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A Microarray Analysis of the Host Response to Infection with *Francisella tularensis*

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CONTENTS

ABSTRACT

Francisella tularensis is a gram-negative bacterium that is the cause of the serious and sometimes fatal disease, tularemia, in a wide range of animal species and in humans. The response of cells of the mouse macrophage cell line J774 to infection with *Francisella tularensis* LVS was analyzed by means of a DNA microarray. It was observed that the infection conferred an oxidative stress upon the target cells and many of the host defense mechanisms appeared to be intended to counteract this stress. The infection was characterized by a very modest inflammatory response.

Tularemia caused by inhalation of *F. tularensis* subspecies *tularensis* is one of the most aggressive infectious diseases known. We used the mouse model to examine in detail the host immune response in the lung. After an aerosol challenge all mice developed clinical signs of severe disease, showed weight loss by day four of infection, and died the next day. Gene transcriptional changes in the mouse lung samples were examined on day one, two, and four of infection. Genes preferentially involved in host immune responses were activated extensively on day four but on day one and two, only marginally or not at all. Several genes upregulated on day four are known to depend on IFN-γ or TNF-α for their regulation. In keeping with this finding, TNF-α and IFN-γ levels were found to be increased significantly in bronchoalveolar lavage on day four.

We undertook an analysis of the transcriptional response in peripheral blood during the course of ulceroglandular tularemia by use of Affymetrix microarrays. Samples were obtained from seven individuals at five occasions during two weeks after the first hospital visit and convalescent samples three months later. In total 265 genes were differentially expressed. The most prominent changes were noted in samples drawn on days 2-3 and a considerable proportion of the upregulated genes appeared to represent an IFN-γ-induced response and also a pro-apoptotic response. Genes involved in the generation of innate and acquired immune responses were found to be downregulated, presumably a pathogen-induced event. A logistic regression analysis revealed that seven genes were good predictors of the early phase of tularemia.

Recently, a large number of methods for the analysis of microarray data have been proposed but there are few comparisons of their relative performances. We undertook a study to evaluate established and novel methods for filtration, background adjustment, scanning, and censoring. For all analyses, the sensitivities at low false positive rates were observed together with a bias measurement. In general, there was a trade off between the analyses ability to identify differentially expressed genes and their ability to obtain unbiased estimators of the desired ratios. A commonly used standard analysis using background adjustment performed poorly. Interestingly, the constrained model combining data from several scans resulted in high sensitivities. For experiments where only low false discovery rates are acceptable, the use of the constrained model or the novel partial filtration method are likely to perform better than some commonly used standard analyses.

PAPERS IN THESIS

This thesis is based on the following papers, which will be referred to in the text by their roman numerals.

I. Patrik Rydén, Henrik Andersson, Mattias Landfors, Linda Näslund, Blanka Hartmanová, Laila Noppa, and Anders Sjöstedt

Evaluation of microarray data normalization procedures using spike-in experiments. Manuscript

II. Henrik Andersson, Blanka Hartmanová, Patrik Rydén, Laila Noppa, Linda Näslund, and Anders Sjöstedt

A microarray analysis of the host response to intracellular infection with *Francisella tularensis* LVS. Manuscript

III. Henrik Andersson, Blanka Hartmanová, Rhonda KuoLee, Patrik Rydén, Wayne Conlan, Wangxue Chen, and Anders Sjöstedt

Transcriptional profiling of host responses in mouse lungs following aerosol infection with type A *Francisella tularensis*. J. Med. Microbiol. 2006; 55: 263-271

IV. Henrik Andersson, Blanka Hartmanová, Erik Bäck, Henrik Eliasson, Patrik Rydén, and Anders Sjöstedt

Transcriptional profiling of the peripheral blood response during tularemia. Manuscript

SAMMANFATTNING

Francisella tularensis är en gramnegativ bakterie som orsakar den allvarliga och ibland dödliga sjukdomen tularemi (harpest) hos olika djurslag och även hos människor. Vi studerade immunsvaret i den murina makrofagcellinjen J774 vid infektion med *F. tularensis* LVS med hjälp av microarrayanalys. Infektionen orsakade en oxidativ stress i värdcellerna och många av försvarsmekanismerna verkade syfta till att motverka denna stress. Utmärkande för infektionen är det marginella inflammatoriska svaret.

Tularemi orsakad av inhalering av *F. tularensis* subsp. *tularensis* är en av dom mest aggressiva infektionssjukdomar som finns. Vi studerade immunsvaret i lunga i den experimentella musmodellen. Efter att ha inandats bakterier utvecklade alla möss kliniska tecken på allvarlig sjukdom, viktminskning vid dag fyra, och dog dagen efter. Vi undersökte genuttrycket i lungan och fann att gener involverade i immunsvaret var aktiverade vid dag fyra, men knappt eller inte alls vid dag ett och två. Många gener som var uppreglerade vid dag fyra är reglerade av IFN-γ eller TNF-α. Vi fann också en ökad mängd av dessa två cytokiner i lungan vid dag fyra.

Vi studerade genuttrycket i blod från patienter med ulceroglandulär tularemi med hjälp av Affymetrix microarrayer. Blodprover togs från sju patienter vid fem tidpunkter under två veckor efter första läkarbesöket samt ett konvalescentprov tre månader senare. Vi fann 265 differentiellt uttryckta gener. Dom största förändringarna noterades vid dag 2-3. En stor andel av dom uppreglerade generna visade på ett IFN-γ-inducerat samt pro-apoptotiskt svar. Flera gener involverade i immunförsvaret var nedreglerade, detta är antagligen inducerat av bakterien. Med logistisk regressionsanalys fann vi sju gener som var bra markörer för tidig tularemi.

På senare tid har många metoder för analys av microarraydata publicerats men få jämförelser har gjorts av hur väl dom fungerar. Vi utförde en utvärdering av existerande och nya metoder, där vi jämförde metodernas känslighet och skattade värden. Generellt sett var där en kompromiss mellan metodernas förmåga att identifiera differentiellt uttryckta gener och att presentera väntevärdesriktiga skattningar. En vanligt använd standardmetod med bakgrundskorrigering fungerade dåligt. Den s.k. “constrained model” som kombinerar data från flera skanningar visade hög känslighet. Denna metod samt den nya metoden “partial filtration” visade sig fungera bättre än dom vanligt använda standardmetoderna.

ABBREVIATIONS

ACP	Acid phosphatase
BAL	Bronchoalveolar lavage
bp	Base pairs
CFU	Colony forming units
CM	Constrained model
DC	Dendritic cells
DE	Differentially expressed
FDR	False discovery rate
FPR	False positive rate
H_2O_2	Hydrogen peroxide
IFN	Interferon
Igl	Intracellular growth locus
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IRF	Interferon regulatory factor
LBP	LPS binding protein
LD_{50}	Lethal dose 50%
LPS	Lipopolysaccharide
LVS	Live vaccine strain
MAPK	Mitogen-activated protein kinase
MHC	Major histocompatibility complex
NDE	Not differentially expressed
NO	Nitric oxide
$ONOO^-$	Peroxynitrite
PAMP	Pathogen-associated molecular pattern
phox	Phagocyte oxidase
PMT	Photomultiplier tube
PRR	Pattern-recognition receptor
Q-PCR	Quantitative Realtime PCR
RLS	Restricted linear scaling
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
TIR	Toll/IL-1R homology
TLR	Toll-like receptor
TNF	Tumor necrosis factor

INTRODUCTION

Microarray

About the technology

The microarray technology is a fairly new technique for measuring the relative gene expression in two or more samples. It has revolutionized the research area on gene expression. The technique was first published by Mark Schena and Pat Brown in Science 1995 (Schena *et al.*, 1995). Since then, the number of publications involving microarrays has increased exponentially (fig. 1).

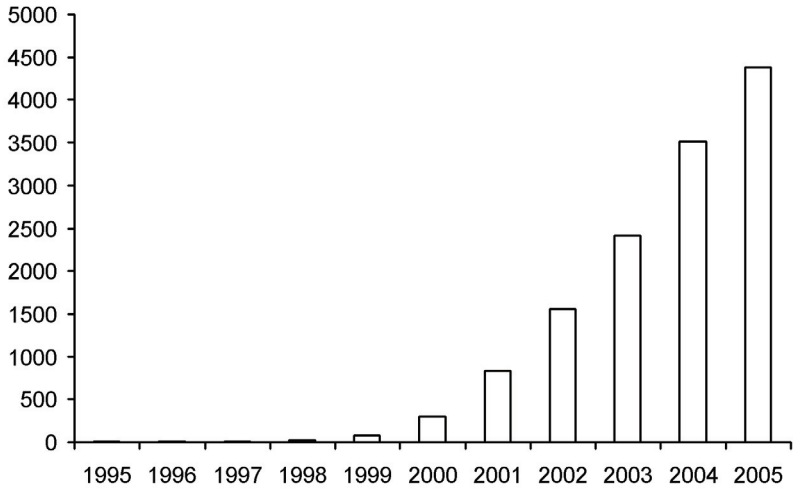

Fig. 1. Increase in number of microarray publications. Bars represent a PubMed search for the word "microarray".

Traditional hybridization assays developed in the 1970s utilize nitrocellulose or nylon membranes and radiolabeled probes (Benton & Davis, 1977; Grunstein & Hogness, 1975), where a handful of genes can be analyzed simultaneously. In a microarray experiment, cDNA (250-5000 bp) or oligonucleotides (25-75 bp) corresponding to genes of interest are attached to a solid support, usually glass. Then RNA from the samples of interest (cell cultures, tissues, blood, with or without treatment) is reverse transcribed into cDNA, and possibly transcribed to cRNA, incorporating a fluorescent

dye. The labeled cDNA (cRNA) is then hybridized to the array. After a stringent wash the array is scanned in a confocal microscopy scanner.

The advantage of microarrays over traditional hybridization assays is that the solid surface is non-porous and non-flexible enabling the deposition of small amounts of material in a precisely defined location, faster hybridization kinetics, smaller reagent volumes and more concentrated samples. The miniaturization allows for parallel study of tens of thousands of genes in a single assay, for example all open reading frames in the human genome. However, the complexity of such a large analysis makes microarray more of a screening method. The candidate differentially expressed genes should always be verified by a second method, for example quantitative real-time PCR (Q-PCR).

There are three major platforms for microarrays. In cDNA arrays each gene of interest is cloned, amplified by PCR and the purified product is printed, or "spotted", onto specially coated microscope slides using a high-precision three-axis robot. In spotted oligonucleotide arrays, the cDNA above is replaced with 30-75 bp oligonucleotides designed and synthesized individually for each gene. In the third platform, patented by the Affymetrix corporation, 25 bp oligonucleotides are built directly on the array, using photolithography.

Data analysis

In the late 1990's there were very few and expensive commercial arrays available, too costly for academic researchers. Therefore most researchers produced their own arrays. The lack of standardized and optimized methods for array manufacturing and hybridization led to production of arrays and results of varying quality. Today most microarray experiments are performed with standardized laboratory procedures. However, the data analysis field still lacks a "gold standard". This makes it difficult to judge the quality of microarray results and to make comparisons between different studies.

Regardless of platforms, microarray data are noisy due to the co-existence of genuine biological variation and experimental noise. Noise can arise from any step; sample

preparation, labeling, hybridization or scanning. Moreover, noise can hide meaningful biological signal and seriously skew the results of downstream data analyses. Therefore, it must be minimized to ensure the accuracy of downstream studies. In order to do so, many normalization strategies have been proposed and evaluated, most have been for data obtained from cDNA microarrays and from Affymetrix GeneChips. Because of differences in array designs, methods that perform well for one platform may not work equally well for the other. It is therefore necessary to validate normalization methods for each platform. The methods used for transforming raw data to normalized log-ratios make up a normalization procedure; it can for example involve different types of filtration, background adjustment, and channel adjustment.

Combined image analysis. Some highly expressed genes may give rise to saturated signals; signals that are higher than the maximum intensity than the scanner can detect. Many researchers choose to adjust scanner settings so that only a fraction of the genes give saturated signals. The tradeoff of using such low settings is that many low expressed genes will have intensities that cannot be separated from the background noise. Dudley *et al* (Dudley *et al.*, 2002) suggested using linear regression to combine data from different scanner sensitivity settings. In this way the linear range of the experiment is increased and stronger and more robust signals can be obtained from low expressed genes without getting saturated intensities from high expressed genes. Consequently, the number of genes that can be evaluated in a single experiment increases. An alternative method for combining data from several scans at different scanner sensitivity settings called the constrained model (CM) was published in 2004 by Bengtsson *et al* (Bengtsson *et al.*, 2004).

Filtration. The image analysis software cannot identify all spots on an array. This can be due to errors in array production so that no DNA was attached, or more commonly because the corresponding gene was not expressed in the samples hybridized to the array. These spots are flagged by the software as not found. Data from these spots can be treated in three different ways; removed from further analysis (complete filtration), left in and treated as data from spots that were found (no filtration), or treated with a novel method we propose in Paper I called partial filtration. In partial filtration the data are treated as missing values during normalization but prior to calculating test-statistics the spots' log-ratios are set to zero. This is based on the assumption that a non-expressed gene is not differentially expressed and can be regarded as having a log-ratio equal to zero.

Background adjustment. One of the biggest problems in the analysis of microarray data is that they are plagued with inhomogeneous backgrounds. On a microarray slide, the measured fluorescence intensity of a spot is a combination of the image background intensity and the intensity determined by the hybridization level of the mRNA samples with the spotted DNA. Background adjustment is necessary to estimate the true hybridization level of the cDNA. The existence of inhomogeneous background can make this task very difficult. Besides the spot intensities, the image analysis also measures the intensities from areas surrounding the spots, so called background intensities. Different methods have been developed to correct for microarray background. Global background adjustment uses the mean or median intensity of the observed background intensities to estimate the background for all spots and consequently it is seldom used in real applications with inhomogeneous backgrounds. Local background adjustment subtracts the spots' background intensities from their foreground intensities. Recently, researchers have suggested that local background adjustment introduce noise to the data, and that this type of analysis should be avoided (Qin & Kerr, 2004). Other methods have been proposed combining global and local backgrounds using different algorithms, for example morphological opening used by the image analysis software Spot (http://spot.cmis.csiro.au/spot /doc/Spot.pdf) or the TV+L1 model (Yin *et al.*, 2005).

For low expressed genes the estimated background may be higher than the spot intensity, resulting in negative values after background adjustment. When working with a single cell type, for example a cell line, as much as 60-70% of the genes may not be expressed at all, or below the detection limit. For these genes the background-adjusted intensity is expected to be distributed around zero, with approximately half of the observations being negative. Since the next step in the analysis usually is log-transformation of the corrected intensities, these values are a dilemma. Genes that are switched on or off in an experiment, giving negative values in one sample but positive in the other, may be of great biological interest. Unfortunately, since these data cannot be log-transformed and subsequently normalized, they are usually excluded from further analysis.

Channel adjustment. This process aims to minimize systematic variation, caused by channel differences, for example caused by differences in labeling efficiency between the two fluorescent dyes, so that biological differences can be more easily

distinguished. Global normalization is the simplest type of channel adjustment, where a constant adjustment is used to force the distribution of the log-ratios to have a median of zero for each slide. The drawback of such a simple approach is that it does not take into account that dye biases can depend on overall spot intensities and spatial locations on the array. Yang *et al*. has proposed a method called print tip MA-lowess (Yang *et al.*, 2002). Today, this is one of the most widely used methods for normalizing cDNA array data. It is based on robust local regression, to accommodate different types of dye biases. In short, the MA-lowess method fits a smooth curve to the MA plot, where M is the average log-ratio and A is the average log intensity. By applying this normalization separately for each subgrid (each subgrid is printed by one pin in the spotter, hence the name print tip), a compensation for spatial differences is achieved.

Statistical tests. Traditional methods, such as t-tests, are not well suited for microarray data. Due to usually small number of replicates the means are easily thrown off by outliers. Furthermore, the t-statistic for some genes may be large by chance due to very small sample variances. Lönnstedt *et al* (Lönnstedt & Speed, 2002) presented the B statistic, an empirical Bayes method which combines the information from all genes in the experiment to estimate prior distributions of the genewise variances. A posterior odds ratio B is then calculated for each gene to decide whether it is differentially expressed. The B statistic is more powerful than averages and t-statistics. It provides a ranking of how likely it is that the genes are differentially expressed but it has a drawback in that it does not produce p-values, and therefore it is difficult to determine a cut-off value.

Francisella

History and classification

Francisella was first isolated by McCoy and Chapin from ground squirrels and rats in Tulare County, California (McCoy & Chapin, 1912). It was renamed several times but was given its current name in honor of Dr. Edward Francis, a microbiologist who

extensively studied the pathogenesis of *Francisella tularensis* and revealed the basic facts about diagnostic methods and clinical forms of tularemia. Inoculation or inhalation of less than 10 bacteria may cause disease (Saslaw *et al.*, 1961a; Saslaw *et al.*, 1961b). Due to this extreme virulence and the ease of aerosol transmission, *F. tularensis* was used in the biological weapons programs in several countries (Kortepeter & Parker, 1999). According to the CDC classification, *F. tularensis* is a category A biological agent (Khan *et al.*, 2000). These organisms are considered to be the most likely biological warfare agents.

Francisella is the only recognized genus of the family *Francisellaceae* (Sjöstedt, 2005b). There are two known species; *F. tularensis* and *F. philomiragia*. *F. tularensis* has four subspecies; *tularensis*, *holarctica*, *mediasiatica* and *novicida*. They differ in virulence, certain biochemical properties and geographical distribution. *F. tularensis* subsp. *tularensis* is the most virulent subspecies with an LD_{50} in rabbits of less than 10 and it is found almost exclusively in North America. It has been found only once in Europe. Seventeen strains isolated from mice and fleas in Slovakia, were classified as *F. tularensis* subsp. *tularensis* (Gurycova, 1998). *F. tularensis* subsp. *holarctica* is distributed throughout the whole Northern hemisphere. It has been found in every European country except Portugal, Iceland and Great Britain (Tärnvik *et al.*, 2004). It is less virulent with a LD_{50} in rabbits of more than 10^6 CFU. Subsp. *tularensis* and *holarctica* are often designated as type A and type B respectively, division proposed by Jellison (Sjöstedt, 2005b). Most cases of human or animal tularemia are caused by these two species. *F. tularensis* subsp. *mediasiatica* and *novicida* are rarely associated with human disease. Subsp. *mediasiatica* is endemic in the Central Asian region of former USSR and *novicida* is mainly found in North America. *Novicida*-like subspecies was recently isolated in Australia from a foot-wound (Whipp *et al.*, 2003), which indicates a wider distribution of this bacterial species than was previously thought.

Francisella is a short, gram-negative, coccoid or rod-shaped, facultative intracellular bacterium. Its length varies from 0,2-0,7 x 0.2 μm for subspecies *tularensis*, *holarctica* and *mediasiatica*, when they are in logarithmic growth phase in liquid medium. *F. philomiragia* and *F. tularensis* subspecies *novicida* are slightly bigger; 0.7 x 1.7 μm. Bacteria during stationary growth phase or in infected tissues are pleomorphic with elongated or filamentous cells. Cells of virulent strains are covered with a capsule. Under the capsule is an outer layer surrounding double-layered cell wall. The capsule,

as well as the cell wall, has an unusually high lipid concentration for gram-negative bacteria. *Francisella* has a unique composition of long-chain fatty acids. *F. tularensis* lipopolysaccharide (LPS) has almost no biological activity except of the ability to activate complement. LPS low toxicity is most likely due to its tetraacylation of lipid A (Vinogradov *et al.*, 2004).

The live vaccine strain

In the former USSR, in the 1940's, a strain of *F. tularensis* subsp. *holarctica* was repeatedly passaged on agar and an attenuated strain, consisting of two variants, was isolated. The more virulent variant was standardized for use as a human vaccine at the US Army Research Institute of Infectious Diseases, Fort Detrick (Eigelsbach & Downs, 1961). It was designated as the live vaccine strain (*F. tularensis* LVS) and was used as vaccine against tularemia until the early 1990's. However, since the attenuation is not defined it is no longer licensed as a human vaccine. *F. tularensis* LVS is attenuated in humans but still relatively virulent in mice, where it causes a disease resembling human tularemia (Elkins *et al.*, 2003). This has made it a very important laboratory strain for studying tularemia since it can be used outside a biosafety level 3 facility. The intraperitoneal LD_{50} is one bacterium but after intradermal challenge the LD_{50} is many logs higher than for type A or type B strains (Anthony & Kongshavn, 1987).

Tularemia

Francisella is one of the most infectious bacteria known (Petersen & Schriefer, 2005). Tularemia can be acquired via several routes and the clinical presentation depends on the route of entry (Tärnvik & Berglund, 2003). It is often transmitted after direct contact with infected animals or their products, especially rabbits and hares. It can also develop after inhalation of contaminated air, ingestion of contaminated food or water or bites with blood-sucking arthropods such as ticks and mosquitoes. It cannot be transmitted from man to man. Farming and hunting activities but also cutting grass and gardening are risky with respect to possible sources of infection (Ellis *et al.*, 2002;

Hornick, 2001; Jensen & Kirsch, 2003). Inhalation of *F. tularensis* gives rise to the most aggressive form of tularemia, the respiratory form that in both animals and humans is associated with considerable mortality (Dennis *et al.*, 2001). Transmission by arthropods causes the ulceroglandular form of the disease and it may also result after direct contact with infected animals. In European countries, the ulceroglandular form is the predominant variant of the disease and in Sweden it constitutes >90% of the cases (Tärnvik & Berglund, 2003). A third, rather rare form is oropharyngeal tularemia, which occurs after oral ingestion of contaminated food or water. Another unusual form of the disease is the oculoglandular, which leads to conjunctivitis and photophobia. Incubation period for tularemia is usually 3-5 days, but it may vary from 1–21 days.

Strains of *F. tularensis* subsp. *tularensis* are the most virulent. Mortality of tularemia caused with this subspecies was 5-30% before antibiotic treatment was introduced. Today it has declined to 1-2% (Sjöstedt, 2005b; Tärnvik & Berglund, 2003). *F. tularensis* subsp. *holarctica* causes a milder version of tularemia, which is rarely fatal. Tularemia-like disease can develop also after infection with *F. tularensis* subsp. *novicida* and *mediasiatica*. *F. philomiragia* rarely causes human disease, mostly in immunocompromised patients and people close to dying from drowning (Sjöstedt, 2005b).

Tularemia characteristically presents as an acute illness with non-specific flu-like symptoms such as high fever, chills, general body ache, fatigue, headache and nausea. Dry cough and sore throat often develop regardless of route of infection (Tärnvik & Berglund, 2003).At the site of infection, a primary ulcer develops, often from a papule into a pustule (Eliasson *et al.*, 2005). The ulcer is sometimes inconspicuous and usually heals within a week and can be mistaken for an arthropod bite. Although the ulcer heals, the draining lymph nodes enlarge, become palpable and tender, and if proper antibiotic treatment is not instituted within the first two weeks of infection, severe enlargement and sometimes suppuration ensues. A few days after inoculation the bacteria disseminate to liver and spleen. The bone marrow and lung are only colonized to a minor extent and other tissues or organs are free of bacteria. Besides these clinical manifestations, little is known about other aspects of the disease. Generally, changes in peripheral blood are inconspicuous as evidenced by small

increases in mean leukocyte counts and, moreover, mean peak C-reactive protein values are remarkably low for an invasive disease (Syrjala, 1986).

F. tularensis is susceptible *in vitro* to aminoglycosides, tetracyclines, quinolones and chloramphenicol but resistant to β-lactam antibiotics. A protocol for prophylactic measures by medical workers was published by CDC (Dennis *et al.*, 2001). Treatment with aminoglycosides, doxycycline, or ciprofloxacin is recommended for patients.

Virulence and intracellular life of *Francisella tularensis*

F. tularensis behaves as an intracellular pathogen both *in vitro* and *in vivo*. Macrophages are believed to represent main cell type where it replicates. Molecular mechanisms used by *F. tularensis* to mediate its uptake into the host cell are in general unknown. However, it was published recently that *F. tularensis* enters the host cell by a novel process involving unique formation of asymmetric, spacious pseudopod loops in membrane of host cell (Clemens *et al.*, 2005). This process was strongly dependent on the presence of intact complement factor C3 in the serum and complement receptors. Internalization was inhibited by cytochalasin B, which indicates an important role for actin microfilaments. Molecular mechanism of engulfment is most likely mediated by molecules which does not require any bacterial metabolic activity, because both live and formalin-killed or protease-treated *F. tularensis* entered host cells via this process. A possible candidate for such a molecule is LPS. Although LPS from most pathogens is considered as a virulence factor, LPS from *Francisella* is far less potent. The ability of LPS to induce expression of TNF-alpha, IL-1 and NO from mononuclear cells demands 100 or 1000 times higher concentration than for LPS from *Salmonella. typhimurium*, *Bordetella pertussis* or *Escherichia coli* (Ancuta *et al.*, 1996; Sjöstedt, 2003).

After uptake by the host cell, *F. tularensis* first resides inside a phagosome. Other intracellular bacteria have developed different strategies for survival (Amer & Swanson, 2002). Some interfere with the maturation process of the phagosome to prevent the formation of the phagolysosome which has a much more hostile interior environment than the initial phagosome, *e.g. Salmonella* and *Mycobacterium*. Another strategy, used by for example *Listeria*, *Shigella* and *Rickettsia*, is to escape into the

cytoplasm. A third strategy, utilized by *Legionella*, *Brucella* and *Chlamydia*, is to hide inside a nonphagosomal compartment. Recent data suggest that *F. tularensis* is able to interfere with the maturation process of the phagosome to a phagolysosome, escape from the phagosome within a few hours after uptake and reside and multiply in the cytoplasm (Clemens *et al.*, 2004; Golovliov *et al.*, 2003). The mechanism behind this escape is not yet characterized, but may involve degradation of the phagosomal membrane (Golovliov *et al.*, 2003).

Lindgren *et al*. showed that expression of the protein denoted IglC (Golovliov *et al.*, 1997) is required for escape from the phagosome and multiplication of *F. tularensis* (Lindgren *et al.*, 2004a). IglC expression is upregulated when *F. tularensis* is inside macrophages (Golovliov *et al.*, 1997). It is part of an operon with four open reading frames denoted *iglABCD* (Nano *et al.*, 2004). The exact functions of IglC and D are unknown since they show no sequence similarities to any known protein. IglA and B showed a 30% identity to proteins involved in secretion in *Rhizobium leguminosarum*. MglA is a positive regulator of the *iglABCD* operon, and it is essential for *F. tularensis* survival in macrophages (Lauriano *et al.*, 2004).

Acid phosphatases (ACPs) have been implicated as virulence factors for intracellular pathogens through the suppression of respiratory burst (Reilly *et al.*, 1996). Properties of ACP in *F. tularensis* differs from other intracellular bacteria and ACP is not required for intracellular growth (Reilly *et al.*, 1996). *F. tularensis* genome contains type IV pili genes and *F. tularensis* LVS produces fibers similar to type IV pili (Gil *et al.*, 2004).

Electron microscopy analysis has shown that virulent *F. tularensis* has a capsule that is easily lost upon hypertonic conditions or aerosolization (Hood, 1977; Sandström *et al.*, 1988; Sorokin *et al.*, 1996). Stable capsule-deficient mutants have been created, one of which was avirulent in mice (Sorokin *et al.*, 1996). When ingested in polymorphonuclear leukocytes the mutant did not induce an antimicrobial response and survived in the cells. This indicates that the capsule is necessary for full virulence of *F. tularensis*.

Innate immune response and pathogen recognition

The innate immune system is the first line of defense against pathogens and it is mediated by phagocytes including macrophages, neutrophils and dendritic cells (DCs). Professional phagocytes are characterized by their ability to express pattern-recognition receptors (PRRs) that recognize, bind to and trigger internalization of pathogens. After internalization, microbes are located inside the phagosomes. If the microbes do not possess intracellular survival mechanism, the phagosome containing the microbe fuses with the lysosome where it is quickly killed (Hamrick *et al.*, 2000; Polsinelli *et al.*, 1994; Prada-Delgado *et al.*, 2001). PRRs recognize microbial components, known as pathogen-associated molecular patterns (PAMPs) that are essential for the survival of the microorganism and are therefore difficult for the microorganism to alter. Different PRRs react with specific PAMPs, show distinct expression patterns, activate specific signaling pathways, and lead to distinct antipathogen responses. Activation of PRRs lead to the expression of large numbers of antimicrobial molecules, which attack the pathogen by different mechanisms. The basic machineries underlying innate immune recognition are highly conserved among species, from plants and fruit flies to mammals.

A central group of PRRs is Toll-like receptors (TLRs). They are integral membrane glycoproteins that play a crucial role in recognition of and response to pathogens. In response to triggering PAMPs, TLRs activate signaling pathways, which lead to expression of antimicrobial molecules, cytokines and costimulatory molecules (Karin & Lin, 2002). To date, 12 members of the TLR family have been identified in mammals. They contain an extracellular domain that recognizes specific PAMPs, and an intracellular domain homologous to that of interleukin 1 receptor (IL-1R), termed the Toll/IL-1R homology (TIR) domain (Bowie & O'Neill, 2000). TLRs are expressed on various immune cells, including macrophages, dendritic cells (DCs), B cells, specific types of T cells, and even on nonimmune cells such as fibroblasts and epithelial cells. Expression of TLRs is not static but rather is modulated rapidly in response to pathogens, a variety of cytokines, and environmental stresses.

LPS is generally the most potent immunostimulant among the gram-negative bacterial cell wall components. It associates with LPS binding protein (LBP), an acute phase protein present in the bloodstream, and then binds to CD14 expressed on the cell surface of phagocytes. LPS is then transferred to MD-2, which associates with the extracellular portion of TLR4, followed by oligomerization of TLR4 (Poltorak *et al.*, 1998; Shimazu *et al.*, 1999). Consequently, TLR4 mutants are highly susceptible to infection by gram-negative bacteria. Components of Gram-positive bacterial cell walls can also stimulate innate immunity. Although these bacteria do not contain LPS, lipoteichoic acid (LTA) functions in a similar manner as an immune activator. Lipoproteins and peptidoglycan (PG), which are present in both Gram-positive and Gram-negative bacteria, are also potent immunostimulants. TLR2 plays a major role in detecting Gram-positive bacteria and is involved in the recognition of a variety of microbial components, including LTA, lipoproteins, and PG. Flagellin is the major protein constituent of bacteria flagella. TLR5 is responsible for the detection of flagellin. Bacterial genomic DNA is also an immunostimulant and is recognized by TLR9 (Hemmi *et al.*, 2000; Krieg, 2002). Its stimulatory effect is due to the presence of unmethylated nucleotides.

Stimulation of cells with a TLR ligand recruits adaptor proteins containing a TIR domain, such as myeloid differentiation factor 88 (MyD88), to the cytoplasmic portion of the TLRs through homophilic interaction of their TIR domains. This results in the triggering of downstream signaling cascades, including NF-κB and MAP kinases. NF-κB regulates the expression of many molecules involved in host defense including cytokines, immunoreceptors, cell adhesion, and inducible nitric oxide synthase (iNOS) (Hatada *et al.*, 2000). NF-κB can activate the expression of TNF-α and initiate its secretion. It can also activate expression of IL-1β and induce respiratory burst. MAP kinases are responsible for activating transcription factors that stimulate the synthesis of various inflammatory proteins including TNF-α, IL-1β, IL-6 and iNOS (Chen *et al.*, 1999).

TLRs recognize pathogens at either the cell surface or lysosome/endosome membranes, suggesting that the TLR system is not used for the detection of pathogens that have invaded the cytosol. NOD-LRR proteins are implicated in the recognition of cytosolic bacteria. Proteins in this family possess LRRs (leucine rich repeat) that mediate ligand sensing, a nucleotide binding oligomerization domain (NOD) and a

domain for the initiation of signaling. NOD1 and NOD2 can detect bacterial peptidoglycans and ligand binding results in NF-κB activation.

The complement system is a major driver of our innate immune response and plays an important role in defense against foreign pathogens. It is composed of a large number of proteins that are found in the circulation, in tissues and other body fluids in pro-enzymatic form. Together with phagocytic cells, the complement system is a powerful tool to defend the host against foreign attack. The complement system itself harbors major protective activities against pathogens, by its direct cytolytic, chemotactic, anaphylactic and opsonic activities. By these properties, it is optimally suited to play a major role in the induction of the acquired immune system by bringing antigens in direct and optimal contact with the major players, B and T cells.

The role of ROS and RNS

After uptake of a pathogen, professional phagocytes can produce reactive oxygen species (ROS) and reactive nitrogen species (RNS). They are highly active substances that mediates killing of the pathogen. The highest concentrations can be found inside the phagosomes, but lower levels are present in the cytosol and extracellularly.

ROS can kill pathogens in several ways. They can oxidize DNA, react with unsaturated fatty acids in membranes and thereby destabilizing the lipid bilayer, affect ionic channels, membrane enzymes and transport proteins (Kehrer, 2000). ROS can also alter signaling pathways in the cell, for example MAP kinase and JAK/STAT pathways, thereby modifying the activity of growth factors, protein kinases, protein phosphatases and transcription factors (Kehrer, 2000).

Phagocyte oxidase (phox) is an important source of ROS. It catalyzes the production of large amounts of superoxide. Superoxide rapidly dismutates to form H_2O_2. This can also occur enzymatically by superoxide dismutase (SOD). H_2O_2 is more reactive than superoxide. Phox consists of five constitutively expressed subunits that assembles upon activation (Babior *et al.*, 2002). Two subunits are membrane spanning and the superoxide is formed in certain intracellular compartments such as phagosomes or

extracellularly. Phox is activated through various receptors, for example IgG and CR3 (Shao *et al.*, 2003), and by IFN-γ (Vazquez-Torres *et al.*, 2000).

Xanthine oxidoreductase (XOR) plays a role in host resistance against pathogens (Segal *et al.*, 2000). It is upregulated by proinflammatory cytokines (Umezawa *et al.*, 1997). XOR can reduce O_2 to generate superoxide. It has also been reported to simultaneously generate NO and superoxide under limited oxygen tension, leading to the formation of peroxynitrite ($ONOO^-$) (Harrison, 2002). The highest levels of XOR are found in liver and intestine, but it is present at low levels in all organs.

The mitochondria are another source of superoxide. A side reaction to the respiratory chain has been shown to produce superoxide (Cadenas & Davies, 2000). The release of ROS from mitochondria is increased by NO and TNF-α respectively (Cadenas *et al.*, 2001). Monoamine is another source of ROS in the mitochondria (Cadenas & Davies, 2000). It catalyzes the production of hydrogen peroxide (H_2O_2) and this contributes to the increase in steady state concentration of ROS in the mitochondria and cytosol.

The killing of many pathogens depends on RNS (Bogdan *et al.*, 2000). An immunologically important source of NO is inducible nitric oxide synthase (iNOS). iNOS expression is regulated by cytokines, immune complexes and microbial products (Bogdan, 2001). The activity of iNOS can be regulated at the transcriptional, mRNA or protein level. NF-κB and Stat1-α are critical transcriptional regulators of iNOS. IFN-γ stimulation induces upregulation of iNOS through the activation of Stat1-α.

At low concentrations NO can influence a variety of cellular processes. It is thought to be anti-apoptotic, terminate lipid peroxidation induced by ROS and to act as a pro-inflammatory molecule (Bogdan, 2001). At high concentrations NO is rapidly oxidized to form other RNS (Wink & Mitchell, 1998). N_2O_3 is the major product in an environment with low concentrations of ROS. N_2O_3 nitrosylates amines and thiol groups in proteins. Nitrosylation of bacterial proteins involved in metabolism, respiration or with antioxidant properties might inhibit their function and thereby affect the viability of the bacteria. At high concentrations of both NO and superoxide, such as in activated macrophages, $ONOO^-$ will be the major RNS produced. $ONOO^-$ is a powerful oxidant and nitrant. $ONOO^-$ and derivatives thereof nitrosate tyrosine and tryptophan residues and oxidize methionine in proteins, and similar to nitrosylation of

proteins, this may decrease the viability of bacteria (Radi, 2004). Nitration is an irreversible modification in contrast to nitrosylation and is therefore a more toxic event (Mannick & Schonhoff, 2002). Lipid peroxidation is another effect induced by $ONOO^-$ that disrupts the membrane integrity of the cell and thereby enhances the access of toxic radicals to the interior of the bacterial cell (Bogdan, 2001). Further, $ONOO^-$ damages DNA.

At an early stage of infection, NO acts as a pro-inflammatory molecule, inducing vasodilation and the recruitment of neutrophils (Bogdan, 2001). NK-cell development and cytotoxicity also partly rely on NO (Cifone *et al.*, 2001). But RNS also acts as a suppressor of the immune response. For example, RNS inhibit T cell proliferation and induces apoptosis of T cells since lymphocytes have a minimal capacity to replenish their glutathione content (Kroncke *et al.*, 2001). High concentrations of NO down-regulate the expression of different adhesion molecules and thereby inhibit the recruitment of inflammatory cells to the infection site (Bogdan, 2001).

Response to intracellular pathogens

Antigens from intracellular pathogens residing in the cytosol are transported to the cell surface and presented by MHC class I molecules. They are recognized by $CD8^+$ T cells. $CD8^+$ T cells release perforin which creates holes in the antigen presenting cells membrane, granzymes which are proteases that act intracellularly to trigger apoptosis, and often IFN-γ. A membrane-bound effector molecule expressed on $CD8^+$ T cells is Fas ligand. It binds to Fas on the antigen-presenting cell and induces apoptosis.

Antigens from intracellular pathogens residing in intracellular compartments, such as phagosomes, are presented on the cell surface by MHC class II molecules that are recognized by $CD4^+$ T cells. There are two functional types of $CD4^+$ T cells; T_H1 and T_H2. IL-12 is a key cytokine in driving the development of T_H1 cells. They are specialized to activate macrophages that are infected by a pathogen. T_H1 cells express membrane-bound CD40 ligand and/or Fas ligand and secrete IFN-γ. CD40 ligand together with IFN-γ activates the target cell to kill the pathogen, Fas ligand induces apoptosis of the infected cell. IFN-γ induces iNOS, limits of the availability of iron, upregulates MHC class II molecules and enhances endosomal trafficking that

promotes the formation of a phagolysosome and proper assembly of the phagocyte oxidase. T_H2 cells are specialized for B cell activation. T_H2 cells secrete B cell growth factors IL-4 and IL-5. The principal membrane-bound effector molecule expressed by T_H2 cells is CD40 ligand, which induces B cell proliferation.

NK cells are important in innate immunity to intracellular pathogens. NK cells are activated in response to interferons and macrophage-derived cytokines, such as IL-12. IL-12 in synergy with TNF-α can elicit the production of large amounts of IFN-γ by NK cells. This is crucial in controlling some infections before T cells have been activated to produce this cytokine.

Host immune response during tularemia

The ability of *F. tularensis* to multiply intracellularly has been well documented both *in vivo* and *in vitro* and in professional as well as non-professional phagocytes (Conlan & North, 1992; Golovliov *et al.*, 1995; Merriott *et al.*, 1961; Stenmark *et al.*, 1999).

TNF-α and IFN-γ are needed for immune mice to clear *F. tularensis* LVS infection at high inocula (Sjöstedt *et al.*, 1996) and they are critically needed during the early phase of a primary infection (Leiby *et al.*, 1992; Sjöstedt *et al.*, 1996). Murine macrophages activated by TNF-α and IFN-γ are able to kill phagocytosed *F. tularensis* (Fortier *et al.*, 1992). The viability of non-activated macrophages is less than 50% whereas IFN-γ treated cells are over 70% viable. IFN-γ, TNF-α, and IL-12p70 (composed of the subunits p40 and p35) are all produced during a primary infection. Mice deprived of either IFN-γ or TNF-α do not restrict the replication of the bacteria during the early phase of the infection (Sjöstedt *et al.*, 1996). The LD_{50} of TNF-α deprived mice is several thousand times lower compared to immunocompetent mice. IFN-γ gene-deficient mice succumb to even one single bacterium of *F. tularensis* LVS. IL-12p70- or IL12p40-deficient mice control the initial phase of the infection but are unable to eradicate the bacterium (Elkins *et al.*, 2002). In contrast, IL12p35-deficient mice clear the infection, indicating that clearance of *F. tularensis* LVS is dependent on the IL12p40 subunit rather than IL-12p70.

F. tularensis LVS infection of monocytic cells results initially in activation of intracellular signaling and cytokine secretion (Telepnev *et al.*, 2005). This includes increased phosphorylation of IκBα, cJun and p38. However, this initial activation is soon followed by subsequent downregulation. This downregulation is dependent on the *F. tularensis* protein IglC, since it is not observed during an infection with a Δ*igl*C mutant or heat killed bacteria. Infection with live or heat killed *F. tularensis* LVS or Δ*igl*C initially stimulates secretion of high levels of TNF-α, but while the TNF-α concentrations continuously increase in cells infected with the mutant strain or killed bacteria, there is a significant decrease in TNF-α concentrations in cells infected with live *F. tularensis* LVS (Telepnev *et al.*, 2005). Live but not heat killed *F. tularensis* or Δ*igl*C can also inhibit production of TNF-α and IL-1β in response to *E. coli* LPS or BLP stimulation (Telepnev *et al.*, 2003). Telepnev *et al*. showed that this inhibition was due to intracellular *F. tularensis* blocking multiple intracellular signaling pathways, including those involving NF-κB, cJun and p38.

iNOS-derived NO is a major mediator of IFN-γ induced killing of *F. tularensis*. IFN-γ-activated macrophages from $iNOS^{+/+}$ mice kill 97% of internalized bacteria within 6 hours, as compared to 28% by macrophages from $iNOS^{-/-}$ mice, or wild type macrophages treated with NMMLA, a competitive inhibitor of iNOS (Lindgren *et al.*, 2005). After 24 hours, macrophages from $iNOS^{-/-}$ mice have eradicated most of the bacteria. Phox-dependent mechanisms were shown to be partly responsible for this retained bactericidal activity. Furthermore, $ONOO^-$ was shown to play a major role in the IFN-γ-induced killing in macrophages, and iNOS-dependent NO production contributes significantly to the formation of $ONOO^-$. Phox is important for the control of murine tularemia. In $p47^{phox-/-}$ mice, the LD_{50} by intradermal infection was found to be almost 200-fold lower than in $p47^{phox+/+}$ mice (Lindgren *et al.*, 2004b). However, $p47^{phox-/-}$ mice are still able to restrict infection and eradicate the bacteria.

During the innate phase of *F. tularensis* LVS infection in mice, neutrophils suppress the replication and are critically required to resolve an infection with *F. tularensis* LVS (Sjöstedt *et al.*, 1994). The presence of either $CD4^+$ or $CD8^+$ T cells is sufficient for eradication of infection while the absence of both subsets renders the mice unable to clear the bacteria during the late phase of infection (Conlan *et al.*, 1994). The fact that mice depleted of $CD4^+$ T cells produce a poor IgG antibody response compared to infected wild-type mice, but still clear the infection, indicates that antibodies are of minor importance. Nevertheless, transfer of *F. tularensis*-specific antibodies to naïve

mice prior to challenge protects mice against an otherwise lethal dose of the *F. tularensis* LVS or a virulent strain of subsp. *holarctica* (Stenmark *et al.*, 2003). Moreover, B cells contribute to protection in an antibody-independent manner, for example by antigen presentation and secretion of cytokines and chemokines (Elkins *et al.*, 1999). Normally, mice clear a primary infection with *F. tularensis* LVS within 2-3 weeks after inoculation and then develop protective immunity to *F. tularensis* LVS. $CD4^+$ and $CD8^+$ T cells are required for protection only when the rechallenge dose of *F. tularensis* LVS exceeds a certain threshold, indicating that other mechanisms are also active in the protective immunity to *F. tularensis* LVS (Conlan *et al.*, 1994). $Thy1.2^+$ $CD4^-$ $CD8^-$ cells may contribute to the protection. The role of γ/δ T cells in primary infection appears to be minimal, however γ/δ T cell KO mice are slightly more susceptible to a secondary infection (Yee *et al.*, 1996). There is a large polyclonal expansion Vγ9Vδ2 T cells in the peripheral blood of tularemia patients, but not in the blood of individuals vaccinated with LVS (Poquet *et al.*, 1998).

AIMS OF THE THESIS

- To evaluate existing methods and develop new tools for the analysis of microarray data

- To characterize the events that occur inside cells infected with *Francisella tularensis* LVS

- To get a better understanding of the extreme virulence of type A *F. tularensis* strains during aerosol infection

- To study the pathogenesis of human tularemia and identify potential markers for the early phase of the disease

RESULTS AND DISCUSSION

Performance of different methods for microarray data analysis (Paper I)

A large number of methods for analysis of microarray data have been proposed, but the relative merits of these methods are generally not easy to assess. To evaluate the performance of different analysis procedures we used eight in-house produced microarrays with approximately 10,000 spots from 20 artificial genes, 4,000 of which transcripts were spiked into the mRNA before labeling at different concentrations, *i.e.* differentially expressed (DE) genes.

An analysis of microarray data involves a series of actions, for example; image analysis, filtration, background adjustment, merging data from several scans, dye normalization, treating negative intensities, censoring, obtaining test-statistics and determining the cut-off value. At each step there are a number of possible methods making the total number of possible analyses very large. We simultaneously evaluated seven scanning procedures, three filtration methods, two ways of background adjustment and four censoring approaches. The majority of these methods are well established, but we also introduced some novel methods. Altogether 252 analyses were evaluated.

Evaluation measures

It is necessary to introduce measures that can be used to characterize the analyzed data. Microarray experiments are often used as screening methods with the objective to select a sample of genes where most of the selected genes are DE, *i.e.* a sample with small *false discovery rate* (FDR). The FDR is the proportion of not differentially expressed (NDE) genes among the genes classified as DE. A gene is classified as DE if its test-statistic is above a user determined cut-off. Commonly, microarray experiments are used to study thousands of genes although only a small fraction of the genes are DE. The *false positive rate* (FPR) is the proportion of NDE genes that are falsely included in the selected sample. A very small FPR needs to be considered if the

goal is to have a relatively small FDR. *Sensitivity* is the proportion of DE genes that are correctly classified. The sensitivity at a fixed small FPR is one measure that can be used to rank the analyses. In Paper I we used a fixed FPR of 0.05%, 0.1% and 0.5%.

An analysis' ability to correctly estimate the ratios of the DE genes is another relevant measure. We introduce two measures, the *reflected bias* and the *selected bias.* Consider an experiment that contains r DE-genes, where the *k*th gene is replicated n_k times and has the true log-ratio Ω_k. The reflected bias is estimated by:

$$\hat{b}_{DE} = \frac{\sum_{k=1}^{r}\sum_{i=1}^{n_k} sign(\Omega_k)\left(\bar{M}_{ki} - \Omega_k\right)}{n_{DE}}$$

where $\bar{M}_{ki}$ are the average normalized log-ratios and where n_{DE} is the total number of DE-spots on the array. The selected bias is estimated similarly as the reflected bias although only genes classified as DE are used to estimate the bias. For some problems, the selected bias may be a more relevant measure than the reflected bias.

We postulate that the two most important properties are:

I. High sensitivity in conjunction with an acceptable FDR.

II. The expected values of the average normalized log-ratios of DE-genes should be close to the true log-ratios and the bias should be independent of the genes mRNA concentrations.

Although property I is generally the most important, the magnitude of the regulations can be important for understanding the biology. Combining results from studies using different techniques (*e.g.* cDNA-microarrays and Q-PCR) can be difficult if the estimates are biased. Furthermore, if the average normalized log-ratios do not have a strong linear relationship to the true log-ratios so that the bias is dependent on the genes' mRNA-concentrations (*e.g.* the bias is higher for low expressed genes than for high expressed genes) then results from high-level analyses such as clustering or classification can be misleading.

Combined image analysis

Combined image analysis was used to obtain data from the scanned images. The arrays were scanned at four settings (laser power/PMT): 70/70, 80/80, 90/90, and 100/100, where the numbers are percentages of maximum values. Here, the images from each of the lower scans (70, 80, or the 90 scan) were analyzed together with images from the 100 scan. For one array, additional image analyses were done in the "standard way" where images from scans were analyzed separately. The percentage of spots found by the two image analyses at the 70, 80, 90, and 100 scans were for the standard analysis 48%, 51%, 56%, and 59% and for the combined analysis 59%, 59%, 63% and 63%. This suggests that even if only data from a low scan will be used in further analyses, combined image analysis using additional images from a higher scan will give better spot finding and thereby improve the quality of the low scan data.

Scanning procedures

In addition to analyzing the data from each of the four scans separately we used restricted linear scaling (RLS), a slight modification of the linear scaling algorithm suggested by Dudley *et al*. (Dudley *et al.*, 2002), to combine the data from the 70, 80 and 90 scan and the 80, 90 and 100 scan respectively. We also used the constrained model (CM) with the 70 scan as baseline to combine data from all four scans. The 80 scan can be thought of as a standard scan since it was the highest scan where only a small fraction (< 0.2%) of the intensities were saturated.

The CM-procedure demonstrated on average the highest sensitivities followed by the 80 scan procedure, and the RLS-procedures ranked in the middle, while the remaining single-scan procedures had the lowest sensitivities. The CM-procedure was robust in the sense that it was able to obtain relatively high sensitivities for normalizations where most other scanning procedures performed poorly.

Filtration

Intensities from spots not found by the image analysis software were treated in three different ways:

I. **Complete filtration:** the intensities were treated as missing values.
II. **Partial filtration:** the intensities were treated as missing values during normalization, but prior to calculating test-statistics, the spot's log-ratios were set to zero.
III. **No filtration:** the intensities were treated as intensities from spots that were found.

Complete filtration is commonly used while partial filtration is a novel method. The idea behind partial filtration is that spots called "not found" commonly arise from genes that are not expressed in either channel and can therefore be regarded as NDE genes.

Normalization procedures using no filtration had generally relatively low sensitivities. Interestingly, normalization using partial filtration together with local background adjustment was able to obtain relatively high sensitivities for all scanning procedures. Normalizations using complete filtration had in many cases lower sensitivities than those using partial filtration. However, using the CM with complete filtration had the single highest sensitivity at the 0.05% FPR.

Background adjustment

The analyses did either not apply any background adjustment or applied the "standard" local background adjustment. Background adjustment had a large impact on the results, however, positive for some methods but negative for other. Methods using complete filtration worked better with no background adjustment, while methods using partial filtration benefited from using local background adjustment.

Censoring

A-spot intensities were censored such that all intensities below a user-defined censoring value λ were increased to this value. The censoring values 1, 8, 64, and 512 were used (1 equals no censoring). The idea behind censoring is to moderate the variance of the weakly expressed genes. It still remains to be determined how to select an optimal censoring value.

For analyses using background adjustment the use of optimal instead of no censoring resulted in considerable higher sensitivities. Arguably, using the 80 scan with complete filtration, local background adjustment and censoring value 64 had the overall best performance. Interestingly, this is the standard analysis method with additional censoring indicating that censoring can be very powerful.

Bias

Most analyses had a negative reflected bias, *i.e.* underestimated the magnitude of regulation, and produced ratios often less than 50% of the true ratio. Low expressed genes had generally higher bias than high expressed genes. Furthermore, the magnitude of regulation affects the bias so that strongly regulated genes had higher bias than moderately regulated genes. Analyses using background adjustment had significantly lower biases than analyses not using background adjustment. For analyses using background adjustment, the 70 and 80 scan procedures gave the lowest biases among the scanning procedures. The CM procedure had high bias independently of which normalization procedure was used. In general, methods using complete filtration had lower biases than analyses using partial filtration or no filtration. As expected, the bias increased with increased censoring values.

Concluding remarks (Paper I)

In conclusion, there was a trade off between the ability of the analyses to identify DE genes and their ability to provide unbiased estimators of the desired ratios. No single analysis achieved both low bias and high sensitivity. Strong regulation and high mRNA levels contributed to high sensitivities. For the moderately regulated genes the "standard analysis" (80 scan, local background adjustment, complete filtration and no censoring) had very low sensitivities. We believe that using censoring together with background adjustment is very promising and that these results could serve as an inspiration for further research. The results show that the CM procedure generates among the highest sensitivities for most normalization procedures. In the case bias is not a major problem, we strongly recommend the use of the CM procedure. On the other hand, if only images from one scanning are available then partial filtration is a good alternative. It has the advantages of not being sensitive to how the scanning is done and that the biases of the correctly classified DE-genes are relatively low (*i.e.* low selected bias).

Response of murine macrophages to infection with Francisella tularensis LVS (Paper II)

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. The global transcriptional response elicited in the murine macrophage-like cell line J774.A1 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 post infection.

Transcriptional response

Microarray data were filtered to include only those spots for which the infected/non-infected ratio was at least 1.7-fold up- or down and with a significance of $p < 0.05$. However, an increase in p value cut-off had little influence on the outcome, suggesting that most genes above the cut-off ratio were significant. A total of 229 clones corresponding to 217 unique genes fulfilled the criteria for being differentially regulated. Of these clones, 23% were found to be regulated at 0 min after start of infection, 15% at 30 min, 22% at 60 min, 35% at 120 min, and 10% at 240 min. Only 6% of the differentially expressed clones were differentially regulated at more than one time point.

Stress response

We found several pieces of evidence that the *F. tularensis* infection resulted in an oxidative stress on the J774 cells. It included the upregulation of c-fos, a transcription factor under the control of the mitogen-activated protein kinase pathway (MAPK), which has been shown to be regulated in response to hypoxia and certain other forms of stress (Yuan *et al.*, 2004). Tropomyosin 2 was upregulated at 60 min and it has also been identified as an oxygen sensor (Thorne *et al.*, 2004). Phosphatase-1 (PP1) promotes the recycling of protein factors and affects the energy state of the cell (Ceulemans & Bollen, 2004). PP1 also controls the activation of MAPK and thereby indirectly MAPK-dependent anti-bacterial mechanisms (Mitsuhashi *et al.*, 2003). We observed an upregulation of cytochrome b, which has a crucial role in the generation of energy transduction and release of ROS in the mitochondria (Crofts, 2004). Important parts of the cellular anti-oxidative response are the reduced form of glutathione (GSH) and enzymes protective against ROS (Inoue *et al.*, 2003). However, we detected an upregulation of glutaminase and gamma-glutamyl transferase 1. They contribute to the depletion of glutathione in the cell (Newsholme & Calder, 1997; Jean *et al.*, 2003; Whitfield, 2001). Upregulation of these enzymes may lead to an increased oxidative stress. Most likely, bacterial factors rather than host factors are responsible for this activation.

Hydroxylation of tryptophan can lead to the generation of anti-bacterial molecules and/or counteraction of the oxidative stress imposed on the infected cell. We observed upregulation of enzymes essential for the formation of BH4, a co-factor essential for the hydroxylation of aromatic amino acids (Thony *et al.*, 2000).

Rapid induction followed by a downregulation of TNF-α

We observed that TNF-α was upregulated at 0 min, but already at 30 min it was downregulated to slightly below basal level and this low level persisted during the remaining time points. Previous studies have shown that *F. tularensis*-infected cells are unable to secrete TNF-α and no activation of NF-κB and MAPK occurs (Telepnev *et al.*, 2003).

Our analysis may have identified molecular mechanisms behind the downregulation of TNF-α since tristetraprolin (TTP) was found to be upregulated concomitantly with TNF-α. TTP has been shown to inhibit TNF-α production from macrophages by destabilizing its messenger RNA (Carballo *et al.*, 1998) and is induced by the same agents that stimulate TNF-α production, including TNF-α itself. Moreover, cytochrome P450 2E1 (CYP2E1) was found to be strongly upregulated at 0 min, followed by a significant downregulation at 30 min and >100-fold downregulation at 120 and 240 min. CYP2E1 has been suggested to activate ERK1/2, NF-kB, and p38 in response to TLR-stimulation (Cao *et al.*, 2005) and the activation of p38 has been shown to stabilize TNF-α transcripts, resulting in increased TNF-α production. Thus, the regulation of CYP2E1 may specifically contribute to the lack of TNF-α during the later phases of the *F. tularensis* infection.

The proteasome

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, transcription and signal transduction, for example activation of NF-κB by degradation of its inhibitor IκB (Yaron *et al.*, 1997). Several genes belonging to the complex were found to be downregulated during the course of the infection. The proteasome has been found to exert anti-bacterial effects against cytosol-located *Salmonella* (Perrin *et al.*, 2004). Since *F. tularensis* is an example of a cytosol-located pathogen (Golovliov *et al.*, 2003), the inhibition of the proteasome may benefit the bacterium.

Effects of an antioxidant or an inhibitor of inducible nitric oxide synthase on the cytopathogenic effect of the *F. tularensis* infection

Previous work has demonstrated that J774 cells exhibit a cytopathogenic effect within 12 hours after start of the *F. tularensis* LVS infection and this cytopathogenic effect eventually leads to apoptosis of the infected cells (Lai & Sjöstedt, 2003). Considering that the microarray analysis indicated that the infection resulted in a considerable oxidative stress upon the infected cells, we studied if the cytopathogenic effects were affected by addition of an anti-oxidant, NAC or an inhibitor of inducible nitric oxide synthase, NMMLA. The relative levels of LDH release were used as markers for the cytopathogenicity. We observed significantly lower levels of LDH in the presence of NMMLA or NAC. This indicates that the production of reactive oxygen and nitrogen species each significantly contributes to the cytopathogenic effect. Concomitantly, with the decreased cytopathogenic effects after addition of NMMLA or NAC, the treatments led to slightly, but significantly lower bacterial numbers after 15 hours of infection indicating that the intracellular redox environment affects the growth of *F. tularensis*.

Concluding remarks (Paper II)

We observed only limited differential regulation of genes at any of the time points, indicating that *F. tularensis* displays a "stealth"-like behavior. Little evidence for induction of a proinflammatory program was observed. This conspicuous lack of an

inflammatory response during the infection is likely to be part of the overall intracellular survival strategy of *F. tularensis*. Collectively, our results suggest that the *F. tularensis* infection confers an oxidative stress upon the target cells and many of the host defense mechanisms appear to be related to counteracting the oxidative stress and mobilizing the energy needed.

Transcriptional profiling of host responses in mouse lungs following aerosol infection with type A Francisella tularensis (Paper III)

Inhalation of type A *F. tularensis* gives rise to the most aggressive form of tularemia. It is associated with considerable mortality in both experimental animals and humans (Dennis *et al.*, 2001). This study was undertaken in order to better understand the extreme virulence of type A *F. tularensis* strains. Transcriptional changes in mouse lung on day one, two and four 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.

Pathology

All mice subjected to an aerosol challenge (~20 CFU) of type A *F. tularensis* developed clinical signs of illness including weight loss at day four and died of the infection by day five. Consistent with previous observations (Conlan *et al.*, 2003), histopathologic examination of the lung from mice killed on day four of infection revealed acute suppurative and necrotic bronchopneumonia, which involved one or more lung lobes, and acute to subacute vasculitis and perivasculitis.

Transcriptional response

A total of 456 clones representing 424 unique genes were found to be differentially expressed. 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. The proportion of upregulated genes on day one, two and four 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.

IFN-γ and TNF-α

During the first two days of infection, very few inflammatory changes were observed in the lungs whereas by day four, histology revealed localized massive, acute inflammation and intense vasculitis. The expression of IFN-γ mRNA was slightly or not at all upregulated during day one and two. TNF-α mRNA expression was moderately downregulated at these two time points. On day four, however, both cytokines were strongly upregulated. In support of these data, both TNF-α and IFN-γ proteins were significantly upregulated in the bronchoalveolar lavage (BAL) fluid on day four after aerosol exposure.

A number of genes that were strongly upregulated on day four of infection are known to be dependent on IFN-γ for their transcription. These include β_2 microglobulin, guanylate nucleotide binding protein 2 (GBP-2), IFN-γ-induced GTPase and T cell specific GTPase. All of these factors are known to be regulated in response to other intracellular infections and the latter two are active in phagocytic and secretory pathways. Other upregulated IFN-γ-regulated factors included pre-B cell colony-enhancing factor and spi2a. Both have diverse biological functions among others, regulation of inflammation. Factors known to be regulated by TNF-α included LPS-induced TN factor (Litaf, TBX-1) and tissue plasminogen activator (Plat). A tissue inhibitor of metalloproteinases, timp3, was upregulated throughout the infection. It can control levels of TNF-α by inhibition of the TNF-α-converting enzyme (TACE) (Black, 2004).

Oxidative stress

In accordance with the results from the macrophage-like cell line in Paper II we observed a regulation in mouse lung of genes causing a decreased resistance to oxidative stress. Transglutaminase 2 was upregulated at all three time points during infection and peroxisome proliferative activated receptor gamma coactivator 1 beta (Ppargc1b) was found downregulated on day two and four. Downregulation of Ppargc1b may contribute to a diminished ability to withstand reactive oxygen species since it is critically required in mitochondrial metabolism and respiration (St-Pierre *et al.*, 2003). In contrast to the J774 cells, tropomyosin was downregulated throughout the course of infection. This may contribute to a decreased cellular adaptation to oxidative stress (Thorne *et al.*, 2004).

Concluding remarks (Paper III)

Overall, the findings in Paper III 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 bronchoalveolar lavage fluid and concomitant activation of pathways known to be regulated by these cytokines. Despite this apparently appropriate host immune response, type A *F. tularensis* infection is uniformly lethal in 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 harbor more than 10^8 CFU 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 four of infection. One of the reasons for the extremely rapid replication of type A *F. tularensis* may be unrestricted growth in macrophages that are unable to activate effective antibacterial defense 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.

Gene expression in human peripheral blood during tularemia (Paper IV)

To better understand the pathogenesis of ulceroglandular tularemia we performed a large-scale microarray analysis of the host response in peripheral blood during the first two weeks of the infection. The response was analyzed using Affymetrix U133A microarrays comprising 14,500 genes. Samples were obtained from each of seven individuals at five time points during the first two weeks of infection and a convalescent sample approximately three months later. RNA was prepared from each sample and analyzed together with a pool of RNA from eight healthy volunteers.

Transcriptional response

Data were filtered to include only those spots for which the ratio was at least two-fold up- or downregulated compared to non-infected cells and with a B statistic above zero. Overall, 265 differentially expressed genes, compared to the pool from healthy volunteers, were detected in samples collected during the two-week period, and 95 of these were regulated at more than one time point but not in the convalescent samples. In the convalescent samples, 31 of the genes were differentially expressed. This number was lower than in samples obtained at any time point during the initial two-week period. We categorized differentially expressed genes with clear identities and characterized functions; in total 220 genes. The largest categories were Nucleic acid binding (45 genes), Transcription factors (21 genes), Oxidoreductase activity (19 genes), Receptors (18 genes), Hydrolase activity (15 genes), Defense/Immunity (11 genes).

Cytokines and chemokines

It is possible that certain intracellular pathogens attempt to subvert innate and acquired immune mechanisms by specifically down-regulating genes critically required for generation of an effective anti-bacterial response. We found several examples of transcriptional adaptation that may interfere with the generation of an effective

immune response. For example, the gene encoding the chemokine receptor CCR7, important for recruitment of migration of T and B cells into secondary lymphoid organs was downregulated, as well as the CD3 epsilon chain, the IL-2B receptor, and the T cell receptor CD8A, all of which are necessary for the generation of the adaptive cell-mediated immunity.

A number of IFN-regulated genes or genes affecting IFN-γ were found to be upregulated, for example G1P2, GBP-1, GCH1, IFI16, IFIT3, TAP1, and STAT1. These factors are important for the activation of multiple arms of the innate and adaptive immune response and probably reflect a normal adaptation to an infection.

We performed Q-PCR analysis of cytokine gene expressions. We found a significant change in mRNA levels for IFN-γ, TNF-α, IL-1β and IL-15. Due to their transcriptional upregulation, they were chosen for analysis of serum levels. An increase of IFN-γ levels was observed at the 2-3 day time point but besides one individual sample, not thereafter. Levels of TNF-α, IL-1β, and IL-15 were below the detection limits in all samples.

Stress response

A number of factors involving host defense mechanisms to protect against oxidative damages were upregulated. They include glutaredoxin (GLRX) and MSRB2, which appear to function as repair mechanisms during oxidative damage during infection.

Apoptosis

Previous studies demonstrated that *F. tularensis*-infected murine macrophages are killed by apoptosis (Lai *et al.*, 2001; Lai & Sjöstedt, 2003). The apoptosis is mediated via a pathway similar to the intrinsic pathway with involvement of the mitochondria and subsequent activation of caspase-9 and caspase-3 (Lai & Sjöstedt, 2003). Also during human tularemia, we found evidence of a pro-apoptotic response as evidenced by strong upregulation of an activator of caspase-mediated apoptosis, TNFSF10

(TRAIL), a cytokine that belongs to the TNF ligand family. Also caspase 1 was upregulated at several time points. Sequential activation of caspases plays a central role in the execution-phase of cell apoptosis (Ghayur *et al.*, 1997). Modulation of their gene expression has been found to be essential for the pathogenicity of *Salmonella* infection (Hersh *et al.*, 1999). We observed a persistent downregulation of SGK. It plays a crucial role in promoting cell survival via an NF-κB-dependent mechanism (Zhang *et al.*, 2005). On the other hand, TOSO was downregulated. It is an inhibitor of apoptosis induced by members of the TNF receptor family and Fas (Hitoshi *et al.*, 1998). The overall picture fits with that of a pro-apoptotic response. Whether this benefits the pathogen or the host is unclear but in the murine model it was suggested that the macrophage-mediated apoptosis favors *F. tularensis* since it allows the bacterium to escape by a silent mechanism from heavily infected cells deplete of nutrients and infect neighboring cells replete of nutrients (Sjöstedt, 2005a). The apoptotic nature of host cell death also fits with the general stealth-like nature of tularemia.

T cell response

TRGV9 is a gene expressed by a subset of γδ T cells, so called Vγ9Vδ2 T cells. It has previously been demonstrated that tularemia patients exhibit a long-lasting and very prominent increase of this cell type (Poquet *et al.*, 1998). TRGV9 RNA levels were much higher in the patient convalescent samples obtained 3 months after recovery than in the pooled control RNA sample. This agrees with the previous demonstration that markedly higher levels of Vγ9Vδ2 T cells persisted at least 18 months after tularemia (Kroca *et al.*, 2000).

Common response to pathogens

A core cluster of some 500 genes has been suggested to constitute a general “alarm-signal” for infection and the differential regulation of these genes have been identified to occur in macrophages, DC, and PBMC during many different forms of infection (Jenner & Young, 2005). On the Affymetrix U133A arrays, 260 of the 511 core-cluster

genes were present and transcripts from 104 “alarm-signal” genes were detected in our experiments. Only 16 were found to be differentially expressed, 14 of which were upregulated. Two genes were found to be downregulated, BCL2-associated athanogene at days 2-3 and 6-7, and Pim-1 at days 6-7.

Classifiers for tularemia

We performed a logistic regression analysis to identify genes that best classify tularemia patients at the first time point, day 2-3, before the start of treatment. We found 19 genes that gave a good separation between this time point and the convalescent samples. Of these, 13 genes gave a complete separation and six of these were among the aforementioned “alarm-signal” genes. Since they have been identified as indicators of infection in general, we did not consider them as good indicators of tularemia. The remaining, which can be considered as putative diagnostic markers of tularemia, were STAT1, SECTM1, TNFAIP6, TNFSF10, CD3E, MSRB2 and IL2RB. Since several of the genes encode factors known to be involved in innate immune mechanisms, it can be assumed that they are regulated in response to other infections as well. However, the concomitant regulation of these seven genes may be a strong diagnostic indicator of tularemia. The usefulness of these genes for prediction of tularemia should be validated on a larger patient material.

Concluding remarks (Paper IV)

The results provide a picture of pathogenic mechanisms active during human infection and identify issues that require future investigations. As in the mouse model in Paper III, these findings indicate that many of the genes prominently upregulated during the early phase of human tularemia are dependent on IFN-γ for their expression. We observed downregulation of a number of factors known to be critically required for effectuating various facets of an immune response. Thus, it is possible that specific downregulation of key immune effector genes counteracts optimal bactericidal mechanisms triggered via the expansion of a protective T_H1 immune response and allows persistence of the infection. Moreover, serum levels of proinflammatory

cytokines were not increased at all with the exception of IFN-γ that showed an initial, transient increase. The findings fit with the view that *F. tularensis* has a stealth strategy to ensure intracellular survival and with the rather inconspicuous clinical manifestations during the disease (Sjöstedt, 2005a). It should be noted that all patients received antibiotic therapy during the first six days. The treatment may have contributed to the minimal inflammatory response thereafter. Altogether, our results provide a useful foundation for explaining the pathogenesis of ulceroglandular tularemia. Large-scale transcriptional analysis allows for an effective identification of diagnostic and predictive biomarkers and if such markers could be included in rapid, simple assays, they will provide important information to assist in decisions regarding institution of early therapeutic regimens in tularemia patients.

CONCLUSIONS

- There is a trade-off between a microarray analysis method's ability to identify differentially expressed genes and its ability to provide unbiased estimators of the desired ratios
- In the case bias is not a major issue, the preferred method is either the constrained model or the novel method partial filtration

- The adaptive transcriptional response to *Francisella tularensis* LVS in macrophages is very modest
- *F. tularensis* LVS infection confers an oxidative stress upon the target cells and many of the host defense mechanisms appear to be intended to counteract this stress

- A vigorous cellular inflammatory response ensues in *Francisella*-infected mouse lungs, however not until after day two of infection
- Genes involved in host immune responses were activated extensively on day four, many of which are dependent on TNF-α and IFN-γ, together with concomitant increases of IFN-γ and TNF-α in BAL fluid

- Several genes upregulated during the early phase of human tularemia are also dependent on IFN-γ for their expression
- The most prominent changes were noted in samples drawn on days 2-3
- A number of factors known to be critically required for effectuating various facets of an immune response were downregulated
- Serum levels of proinflammatory cytokines were not increased at all with the exception of IFN-γ that showed an initial, transient increase
- Seven genes were found to be good predictors of the early phase of tularemia

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