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Original article

Gene expression following low dose inhalational *Francisella tularensis* (SchuS4) exposure in Balb/c mice and the potential role of the epithelium and cell adhesion

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Abstract

Interactions between *Francisella tularensis* and the host are slowly being elucidated. Microarray technology was used to further characterise the response of Balb/c mice after inhalation of the virulent *F. tularensis*, SchuS4. The validated array data revealed changes in expression of 476 genes across a 96 h time course following infection ($p \leq 0.05$). These data confirm down-regulation of the toll-like receptor pathway (TLR3, 4, 5, 7 and 8), and the induction of IFN-γ inducible genes (T-cell specific GTPase, β2 microglobulin and interleukin 21). The overall response appears to be two staged with an initial up-regulation of genes involved in apoptosis, TNFα production and antigen presentation. This is followed by a large alteration of expression at 96 h as the host succumbs to infection. A key regulatory time-point has been identified at 24 h post challenge, where several transcriptional events may predicate the progression of infection; these include transcriptional regulators of inflammation and proteolytic pathways. Pathway analysis indicates a novel role for cell–cell adhesion and extracellular matrix modulation in infection. Transcripts representing cellular junctions, focal adhesion and adherens junctions changed following infection. Additionally, aspects of extracellular matrix remodelling have been confirmed at the protein level, suggesting an important role of the respiratory epithelium in host response to *F. tularensis* warranting further study.

Keywords: *Francisella tularensis*; Tularaemia; Microarray; Balb/c; Lungs; Respiratory epithelium

1. Introduction

Francisella tularensis is a Gram negative, pleomorphic, aerobic coccobacillus, which is non-motile and 0.1–1 × 0.1–3 μm long. First isolated in 1911 [1] and initially classified *Bacterium tularense*, the taxonomic characterisation of the bacterium has developed over time and modern classification places the bacterium as the only member of the family *Francisellaceae*. The genus "*Francisella*" is split into three species; *tularensis*, *novicida* and *philomirgia*. Although *F. tularensis* infection is classically zoonotic, in recent years there has been an increase in research due to the potential to use *F. tularensis* as a bioweapon. *F. tularensis* has been weaponised in the past by both the former USSR and USA [2] and it is the subspecies *tularensis* that poses the highest biological threat, due to its very low infectious dose when aerosolised; the LD_{50} in man being less than 10 colony forming units [3].

Virulent strains of *F. tularensis* subsp. *tularensis* are lethal in mice, guinea pigs, rabbit, hamsters and rats [4]. The model of preference for studying *F. tularensis* has been the inbred mouse, due to its consistency in modelling disease. Many murine studies [5,6], along with individual cell types [7,8], have been used to determine key findings to identify mechanisms involved in infection. This includes several studies involving the use of microarrays to probe the whole genome (or sub-sections) in response to *F. tularensis* in vitro or in vivo

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[9–12]. All of these studies have focussed heavily on the macrophage derived immune response to *F. tularensis* and not necessarily on the holistic response to infection and unique signals, aside from general inflammation. However, the bacterium has been implicated in direct infection of several cell types ranging from epithelial cells to dendritic cells [8,13]. The summation of these host response studies still cannot elucidate a complete molecular mechanism of infection for phagocytic cells, or alternative host cell types. The progression of infection from work on immune cells points towards *F. tularensis* interacting with host cell toll-like receptor 2 (TLR2) to initiate the innate immune response [6]. This interaction has so far been linked to two bacterial lipoproteins; TUL4 and FTT1103 [14]. Interestingly *F. tularensis* lipopolysaccharide (LPS), unlike other bacterial LPS, does not bind directly to TLR4 as would be expected through the LPS lipid A portion to produce a pro-inflammatory cascade.

Following cellular interaction *F. tularensis* induces novel asymmetric spacious pseudopod loops in a manner dependant on host cells having receptors for complement factor C3 [7]. The pseudopod loops close to form a spacious phagosome and within minutes is remodelled to form a tight phagosome termed the *F. tularensis*-containing phagosome (FCP) [7]. Whilst the precise mechanism is still unclear, but it is apparent that *F. tularensis* can escape the phagosome primarily due to *iglC* and its regulator MglA allowing the bacterium to enter the cytosol for replication [15]. Following replication the bacteria escape the host cell via apoptosis and pyroptosis [16]. The host inflammatory response through apoptosis and pyroptosis has been recently reviewed by Henry and Monack [17]. In brief, the host cell recognises pathogen associated molecular patterns (PAMPs), released into the cytosol by the bacteria, through specific receptors. The specific sensor for *F. tularensis* is as yet unknown. This in turn activates the inflammasome and thus caspase 1. Caspase 1-mediated cell death results in discrete pores in the plasma membrane leading to osmotic lysis [18]. Caspase 1 also processes the proforms of the inflammatory cytokines IL1β, IL18 and IL33 into their active states. These pro-inflammatory cytokines in turn induce other cytokines such as IFN-γ, IL-4, 5 and 13 [5,19]. Thus the bacteria are released from the infected cell and host inflammatory cascades activated to deal with infection.

The work presented here uses microarray technology to describe the host response to aerosolised *F. tularensis* (SchuS4) in a Balb/c mouse model. We propose a key determinant time point at 24 h post challenge which may prove instrumental in directing the outcome of infection. Furthermore, we suggest an important, novel role for the epithelium and cellular adhesion in the progression of an in vivo infection. The elucidation of genes changing in response to *F. tularensis* will enable a greater understanding of the mechanisms of response to infection and offers the potential to augment the development of therapeutics to *F. tularensis*.

2. Materials and methods

2.1. Animals

All work was conducted under the terms of a licence granted in accordance with the UK Animal (Scientific Procedures) Act, 1986. Female BALB/c mice (Charles River Laboratories Ltd., Margate, Kent, UK) aged 6–8 weeks, were habituated to the experimental animal unit for 1 week prior to inhalational challenge. Environmental conditions were maintained at 21 °C ± 2 °C and 55% ± 10% humidity with lighting set to mimic a 12/12 h dawn to dusk cycle. Mice were housed in polycarbonate cages (six animals per cage) with steel cage tops and corncob bedding (International Product Supplies, Wellingborough, UK). The mice were fed a Teklad TRM 19% protein irradiated diet ad libitum (Harlan Teklad, Bicester, UK) and given fresh water daily. All animals weighed 19.6 ± 0.9 g prior to challenge.

2.2. F. tularensis

F. tularensis subsp. *tularensis* SchuS4 (originally isolated from a human ulcer, Ohio, 1941) was used for all infections following growth in modified cysteine partial hydrolysate (MCPH) broth. Bacteria were plated on blood cysteine glucose agar (BCGA) plates and incubated at 37 °C for bacterial enumeration.

2.3. F. tularensis *exposures*

For bacterial exposures, 10 ml of *F. tularensis* culture was aerosolised using a Henderson Apparatus over an exposure time of 10 min as described previously [20]. The aerosol was delivered at a flow rate of 12 L/min with impinger samples from the exposure apparatus plated on BCGA to calculate retained dose. Using the known flow rate of the Henderson exposure apparatus (66 L/min), bacterial counts from these samples were then converted to bacterial counts per litre of air. The breathing rate of the animals in the apparatus (approximately 20 ml of air per minute) was then added to the calculation along with the length of exposure (10 min) to yield an estimated delivered dose expressed in cfu per animal. Previous studies have determined that aerosol uptake in obligate nasal breathers, such as the mouse, is approximately 40% [21]. Using this conversion factor the estimated retained dose can be calculated for each exposure.

Two animal exposures were carried out; the first for microarray studies and the second to confirm the microarray data by measuring protein levels. The initial agent exposure challenged six animals per time point (4 time points), exposed to a full body challenge of *F. tularensis*. Sham exposures were completed on a further three animals per time point to aerosolised culture medium only; MCPH broth, under full experimental conditions. Following exposure the animals were culled over an experimental time-course, six animals per time-point at 1, 24, 48 and 96 h post exposure respectively. Due to the low dose delivered (~10 cfu) all animals were culled