Transcriptional profiling of host responses in mouse lungs following aerosol infection with type A *Francisella tularensis*

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Tularaemia caused by inhalation of type A *Francisella tularensis* bacteria is one of the most aggressive infectious diseases known, but the reasons for the very rapid spread of the organism from the lungs to internal organs and the ensuing mortality are unknown. The present study used the mouse model to examine in detail the host immune response in the lung. After an aerosol challenge with 20 c.f.u. of the type A strain FSC033, all mice developed clinical signs of severe disease, showed weight loss by day 4 of infection and died the next day. Histopathological findings in the lung revealed acute inflammation and intense vasculitis and perivascularitis on day 4. Gene transcriptional changes in the mouse lung samples were examined on days 1, 2 and 4 of infection using a cDNA microarray with 20 600 mouse clones representing 18 500 genes. In total, 424 genes were found to be differentially expressed, some of which were both up- and downregulated at different time points, 192 of which were upregulated and 234 of which were downregulated for at least one time point. A high percentage of selected genes identified by the microarray analysis were confirmed to be differentially regulated by quantitative real-time PCR. Categorization of the differentially expressed genes showed that those preferentially involved in host immune responses were activated extensively on day 4 but hardly or not at all on days 1 and 2. Further analysis revealed that several of the genes upregulated on day 4 are known to depend on gamma interferon or tumour necrosis factor alpha for their regulation. In keeping with this finding, tumour necrosis factor alpha and gamma interferon levels were found to be increased significantly in bronchoalveolar lavage on day 4.

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INTRODUCTION

An important host defence mechanism against infection is the uptake of pathogenic microbes through phagocytosis and subsequent killing by phagocytic cells. To counteract this, some pathogens have evolved strategies to avoid phagocytosis by exhibiting contact-dependent antiphagocytic activities. Other pathogens survive and proliferate within phagocytes by evading or diverting the intracellular host killing mechanisms, for example, through escape from the phagosome by lysing the vacuolar membrane (*Listeria*, *Shigella*) (Cornelis *et al.*, 1998), blocking the fusion of phagosomes with lysosomes (*Legionella*) or resisting the

antimicrobial activity of phagosomes by manipulating their assembly (*Mycobacteria*, *Salmonella*) (reviewed by Aderem & Underhill, 1999).

The response to intracellular infections is often studied *in vitro* using infected, purified host cell populations, such as macrophages or dendritic cells, that are the primary targets for the infectious agents as models. However, intracellular infections elicit much broader responses from the host since they usually trigger a vigorous inflammatory response. This response profoundly affects tissues and cells adjacent to infectious foci and sometimes also results in systemic effects. Easily accessible samples to analyse local and systemic effects of infection-induced inflammation include peripheral blood cells or infected whole organ samples. By analysing transcriptional and translational profiles from such mixed

Abbreviations: BAL, bronchoalveolar lavage; LVS, live vaccine strain; Q-PCR, quantitative real-time PCR.

cell populations at various times during the infectious process, a detailed picture of the overall effects of infection on the host can be obtained. Such an analysis will help us to understand the pathogenesis of the disease and to identify pathogen-specific inflammatory profiles.

Tularaemia is a zoonotic disease caused by the intracellular bacterial pathogen *Francisella tularensis*. In nature, it occurs predominantly among hares, rabbits and rodents, and spreads via direct contact with primary hosts or via arthropods or by aerosols to humans (Oyston *et al.*, 2004). A characteristic feature of *F. tularensis* is its ability to withstand the antimicrobial effects of macrophages and to proliferate effectively intracellularly (Sjöstedt, 2003). Most experimental models of tularaemia have used attenuated strains such as the live vaccine strain (LVS). However, recent work has shown that there are key differences between the pathogenesis of the infection caused by LVS and virulent strains of *F. tularensis* (Chen *et al.*, 2003; Conlan *et al.*, 2003). For example, sublethal infection with LVS confers protection against aerosol challenge with LVS, but not virulent strains of the pathogen (Conlan *et al.*, 2005). Finally, immunodeficient mice such as those unable to produce gamma interferon (IFN- γ) or nitric oxide from inducible nitric oxide synthase or mice with broad immunodeficiencies such as SCID mice or neutropenic mice are rendered much more susceptible to LVS, but not to virulent strains (Chen *et al.*, 2004). This indicates that immunocompetent mice are virtually defenceless against aerosol or intradermal challenges with even the lowest inocula of virulent *F. tularensis* strains. The present study was undertaken in order to better understand the extreme virulence of type A *F. tularensis* strains. It characterizes the transcriptional profile of the murine host response in the lung during the first 4 days of an infection with a highly virulent strain of the pathogen.

METHODS

Mice. Specific-pathogen-free female C57BL/6 mice (8–12 weeks of age) were purchased from Charles River Laboratories. Mice were housed under specific-pathogen-free conditions in a federally licensed animal biosafety level 3 facility (National Research Council of Canada, Ottawa) with free access to sterile water and certified mouse chow. The animals 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.

***F. tularensis* and experimental infections.** Stocks of type A *F. tularensis* strain FSC033/snMF, originally isolated from a squirrel in Georgia, USA (Johansson *et al.*, 2000), were prepared as previously described (Conlan *et al.*, 2003). For aerosol exposure, thawed *F. tularensis* stocks were diluted in Mueller–Hinton broth containing 20% (v/v) glycerol to maintain infectivity at the high relative humidity (70–80%) employed. Aerosols of type A *F. tularensis* strain FSC033/snMF were generated with a Lovelace nebulizer operating at a pressure of 40 p.s.i. (276 Pa) to produce particles in the 4–6 μ m range required for inhalation and retention in the alveoli (Fitzgeorge *et al.*, 1983). Mice were exposed to these aerosols for 7 min using a customized commercial nose-only exposure apparatus

(In-tox Products, Albuquerque, NM, USA), resulting in the implantation of 10–20 organisms into the lungs (Chen *et al.*, 2003; Conlan *et al.*, 2003).

Bronchoalveolar lavage (BAL). Groups of six mice were killed on days 0, 1, 2 and 4 by CO₂ asphyxiation. The trachea was exposed through a midline incision and cannulated with a plastic catheter. The lungs were lavaged by instillation of five 1.0 ml aliquots of PBS supplemented with 3 mM EDTA (Chen *et al.*, 1992). The lavage fluid was centrifuged at 2450 g for 7 min and the supernatant was collected, filter-sterilized and stored at –80 °C for cytokine analysis.

Total RNA isolation and quality confirmation. Groups of six mice were sacrificed by CO₂ asphyxiation on days 0, 1, 2 and 4 after aerosol exposure. Uninfected mice were sacrificed as controls. Lungs from individual mice were dissected from the thoracic cavity and immediately placed in 1.0 ml RNeasy lysis buffer (Qiagen) and stored at –80 °C until needed.

Total RNAs were isolated from the lungs using an RNeasy isolation kit (Qiagen) and stored at –70 °C until use. RNA samples with a ratio of A₂₆₀ to A₂₈₀ between 2.0 and 2.1 were used for experiments. The quality of the RNA was also checked by running 300 ng of each sample on a Lab-On-A-Chip (Caliper Technologies Corp.) which was evaluated on a bioanalyser (Agilent Technologies); only non-degraded RNA samples were used for microarray and quantitative real-time PCR (Q-PCR) experiments.

Microarray construction. In-house-produced cDNA arrays consisting of 20 600 mouse clones derived from two different clone sets were used. These include a 15 000 mouse cDNA set from the National Institute of Aging (Kargul *et al.*, 2001; <http://lgsun.grc.nia.nih.gov/cDNA/15k.html>) and a 5400 cDNA clone set obtained from Research Genetics. Universal Scorecard (Amersham), five plant clones (*Pinus sylvestris*) and 18 housekeeping genes (cloned in-house) were included as controls. The clones were amplified by PCR and the purified PCR products were dissolved in 50% DMSO and then spotted on microscope slides using a Microgrid II arrayer (Biorobotics).

Labelling and hybridization. RNA concentration was measured with Nanodrop (NanoDrop Technologies) and adjusted to approximately 25 μ g per sample. Synthesis and labelling of cDNA was performed according to a protocol available at <http://www.umu.se/climi/bact/Microarray/index.html>. Briefly, aminoallyl-dUTP (Amersham) was incorporated into cDNA during reverse transcription using Superscript II enzyme (Gibco-BRL). cDNA was then indirectly labelled with Cy3 or Cy5 fluorescent dyes (Amersham). A test sample from one mouse and a reference sample from a pool of untreated mice were labelled with Cy3 and Cy5 dye, respectively. The fluorophores were reversed on every other array to compensate for dye bias. Labelled cDNA from test and reference samples was mixed and purified. cDNA was then dissolved in DIG Easy Hyb solution (Roche) supplemented with tRNA (Sigma) and fish sperm DNA (Sigma). Hybridization was performed at the temperature recommended for the DIG Easy Hyb, 37 °C, overnight in a Genetac Hybridization Station (Genomic Solutions). Slides were washed sequentially with 0.1 $\times$ SSC, 0.1% SDS followed by 0.1 $\times$ SSC.

Statistical analysis. Between nine and 11 replicated arrays were performed for each of the three time points examined. Arrays were scanned using a Scanarray 4000XL (Perkin Elmer) at three different intensity settings. The images were analysed with the software ScanArray Express (Perkin Elmer). Median signals were obtained and five or six replicated arrays of good quality were used for statistical analysis. Background correction was performed by subtracting the local background signals from the foreground signals. The background-corrected signals from the three scans of each array were combined using a method similar to that of Dudley *et al.* (2002).

Data from the two channels on each array were normalized using print-tip MA-loess (Yang *et al.*, 2002). The normalized data were analysed using the *B* statistic suggested by Lonnstedt & Speed (2002). Differentially expressed genes were selected according to ratio and *B* values.

Q-PCR. RNA from the same control and test samples used in the microarray experiments was reverse transcribed into cDNA. Specific primers for selected genes were designed using the Primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi).

Q-PCR was performed using SYBR green I PCR kit (Applied Biosystems) in an ABI Prism 7900HT Sequence Detection System (Applied Biosystems). Each reaction contained 12.5 µl SYBR green I PCR kit, 250 nM reverse and forward primers and 5 µl cDNA. The total volume of the reaction was adjusted with water to 25 µl. PCR was performed in MicroAmp 96-well plates (Applied Biosystems) capped with MicroAmp optical adhesive seal. The reactions were incubated at 50 °C for 2 min, 10 min at 95 °C, followed by 45 cycles of 15 s at 95 °C and 1 min at 60 °C. The PCRs were subjected to a heat dissociation protocol present in the ABI SDS 2.0 software (Applied Biosystems).

BAL tumour necrosis factor alpha (TNF-α) and IFN-γ measurements. The levels of TNF-α and IFN-γ in undiluted BAL supernatants were measured by the Beadlyte mouse cytokine detection kit (Upstate Biotechnology) on a Luminex 100IS system (Luminex Corp.) according to the manufacturers' instructions. The lower limit of detection was 4.5 pg ml⁻¹ for TNF-α and 3.1 pg ml⁻¹ for IFN-γ.

RESULTS

Aerosol infection with type A *F. tularensis*

As expected from our previous experience with this retained-low-dose (~20 c.f.u.) aerosol model of type A *F. tularensis* infection (Conlan *et al.*, 2003), all mice developed clinical signs of illness including weight loss at day 4 and died of the infection by day 5 (Fig. 1a). Consistent with our previous observations (Conlan *et al.*, 2003), histopathological examination of the lung from mice killed on day 4 of infection revealed acute suppurative and necrotic bronchopneumonia, which involved one or more lung lobes (Fig. 1b, c), and acute to subacute vasculitis and perivascularitis.

Microarray analysis of aerosol infection with type A *F. tularensis*

In this study, transcriptional changes in mouse lung on days 1, 2 and 4 after aerosol exposure with type A *F. tularensis* were examined and compared to controls obtained from untreated mice using spotted arrays with 20 600 mouse clones representing 18 500 genes. A total of 9–11 pairwise comparisons were performed between *F. tularensis*-infected and control mouse lungs for each time point. Five to six replicated arrays of good quality were used for further analysis. After normalization of the microarray intensity using MA-loess to eliminate the potential bias due to incorporation efficiency, fluorescence yield and the laser power used in the scanning, statistical analyses were performed using S-Plus 6.1 software (Insightful Corp.) by calculating *B* values. The ratio between the treated sample and reference sample had to be up- or downregulated at least twofold with a corresponding *B* value higher than zero for clones to be

Fig. 1. (a) Survival (■, solid line) and body weight loss (●, dashed line) of C57BL/6 mice after aerosol infection with ~20 c.f.u. type A *F. tularensis*. (b) Lung from a mouse killed at day 4 after aerosol inoculation with ~20 c.f.u. type A *F. tularensis* showing the presence of many degenerative and necrotic neutrophils together with macrophages and other cellular debris in the alveolar spaces. (c) Lung from a control mouse showing the normal pulmonary structure. Micrographs in (b) and (c) were stained with haematoxylin and eosin; original magnification $\times 200$.

selected as differentially expressed. A total of 456 clones representing 424 unique genes fulfilled these criteria. Among them, 222 clones representing 192 genes were found to be upregulated and 236 clones representing 234 genes were downregulated for at least one time point. Some genes were both up- and downregulated at different time points. The proportion of upregulated genes on days 1, 2 and 4 after infection was 18, 24 and 57%, respectively, whereas 35, 36 and 30%, respectively, were downregulated. Only five genes (1.2%) were differentially expressed at more than one time point.

We categorized a total of 167 differentially expressed genes with clear identities and characterized functions. We grouped the differentially expressed genes into the following categories: (i) metabolism (44 %), (ii) signal transduction (6 %), (iii) growth (12 %), (iv) response to pathogens (5 %), (v) ion homeostasis (5 %) and (vi) miscellaneous functions (28 %).

Cluster analysis of differentially expressed genes

Differentially expressed genes with ratios >2 or <0.5 and B values greater than zero were selected for cluster analysis. Replicated clones and clones with missing values were excluded. Genes were categorized as upregulated (1), downregulated (-1) or unchanged (0), and then subjected to SOM (self-organising map) cluster analysis. The results fitted into 22 out of 27 possible clusters ($3 \times 3 \times 3$). Six clusters contained more than 10 genes, constituting 84 % of the genes (Fig. 2). Genes included in cluster 1 participate in host defence responses to infection and they were activated extensively on day 4 but hardly or not at all on days 1 and 2. Genes in cluster 2 are involved in cellular killing mechanisms. These genes were downregulated on day 1 and unchanged on days 2 and 4. We hypothesize that this is due to a bacteria-triggered downregulation to avoid killing mechanisms of the host cell. In keeping with this, we have previously demonstrated that macrophages infected *in vitro* with LVS rapidly downregulate intracellular signalling and cytokine secretion in macrophages (Telepnev *et al.*, 2003, 2005). Cluster 7 contains genes that participate in regulation of transcription and metabolism. They were upregulated on

day 1 and unchanged on days 2 and 4. This may reflect the stimulation of cell expression by bacteria to obtain resources necessary for their proliferation.

Validation of microarray data with Q-PCR and ELISA

Genes identified as being differentially expressed by microarray analysis based on B values greater than 2 and gene annotation were subjected to Q-PCR analysis for confirmation. RNA samples from the same mice that were used for microarray analysis were used for Q-PCR analysis. These genes were chosen because they (i) encode critical mediators of lung inflammation, (ii) have distinct patterns of expression at various time points and (iii) are from different functional categories (Table 2). The correlation between microarray data and Q-PCR results in terms of the magnitude and direction of gene expression patterns for all genes validated was significant ($r^2 = 0.6795$, $P < 0.0001$), indicating good agreement between the two assays for the identification of differentially regulated genes (Table 1 and Fig. 3). Q-PCR analyses were performed for 27 genes and three time points. All of these genes had been found to be significantly differentially expressed at least for one time point by the microarray analysis with the exception of $\text{TNF-}\alpha$ and $\text{IFN-}\gamma$, which were expressed at such low levels that they were below the detection limit. They were included because they are known to be important for the development of protective Th1 immune responses. All of these genes were confirmed as differentially expressed at least for one time point ($P < 0.05$) by Q-PCR and, of these, 21 were

Fig. 2. SOM (self-organising map) clustering of genes up- and downregulated in the course of aerosol infection with type A *F. tularensis*. n , Number of genes in each cluster. Each line shows results for an individual gene.

Table 1. Validation by Q-PCR of selected genes that were regulated in the lungs by type A *F. tularensis* according to microarray

Values represent ratios between the level in the indicated sample and that of untreated control mice. Microarray (MA) results with *B* values ≥ 2 and Q-PCR results with $P > 0.05$ are considered significant and are marked in bold. BDL, Below detection level by microarray.

UniGene code	Gene product	Day 1		Day 2		Day 4	
		MA	Q-PCR	MA	Q-PCR	MA	Q-PCR
Mm.163	β_2 Microglobulin	0.99	1.02	1.07	0.89	4.24	2.75*
Mm.29742	CD24a antigen	0.98	1.75*	0.91	1.51	3.06	3.27*
Mm.2375	Creatine kinase, muscle	0.35	0.10*	0.27	0.06*	0.56	0.01*
Mm.29028	Death-associated protein 3	0.39	0.79	0.93	0.66	0.66	0.33*
Mm.24038	Guanylate nucleotide-binding protein 2	1.19	1.22	1.34	1.09	21.10	18.95*
Mm.196110	Haemoglobin alpha, adult chain 1	1.09	1.09	1.42	1.51*	0.24	0.20*
Mm.18603	Histidine decarboxylase	0.74	1.11	0.70	1.33	5.35	1.46
Mm.240327	IFN- γ	BDL	2.19	BDL	0.95	BDL	10.71*
Mm.33902	IFN- γ -induced GTPase	1.24	0.75	0.89	0.88	14.95	16.19*
Mm.246505	Kinesin family member 14 (Kif14)	BDL	0.66	0.36	0.33*	1.00	0.22*
Mm.294753	LPS-induced TNF factor	0.69	1.20	1.09	1.48	3.27	4.14*
Mm.34102	Ornithine decarboxylase, structural 1	0.97	0.92	1.05	0.82	2.48	2.54*
Mm.131572	P21 (CDKN1A)-activated kinase 7	0.93	1.10	1.06	0.88	0.25	0.13
Mm.32269	Peroxisome proliferative activated receptor	1.29	0.71	1.31	0.56	0.32	0.26*
Mm.154660	Plasminogen activator, tissue	1.97	1.65*	0.47	1.36	11.43	16.65*
Mm.202727	Pre-B-cell colony-enhancing factor 1	0.95	1.47	1.02	1.18	3.47	5.06*
Mm.37294	Prolyl endopeptidase	0.35	1.13	2.57	1.20	0.81	0.34*
Mm.289832	Proteasome (prosome, macropain) 26S subunit, ATPase 3	3.30	0.86	0.55	0.86	0.36	0.51
Mm.17932	Purine-nucleoside phosphorylase	0.91	1.11	0.84	1.00	4.78	3.84*
Mm.2128	S100 calcium-binding protein A9 (calgranulin B)	BDL	0.64*	0.66	1.38	9.20	27.73*
Mm.264709	Serine (or cysteine) proteinase inhibitor	0.45	0.56	1.40	1.04	15.80	25.14*
Mm.311655	Special AT-rich sequence-binding protein 1	0.97	0.15*	0.35	0.11*	0.80	0.08*
Mm.15793	T-cell-specific GTPase	1.34	0.99	1.09	1.02	29.49	19.95*
Mm.4871	Tissue inhibitor of metalloproteinase 3	0.86	1.92	0.99	2.13*	4.34	6.84*
Mm.330731	Transglutaminase 2, C polypeptide	1.01	1.17	0.98	1.36	3.05	2.97*
Mm.646	Tropomyosin 2, beta	0.33	0.06*	0.67	0.05*	0.76	0.04*
Mm.1293	TNF- α	BDL	0.30*	BDL	0.68*	BDL	4.57*

*Significant after Bonferroni correction for mass significance.

significant after a Bonferroni correction for mass significance. Of the remaining samples for these genes that were not identified as differentially expressed by microarray analysis, 18 were found to be significantly regulated ($P < 0.05$) by Q-PCR using the Bonferroni correction.

We also validated the expression of TNF- α and IFN- γ mRNA by quantification of these cytokines in the lung. In support of the gene expression data, both TNF- α and IFN- γ proteins were significantly upregulated in the BAL fluid on day 4 after aerosol exposure to type A *F. tularensis* (Fig. 4).

DISCUSSION

Inhalation of type A *F. tularensis* gives rise to the most aggressive form of tularaemia that is associated with considerable mortality in both experimental animals and

humans (Dennis *et al.*, 2001). The contribution of the pathogenesis in the lung to the outcome of the disease is, however, enigmatic. Thus, in mice it was observed that the bacterial burden and tissue pathology were much more severe in liver and spleen than in the lung after an aerosol infection with type A *F. tularensis* (Conlan *et al.*, 2003). In humans, too, an early phase of infection has been reported to occur, characterized by overt clinical symptoms but no X-ray abnormalities (Goodpasture & House, 1928; Overholt *et al.*, 1961; Saslaw *et al.*, 1961). The typical manifestations of pneumonia appear to occur during the later stages of human infection (Goodpasture & House, 1928; Overholt *et al.*, 1961). Thus, the early phase of murine respiratory tularaemia appears to mimic the early course of human respiratory tularaemia. Therefore, characterization of the anti-*Francisella* immune response in the mouse lung may be of general relevance for understanding the pathogenesis of tularaemia in other species.

Table 2. Gene ontology for genes regulated in the lungs by type A *F. tularensis* according to Q-PCR

UniGene code	Product	Symbol	Gene ontology	
			Biological process	Molecular function
Mm.163	β_2 Microglobulin	B2m	Antigen presentation	MHC class I receptor
Mm.29742	CD24a antigen	Cd24a	—	—
Mm.2375	Creatine kinase, muscle	Ckm	Phosphocreatine metabolism	Creatine kinase activity
Mm.29028	Death-associated protein 3	Dap3	Regulation of apoptosis	Ribosome
Mm.24038	Guanylate nucleotide-binding protein 2	Gbp2	Immune response	GTPase activity
Mm.196110	Haemoglobin alpha, adult chain 1	Hba-a1	Oxygen transport	Oxygen transporter
Mm.18603	Histidine decarboxylase	Hdc	Carboxylic acid metabolism	Lyase
Mm.240327	IFN- γ	Infg	Immune response	Signal transducer
Mm.33902	IFN- γ -induced GTPase	Igtp	—	GTPase activity
Mm.246505	Kinesin family member 14 (Kif14)	Kif14	—	—
Mm.294753	LPS-induced TNF factor	Litaf	—	Protein binding
Mm.34102	Ornithine decarboxylase, structural 1	Odc1	Polyamine biosynthesis	Carboxy-lyase activity
Mm.131572	P21 (CDKN1A)-activated kinase 7	Pak7	Anti-apoptosis	Protein kinase
Mm.32269	Peroxisome proliferative activated receptor	Ppargc1b	—	Nucleic acid binding
Mm.154660	Plasminogen activator, tissue	Plat	Proteolysis and peptidolysis	Serine-type endopeptidase
Mm.202727	Pre-B-cell colony-enhancing factor 1	Pbef1	Nucleotide biosynthesis	Transferring glycosyl groups
Mm.37294	Prolyl endopeptidase	Prep	Proteolysis and peptidolysis	Hydrolase activity
Mm.289832	Proteasome 26S subunit, ATPase 3	Psmc3	Transport/protein catabolism	Nucleotide binding
Mm.17932	Purine-nucleoside phosphorylase	Pnp	—	Purine-nucleoside phosphorylase
Mm.2128	S100 calcium-binding protein A9	S100a9	—	Calcium ion binding
Mm.264709	Serine (or cysteine) proteinase inhibitor	Serpina3g	—	Endopeptidase inhibitor
Mm.311655	Special AT-rich sequence-binding protein 1	Satb1	Histone methylation	Transcription factor
Mm.15793	T-cell-specific GTPase	Tgtp	—	GTP binding
Mm.4871	Tissue inhibitor of metalloproteinase 3	Timp3	—	Metalloendopeptidase inhibitor
Mm.330731	Transglutaminase 2, C polypeptide	Tgm2	Proteolysis and peptidolysis	Glutamyltransferase
Mm.646	Tropomyosin 2, beta	Tpm2	Muscle contraction/muscle development	Actin binding
Mm.1293	TNF- α	Tnf	Innate immune response	Signal transducer

—, Not known.

Fig. 3. Comparison of ratios obtained from cDNA microarray experiments and Q-PCR experiments for a subset of genes differentially expressed at 4 days after aerosol infection with type A *F. tularensis*. There was a strong correlation between the microarray analysis and Q-PCR data ($r^2 = 0.6795$, $P < 0.0001$).

Previous work has demonstrated that mice that inhale a low dose of type A *F. tularensis* strain FSC033 develop clinical signs of disease by day 3 and die within 5 days (Conlan *et al.*, 2003). Around day 3 of infection, in conjunction with the onset of clinical symptoms, very large numbers of bacteria ($\geq 10^8$ c.f.u. per organ) are present in lung, liver and spleen (Conlan *et al.*, 2003). The present data on gene regulation and cytokine levels in the lungs of the infected mice correlate well with the clinical presentation. During the first 2 days of infection, very few inflammatory changes were observed in the lungs whereas, by day 4, histology revealed localized massive, acute inflammation and intense vasculitis. Also, levels of IFN- γ and TNF- α in BAL rose dramatically from day 2 to day 4.

The failure to detect TNF- α and IFN- γ transcripts by the microarray demonstrates an obvious limitation of this technique. When constitutive levels of a given gene are very

Fig. 4. Cytokine levels in BAL fluids from *F. tularensis*-infected mice. Levels of TNF- α and IFN- γ in BAL fluids from mice killed at 0, 1, 2 and 4 days after aerosol inoculation with type A *F. tularensis* were determined by Beadlyte mouse cytokine detection kit on a Luminex 100IS system. Each dot on the graph represents the result from an individual mouse at that time point ($n=6$).

low, even a marked upregulation may not be detected by the DNA microarray since the increased levels are still below the detection limit. Both cytokine genes are known to be expressed at low constitutive levels (Fink *et al.*, 1998; Whelan *et al.*, 2003).

A number of genes that were strongly upregulated on day 4 of infection are known to be dependent on IFN- γ for their transcription. These include β_2 microglobulin, a glycoprotein constituting a substantial part of the major histocompatibility complex class I (MHC I) molecule. It is also involved in iron-uptake metabolism (Waheed *et al.*, 2002). Its transcription is in part dependent on nuclear factor kappa-B (NF- κ B) and interferon regulatory factors (IRFs) and is upregulated by cytokines such as TNF- α , IFN- α , IFN- β and IFN- γ . Guanylate nucleotide-binding protein 2 (GBP-2) was also found to be strongly upregulated on day 4 of infection. It is a member of the p65 GBP family, one of four families of IFN-inducible GTPases. Its expression in macrophages is induced by IFN- γ and lipopolysaccharide (LPS). It was found to be strongly induced in the livers of mice infected with another intracellular bacterial pathogen, *Listeria monocytogenes*, in an IRF-1-dependent manner (Boehm *et al.*, 1998).

Additionally, IFN- γ -induced GTPase was slightly downregulated on days 1 and 2 but, together with T-cell-specific GTPase, strongly upregulated on day 4. Both are members of

the p47 group of interferon-induced GTPases. They are active in phagocytic and secretory pathways and have been shown to be induced during infection by the intracellular pathogens *Toxoplasma gondii* (Collazo *et al.*, 2002) and *L. monocytogenes* (Macmicking, 2005; McCaffrey *et al.*, 2004). Pre-B-cell colony-enhancing factor was upregulated on day 4 of infection. Originally, it was found to act as a growth factor for B cells but was later identified as an inflammatory cytokine, the expression of which is enhanced by IFN- γ . Spi2a was downregulated on day 1 but strongly upregulated on day 4. It belongs to a family of intracellular and extracellular serine and cysteine proteinase inhibitors called serpins. Their target enzymes regulate very diverse processes, including apoptosis, inflammation and neoplasia (Gettins, 2000). Its expression has been shown to be strongly induced by IFN- γ and *in vivo* by intracellular bacteria such as *Mycobacterium bovis* bacille Calmette-Guérin (BCG), *L. monocytogenes* and *Salmonella typhimurium*, and by bacterial products like LPS or peptidoglycan (Hamerman *et al.*, 2002).

Some genes upregulated on day 4 of infection are known to be induced by TNF- α . For instance, LPS-induced TN factor (Litaf, TBX-1) is a transcription factor, responsible for TNF- α expression after LPS stimulation (Myokai *et al.*, 1999). It was upregulated at all three time points during infection. The same type of upregulation was also observed for tissue plasminogen activator (Plat), which is a serine protease that converts plasminogen to plasmin. Upregulation of host plasminogen activators has also been observed during other bacterial infections. For example, urokinase (one of two mammalian plasminogen activators) was enhanced during infection with *Staphylococcus aureus* and *Borrelia burgdorferi* (Lähtenmäki *et al.*, 2005). Induction of plasminogen activators is regulated by a number of cytokines such as TNF- α and interleukin 1 and 2.

Tissue inhibitors of metalloproteinases (Timp)s are natural inhibitors of proteolytic activity of matrix metalloproteinases (Lambert *et al.*, 2004). Timp3 was upregulated at all three time points investigated. It is located in the extracellular matrix and can control levels of TNF- α by inhibition of the TNF- α -converting enzyme (TACE) (Black, 2004).

The S100 calcium-binding protein A9 (S100A9) is one of three calcium-binding proteins from the S100 family, which constitutes a group of proinflammatory proteins released from phagocytic cells, especially neutrophils (Roth *et al.*, 2003). Expression of S100A9 is induced by LPS and oxygen species and induces release of neutrophils from bone marrow and recruitment of leukocytes (Vandal *et al.*, 2003). Elevated expression of S100A9 was found in severe acute respiratory syndrome patients (Reghunathan *et al.*, 2005). In our inhalation tularaemia model, it was downregulated on day 1 but upregulated on days 2 and 4.

Transglutaminase 2 belongs to the thiol- and Ca^{2+} -dependent acyl transferases. When it is aberrantly activated in tissues and cells, dysregulated inflammation results and

this may relate to a variety of diseases, including neurodegenerative and autoimmune diseases. It was upregulated at all three time points during infection. This enzyme has a mitochondrial location and causes selective depletion of mitochondrial glutathione, leading to a decreased ability to resist oxidative stress (Newsholme & Calder, 1997). Down-regulation of peroxisome proliferative activated receptor gamma coactivator 1 beta (Ppargc1b), which was observed on days 2 and 4, may also contribute to a diminished ability to withstand reactive oxygen species because it is critically required in mitochondrial metabolism and respiration (St-Pierre *et al.*, 2003). It is one of many co-activators of nuclear receptors and interacts with and enhances the activity of hepatic nuclear factor-4, peroxisome proliferative activated receptor- α and the glucocorticoid receptor. Tropomyosin was downregulated at all three time points. It is part of the cytoskeleton, but it has also been identified as an oxygen sensor and its downregulation may contribute to a decreased cellular adaptation to oxidative stress (Thorne *et al.*, 2004).

Overall, the findings in the current study demonstrated that a vigorous cellular inflammatory response ensues in *Francisella*-infected mouse lungs as evidenced by a marked increase of IFN- γ and TNF- α in BAL fluid and concomitant activation of pathways known to be regulated by these cytokines. Thus, experimental inhalation tularaemia appears to result in an innate Th1 type immune response. Despite this apparently appropriate host immune response, type A *F. tularensis* infection is uniformly lethal in inbred and outbred mice regardless of their genetic background (Conlan *et al.*, 2003). One notable observation is that these events appear to be initiated after the second day of infection and it is known that the target organs harbour more than 10^8 c.f.u. of bacteria a day later. Hence, it is possible that bacterial replication is so rapid that it overwhelms even the prominently activated antimicrobial host immune mechanisms observed on day 4 of infection. It has been observed that immune activation mediated *in vitro* via toll-like receptor 2 and 4 is inhibited in *F. tularensis*-infected human and mouse monocytic cells (Telepnev *et al.*, 2003, 2005). The relevance of these findings to the situation *in vivo* is not known, however, and the findings on day 4 indicate clearly that there is no general suppression of immune responses in the infected mice. This does not exclude the possibility that the effects observed *in vitro* are still relevant to the situation in an individual cell *in vivo*. Thus, one of the reasons for the extremely rapid replication of type A *F. tularensis* *in vivo* may be unrestricted growth in macrophages that are unable to activate effective antibacterial defence mechanisms or that virulent strains of *F. tularensis* are able to modulate the host response by delaying the generation and secretion of IFN- γ and TNF- α .

The fact that the mouse model of inhalation tularaemia results in 100 % mortality even at low dose might be considered aberrant compared with the human disease. However, this is also the case in other models of type A

tularaemia such as the rabbit or the monkey, in which the bacterium causes an extremely aggressive infection and the lethal doses are very low (Baskerville & Hambleton, 1976; Baskerville *et al.*, 1978). Even in humans, inhaled inocula of <100 c.f.u. cause severe clinical disease (Saslaw *et al.*, 1961) with mortality of 30–60 % in the absence of effective chemotherapy. Therefore, the findings of the present study might well mimic the pathogenesis of tularaemia in other host species.

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