Immune Cell Responses to a Live Attenuated Tularemia Vaccine

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Abstract

Francisella tularensis (*Ft*), a facultative intracellular bacterium causing tularemia, poses a minimal community risk in the United States, but is a major bioterrorism threat due to its weaponization potential. A 1970 World Health Organization study estimated that aerosolizing 50 kg of *Ft* over a city of 5 million people could kill 19,000 and incapacitate 250,000. A 1997 study also projected costs of $5.5 billion ($11.1 Billion in 2025) per 100,000 people infected. *Ft* is a Tier 1 Select Agent according to the Center for Disease Control and Prevention, and while it is treatable with antibiotics, the potential for antibiotic resistance and distribution challenges may limit their effectiveness.

Various groups in the United States have tried to develop an *Ft* vaccine to remedy this deficiency, but none have yet achieved FDA approval. We are proposing *Ft* SCHU S4 Δ*clpB* (ATI-1701, referred to here as Δ*clpB*) as a live attenuated vaccine for tularemia. Animal studies to date have shown Δ*clpB* to be safe and effective, with as few as six CFU Δ*clpB* protecting up to 100% of mice from subsequent lethal challenge. While most vaccines' effects are measured by humoral responses, *Ft*'s intracellular nature implicates that measurements of immunocytes' responses may be useful for further development. This study explores immune responses of THP-1 macrophages and peripheral blood mononuclear cells (PBMCs) to Δ*clpB*. Specifically, these experiments investigate which immunity-related molecular pathways Δ*clpB* activates in PBMCs. These results offer insights to help find potential mechanisms of immunity, which are used to help bridge the gap between preclinical and clinical studies of ATI-1701 as a vaccine candidate.

Background

Cell Models

THP-1 cells and peripheral blood mononuclear cells (PBMCs), were used to study vaccine stimulation through changes in genetic markers, cell viability, and molecular responses. THP-1 cells were used to establish *in vitro* methods before moving into PBMCs.

Relevant Considerations

Ft SCHU S4 does not trigger TLR responses in HEK293 cells (1). Other studies show *Ft* may not upregulate some inflammatory genes. *Ft*-infected macrophages release Prostaglandin E2 (PGE2), blocking T cell proliferation and promoting an anti-inflammatory Th2 response (2).

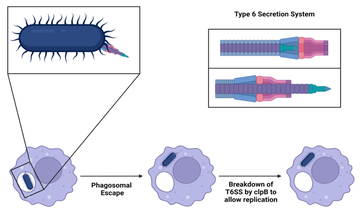

Figure 1: Normal operation of the T6SS. T6SS assembles, injects membrane-disrupting proteins for phagosomal escape, then ClpB disassembles contracted sheaths for recycling. **Δ*clpB* impact:** Impaired sheath disassembly reduces T6SS cycling efficiency, severely attenuating intracellular replication.

Key Genes & Proteins

TNF-α: Pro-inflammatory cytokine primarily involved in innate immunity, promotes inflammation and apoptosis. ***IFN-γ***: Commonly upregulated in tularemia, considered a key player in *Ft* infection (3). ***IL-10***: Anti-inflammatory, regulates response by inhibiting pro-inflammatory cytokines and promoting anti-inflammatory genes like Bcl-3 (4). ***IL-1β***: Pro-inflammatory marker downstream of TLRs, often downregulated by *Ft* (5). **CD64**: A high-infinity IgG Fc-gamma receptor. This glycoprotein is found on macrophages and monocytes and is essential in the response of the innate immune system (6).

Results

RT-qPCR

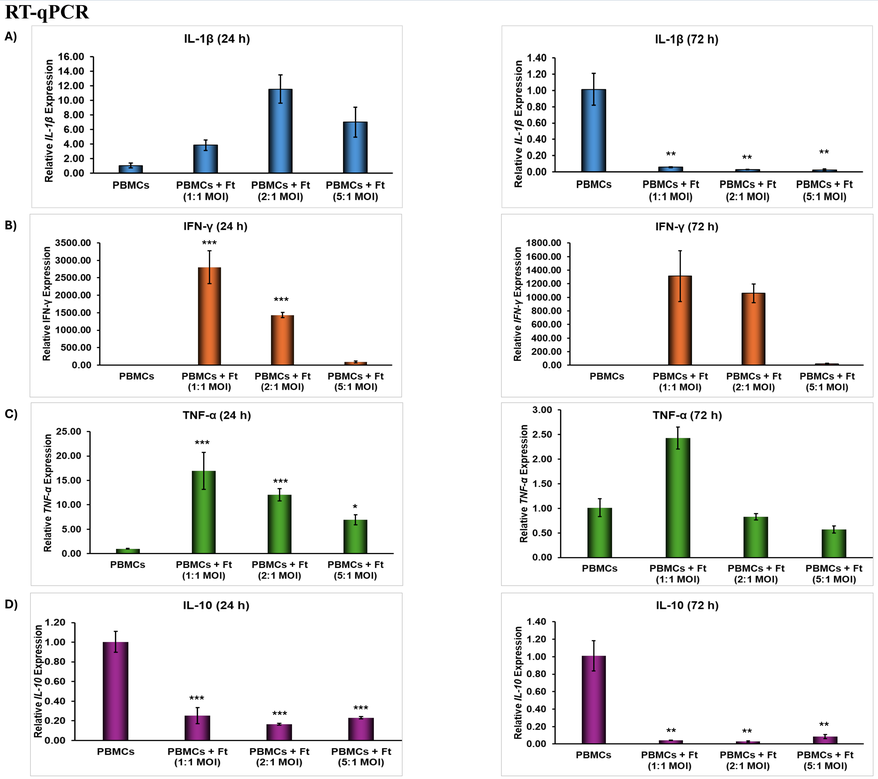

Figure 4: Inflammatory Response of PBMCs to *Ft* Δ*clpB* as Measured by RNA Expression
A panel of inflammatory markers prominent in the tularemia literature was selected and investigated for how they reacted to LPS and *Ft* Δ*clpB*. LPS robustly induced *IL-10* and *IL-1β* (not shown), indicating that the cells were responsive to inflammatory stimuli. **A)** *IL-1β* is upregulated at 24 h but suppressed by 72 h. **B)** *IFN-γ*, a prominent inflammatory marker related closely to tularemia in literature, is significantly induced at both 24 and 72 h, though the effect fades with time and increasing MOIs. **C)** *TNF-α* follows the same trend as *IFN-γ*, though the response is less robust than seen with *IFN-γ*. **D)** *IL-10* is significantly suppressed across all timepoints, likely due to downstream effects of *IFN-γ*. The normality of collected data was assessed using Shapiro-Wilk testing and comparisons between groups were made using one-way ANOVA for parametric datasets or Kruskal-Wallis testing for non-parametric datasets. Statistically significant changes compared to controls were indicated as follows: * = $p < 0.05$; ** = $p < 0.005$; *** = $p < 0.0005$ (n=2) .

Time Lapse Microscopy

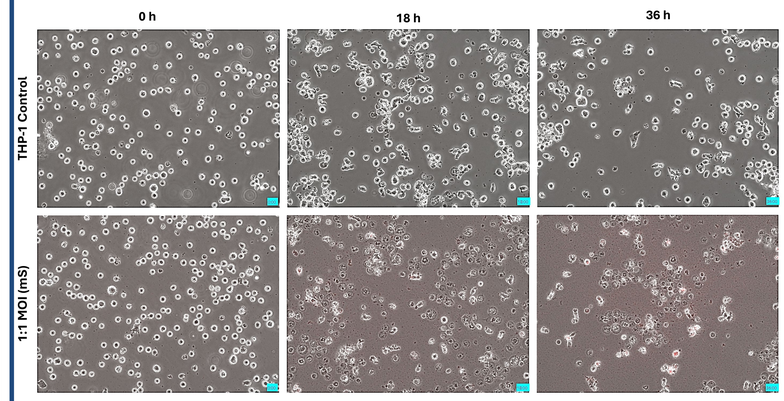

Figure 6: THP-1 Cells infected with the mScarlet-expressing strain of Δ*clpB*
THP-1 cells were either infected at a 1:1 MOI, 2:1 MOI (not shown) or were uninfected as controls. mS infection was visualized with Texas Red and host cells were visualized using bright field microscopy. Images were then merged in an overlay to visualize both fields together. Results show a notable decrease in the number of cells in the 1:1 MOI compared to control. THP-1 cells infected with the mScarlet-expressing Δ*clpB* bacteria emitted strong signals under Texas Red, further suggesting successful infection in agreement with flow cytometry results.

Conclusions

Genetic Inflammatory Response

- *IFN-γ*: Proportionally enormous spike at 1:1 MOI, 3000x higher than control, then decreases at 2:1 and 5:1 MOIs in both 24 and 72-hour experiments.
- *TNF-α*: Spikes at 1:1 MOI (lesser magnitude than *IFN-γ*), follows similar decreasing trend, possibly due to direct regulation by *IFN-γ* (3). This trend holds at 72 h.
- *IL-10*: Suppressed in both 24 and 72-hour experiments; 24-hour suppression may be due to up-regulation of anti-inflammatory PGE2, expressed in the first 24 hours of infection (2). A *TNF-α* spike could also counteract the *IL-10* pathway through its induction of STAT3 dephosphorylation in human monocytes (8). At 72 h, down-regulated due to the immunosuppressive nature of *Ft* (2), a *TNF-α* spike (7) or cell death.
- *IL-1β*: Activated and up-regulated at 2:1 and 5:1 MOIs, but less significantly than other markers, perhaps due to a suppressive effect on TLR2. At 72 h, significantly down-regulated as compared to 24-hour results, likely due to cell death, the immunosuppressive nature of *Ft* (2) or the suppressive effect of activated TLR2 (5).

Flow Cytometry

- Expression of mS in THP-1 cells rose in a dose-dependent manner with the addition of mS bacteria. The true expression change is likely understated due to sub-optimal detection of the mScarlet dye by the cytometer's PE channel.
- CD64 expression on THP-1 cells decreases with increasing MOI. This may be due to receptor internalization during phagocytosis, though active immunosuppression by *Ft* cannot be ruled out. We are also investigating a potentially novel paracrine regulation mechanism via Δ*clpB*.

Time Lapse Microscopy

- ICC imaging, in conjunction with the flow cytometry results, suggest successful infection of THP-1 cells with Δ*clpB*. The 2:1 MOI did not show an apparent difference in infection rate, but more work needs to be done to quantify those data.
- Cell counts in higher MOIs were lower than those seen in the Control groups. More study is needed to establish if this is due to cell death or growth inhibition imposed by paracrine effects of Δ*clpB*.

General Takeaway:

- This data suggest that PBMCs demonstrate a robust immune response to Δ*clpB*. Additionally, Flow cytometry and immunocytochemistry suggest that Δ*clpB* successfully infects THP-1 cells. Additional experiments will attempt to replicate these results in PBMCs.

Methods

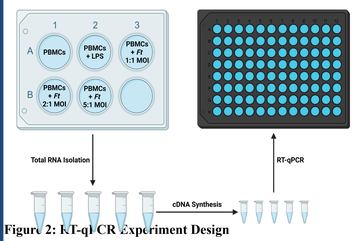

Figure 2: RT-qPCR Experiment Design

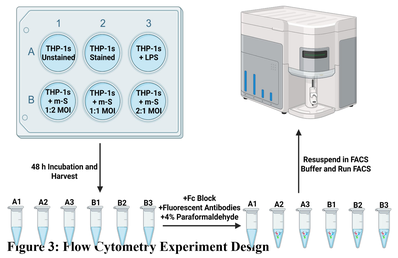

Figure 3: Flow Cytometry Experiment Design

Time Lapse Microscopy Methods for THP-1 Analysis

1. 10^6 THP-1 cells were plated for each treatment group for analysis after 48 hours and a corresponding number of mS bacteria were plated to match the corresponding MOIs:
   a. THP-1 Control
   b. THP-1s + mS, 1:1 MOI
   c. THP-1s + mS, 2:1 MOI (not shown here)
2. Cells were imaged in a six-well plate every 30 minutes for 48 hours. A temperature of 37°C and 5% CO_2 were maintained using a Hokai HIT stage-top incubator.
3. Images were captured using bright field and Texas Red channels at 20x magnification on a Keyence BZ-X810 fluorescent microscope. Each channel was combined in overlay for final visualization.

Flow Cytometry

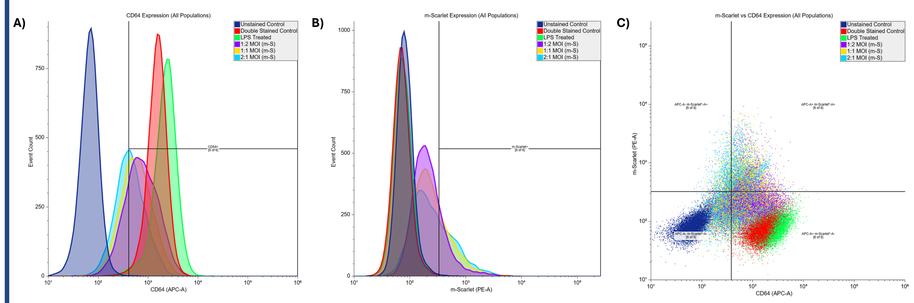

Figure 5: Flow Cytometry Results Using mScarlet-expressing Δ*clpB*
Flow cytometry was carried out on THP-1 cells infected with a Δ*clpB* strain expressing the fluorescent protein mScarlet (mS). **A)** CD64 surface expression decreases dose-dependently with tularemia. This would be consistent with internalization of the marker after engulfing the bacteria, or paracrine downregulation of the surface protein by *Ft*. More work needs to be done to see which mechanisms are being induced. LPS stimulation slightly increased CD64 expression relative to the stained, unstimulated THP-1 Control. **B)** Expression of mS increases dose-dependently in THP-1 cells, as expected. The shift in peaks is small, and there is variation in the expression levels of mS. The small shift in peaks may be due to either weak signals relative to antibody staining (as seen in panel **A**) or that the cytometer used caught the tails of mS's excitation and emission peaks for stimulation and detection. LPS stimulation did not shift the peak, and CD64 staining did not bleed into the PE channel via spectral overlap. **C)** CD64 and mScarlet expression, plotted together on a scatter plot, suggest an inverse correlation between CD64 and mScarlet, and therefore, a similar correlation between CD64 and Δ*clpB* infection. More work must be done to confirm the mechanism by which Δ*clpB* infection seems to downregulate CD64.

Acknowledgements

This work was funded by the United States Air Force Academy Cooperative Agreement FA7000-23-2-00007.

The *Francisella tularensis* Δ*clpB* and Δ*clpB* mScarlet strains used for experiments were generously provided by H. Carl Gelhaus, Appili Therapeutics, and the Anders Sjöstedt lab.

Figures 1 and 2 were made using Biorender.com.

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References Cited