A microarray analysis of the murine macrophage response to infection with *Francisella tularensis* LVS

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The response of cells of the mouse macrophage cell line J774 to infection with *Francisella tularensis* LVS was analysed by means of a DNA microarray representing approximately 18 500 genes (20 600 clones). The adaptive response was modest at all time points, and at most, 81 clones were differentially regulated from the time point of uptake of bacteria (0 min) up to 240 min later. For all five time points, 229 clones fulfilled the criteria of being differentially regulated, i.e. the ratio between infected versus non-infected cells was at least 1·7-fold up- or down-regulated and $P < 0\cdot05$. It was found that many of the differentially regulated genes are known to respond to stress in general and to oxidative stress specifically. However, at 120 min it was observed that genes that lead to depletion of glutathione were upregulated. Possibly, this was a result of mechanisms induced by *F. tularensis*. Generally, there was a conspicuous lack of inflammatory responses and, for example, although tumour necrosis factor alpha (TNF- α) was upregulated at 0 min, a significant down-regulation was noted at all subsequent time points. When cells were treated with an inhibitor of inducible nitric oxide synthase (iNOS) or the antioxidant *N*-acetylcysteine (NAC), the infection-induced cytopathogenic effect was significantly inhibited. Together, the results suggest that *F. tularensis* LVS infection confers an oxidative stress upon the target cells and that many of the host-defence mechanisms appear to be intended to counteract this stress. The infection is characterized by a very modest inflammatory response.

Received 3 February 2006

Accepted 9 May 2006

INTRODUCTION

Macrophages have essential roles in host defence to infection, as they often mediate the killing of microbes and also initiate, maintain and resolve host inflammatory responses by releasing cytokines and chemokines. They also serve as a habitat for many intracellular pathogens, a group of pathogens that utilize the intracellular environment as a safe haven to avoid extracellular defence mechanisms. Initially, macrophages may perish due to unrestricted growth of intracellular pathogens, but soon they start to mobilize an antimicrobial host defence. To survive within or escape from the hostile environment of an activated macrophage, pathogenic bacteria have evolved sophisticated strategies for evasion or neutralization of intracellular host-defence mechanisms (Cossart & Sansonetti, 2004). Our understanding of the strategies of the pathogens has advanced considerably during the last few years, in part due to a rapid

accumulation of genomic information. Although each intracellular bacterium seems to have evolved a number of such strategies, a common theme seems to be that type III or type IV secretion systems are essential for the intracellular survival of many of the pathogens (Cascales & Christie, 2003; Tampakaki *et al.*, 2004). This has been observed in *Salmonella*, *Yersinia*, *Chlamydia*, *Brucella* and *Legionella*. There are notable exceptions in this regard. *Francisella tularensis*, for example, is a facultative intracellular bacterium that is highly virulent and very well adapted to the intracellular habitat, but lacks production of exotoxins and possesses no type III or type IV secretion systems.

F. tularensis is a Gram-negative bacterium that has been associated with disease in a wide range of animal species. In humans, the bacterium is the cause of the serious and sometimes fatal disease tularemia (Tarnvik & Berglund, 2003). The disease has been reported in many countries of the northern hemisphere, but never from the southern hemisphere (Oyston *et al.*, 2004). However, an isolate of *F. tularensis* has been reported from Australia (Whipp *et al.*, 2003). Terrestrial and aquatic mammals, such as ground squirrels, rabbits, hares, voles, muskrats, water rats and

Abbreviations: FADD, Fas-associated death domain; LDH, lactate dehydrogenase; MAPK, mitogen-activated protein kinase; NAC, *N*-acetylcysteine; NMMLA, NG-monomethyl-L-arginine; Q-PCR, quantitative PCR; SOM, self-organizing map; SUMO, small ubiquitin-like modifier.

other rodents, have been suggested to serve as reservoirs. There are four recognized subspecies of *F. tularensis*: subspecies *holarctica*, *tularensis*, *mediasiatica* and *novicida* (Sjöstedt, 2005). In humans or rabbits, strains belonging to subspecies *tularensis* cause the most severe form of disease, but isolates belonging to all subspecies are highly virulent in mice. The aggressive features of the disease caused by strains of subspecies *tularensis* have been an important basis for the designation of *F. tularensis* as a category A agent. Comparative genetic analyses have revealed that there is a significant degree of genetic identity between isolates belonging to each subspecies (Tärnvik *et al.*, 1992). Little is known that explains the high virulence of *F. tularensis*. Recently, the genome of the SCHU S4 strain belonging to the highly virulent subspecies *tularensis* has been sequenced (Larsson *et al.*, 2005). The analysis has revealed only a few mechanisms that explain the adaptation of *F. tularensis* to the intracellular habitat. A notable feature is the presence of a 33.9 kb duplicated region which each comprises 25 genes with no γ -proteobacterial homologues (Nano *et al.*, 2004). Of these genes, members of an operon denoted *igl* have been shown to be essential for the intracellular growth of subspecies *holarctica* (Golovliov *et al.*, 2003) and *novicida* in macrophages and amoebae (Lauriano *et al.*, 2004). There is an attenuated strain of subspecies *holarctica* that has been widely used for human vaccination, the LVS strain. The vaccine strain is attenuated in humans and confers effective immunity to tularaemia, but it is virulent in mice and replicates intracellularly almost as effectively as wild-type strains (Tärnvik *et al.*, 1992).

Due to our lack of understanding of the virulence mechanisms that allow *F. tularensis* to rapidly replicate intracellularly, we asked whether an analysis of the specific changes in host-gene mRNA populations during infection with the pathogen could yield clues to characterize and elucidate the events that occur inside infected cells. To this end, we performed an analysis based on large-scale DNA microarrays of the host-gene adaptation during an infection with the vaccine strain *F. tularensis* LVS.

METHODS

Bacterial strain. *F. tularensis* LVS was cultured on modified Thayer–Martin (McLeod) agar plates at 37 °C in 5% CO₂. The bacteria were suspended in PBS to OD₆₆₀ = 1.0 (OD₆₆₀ 1.0 is approximately equal to 2 × 10⁹ bacteria ml⁻¹), pelleted by centrifugation and resuspended in PBS to 1 × 10¹¹ bacteria ml⁻¹. Then they were diluted to the appropriate density before use in cell-infection experiments.

Mammalian cell culture and infection. The mouse macrophage-like J774A.1 (ATCC TIB-67) cell line was grown in DMEM supplemented with 2 mM Glutamax I and 10% heat-inactivated fetal calf serum (FCS) (all from Invitrogen) in a humidified 5% CO₂ atmosphere at 37 °C.

Macrophages were seeded 20 h prior to infection in tissue-culture plates at a density of 5 × 10⁶ cells per 92 mm well (8 ml of culture medium) in DMEM lacking antibiotics. Two hours before infection, the cells were washed once with prewarmed wash medium (37 °C), i.e.

DMEM containing 1% FCS and incubated for 2 h at 37 °C with fresh DMEM containing 10% FCS. Heat-inactivated mouse serum was added to the monolayers, including wells containing control cells, to a final concentration of 1%. The macrophages were infected with *F. tularensis* LVS to give an m.o.i. of 500 bacteria per eukaryotic cell. The cells were incubated at 37 °C for 15 min, then washed three times with wash medium and further incubated with fresh culture medium containing 2 µg gentamicin ml⁻¹ for the indicated time (0–240 min). Total RNA from non-infected and infected J774 cells was extracted by addition of 2 ml TRIzol Reagent (Invitrogen) per well following the instructions of the manufacturer.

Assay of intracellular bacterial multiplication. To find an appropriate m.o.i., i.e. ratio of bacterial cells and J774A.1 cells, we applied an immunofluorescent technique developed to differentiate intracellular from extracellular bacteria. A mouse mAb specific to *F. tularensis* LPS (Grunow *et al.*, 2000) was used, and 500 cells were counted. At an m.o.i. of 500, a mean of 98% (range 94–99%, seven experiments) of the J774A.1 cells were found to contain intracellular bacteria. This m.o.i. was generally used in the study. Using the same technique, we determined that, on average, there were 2.7 intracellular bacteria per cell.

Microarray construction. In-house-produced cDNA arrays consisting of 20 600 mouse clones derived from two different clone sets were used. These included a 15 000 mouse cDNA set from the USA 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 Biosciences), 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, then spotted on microscope slides using a Microgrid II arrayer (Genomic Solutions).

Labelling and hybridization. A detailed protocol for cDNA synthesis and labelling can be found at <http://www.umu.se/climi/bact/Microarray/index.html>. In brief, first-strand cDNA synthesis was performed using Superscript II (Invitrogen) incorporating amino-allyl-dUTP (Amersham Biosciences). Total RNA (25 µg) was used in each reaction. In a second step, a fluorophore, Cy-3 or Cy-5 (Amersham Biosciences), was coupled to the amino-allyl groups, and one fluorophore was used to label cDNA from non-infected cells, the other to label cDNA from infected cells. Cy-3 and Cy-5 were switched in every other experiment to compensate for differences between the two fluorophores. The labelled cDNA was purified, and cDNA from non-infected cells and infected cells was mixed and dissolved in DIG Easy Hyb (Roche) supplemented with tRNA (Sigma-Aldrich) and fish sperm DNA (Sigma-Aldrich). Hybridization overnight at 37 °C and washing was performed in a Genetac hybridization station (Genomic Solutions). Washing was performed at 50 °C in 0.1 × SSC, 0.1% SDS, followed by 0.1 × SSC.

Statistical analysis. Each array was scanned using a Scanarray 4000XL (Perkin Elmer) at three different intensity settings, and the images were analysed using the software Quantarray (Perkin Elmer) and median signals were recorded. For each time point, six to eight replicated arrays were used. Background correction was performed using signals from negative control clones. The background-corrected signals from the three scans of each array were combined using a method similar to that described by Dudley *et al.* (2002). Data from the two channels on each array were normalized using MA-Loess. The normalized data were analysed using the modified *t* statistic as described previously (Baldi & Long, 2001). Selection of differentially expressed genes was made using *P* values and ratios.

Quantitative PCR (Q-PCR). Q-PCR was performed in the ABI Prism 7900HT Sequence Detection system (Applied Biosystems)

using the SYBR green I PCR kit (Applied Biosystems) as recommended by the manufacturer. Each reaction contained 12.5 µl SYBR green mix, 250 nM forward and reverse primers and 5 µl cDNA, and the total volume was adjusted with water to 25 µl. The reactions were performed in MicroAmp 96-well plates (Applied Biosystems) capped with MicroAmp optical adhesive seals. 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 PCR reactions were subjected to a heat-dissociation protocol present in the ABI SDS 2.0 software (Applied Biosystems).

Assay of cytopathogenicity and bacterial growth in the presence of an antioxidant or an inhibitor of inducible nitric oxide synthase (iNOS). Cells (4×10^4) were seeded into each well in 96-well tissue-culture plates and incubated overnight. After 60 min of stimulation with 1 mM NG-monomethyl-L-arginine (NMMLA) (Sigma-Aldrich) or 0.5 mM *N*-acetylcysteine (NAC) (Sigma-Aldrich), cells were infected with *F. tularensis* as described above. After washing, the cells were incubated in medium containing 2 µg gentamicin ml⁻¹ and NMMLA or NAC for the indicated time (0–15 h).

Cytopathogenicity was evaluated by measuring lactate dehydrogenase (LDH) activity released in the medium. Medium (50 µl) was transferred to a 96-well plate for LDH activity analysis using the CytoTox96 non-radioactive assay (Promega) according to the manufacturer's recommendations. To determine the maximum LDH activity, cells were lysed in 50 µl 0.1 % sodium deoxycholate in PBS and cell lysates were added to the growth medium. Quantification was performed by measuring A_{492} . The absorbance from medium alone was subtracted from the sample absorbance values.

For determination of numbers of intracellular bacteria, cells were lysed with 0.1 ml of 0.1 % sodium deoxycholate in PBS. After addition of 0.9 ml PBS, portions of each lysate, serially diluted in PBS, were plated on modified Thayer–Martin agar for determination of viable bacteria.

RESULTS AND DISCUSSION

Temporal analysis of host-cell gene expression during an *F. tularensis* infection

The global transcriptional response elicited in the murine macrophage-like cell line J774A.1 was determined using cDNA microarray hybridization. For each time point, material was hybridized from non-infected control cells and from *F. tularensis*-infected cells to microarrays containing 20 600 mouse probes, representing 18 500 genes. Samples were obtained at 0, 30, 60, 120 and 240 min after washing of cells. Previously, we have shown that more than 95 % of the bacteria have a cytoplasmic location at 120 min (Golovliov *et al.*, 2003). Thus, the escape from the phagosome had occurred during the study period of 240 min.

Six hybridizations were performed for each time point. All data were normalized using the S-Plus 6.1 software (Insightful). Data were filtered to include only those spots for which the infected/non-infected ratio was at least 1.7-fold up- or down-regulated compared to non-infected cells and with a significance of $P < 0.05$.

A total of 229 clones corresponding to 217 unique genes fulfilled the criteria for being differentially regulated. Of these clones, 23 % (53 clones) were found to be regulated at

0 min after the start of infection, 15 % (34 clones) at 30 min, 22 % (50 clones) at 60 min, 35 % (81 clones) at 120 min, and 10 % (23 clones) at 240 min. Only 6 % (14 clones) of the differentially expressed clones were differentially regulated at more than one time point. The functional categories of proteins encoded by the differentially expressed genes could be determined for 67 clones, corresponding to 54 unique genes. These are depicted in Table 1(a, b) and categorized according to functional categories described by the Gene Ontology Consortium (<http://www.geneontology.org>).

The addition of bacteria to the cultures triggered an immediate adaptive response of the J774A.1 cells (0 min). Upregulation was observed of *c-fos*, which is a transcription factor under the control of the mitogen-activated protein kinase (MAPK) pathway, and *c-fos* has also been shown to be regulated in response to hypoxia and certain other forms of stress (Yuan *et al.*, 2004). In line with this, its differential regulation may be an immediate adaptation of the cells in response to the attachment and internalization of the bacteria.

At later time points also, a number of genes known to be part of stress responses in general and of a response to oxidative stress specifically, were differentially regulated. One such example is tropomyosin 2, which was upregulated at 60 min. The encoded protein is part of the cytoskeleton, but it has also been identified as an oxygen sensor (Thorne *et al.*, 2004). The latter function may contribute to the cellular adaptation during infection. Phosphatase-1 (PP1) is a ubiquitous serine/threonine enzyme that regulates a variety of cellular processes through the dephosphorylation of dozens of substrates. The activity is believed to promote the recycling of protein factors and to affect the energy state of the cell (Ceulemans & Bollen, 2004). It also controls the activation of MAPK and thereby indirectly MAPK-dependent antibacterial mechanisms (Mitsuhashi *et al.*, 2003). An upregulation was observed at 120 min. Cytochrome *b* has a crucial role in the activity of the *bc₁* complex, one of several complexes that contribute to the energy transduction in the mitochondria (Crofts, 2004). Its upregulation at 120 min indicates that there is a requirement for additional energy, and this may be needed to execute the antibacterial response and the adaptation required during infection.

The energy metabolism in the mitochondria leads to significant conversion of oxygen to reactive metabolites (ROS). To protect the mitochondria from ROS-mediated damage, they are highly enriched with antioxidants, including the reduced form of glutathione, GSH, and enzymes protective against ROS, such as superoxide dismutase (SOD) and glutathione peroxidase (Inoue *et al.*, 2003). However, immediately after uptake of bacteria, we detected an upregulation of a gene encoding a glutaminase. This enzyme has a mitochondrial location, which leads to selective depletion of mitochondrial glutathione, thereby leading to a decreased capability to resist oxidative stress (Newsholme & Calder, 1997). Also, another gene encoding an enzyme that regulates

Table 1. Proteins encoded by genes up- or down-regulated in microarray analysis divided into functional categories

Ratios above 1.7 or below 0.6 are marked in bold. Values represent ratios between the indicated sample and that of untreated control cells.

Category	Accession no.	Description	Time (min)				
			0	30	60	120	240
(a) Upregulated genes							
Cancer	BG071032	Tentative phosphoprotein p53	1.3	1	1	1.1	2
Cell cycle regulator	BG066013	Cyclin ania-6b (cyclin L2)	2.3	1	0.8	0.7	0.7
	BG067002	Cyclin T2	0.9	2.2	0.9	0.9	1.3
Cytokine/chemokine	NM_013693	TNF- α^*	1.8	0.9	0.7	0.9	0.9
Cytoskeleton	BG087093	Tropomyosin 2, beta	1.5	0.9	2.7	0.7	1.3
	AU044038	Tentative tankyrase-related protein	2.3	1.5	0.7	0.9	0.8
Enzyme	BG072319	Tentative interferon-induced protein (Sp100)	1	1	0.9	2.2	1.2
	BG069876	Fatty acid-Coenzyme A ligase	1.1	1	1	2.1	1
	BG080489	Protein phosphatase 1	1	1	0.8	2.2	0.9
	BG072031	6-Pyruvoyl-tetrahydropterin synthase	2.2	0.8	0.7	1.7	1
Golgi apparatus	U45977	UDP-Gal:betaGal beta 1,3-galactosyltransferase	1.4	1.4	2.2	1	2.5
Mitochondrial	AW551184	Cytochrome <i>b</i>	1.3	1.1	0.8	2.3	0.9
	BG075159	Glutaminase, kidney isoform, mitochondrial precursor	2	0.8	1.3	0.5	0.9
Nuclear protein	BG065936	Nuclear VCP-like	2.5	0.9	1.3	1	0.9
Ribosomal	L31609	Ribosomal protein S29	2.2	1.3	0.8	0.9	1.2
Signal transduction	AI413379	p53 apoptosis-associated target	1.3	1	1	2	0.8
	BG078820	Platelet-derived growth factor receptor, alpha polypeptide	0.8	1	3.3	1.3	1.2
	AI893411	Zinc-finger protein 36/tristetraprolin	2.2	1.4	0.6	0.9	1.1
	BG071162	Fas-associating protein with death domain (Fadd)	0.9	0.8	2.3	0.9	0.9
Transcription	AW543818	Cofactor required for Sp1 transcriptional activation	0.9	1.2	2	1.2	1.2
	BG070196	c-fos	2.2	0.9	0.8	0.9	1.1
Transport	AI428862	Solute carrier family 22, member 2	2	1	1	0.8	1.2
(b) Down-regulated genes							
Cancer	BG067858	Tentative TPR to MET oncogene	1.2	0.5	1.4	0.9	1.1
Cell cycle regulator	AI451894	Cyclin G2	0.9	0.6	2.6	1	1.2
Cytochrome	AI323829	Cytochrome P450, 2e1	2.2	0.5	0.7	1.2	0.8
Cytoskeleton	BG070044	Telokin; myosin, light polypeptide kinase	0.4	1	1.1	0.9	1.7
Enzyme	AI452085	Tyrosine kinase TYK2	1.1	1	0.5	1.2	1.1
	BG083949	Sterol-C4-methyl oxidase-like	1.1	1	1	0.5	1.1
	BG086677	<i>Homo sapiens</i> sentrin/SUMO-specific protease (SEN7)	0.8	0.9	1	0.6	0.4
	AI449648	Tentative prostaglandin E synthase 2	0.8	1	0.4	0.9	1
	BG083405	Acetyl-Coenzyme A acetyltransferase 1	1.1	0.5	0.8	0.7	0.7
	AI323672	Gamma-glutamyl transferase 1	0.7	1.2	0.5	1.7	1.2
	J00423	Hypoxanthine guanine phosphoribosyl transferase	1	1.3	1	0.5	1
	AI449384	Sialyltransferase 8 (alpha-2,8-sialyltransferase) D	0.9	0.5	1	0.8	1
Golgi apparatus	BG087731	Golgi vesicular membrane-trafficking protein p18	1	1	1.2	0.5	0.8
Intracellular traffic	AW539497	SEC22 vesicle-trafficking protein-like 1 (<i>Saccharomyces cerevisiae</i>)	0.7	1.1	1	0.4	1.2
Lysin	AW556797	Protein kinase, lysine deficient 4	0.9	1.1	1	0.4	1.5
Nuclear protein	BG064733	Nuclear receptor binding protein	0.5	0.9	1.2	1.5	0.7
	BG075398	Tentative nuclear localization signals binding protein 1	0.9	0.5	1.4	1.2	1.2
Nucleic acid binding	BG088454	Heterogeneous nuclear ribonucleoprotein U	1.1	1	1.1	0.5	0.8
RNA binding	BG069460	Hermes; RNA binding protein gene with multiple splicing	0.6	0.3	1	1.4	0.9
Signal transduction	BG088461	p75NTR-associated cell death executor (Nade)	1	1.9	0.3	1.1	0.7
	BG064955	DNA segment, Chr 10, ERATO Doi 610	0.4	1.2	1	0.6	0.8
	BG080936	ADP-ribosylation factor GTPase activating protein 3	0.4	1.2	0.6	1.4	1.3
	AI324201	Probable serine/threonine protein kinase	0.4	1.2	0.7	1.1	1.2
Structural protein	BG071624	Beta-defensin 3 (Bd3)	1.1	1	1	0.5	1
	M10624	Ornithine decarboxylase, structural	1.4	0.7	1	0.5	1

Table 1. cont.

Category	Accession no.	Description	Time (min)				
			0	30	60	120	240
Translation	BG077018	Eukaryotic translation initiation factor 1A	1.1	0.9	1.4	1	0.5
	BG083267	Eukaryotic translation initiation factor 2, subunit 3	1.5	1	1.2	1	0.5
	BG088134	Eukaryotic translation initiation factor 3, subunit 7 (zeta)	0.8	1.1	0.5	1.2	0.8
Ubiquitin/proteasome	AI427491	Ubiquitin specific protease 22	1.1	0.9	1.2	1.2	0.4
	AA409615	Proteasome 26S subunit, non-ATPase, 12	0.5	1	0.9	0.9	1.2

*Based on a mean of 21 replicates on each array.

glutathione, gamma-glutamyl transferase 1, which hydrolyses the gamma-glutamyl linkage of glutathione (Jean *et al.*, 2003; Whitfield, 2001), was found to be upregulated at 120 min. The reason behind the upregulation of these glutathione-regulating enzymes is unclear, but again underscores the critical role of intramacrophage oxidative levels during *F. tularensis* infection. The lack of upregulation of antioxidant mechanisms indicates that bacterial factors are more likely than host factors to be the driving force at these time points.

Tetrahydrobiopterin (BH4) is an essential cofactor in the hydroxylation of the aromatic amino acids phenylalanine, tyrosine and tryptophan. 6-Pyruvoyl-tetrahydropterin synthase, found to be upregulated at 0 and 120 min, is necessary for the formation of BH4 (Thony *et al.*, 2000). This indicates that tryptophan hydroxylation occurs during this phase of infection. Tryptophan catabolites have been found to be critical in modulating the balance between responsiveness to pathogens and tolerance to non-harmful antigens (Moffett & Namboodiri, 2003). Degradation of tryptophan through the kynurenine metabolic pathway has been identified as an important antibacterial mechanism in macrophages and dendritic cells. Tryptophan catabolites also indirectly help to replenish levels of NAD⁺, which are depleted by oxidative stress. Thus, hydroxylation of tryptophan may serve one or both of two important functions, the generation of antibacterial molecules and/or the counteracting of the oxidative stress imposed on the infected cell.

The microarray analysis allowed a detailed study of the regulation of tumour necrosis factor alpha (TNF- α), since it harbours 21 copies of the gene. The gene was upregulated at 0 min, but already at 30 min it was down-regulated and levels were slightly below the basal level, and this low level persisted during the remaining time points (Fig. 1). It should be noted that the basal level is equivalent to only a few copies of the gene, so the assay is relatively insensitive for measuring down-regulation of the TNF- α gene. These findings correlate well with our previous demonstration that *F. tularensis*-infected cells are unable to secrete TNF- α , and also that no activation of NF- κ B and MAPK is present during the infection (Telepnev *et al.*, 2003).

Tristetraprolin (TTP), the prototype of a class of Cys-Cys-His zinc-finger proteins, has been shown to inhibit TNF- α production from macrophages by destabilizing its messenger RNA. The biosynthesis of TTP is induced by the same agents that stimulate TNF- α production, including TNF- α itself. TTP works as a component of a negative-feedback loop that interferes with TNF- α production by destabilizing its messenger RNA (Carballo *et al.*, 1998). TTP was found to be upregulated concomitantly with TNF- α , and this could be one mechanism for the subsequent down-regulation of TNF- α . Another gene that tentatively could be involved in TNF- α regulation is cytochrome P450 2E1 (CYP2E1). It was found to be 2.2-fold upregulated at 0 min, followed by a twofold down-regulation at 30 min. At 120 and 240 min, the levels of CYP2E1 expression were undetectable by the microarray. In response to Toll-like receptor stimulation, CYP2E1 has been suggested to activate ERK1/2, p38 and NF- κ B (Cao *et al.*, 2005). The activation of p38 was found to stabilize TNF- α transcripts, resulting in increased TNF- α production. Thus, its rapid induction, followed by a down-regulation, may be one key to the lack of TNF- α during the later phases of the *F. tularensis* infection.

Fig. 1. TNF- α expression in murine macrophage-like cell line J774A.1 cells during infection with *F. tularensis*. Black bars represent the mean of 21 replicates on each array; white bars represent the mean of six Q-PCR replicates. Error bars indicate SEM.

The down-regulation of a so-called small ubiquitin-like modifier (SUMO)-specific protease at 240 min was noteworthy. Modification of SUMO proteins plays an important role in the function, compartmentalization and stability of target proteins (Hilgarth *et al.*, 2004). The modifications are reversed by a class of proteases known as the SUMO-specific proteases. Thus, regulation of such proteins may have broad effects on the infected cells. Furthermore, proteasome 26S is a multisubunit complex that degrades proteins that have been targeted for destruction by the ubiquitin pathway (Ferrell *et al.*, 2000). It plays a central role in regulating essential cellular processes, such as antigen processing for presentation by the MHC class I pathway (Niedermann, 2002), and transcription and signal transduction, for example activation of NF- κ B by degradation of its inhibitor I κ B (Yaron *et al.*, 1997). The proteasome 26S subunit 12 was found to be down-regulated at 0 min. The gene encoding the functionally related ubiquitin-specific protease 22 was found to be down-regulated at 240 min. The proteasome and the ubiquitin system have been shown to be of special importance for host protection against intracellular bacteria. The proteasome has been shown to associate with *Salmonella typhimurium* bacteria localized in the cytosol and to exert antibacterial effects (Perrin *et al.*, 2004). Conversely, inhibition of proteasome function results in higher bacterial numbers. Since *F. tularensis* is an example of a cytosol-located pathogen (Golovliov *et al.*, 2003), the inhibition of the proteasome and the ubiquitin system may benefit the bacterium.

For Fas, and possibly other death receptors, homotypic death effector domain interactions connect the Fas-associated death domain (FADD) protein to caspase-8 and caspase-10 to mediate formation of the death-inducing signal complex (Barnhart *et al.*, 2003). FADD was upregulated at 60 min.

All genes that were differentially regulated upon infection with *F. tularensis* were subjected to cluster analysis and illustrated by self-organizing map (SOM) algorithms (Genespring 6.1, Silicon Genetics). This clustering further supports the hypothesis that there is a co-regulation of a considerable number of enzymes during the infection. For example, there was predominance of enzymes among the genes of clusters 1.1 and 2.3 (Fig. 2). Another notable finding was that cluster 2.1 contained mostly signal transducers and structural proteins. It is also obvious from these maps that most genes were transiently regulated during *F. tularensis* infection. Possibly, this rapid regulation reflects the need for the cell to quickly adapt to the various phases of the infection.

To further analyse the relation between the kinetics of regulation and the functional roles of the genes, significantly regulated genes were selected and for each gene, the Euclidean distance between responses over time was calculated. Significantly regulated genes (ratio > 1.7 and $P < 0.05$) and with known gene functions (54 cDNAs) are displayed in hierarchical cluster format (Fig. 3).

Statistical analysis of estimated numbers of false-positive and false-negative signals

Even though the criterion for differential regulation was rather stringent ($P < 0.05$) and six to eight arrays were analysed for each time point, there will still be a considerable number of false-positive signals due to the large number of clones tested. In theory, if the underlying assumptions were correct and the estimated P values were accurate, we expected up to 1000 false-positive values for 20 000 clones and a P value cutoff of 0.05. The magnitude of this estimation should be similar even if the assumption of normality was not fulfilled. With a small number of truly differentially expressed genes (1 % = 200 clones), the percentage of false-positive signals compared to all positive signals may be high. This calls for confirmation of the microarray results by an independent method such as Q-PCR.

Confirmation of microarray data by Q-PCR analysis

The relative expression of selected differentially regulated host-cell genes was analysed by Q-PCR on the same samples as those analysed by microarray analysis (Table 2). Genes that had been identified as significantly induced or repressed by the microarray analysis, representing 40 genes and 59 time points, were analysed. A majority of the samples were verified by the Q-PCR as up- or down-regulated, albeit with magnitudes different from those recorded by the microarray analysis. For example, the cytochrome P4502E1 levels were found by the Q-PCR to be more than sevenfold upregulated at 0 min and almost fourfold down-regulated at 30 min, i.e. ratios of much higher magnitude than those recorded by the microarray analysis (2.2- and twofold, respectively). This indicates that the microarray data do not represent a strict quantification of the gene levels, but rather serve as a powerful method for identifying differentially regulated genes on a large scale.

Effects of an antioxidant or an inhibitor of iNOS on the cytopathogenic effect of *F. tularensis* infection

Previous work has demonstrated that J774 cells exhibit a cytopathogenic effect within 12 h of the start of *F. tularensis* LVS infection, and that this cytopathogenic effect eventually leads to apoptosis of the infected cells (Lai & Sjostedt, 2003). Considering that the microarray analysis indicated that the infection resulted in a considerable oxidative stress upon the infected cells, we asked if treatments that diminish oxidation had any effect on the cytopathogenicity. The activation of enzymes producing oxygen species results in the direct formation of toxic molecules such as superoxide and hydrogen peroxide, or, if nitric oxide is produced concomitantly, in the formation of highly cytopathogenic species such as peroxynitrite. Therefore, we studied whether the cytopathogenic effects were affected by the addition of an antioxidant, NAC, or by the addition of an iNOS inhibitor, NMMLA. We observed that the relative levels of LDH release, which were

Fig. 2. Comparison of gene expression patterns upon infection with the *F. tularensis* LVS strain by SOM cluster analysis. The SOM cluster analysis was performed on clones ($n=67$) that were shown to be at least 1.7-fold differentially regulated at one or more time points ($t=0, 30, 60, 120$ and 240 min).

used as a marker of cytopathogenicity, were significantly lower ($P < 0.05$) in the presence of NMMLA or NAC (Table 3). The results indicate that the production of both reactive oxygen and reactive nitrogen species significantly contributes to the cytopathogenic effect. Concomitantly with the decreased cytopathogenic effects after the addition of NMMLA and NAC, it was also observed that the treatments led to slightly, but significantly, lower bacterial numbers (Table 4) after 15 h of infection, indicating that the intracellular redox environment affects the growth of *F. tularensis*.

It is interesting to note that during the period studied, little evidence for the induction of a proinflammatory programme was observed. This conforms well to previous findings showing that TNF- α is only secreted during the initial phase of infection. In fact, previously published evidence indicates that this lack of secretion is directly or indirectly dependent on the expression of a 23 kDa protein

of *F. tularensis* denoted IgIC (Telepnev *et al.*, 2003). A mutant of *F. tularensis*, lacking expression of this protein, induced high levels of TNF- α . The studies have indicated that the MAPK and NF- κ B pathways seem to be blocked during the infection (Telepnev *et al.*, 2003). Thus, the conspicuous lack of an inflammatory response during the infection is likely part of the overall intracellular survival strategy of *F. tularensis*.

After phagocytosis, intracellular microbes reside in a phagosome that eventually fuses with a lysosome, forming an organelle with powerful antimicrobial effects. However, several important intracellular pathogens have developed mechanisms to escape from the phagosome into the cytoplasm, thereby reaching a habitat that seems ideal for multiplication, with few or no antimicrobial mechanisms and a rich supply of nutrients. We and others have shown that almost all *F. tularensis* bacteria are capable of escaping from the phagosome of monocytic cells within 120 min of

Fig. 3. Microarray data were analysed from samples obtained from *F. tularensis*-infected cells at 0, 30, 60, 120 and 240 min. Significantly regulated ($P < 0.05$) genes with a ratio of at least 1.7 and with a known gene function (54 cDNAs) were selected, and for each gene, the Euclidean distances between the expression levels were calculated. Upregulation is shown in red and down-regulation in green.

invasion of human (Clemens *et al.*, 2004) or mouse (Golovliov *et al.*, 2003) monocytic cells. Thus, this time point most likely represents a critical phase of the infection

and the observed upregulation of a number of enzymes with important cellular functions may reflect this. Surprisingly, we observed only limited differential regulation of genes at

Table 2. Q-PCR analysis of a subset of genes found to be differentially expressed by microarray

Values represent ratios between the indicated sample and that of untreated control cells.

Accession no.	Description	Time (min)				
		0	30	60	120	240
BG067002	Cyclin T2		0.5			
NM_013693	TNF- α	2.67	0.99	0.76	0.68	0.95
BG087093	Tropomyosin 2, beta			2.03		
BG069876	Fatty acid-Coenzyme A ligase				1	
BG080489	Protein phosphatase 1				1.8	
BG072031	6-Pyruvoyl-tetrahydropterin synthase	1.14			1.46	
U45977	UDP-Gal:betaGal beta 1,3-galactosyltransferase			1.1		0.93
AW551184	Cytochrome <i>b</i>				1.72	
BG075159	Glutaminase, kidney isoform, mitochondrial precursor	1.77			0.88	
L31609	Ribosomal protein S29	1.05	1.08	1.18	1.06	0.99
BG078820	Platelet-derived growth factor receptor, alpha polypeptide			2.78		
AI893411	Zinc-finger protein 36/tristetraprolin	1.75				
BG071162	FADD			1.66		
AW543818	Cofactor required for Sp1 transcriptional activation			0.9		
BG070196	c-fos	2.14				
AI428862	Solute carrier family 22, member 2	1.79				
AI451894	Cyclin G2		0.69	1.86		
AI323829	Cytochrome P450, 2e1	7.48	0.26	0.25	0.01	1e-4
BG070044	Telokin; myosin, light polypeptide kinase	1.11				0.83
AI452085	Tyrosine kinase TYK2			1		
BG086677	<i>Homo sapiens</i> sentrin/SUMO-specific protease (SENP7)					0.73
BG083405	Acetyl-Coenzyme A acetyltransferase 1		0.9			
AI323672	Gamma-glutamyl transferase 1			0.27	1.47	
J00423	Hypoxanthine guanine phosphoribosyl transferase				0.89	
AI449384	Sialyltransferase 8 (alpha-2,8-sialyltransferase) D		0.29			
BG087731	Golgi vesicular membrane trafficking protein p18				0.85	
AW539497	SEC22 vesicle trafficking protein-like 1 (<i>S. cerevisiae</i>)				1	
AW556797	Protein kinase, lysine deficient 4				0.65	
BG088454	Heterogeneous nuclear ribonucleoprotein U				0.83	
BG069460	Hermes; RNA binding protein gene with multiple splicing		0.76			
BG088461	p75NTR-associated cell death executor (Nade)		0.81	0.26		
BG080936	ADP-ribosylation factor GTPase activating protein 3	0.86				
BG071624	Beta-defensin 3 (Bd3)				0.73	
M10624	Ornithine decarboxylase, structural				1.28	
BG077018	Eukaryotic translation initiation factor 1A					0.82
BG083267	Eukaryotic translation initiation factor 2, subunit 3					0.94
BG088134	Eukaryotic translation initiation factor 3, subunit 7 (zeta)			1.2		
AI427491	Ubiquitin specific protease 22					0.78
AA409615	Proteasome 26S subunit, non-ATPase, 12	0.79				

any of the time points, indicating that *F. tularensis* displays a 'stealth'-like behaviour.

Other experimental models of infection have been analysed with large-scale microarray analysis, and a few studies have observed a similar restricted transcriptional response to infection. For example, infection of murine RAW 264.7 macrophages with *Brucella abortus* resulted in the identification of 140 genes out of some 6000 that were found to be differentially transcribed (Eskra *et al.*, 2003). Also, in *Trypanosoma cruzi*-infected fibroblasts, a minimal transcriptional

response was observed, and only 112 out of 27 000 genes were significantly regulated up to 24 h of infection (Vaena de Avalos *et al.*, 2002). Similarly, fibroblasts infected with *Toxoplasma gondii* responded by a differential regulation of <1 % of the genes during the first 2 h (Blader *et al.*, 2001). At 24 h, approximately 2.6 % of the genes were affected. In contrast to the modest transcriptional responses observed in these models, some 13 % of the genes were differentially regulated in murine RAW 264.7 macrophages at 4 h in response to an infection with *S. typhimurium* (Rosenberger *et al.*, 2000). Interestingly, a very similar picture was

Table 3. LDH release in *F. tularensis* LVS-infected J774 cell cultures

Cells were treated with 1 mM NMMLA or 0.5 mM NAC for 60 min then infected with *F. tularensis* LVS at an m.o.i. of 500. Extracellular bacteria were removed by washing and cells were incubated for the indicated time in medium containing NMMLA or NAC. The experiment shown is representative of three experiments.

Time (h)	Treatment		
	None	NMMLA	NAC
6	0.303 ± 0.049*	0.193 ± 0.020	0.219 ± 0.022
15	0.611 ± 0.023	0.485 ± 0.065†	0.490 ± 0.059†

*Data are presented as mean ± SEM A_{492} based on eight wells.

† $P < 0.05$ compared to control cell cultures.

observed when purified *S. typhimurium* LPS was used in the latter study. This implies that the very potent effects of enterobacterial LPS may be a key catalyst of the host transcriptional response to infection with bacteria that possess LPS of this type. In contrast, the LPS of *F. tularensis* is distinct from enterobacterial LPS and has very weak pro-inflammatory activity (Sandstrom *et al.*, 1992; Vinogradov *et al.*, 2002). Another factor that in a very important way affects the outcome of the host response is the origin of the eukaryotic cells. The response of the murine macrophage cell line used in the present study may differ in many ways from that seen when monocytic cells of human origin are used.

Collectively, the results suggest that *F. tularensis* infection confers an oxidative stress upon the target cells, and many of the host-defence mechanisms appear to be related to counteracting the oxidative stress and mobilizing the energy needed. When antioxidants were added to the infected cells, a decrease of the cytopathogenic response was observed,

Table 4. Growth of *F. tularensis* LVS in J774 cells with NMMLA or NAC

Cells were treated with 1 mM NMMLA or 0.5 mM NAC for 60 min then infected with *F. tularensis* LVS at an m.o.i. of 500. Extracellular bacteria were removed by washing and cells were incubated for the indicated time in medium containing NMMLA or NAC. The experiment shown is representative of three experiments.

Time (h)	Treatment		
	None	NMMLA	NAC
0	5.33 ± 0.07*	5.29 ± 0.14	5.40 ± 0.08
6	4.91 ± 0.16	4.73 ± 0.12	4.75 ± 0.07
15	6.36 ± 0.08	5.84 ± 0.06†	5.72 ± 0.07†

*Data are presented as mean ± SEM log₁₀(c.f.u. per well) based on five wells.

† $P < 0.05$ compared to control cell cultures.

indicating that the production of oxidative species contributes to the cytopathogenic effect. Another striking feature was the relatively low numbers of regulated genes detected, and in particular, a conspicuous lack of an inflammatory response that most likely is dependent on *F. tularensis*-specific mechanisms. Based on the results, it seems logical to investigate whether activation of inflammatory responses in the infected cells will result in control of the infection.

ACKNOWLEDGEMENTS

Grant support was obtained from the Swedish Medical Research Council, the Medical Faculty, Umeå University, Umeå, Sweden, and DARPA, USA.

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