia.

## 2. Materials and methods

### 2.1. Bacterial strains

A squirrel isolate of type A *F. tularensis* strain, FSC 033, and an isolate, FSC 237, of the clinical strain, SCHU S4, was obtained from the *Francisella* Strain Collection of the Swedish Defense Department. Working stocks of both isolates were prepared as previously described (Twine *et al.*, 2006).

### 2.2 Infection of mice

Specific-pathogen-free female BALB/c and C57BL/6 mice were purchased from Charles Rivers Laboratories (St. Constant, Que.), and entered experiments when they were 6-8 weeks

old. Mice were maintained and used in accordance with the recommendations of the Canadian Council on Animal Care Guide to the Care and Use of Experimental Animals. ID inocula (50  $\mu$ l / mouse) were injected into a fold of skin in the shaved mid-belly. Mice were exposed to aerosols of the pathogen using a customized commercial nose-only exposure apparatus (In-tox Products, Albuquerque, NM) as previously described (Conlan *et al.*, 2003, 2005; Chen *et al.*, 2003, 2004; Shen *et al.*, 2004). All work involving type A *F. tularensis* was performed in a federally-licensed, and Select-Agent-approved small animal containment level 3 facility. Mice that became terminally ill were killed by CO<sub>2</sub> asphyxiation and, based on experience, it was assumed that they would have died naturally one day later.

### 2.3 Quantitative bacteriology

At various times of infection, mice were killed by CO<sub>2</sub> asphyxiation. For bacteriology, livers, spleens, and lungs were removed, minced with scissors, and homogenized using aerosol-proof homogenizers. Additionally, for mice challenged ID, a 1 cm<sup>2</sup> piece of skin surrounding the inoculum site was removed and processed as above. Organ homogenates were diluted in sterile saline and plated for CFU counts.

### 2.4. Cytokine and chemokine determinations

For cytokine/chemokine analysis, groups of mice (n=3/group) were killed on days 1-4 post-challenge. Sera were prepared from blood collected by cardiac puncture, and were filter sterilized (0.22  $\mu$ m) and stored at -20°C until needed. Serum levels of cytokines and chemokines were determined using Beadlyte® Mouse 21-plex Cytokine Detection System on a Luminex® 100 IS system (Luminex, Austin, TX) as specified by the manufacturer. Analyses were done in duplicate, and the cytokine/chemokine concentrations were calculated against the standards using Beadview® software version 1.03.

Spleen, liver, lungs, and skin were dissected and immersed immediately in RNAlater (Qiagen, Germantown, MD) and stored as above until needed. Relative amounts of cytokine and chemokine mRNA in infected tissues over the course of infection were estimated by real-time PCR essentially as described elsewhere (Johnson *et al.*, 2003; Andersson *et al.*, 2006). Briefly, total RNA was extracted from tissues, cDNA prepared, amplified and quantified using primers and probes designed with the Primer3 program available at [http://frodo.wi.mit.edu/cgi-bin/primer3/primer3\\_www.cgi](http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Levels of PCR product were normalized to a housekeeping gene (beta-2-microglobulin ( $\beta$ 2m), and data presented as the average of relative expression values compared to those in the corresponding tissues of uninfected mice.

## 3.0 Results

### 3.1 Infection kinetics following ID or aerosol challenge with type A isolates of *F. tularensis*

BALB/c and C57BL/6 mice were infected ID with 10<sup>3</sup> cfu of type A isolate FSC 033. Additional groups of BALB/c mice were infected by aerosol with 10<sup>1</sup> or 10<sup>3</sup> of SCHU S4 (FSC237). The ensuing kinetics of these infections are shown in Table 1. Regardless of challenge dose or route, infection disseminated to the liver and spleen by day 2 and over the next 2 days grew rapidly therein. BALB/c and C57BL/6 mice appeared equally susceptible to ID challenge in that each died 3 to 4 days after challenge with similar bacterial burdens in their organs. However, BALB/c mice invariably showed overt signs of infection much earlier than C57BL/6 mice. BALB/c mice always exhibited clear signs of infection (ruffled fur) by day 2 of infection that became progressively worse on each successive day (lethargy, hunching, anorexia, cold to the touch, conjunctivitis) whereas C57BL/6 mice only became obviously sick during the final day of infection by which time BALB/c mice appeared moribund. Moreover, overt inflammation at the skin inoculation site was much more pronounced in BALB/c versus

C57BL/6 mice by day 4 of infection (not shown). BALB/c mice challenged with low or high dose aerosols of SCHU S4 also showed progressive signs of infection from day 2 onwards, but mice challenged with the higher dose died 1-2 days earlier. The data presented above is consistent with that we have previously generated for BALB/c and C57BL/6 mice challenged ID and by aerosol with low doses of FSC 033 or SCHU S4 (Conlan *et al.*, 2003,2005;Chen *et al.*, 2003, Twine *et al.*,2006). Similar results were reported by others for another distinct type A isolate (Wu *et al.*, 2005).

### 3.2 Changes in serum cytokine and chemokine levels during primary tularemia

The levels of a panel of 21 cytokines and chemokines in the sera of representative mice (n=3 / group) from the experiments depicted in Table 1 were measured on various days post-challenge, and the most pronounced increases are shown in figure 1. In all cases by day 4 of infection, statistically-significant (P<0.05) and often substantial increases were observed in the levels of IFN- $\gamma$  (100-700-fold), TNF- $\alpha$  (7-120-fold), IL-6 (400-6300-fold), IL-1 $\beta$  (2.5-16-fold), IL-12p70 (2-9-fold), KC (40-980-fold), MCP-1 (65-1950-fold), and RANTES (8-14-fold) in the serum (range in parentheses in the maximum average increase recorded on any day for each of experiments 1-4 shown in Table 1). Some of these changes were evident as early as day 1, and most changes were evident by day 2 of infection. In all cases, serum IFN- $\gamma$  levels peaked on day 3 of infection whereas all other cytokines and chemokines, except IL-12p70, peaked at day 4. Fold-increases in IFN- $\gamma$  levels peaked aerosol versus higher ID challenged mice, whereas the opposite was the case for IL-6, KC, and MCP-1. No such clear distinctions were evident with TNF $\alpha$ , IL-1 $\beta$ , IL-12p40, or RANTES. There were no obvious differences in serum cytokine and chemokine levels between BALB/c and C57BL/76 mice challenged ID. Among the other cytokines and chemokines examined, IL-9 was significantly elevated in three of the four experimental groups (peak increase 6-9-fold), and IL-10 (peak increase 2-5-15-fold) and VEGF (peak increase 3.5-7.0-fold) in ID challenged mice only (not shown). In contrast, decreases in IL-5 (2-3-fold) and IL-13 (2-5-14-fold) were observed in all experimental groups, but these were not always statistically significant (not shown). Finally, IL-1 $\alpha$ , IL-2, IL-3, IL-4, IL12p40, IL-17, GM-CSF, or MIP-1 $\beta$  levels were either unchanged, or only slightly increased or decreased in one or two experiments (data not shown). For simplicity, we have assumed that the latter results were random noise.

### 3.3 Changes in tissue cytokine and chemokine mRNA levels during primary tularemia

In addition to serum protein levels, mRNA levels for a semi-overlapping series of 17 cytokines and chemokines in liver, spleen, lungs, and, for ID challenged mice the skin were determined by qPCR (n=3 mice /group). We considered an increase in the mRNA level of  $\geq 2$ -fold on two or more days of infection, or a single day increase of  $\geq 5$ -fold to be potentially relevant, and any other results were considered to be noise. By these criteria, in the liver (Figure 2), mRNA for IFN- $\gamma$  (82-218-fold), TNF- $\alpha$  (15-79-fold), IL-1  $\beta$  (4-9-fold), IL-6 (36-78-fold), IL-12p35 (8-600-fold), IL-12p40 (2-10-fold), MIP-2 (125-6295-fold), KC (22-217-fold), MCP-1 (294-1458-fold), MIP-1 $\alpha$  (19-31-fold), MIP-1 $\beta$  (16-31-fold), IP-10 (96-201-fold), and iNOS (15-91-fold) were elevated in all four groups of challenged mice (range in parentheses in the maximum average increase recorded on any day for each of experiments 1-4 shown in Table 1). In contrast, IL-12p-40 was only sporadically elevated in all four groups, and IL-10 mRNA was only consistently over-expressed in BALB/c mice (2-6-fold; not shown). Liver mRNA for IL-4 was consistently over-expressed (3-20-fold) in three of the four groups, whereas RANTES mRNA was only elevated on day 4 (9-fold) in C57BL/6 mice challenged ID with FSC033, or (2-3-fold) on three out of four days in BALB/c mice challenged with a low dose aerosol of SCHU S4 (not shown). IL-2 mRNA was never increased on any day of infection in any experiment (not shown). As with serum protein levels, liver mRNA levels for IFN- $\gamma$  peaked on day 3 of infection in all cases. Liver mRNAs for IL-6, MIP-2, MCP-1, and iNOS all peaked on day 4 of infection. Liver mRNA for IL-12p35, MIP-2 and MCP-1 peaked at higher levels

in ID versus aerosol challenged mice. Levels of IL-4, IL-12p35, and iNOS mRNAs peaked higher in C57BL/6 mice.

In the spleen (Figure 3), mRNA levels for IFN- $\gamma$  (37-150-fold), IL-4 (2-12-fold), IL-6 (85-1226-fold), MIP-2 (92-699-fold), KC (72-184-fold), MCP-1 (121-345-fold), IP-10 (10-17-fold), and iNOS (13-187-fold) were elevated in all four groups (range in parentheses in the maximum average increase recorded on any day for each of experiments 1-4 shown in Table 1). MIP-2 mRNA peaked at a higher level in C57BL/6 mice versus BALB/c mice challenged ID with FSC 033 and in mice challenged by aerosol with  $10^3$  versus 10 cfu of SCHU S4. IL-10 mRNA was only consistently elevated (4-33-fold) in the spleens of BALB/c mice, whereas mRNA for IL-1 $\beta$  (3-7-fold), IL-12p35 (5-19-fold), MIP-1 $\alpha$  (5-11-fold), MIP-1 $\beta$  (3-16-fold), and RANTES (5-9-fold) was elevated in the spleens in three of the four experimental groups (not shown). Spleen mRNA for IL-12p40 (3-4-fold) was only consistently elevated in BALB/c mice challenged by aerosol with  $10^3$  cfu of SCHU S4, whereas TNF- $\alpha$  mRNA (3-10-fold) was only consistently expressed in mice challenged with a 100-fold lower dose aerosol (not shown). MIP-2 mRNA peaked at a higher level in C57BL/6 mice versus BALB/c mice challenged ID with FSC 033 and in mice challenged by aerosol with  $10^3$  versus 10 cfu of SCHU S4. Splenic IL-2 mRNA was never elevated (not shown).

In the lungs, mRNA levels for IFN- $\gamma$ , (6-67-fold), IL-6 (57-185-fold), IL-10 (7-54-fold), IL-12p40 (3-12-fold), MIP-2 (18-35-fold), KC (7-26-fold), MCP-1 (8-75-fold), MIP-1 $\alpha$  (6-23-fold), and IP-10 (66-142-fold) were elevated on at least one day of infection in all groups (range in parentheses in the maximum average increase recorded on any day for each of experiments 1-4 shown in Table 1). In most cases, lung cytokine and chemokine mRNA were elevated earlier and at higher levels in mice challenged by aerosol versus ID, presumably reflecting the fact that it takes 2-3 days for the pathogen to disseminate from the skin to the lungs. However, mRNA levels for IL-10 and IP-10 were raised to similar levels on days 2-3 in all groups. Levels of mRNA for IL-1 $\beta$  (3-4-fold) were elevated in three of four groups, and MIP-1 $\beta$  mRNA (4-20-fold) was elevated in all groups except C57BL/6 mice challenged ID. TNF- $\alpha$  mRNA was over-expressed in the lungs of BALB/c mice challenged ID with  $10^3$  cfu of *F. tularensis* strain FSC 033 (2-28-fold), or by aerosol with 10 cfu of SCHU S4 (3-7-fold). Levels of iNOS mRNA were raised a maximum of 6-15-fold only in C57BL/6 mice challenged ID with  $10^3$  cfu of strain FSC 033 or BALB/c mice challenged by aerosol with  $10^3$  cfu of SCHU S4. Levels of mRNA for IL-2, IL-4, IL-12p35, and RANTES were never over-expressed in any group.

In two experiments (experiments 1 and 2 in Table 1), cytokine/chemokine mRNA levels in the skin following ID challenge of BALB/c and C57BL/6 mice with  $10^3$  cfu of FSC 033 were measured. In the skin mRNA levels for IL-1 $\beta$  (30-48-fold), IL-6 (38-204-fold), IL-12p35 (5-53-fold), MIP-2 (4-28-fold), KC (103-104-fold), MCP-1 (13-180-fold), IP10 (27-119-fold), MIP-1 $\alpha$  (31-131-fold), MIP-1 $\beta$  (35-437-fold), RANTES (5-8-fold) were over-expressed in both mouse strains, (range in parentheses in the maximum average increase recorded on any day of infection). IFN- $\gamma$  mRNA (8-338-fold) and IL-10 mRNA (3-6-fold) was consistently over-expressed only in BALB/c mice whereas the opposite was the case for IL-12p40 mRNA (4-23-fold), TNF- $\alpha$  mRNA (3-fold), and iNOS mRNA (8-fold; figures in parentheses show day-to-day range). IL-6, IL-12p35, MCP-1, MIP-1 $\alpha$ , MIP-1 $\beta$  mRNA levels peaked at higher levels in BALB/c versus C57BL/6 mice. This was not the case with mRNA for IL-1 $\beta$ , KC or RANTES which were upregulated to similar peak levels in both mouse strains. IL-2 mRNA was never over-expressed.

#### 4. Discussion

Because human infection with type A *F. tularensis* is clinically rare, it is currently impossible to demonstrate the efficacy of anti-*Francisella* drugs and vaccines in human clinical trials.

Instead, licensure of medical countermeasures will need to rely on the application of FDA's Animal Rule which allows the demonstration of efficacy in animal models. The Animal Rule requires a detailed understanding of the mechanisms of pathogenesis of experimental tularemia. Tularemia pathogenesis involves cellular and molecular immune responses occurring at various sites within the infected host. The cells involved have been reasonably well identified in murine models of tularemia caused by attenuated *F. tularensis* LVS and clinical isolates of the pathogen (Elkins *et al.*, 2003; Cowley *et al.*, 2005; Conlan *et al.*, 2002, 2005; Chen *et al.*, 2004; Fulop *et al.*, 2001; Wu *et al.*, 2005). These studies have revealed that various innate host defenses and B cells and the specific antibodies they produce are able to combat infection with LVS. In contrast, type A strains of *F. tularensis* appear to be completely resistant to murine innate and humoral immune defenses and can only be killed by specific T-cell-mediated immunity under the influence of IFN- $\gamma$ . As confirmed by the current study, type A strains of *F. tularensis* rapidly kill mice before protective CMI responses can be generated. All common mouse strains appear to be equally susceptible to primary infection with type A *F. tularensis*, but only some of them (e.g. BALB/c but not C57BL/6 mice) develop early clinical signs of infection and can be protected by prior vaccination with LVS (Chen *et al.*, 2003; Conlan *et al.*, 2003, 2005; Shen *et al.*, 2004; Wu *et al.*, 2005). The reasons for these discrepancies remain unknown, but could reflect differences in host molecular inflammatory responses to the pathogen.

Very little is known about the molecular immune response to primary infection by type A *F. tularensis*, and how this is affected by host and pathogen genetic background, or route and dose of infection. The current study was undertaken to begin addressing these key issues. It showed that for a selection of cytokines and chemokines, the response to primary infection was qualitatively similar regardless of the aforementioned variables. In all variations of primary murine tularemia examined, increases occurred in serum protein levels of IFN- $\gamma$ , TNF- $\alpha$ , IL-6, KC, RANTES and MCP-1 and in liver, spleen, and lung, mRNA for IFN- $\gamma$ , IL-6, MCP-1, MIP-2, KC, and IP-10. In the skin, mRNA for IL-1 $\beta$ , IL-6, IL-12p35, MIP-2, MCP-1, KC, IP-10, MIP-1 $\alpha$ , MIP-1 $\beta$ , and RANTES was upregulated in both BALB/c and C57BL/6 mice challenged ID. Some cytokines and chemokines showed a more restricted pattern of up-regulation. For instance, mRNA for IL-1 $\beta$ , IL-12p35 and MIP-1 $\beta$  was routinely elevated in the liver and skin only.

Recently, others examined a more limited cytokine and chemokine response in C57BL/6 mice challenged IP with a lethal dose or ID with a sublethal dose of *F. tularensis* LVS (Cole *et al.*, 2006). As in the current study, the latter mice exhibited increases in serum, TNF $\alpha$ , IL-6, IFN $\gamma$ , KC, and MCP-1. In the liver, both studies showed increased expression of mRNA for TNF $\alpha$ , IFN- $\gamma$ , IL-12p35, IL-1 $\beta$ , IP-10, KC, MCP-1, and iNOS. In the spleen, upregulation of mRNA for IFN- $\gamma$ , MCP-1, KC, and IP-10, and iNOS were commonly observed in both studies, as was mRNA for IFN- $\gamma$ , IL-10, MCP-1, KC, and IP-10 in the lungs. Therefore, all of the aforementioned similarities should be considered as robust biomarkers of murine tularemia regardless of the pathogen strain, host genetic background, or challenge route or dose. The same could be true for the other cytokines and chemokines that were shown to be universally up-regulated in all challenge groups in the present study, but were not examined by the Cole *et al.* study. Additionally, both studies recorded increases in serum IL-10, liver mRNA for IL-12p40 and RANTES, spleen mRNA for IL-1 $\beta$ , IL-10, and lung mRNA for IL-1 $\beta$  in C57BL/6 mice. However, compared to the present study, the Cole *et al.* study failed to show increased expression of mRNA for IL-12p40, and iNOS in the lungs, and for IL12p35, RANTES, or IL-4 mRNA in the spleen. These mainly minor discrepancies could be due to differences in the challenge routes employed or could reflect innate virulence differences between LVS and clinical type A strains of the pathogen.

Despite the qualitative similarities in the molecular immune response described above, some kinetic and quantitative differences between mouse challenge groups were evident in the current study. For instance, serum IFN- $\gamma$  levels peaked at higher levels in aerosol versus ID challenged mice, whereas the opposite was the case for IL-6, KC, and MCP-1. In the liver mRNA for TNF- $\alpha$ , and IL-10 was more over-expressed in BALB/c versus C57BL/6 mice whereas the opposite was the case for IL-12p35 and iNOS mRNA. In the spleen, mRNA for MIP-2 and MCP-1 was more over-expressed in C57BL/6 mice. In the skin, mRNA for IFN- $\gamma$ , and IL-10 was only over-expressed in BALB/c mice, whereas the opposite was the case for IL-12p40 and iNOS mRNA. Whether these differences explain why BALB/c mice develop more pronounced inflammation at the site of ID inoculation and more obvious clinical signs of infection remain to be determined.

Most of the cytokines and chemokines up-regulated during primary tularemia are known mediators of acute cellular inflammation which is a hallmark of this disease, and could also contribute directly to the accompanying tissue necrosis. However, determining the relative importance a given cytokine or chemokine to tularemia morbidity and mortality will not be straightforward. For example, not all of them are obviously over-expressed at all sites of infection. Additionally, despite differences in clinical signs, both BALB/c and C57BL/6 mice die at the same time after challenge with type A *F. tularensis*. Furthermore, IFN- $\gamma$  knock out mice develop less severe histopathology than normal mice, but still die at the same time after challenge with a low dose aerosol or ID inoculum of type A *F. tularensis* (Chen *et al.*, 2004). Nevertheless, the current study has begun to refine our understanding of the mechanisms of *F. tularensis* infection necessary for development and regulatory approval of countermeasures against this highly virulent pathogen.

## Acknowledgements

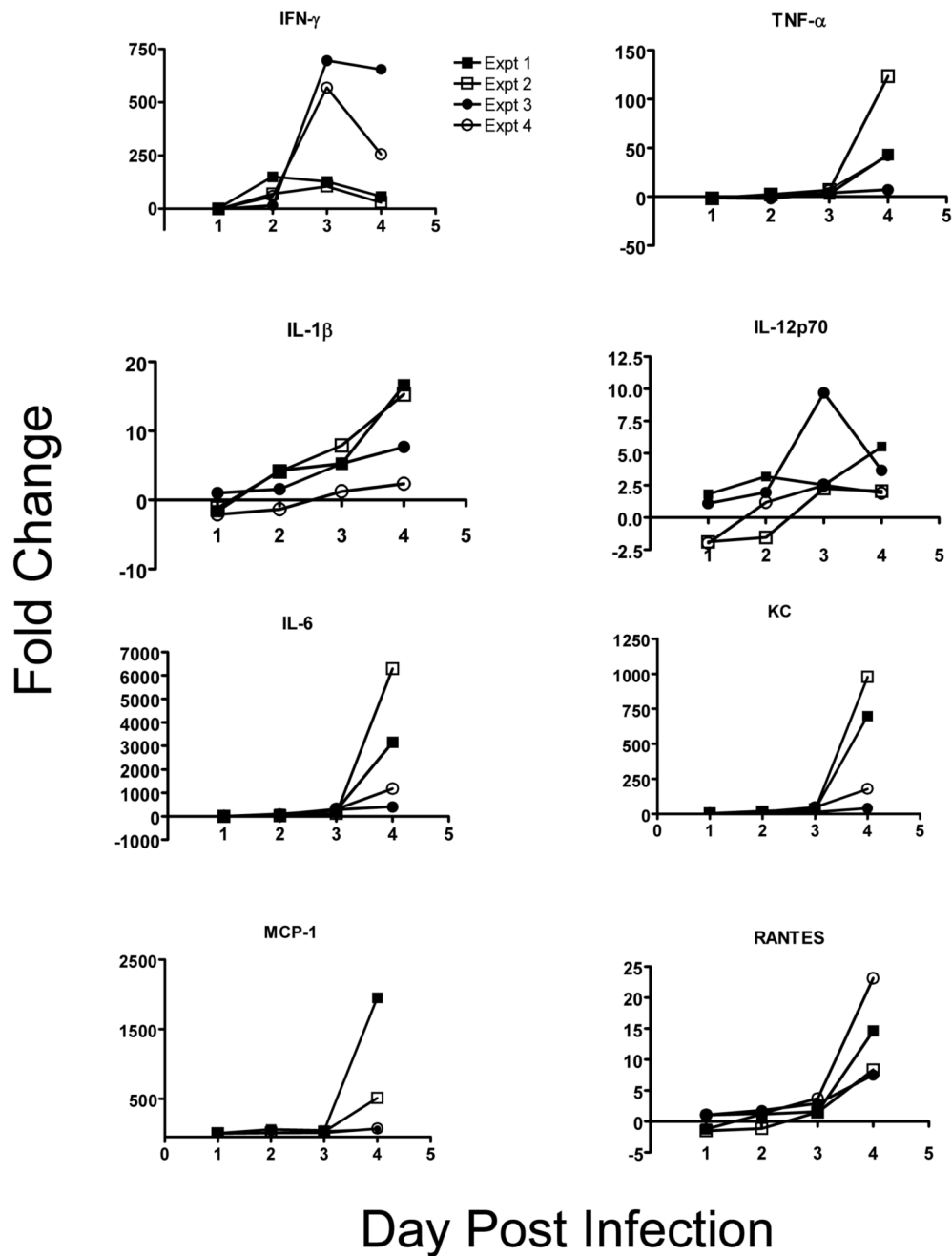
We thank Luc Lemay and Michel Cleroux for technical assistance, and Marianne Casey for administrative assistance. This project has been funded in whole or in part with Federal funds from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Department of Health and Human Services, under Contract No. HHSN26620050041C.

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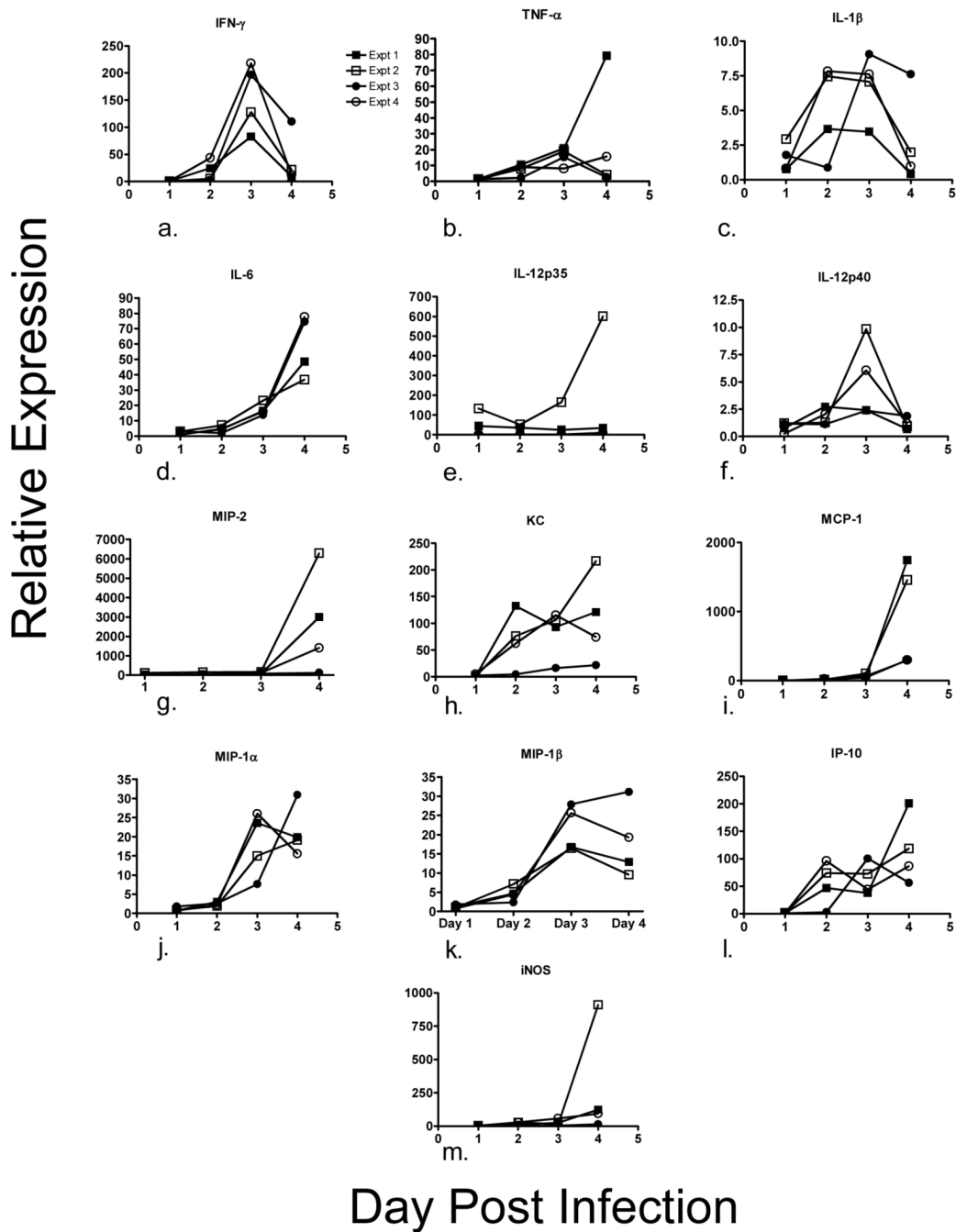
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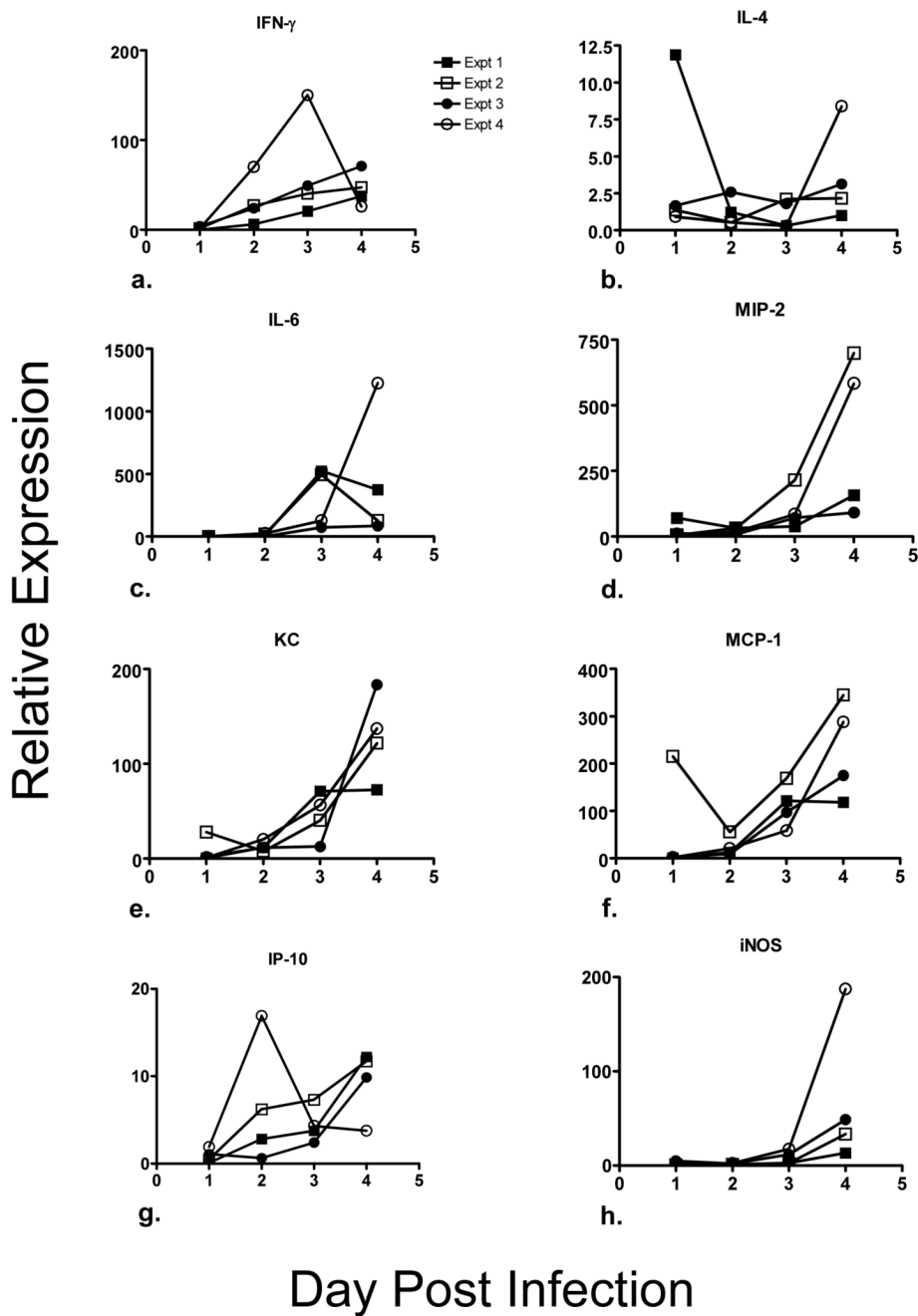




**Figure 1.** Selected serum cytokine and chemokine levels in BALB/c (expts 1,3,4,) and C57BL/6 mice (expts 2) challenged ID (expts 1,2) or by aerosol (expts 3,4) with type A *F. tularensis*. Sera were obtained from 3 mice / group / time-point and examined by Luminex for a panel of 21 cytokines or chemokines. Data were analyzed by t test and were considered significantly different from control sera at P<0.05.



**Figure 2.** Selected cytokine and chemokine mRNA levels in the livers of BALB/c (expts 1,3,4) and C57BL/6 mice (expt 2) challenged ID (expts 1,2) or by aerosol (expts 3,4) with type A *F. tularensis*. Liver mRNA was extracted from 3 mice / group / timepoint and examined by qPCR for a panel of 17 cytokines or chemokines.



**Figure 3.** Selected cytokine and chemokine mRNA levels in the spleens of BALB/c (expts 1,3,4) and C57BL/6 mice (expt 2) challenged ID (expts 1,2) or by aerosol (expts 3,4) with type A *F. tularensis*. Spleen mRNA was extracted from 3 mice / group / timepoint and examined by qPCR for a panel of 17 cytokines or chemokines.

**Table 1**  
Bacterial growth in the organs of mice infected with various concentrations of different strains of type *A. F. tularensis* by ID and aerosol routes.

Expt. No.	Mouse strain	<i>Francisella</i> strain	Route/dose	TTD in days	organ	Log <sub>10</sub> CFU ± SD on day of infection proportion infe n=3-4			
						1	2	3	4
1	BALB/c	FSC033	ID /10 <sup>3</sup> CFU	3-4	skin	6.03 ± 0.15	7.74 ± 0.30	7.51 ± 0.80	8.30 ± 0.10
						3.87 (1/3)	5.0 ± 0.14	7.49 ± 0.85	>10.0 (3/3)
						2.89 (2/3)	5.24 ± 0.14	8.02 ± 1.23	9.70 ± 0.22
2	C57BL/6	FSC033	ID /10 <sup>3</sup> CFU	3-4	lung	1.60 (1/3)	2.45 (2/3)	5.63 ± 1.30	8.86 ± 0.12
						5.66 ± 0.45	7.15 ± 0.18	6.80 ± 0.18	7.60 ± 0.18
						<2.30 (0/3)	6.24 ± 0.99	7.93 ± 0.25	>10.0 (3/3)
3	BALB/c	SCHU S4 (FSC237)	aerosol / 10 CFU	5-6	spleen	1.45 (2/3)	6.77 ± 1.20	8.68 ± 0.06	9.42 ± 0.16
						<1.3 (0/3)	4.08 ± 0.70	6.04 ± 0.39	8.59 ± 0.17
						ND	ND	ND	ND
4	BALB/c	SCHU S4 (FSC 237)	aerosol / 10 <sup>3</sup> CFU	4	skin	<1.6 (0/4)	3.65 ± 1.43	5.65 ± 0.30	7.71 ± 0.18
						<1.6 (0/4)	3.67 ± 1.24	6.14 ± 0.40	8.74 ± 0.32
						4.03 ± 0.19	5.73 ± 0.21	6.77 ± 0.15	7.39 ± 0.32
					liver	ND	ND	ND	ND
						<1.6 (0/4)	5.58 ± 0.41	7.32 ± 0.35	8.93 ± 0.09
						<1.6 (0/4)	4.76 ± 0.63	7.74 ± 1.20	8.83 ± 0.72
					lung	4.76 ± 0.32	6.99 ± 0.25	7.96 ± 0.15	8.39 ± 0.04

TTD, time to death. ND, not done