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Transcriptome analysis of human immune responses following Live Vaccine Strain (LVS) *Francisella Tularensis* vaccination

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Abstract

The live vaccine strain (LVS) of *Francisella tularensis* is the only vaccine against tularemia available for humans, yet its mechanism of protection remains unclear. We probed human immunological responses to LVS vaccination with transcriptome analysis using PBMC samples from volunteers at timepoints pre- and post-vaccination. Gene modulation was highly uniform across all time points, implying commonality of vaccine responses. Principal components analysis revealed three highly distinct principal groupings: pre-vaccination (–144 h), early (+18 and +48 h), and late post-vaccination (+192 and +336 h). The most significant changes in gene expression occurred at early post-vaccination timepoints (≤48 h), specifically in the induction of pro-inflammatory- and innate immunity-related genes. Evidence supporting modulation of innate effector function, specifically antigen processing and presentation by dendritic cells, was especially apparent. Our data indicate that the LVS strain of *F. tularensis* invokes a strong early response upon vaccination. This pattern of gene regulation may provide insightful information regarding both vaccine efficacy and immunopathogenesis that may provide insight into infection with virulent strains of *F. tularensis*. Additionally, we obtained valuable information that should prove useful in evaluation of vaccine lots as well as efficacy testing of new anti- *F. tularensis* vaccines.

Keywords

human; gene regulation; vaccination; bacterial; molecular biology

Introduction

Francisella tularensis, the causative agent of tularemia, is a gram-negative facultative, intracellular bacterium, first isolated in 1911 in association with a plague-like disease among squirrels in Tulare County, California (McCoy and Chapin, 1912; McLendon *et al.*, 2006). *F.*

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tularensis is a CDC Category A threat organism due to its high infectivity rate after exposure to low numbers of organisms (10–50 bacteria), the ease of dispersal, and its potential to cause high morbidity and mortality rates among aerosol-exposed individuals (Isherwood *et al.*, 2005; McLendon *et al.*, 2006; Oyston and Quarry, 2005). Human tularemia presents in ulceroglandular, glandular, oculoglandular, oropharyngeal, pneumonic, and septic forms and is spread through blood-feeding arthropod bites or exposure to infected vermin, soil, or water (Isherwood *et al.*, 2005; Dennis *et al.*, 2001). Most forms of tularemia cause mild acute symptoms of an undifferentiated febrile illness and are treatable by broad spectrum antibiotics (Dennis *et al.*, 2001). Primary pneumonic tularemia is rarely seen in naturally occurring cases; however, the intentional deployment of weaponized or genetically modified/antibiotic-resistant strains of *F. tularensis* presents a public health hazard estimated to result in high incapacitating casualty and mortality rates (Dennis *et al.*, 2001). The economic impact of such an event is believed to be upwards of \$6.4 billion for every 100,000 persons exposed (Kaufmann *et al.*, 1997; Dennis *et al.*, 2001).

The only tularemia vaccine available in the United States is an Investigational New Drug (IND). It is a live attenuated vaccine, comprised of the live vaccine strain (LVS) of *F. tularensis* (Eigelsbach and Downs, 1961). The vaccine is administered by scarification of the volar surface of the forearm and creates a persistent papular/pustular lesion at the inoculation site similar to infection with virulent *F. tularensis* infection (Burke, 1977). The vaccine, in use for 50 years, has substantially lowered the number of laboratory-acquired incidents of tularemia (Oyston and Quarry, 2005; Isherwood *et al.*, 2005; Burke, 1977).

Relatively little is understood regarding protection against tularemia in humans. Much of the data regarding *F. tularensis* immunopathogenesis, as well as the mechanism of protection afforded by the vaccine, comes from murine models. Humoral immunity was previously believed to be important as passive protection against LVS challenge was demonstrated in mice given immune serum from LVS-vaccinated humans (Drabick *et al.*, 1994). However, vaccine-induced humoral responses to LVS may play no role in protection against human pathogenic *F. tularensis* strains (Tarnvik, 1989). Instead, as with most intracellular pathogens, cell-mediated responses are thought to be critical in long-lasting protective immunity (Waag *et al.*, 1992; Waag *et al.*, 1996; Tarnvik *et al.*, 1985; Surcel *et al.*, 1991; Sjostedt *et al.*, 1992). Protection of mice from lethal challenge with LVS develops as early as 2–3 days after intradermal vaccination (Elkins *et al.*, 1992). Similarly, intranasal infection of mice with lethal doses of LVS results in rapid NK cell recruitment and activation in the lungs (Lopez *et al.*, 2004). Recent studies in our laboratory suggest that early innate responses (<48 h) occur in humans after LVS vaccination and may correlate with protective immunity (Fuller *et al.*, 2006). Rapid development of immunity after vaccination in mice also suggests that protective mechanisms are partly attributable to strong initial innate immune responses. The cellular components of these early immune responses, such as NK cells, neutrophils, and DCs, have only recently become the focus of attention in murine and human studies of LVS vaccination (Telepnev *et al.*, 2005; Sjostedt *et al.*, 1994; Malik *et al.*, 2006; Isherwood *et al.*, 2005; Fuller *et al.*, 2006; Elkins *et al.*, 2003; Cole *et al.*, 2006; Barker *et al.*, 2006).

While a FDA-approved vaccine for *F. tularensis* may be on the horizon, the methods used to determine the mechanism of protective immunity have not been re-visited in decades. Here, we examined PBMC samples from vaccinees at varying timepoints in an effort to delineate early immune responses upon which long lasting protection is built. Using modern transcriptome analysis following vaccination, we gained a better understanding of the immunology of LVS vaccination and perhaps the responses necessary for long-lasting immunity. These data will aid in evaluating host response to virulent *F. tularensis* and because LVS is a uniquely live vaccine, the data offer a seldom-observed study of human response to bacterial infection.

Materials and Methods

Collection and preparation of PBMCs

Volunteers were recruited from U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID) personnel at risk of laboratory exposure to *F. tularensis*. A human use clinical protocol to collect peripheral blood samples was approved by institutional review boards at USAMRIID (Human Use Committee FY04-16). Donors provided informed consent and met eligibility criteria. Six healthy adults (3 males and 2 females, 22–54 years of age) received a primary LVS vaccination (*Francisella tularensis* Vaccine, NDBR 101, Lot 4) and donated peripheral blood, 6 days prior to vaccination, 18 h after vaccination, 48 h after vaccination, 8 days after vaccination, and 14 days after vaccination. Inoculum consisted of a single 0.6-mL drop of LVS vaccine (2×10^9 colony-forming units [cfu] per mL) delivered intradermally by bifurcated needle. All vaccinees had positive “takes” (initial formation of small papule which then ulcerates). Vaccinations were deemed clinically successful by microagglutination assays (titers ranged from 1:20 to 1:1280) at day +28 (Massey and Mangiafico, 1974). Blood was collected into heparinized syringes under sterile conditions at the medical clinic and transferred to a biosafety level (BSL)-2 laboratory for processing. PBMCs were purified from whole blood by Ficoll-Paque Plus (GE Healthcare, Piscataway, NJ USA) density gradient centrifugation.

RNA Isolation

The cell pellet was resuspended in 1 ml TRIzol (Invitrogen, Carlsbad, CA, USA) solution and pipetted multiple times to dissolve all clumps and break up the cellular DNA. An additional 2 ml of TRIzol was added to the cell lysate and mixed well. One ml of TRIzol-lysed solutions were then dispensed into 1.5 ml Eppendorf centrifuge tubes and snap frozen in a bath of 95% ethanol and dry ice then transferred to -70°C until RNA was purified. Total cellular RNA was isolated according to the manufacturer's specifications followed by purification with an affinity resin column (Qiagen Inc, Valencia, CA, USA). The quality and concentration of the RNA were determined by measuring the absorbance at 260 and 280 nm. RNA integrity was confirmed by Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). All plastic materials used in the lysing procedure were certified RNase- and DNase-free and all samples were handled with latex glove-covered hands in a BSL-2 laminar flow cabinet to minimize deposition of environmental RNase onto or into sample tubes.

Transcriptome/microarray analysis

RNA samples used in this study were checked for evidence of degradation and integrity and had a minimum A260/A280 ratio of > 1.9 and a minimum 28S/18S ratio of > 1.6 (2100-Bioanalyzer, Agilent Technologies, Palo Alto, CA, USA). The Human Genome U133+2 array, which consists of 54,654 probe sets representing approximately 33,000 human genes was used (Affymetrix, Inc., Santa Clara, CA, USA). GeneChip analysis was performed with Microarray Analysis Suite (MAS) 5.0, Data Mining Tool (DMT) 2.0, and Microarray Database software (Affymetrix, Inc., Santa Clara, CA, USA). All of the probe sets represented on the GeneChip arrays were globally normalized and scaled to a signal intensity of 100.

Various microarray RNA integrity indicators were used to filter samples for quality in the final analysis. Principal component analysis (PCA) was used to quickly identify outlier arrays. Microarray quality control included the following manufacturer's standard parameters: noise (RawQ), consistent number of genes detected as present across arrays, consistent scale factors, and consistent beta-actin and GAPDH 5'/3' signal ratios (Affymetrix, 2000). Only those samples that passed Gene Logic Inc.'s quality control metrics for each of these parameters were added to its Gene Enterprise System® Software for storage and analysis. Gene expression values were floored to 1 and then $\log_2$ -transformed. PCA and analysis of the global correlation matrix were performed based on all probe sets. The covariance PCA and correlation-based

analyses were done within Partek® software (Partek Inc., St. Louis, MO, USA). T-tests were performed for each probe set to identify statistically significant changes in gene expression between pre-vaccination and each of the post-vaccination groups. A gene had to be $p \leq 0.01$ with a fold change of at least 2 in either direction to be considered significantly regulated for individual pair-wise comparisons. Note that the pair-wise comparisons were primarily used to establish relative numbers of genes regulated across time such that adjustment of p-values was not necessary. Potential markers of vaccination were selected statistically using the ANOVA-based pattern-matching techniques described below.

Gene Filtering, Pattern Matching, and Clustering

Five primary patterns of regulation of interest were investigated based on principal trends within the data set. To determine genes that robustly correlated with these five patterns, a three-step process was implemented. First, a one-way ANOVA was performed to calculate F-scores across the five groups of samples. An F-score of 10, which translates to a $p < 0.001$ based on random sampling distributions, was chosen as a cutoff for inclusion such that a score above 10 was deemed evidence that a regulation event had occurred for one or more of the five groups. Individual gene scatter, directly visualized according to group, was used to validate this F-score as robust. Second, the absolute value of the Pearson's correlation was calculated for each probe set relative to the five pre-determined patterns of regulation. A cutoff of 0.95 was chosen to indicate highly correlated probe sets. We visually inspected individual probe sets to validate this cutoff. Finally, we conducted a k-means Euclidean distance clustering of the mean signal intensity for probe sets that passed each of the previous criteria using $k=8, 10, 12$, and 14 clusters after global mean normalization. $K=10$ was chosen as optimal due to generation of clusters by up or down-regulation events and by separation of probe sets into clusters of high degrees of regulation and those of lesser regulation. The distinction of high vs. low degrees of regulation was made as markers of a high degree of regulation would be more likely to validate on another platform than those of lesser regulation. All clustering was performed in Spotfire® DecisionSite™ 8.0 (SpotFire, Inc., Somerville, MA, USA) and ArrayMiner 5.3.2 (SOM; Optimal Design, Brussels, Belgium). Gene annotations utilized Gene (<http://www.ncbi.nlm.nih.gov/entrez/>) and Bioinformatic Harvester databases (Lieber *et al.*, 2004; Lieber *et al.*, 2005).

Results

Response to LVS vaccination is highly comparable from person-to-person

We designed our study to allow a kinetic assessment of global gene regulation upon vaccination with LVS. Five timepoints were chosen representing pre-vaccination (-144 h) as well as early ($+18$ and $+48$ h) and late ($+192$ and $+336$ h) times post-vaccination (Figure 1A). These timepoints allowed assessment of gene-regulation associated with innate (early) and acquired (late) immune responses. The formation of pustules at the inoculation site is accepted as anecdotal evidence of a positive “take” for LVS vaccination and is visual proof of rapid innate immune response to the live bacterial vaccine (Figure 1B, $+18$ h). Rapid induction of this inflammatory response was visible as early as $+0.5$ h and was clearly evident at $+18$ h with all volunteers developing pustules at the inoculation site (Figure 1B). Pustules were resolved by $+672$ h when all volunteers developed positive anti-*F. tularensis* titers (mean $1:364 \pm 526$, and Table 1).

Correlation mapping of all patients, at all timepoints, using all 54,000+ probe sets (i.e., gene fragments) was performed to measure the overall similarity of samples to one another. Visualization by heatmap revealed that no individual sample-to-sample comparison had less than a 0.89 correlation and that average correlations within each group were above 0.95 (Figure 2, enlarged in Supplemental Figure 1). This indicated high correlation across all samples and

that no strong outlier behavior was present on a global gene expression basis. These data signify commonality in human responses to LVS vaccination and lend weight to assertions that the results are applicable to a broad range of vaccinees and their predicted response to the vaccine.

Global expression pattern analysis yields three principal groupings

To determine the relationship between global expression for each patient (Figure 2) and various timepoints, principal component analysis (PCA) was performed using all probe sets on the array (Figure 3A, enlarged in Supplemental Figure 2). Volunteers with similar responses at a given timepoint appeared close to each other on the PCA plot and the converse was also true. The first principal component (x-axis) resulted in an inferred biological component of variability between the early post-vaccination response and the rest of the experiment, with 11% of total experimental variability explained. The second principal component (y-axis) resulted in an inferred biological component of variability between three principal groupings: pre-vaccine (−144 h), early post-vaccination (+18 and +48 h), and late post-vaccination (+192 and +336 h). The chart also reveals an apparent outlier, 22a, the pre-vaccination (−144 h) timepoint for volunteer 22. Further statistical analysis argued that 22a was not a severe outlier and internal controls did not suggest technical errors. Follow-up with this volunteer revealed the volunteer had received another vaccination fewer than 28 days before the pre-vaccination timepoint. Titer results from volunteer 22 at +672 h (day 28) failed to reveal any gross differences in this individual's response to vaccination. In fact, volunteer 22 had the median titer for all five volunteers (Table 1). While this biological variability was noted, volunteer 22 was found to have responses similar to other volunteers at all other timepoints and was not removed from downstream analysis.

While it is true that proteins with stable message are certainly involved in immune responses to vaccination, the impact of genes that show no change are difficult to assess. Therefore, we sought to examine the genes that showed the most change from pre-vaccination to post vaccination and from timepoint to timepoint. Figure 3B represents the relative number of genes that changed greater than twofold (up, down, and up/down combined – total) from one timepoint to another. The highest number of changes came between pre-vaccination vs. 48 h and pre- vs. 18 h post-vaccination, the majority of which were up-regulation events. The least number of changes were observed for 192 h vs. 336 h, then pre- vs. 192 h, and pre- vs. 336 h. These observations point to the very earliest timepoints being the source of the largest gene modulation events and later events having the very least amount of modulation. In further evaluation of the principal groupings resulting from PCA, assessments between each of the three groups, pre-, early post-, and late post-vaccination were performed. There were three to four times as many genes changing in early post-vaccination vs. pre-vaccination than in late post-vaccination vs. pre-vaccination groups. Also, genes were down regulated three times more in early post-vaccination vs. late post-vaccination. This analysis quantifies data from the PCA plot (Figure 3A) and stated that: 1) there were, in fact, three intra-related groups; 2) that the largest difference between any of the groups was between pre-vaccination and early post-vaccination; and 3) late post-vaccination was more similar to pre-vaccination than to early post-vaccination. Together these data corroborate previous studies from our lab and others suggesting that the earliest responses to vaccination or infection, may play key roles in anti-*F. tularensis* immune responses (Telepnev *et al.*, 2005; Malik *et al.*, 2006; Katz *et al.*, 2006; Isherwood *et al.*, 2005; Fuller *et al.*, 2006; Elkins *et al.*, 2003).

Gene expression patterns reveal early peak in LVS-induced gene-modulation

ANOVA-based contrast analysis allowed us to categorize the kinetics of gene modulation during LVS vaccination and search for genes modulated in a defined manner. The ten most prevalent patterns are depicted in Figure 4A. Mean-normalized signal intensity for all five volunteers was plotted (positive or negative) versus the five timepoints assessed, with

individual lines representing individual genes meeting the statistical cutoff to be included in each k-means cluster group (Figure 4B). Despite having relatively high numbers of genes fitting their pattern and meeting the statistical standards set, clusters 1 and 2 were noisy and showed genes without large responses relative to pre-vaccination. In contrast, clusters 3–10 had large relative responses and cluster variability was small. The four most prevalent patterns in order were: “down early” (pattern 4), “up early” (pattern 6), “sustained up” (pattern 3), and “up late” (pattern 5). Gene modulation patterns were further analyzed using self-organizing map (SOM) in which the data were fit to data-defined patterns instead of pre-determined patterns. The six SOMs that best fit the data were similar to patterns 3–6, 8, and 9, with the relative number of genes in each group matching those depicted in Figure 4. These k-means clustering patterns mirrored the fold-change and PCA analysis (Figure 3) in describing the largest differences in gene modulation at 48 h or less (early pre-vaccination). Changes primarily reflect down- (down early) and up- (up early) regulation of genes at ≤ 48 h and subsequent return to pre-vaccination levels at days 8–14. The pattern analysis expounds on the importance of early events by suggesting that of the responses to LVS vaccination, the most ordered and most prevalent are those that occurred at early times post-vaccination.

Immune response genes reflect pre-dominance of early modulation events

The high degree of kinetic similarity among these responding genes suggests activation of a distinct orchestrated biological response to stimuli, in this case, LVS vaccination. Further examination of the biological function of genes in each of the four most prevalent patterns yielded genes involved in six general types of cellular processes: immune-related, cell cycle-related, apoptosis-related, biosynthesis/metabolism, other/ambiguous (genes that are nearly ubiquitous in several processes, e.g. MAPK), and those of unknown function (Figure 5). The most frequently observed pattern, pattern 4 or “down early”, was also the pattern that contained the highest number of immune-related genes. The inverse pattern, pattern 6 or “up early”, also showed a significant bias towards immunity-related gene modulation. The two patterns suggest that a great number of genes are selectively up- or down-regulated in a timeframe more consistent with innate effector activation than acquired immune effector activation. In fact, only pattern 5, “up late,” showed genetic modulation consistent with acquired immune effector activation. Despite its kinetics, pattern 5 had the fewest matching immune-related genes of all patterns examined.

A better indication of the immune events occurring as a consequence of LVS vaccination was obtained by examining specific immune-related genes modulated in each pattern (Table 2–Table 5). Of the 42 genes that fit pattern 3 “sustained up,” approximately nine could be linked directly to immune function (Table 2). Among these were innate immune system defense mechanisms such as regulators of endocytosis/phagocytosis, granule exocytosis, chemotaxis, and inflammatory cytokines. These genes, induced upon vaccination, remained elevated throughout the 336 h of the study, suggesting a continual need for effector mechanisms controlled by these genes.

Pattern 4, “down early,” represents genes that were down-regulated in the early timeframe associated with initiation of immune responses. This group had the largest number of genes matching its pattern. Out of those 56 matches, 21 genes had immune-related functions (Table 3). Among these were regulators of chemotaxis/adhesion, T cell signal transduction, cytoskeletal remodeling, proliferation, and pro-inflammatory cytokine response. Several immune-related genes were just below our stringent threshold (see methods) for inclusion in pattern 4 “down innate” but worthy of note. Included in this group were: *IFNA10/IFNA17*, *CD28*, *CD3E*, *CD3ζ*, *CD3G*, *CD40*, *FASLG* (CD178), *killer cell lectin-like receptor (KLR)-B1*, *KLRC3*, *natural killer cell group (NKG)-7*, *natural killer-tumor recognition sequence (NKTR)*, *STAT3*, *TNF receptor-associated factor (TRAF)-1*, and *TRAF5*. These genes are

intimately associated with pro-inflammatory immune response by NK cells, NKT cells, and TLR-bearing innate immune effector cells such as DCs, monocytes, and neutrophils.

Downregulation of the genes listed in Table 3 suggests an attempt to modify the environment in which immune system activation will take place by dictating the recruitment of cells, the way cells receive stimulatory signals and the cytokine milieu in which cells receive this signal. Downregulation of these genes could suggest negative regulation of pro-inflammatory immune responses at a very early stage. This seems unlikely given the visible immune reaction at the site of vaccination (Figure 1B). Conversely, downregulation may indicate genes that have already been triggered and are now being dampened to prevent an overzealous inflammatory response. It is likely that the protein products of these genes were being produced at +18 to 48 h and thus the transcriptional attenuation observed indicates preparation for subsequent downregulation. Previous reports of innate immune effector cell activation at these timepoints support this hypothesis (Katz *et al.*, 2006; Fuller *et al.*, 2006; Elkins *et al.*, 1992; Elkins *et al.*, 2003).

Despite kinetics suggesting the specific up regulation of genes during acquired immune system activation, pattern 5 “up late,” had the least immune-related gene modulation (Table 4). Only the lymphocyte cell-specific tyrosine kinase *LCK*, was upregulated in a manner consistent with pattern 5. *LCK* plays a vital role in surface receptor signaling in NK, NKT, and T cells. Because transcriptional analysis was performed on total PBMC populations, it is difficult to interpret upregulation of a kinase as ubiquitous as *LCK*. The timeframe in which *LCK* was activated does imply preparation for an immune response. The predominance of biosynthetic and metabolic genes matching this pattern (Figure 5) was consistent with upregulation of a coordinated immune response, whether this was in the form of a secondary innate response or an adaptive response was unclear.

Pattern 6 or “up early” represents genes that are turned on in a timeframe consistent with activation of innate effectors such as mast cells, DCs, neutrophils, macrophages, granulocytes, and monocytes. The immune-related genes in pattern 6 are highly consistent with activation of these innate immune system cells (Table 5). Multiple indicators of innate functional response such as phagocytosis, exocytosis, super oxide formation, antigen processing, cytokine/chemokine production, and signal transduction are among the upregulated genes. Nearly every upregulated gene listed can be directly linked to a specific innate immune system cell and an associated effector function. Genes worthy of note that matched pattern 6 but did not meet cut-off criteria were *CASP4*, *FAS* (*CD95*), *TANK*, and *CRI*, all of which are associated with cytolysis and recognition of pathogens or infected cells. These data are highly consistent with previous work from our lab and others, which noted strong innate immune system responses immediately following LVS vaccination or challenge (Telepnev *et al.*, 2005; Isherwood *et al.*, 2005; Fuller *et al.*, 2006; Elkins *et al.*, 2003).

Genes regulating DC function are strongly activated by LVS vaccination

The substantial decrease in laboratory acquired-tularemia in conjunction with knowledge gained from murine tularemia models suggests that the LVS vaccine is capable of inducing a strong cell-mediated response (Tarnvik *et al.*, 1985; Tarnvik, 1989; Isherwood *et al.*, 2005; Fuller *et al.*, 2006; Elkins *et al.*, 1992; Elkins *et al.*, 2003; Dennis *et al.*, 2001). While the innate immune system may be important for initial response to *F. tularensis* infection, its role in generating long-lasting protective immunity mediated by the adaptive immune system is less clear. The keystone event in linking strong innate responses to well-primed adaptive responses is activation of DCs (Mocikat *et al.*, 2003; Katz *et al.*, 2006; Fuller *et al.*, 2006). Closer examination of genes associated with the professional antigen processing and presentation duties of DC and other nonprofessional antigen presenting cells reveals a strong tendency

towards upregulation at early timepoints followed either by downregulation or sustained high levels of gene expression (Figure 6).

Genes involved in antigen processing and presentation such as *DC-SIGN* (CD209), MHC class I ($\beta 2m$ and *TAP1*), MHC Class II, and CD1 all appear to be upregulated during the first 48 h post-vaccination. This indicated that DCs and other antigen-presenting cells were recognizing components of the LVS vaccine in a manner sufficient to induce upregulation of presentation molecules for naïve $\alpha\beta$ and $\gamma\delta$ T cells. The quality of the DC-T cell activation event was largely dependent on costimulatory and adhesion molecules present on the DC during activation. Costimulatory molecules *B7-1* and *B7-2* (CD80 and CD86) were upregulated at less than 48 h. CD80 was considerably downregulated at 192–336 h while CD86 expression was lower at 192 h but then increased at 336 h. *ICOS*, an important positive regulator of anti-inflammatory lymphocytes was down regulated throughout the timecourse. Upregulation of *ICAM-1*, the ligand for lymphocyte integrin CD11/18, is associated with induction by pro-inflammatory cytokines like *IFN γ* , *TNF α* , *IL-1 α* , and *IL-18*, was noted only at 48 h post-vaccination and was notably downregulated at all other timepoints. *ICAM2*, the ligand for lymphocyte LFA-1 associated with NK cell-mediated clearance and lymphocyte recruitment, was upregulated throughout the timecourse. CD44 and CD58, both known to mediate lymphocyte activation and homing as a result of upregulation by pro-inflammatory cytokines, are also adhesion molecules which were specifically upregulated 18–48 h post-vaccination. For a number of costimulatory/adhesion genes, their ligand/functional counterparts were also upregulated in a complementary fashion (e.g., *CD58/GLIPR*, *LILRA6/DC-SIGN*, *ICAM1/CD36*, *CD44/SPN*) (Table 2–Table 5) again suggesting a finely orchestrated response to LVS vaccination. Recent reports from murine model systems also underscore the importance of DC processing and presentation effector functions in initiation (< 20 h) of anti-*F. tularensis* responses (Katz *et al.*, 2006).

Data suggesting that DC and other innate immune system cells are strongly activated early begs the question of how cells are recognizing LVS components. The greatest surprise in our analysis was the lack of *NOD1/2* (*CARD4* and *CARD15*) regulation in response to vaccination (Figure 7). *F. tularensis* is an intracellular bacterium and therefore pattern recognition motifs that specifically sample the intracellular environment seem likely to be involved in recognition. Yet NOD1/2 signaling did not seem to modulate with respect to vaccination. The data were unable to rule out use of NOD1/2 as recognition receptors for LVS as no measure of protein level was obtained. Murine studies have implied the importance of TLR 2- and 6-mediated recognition of *F. tularensis* by DCs (Katz *et al.*, 2006). In our study, all TLRs were upregulated across all timepoints examined in all volunteers, with moderately higher expression at 18–48 h postvaccination (Figure 6). Several downstream mediators of TLR signaling such as *IRAK3*, *CARD8*, *MYD88*, and *TIRAP* were activated at timepoints immediately post-vaccination (18–48 h) or throughout the course of the study (Figure 6 and Table 5). Thus, human data as well as murine data suggest at least one possible mechanism by which a large number of innate immune system effectors may be activated in response to LVS vaccination (Katz *et al.*, 2006).

Discussion

As modern clinical immunology and molecular biology begin to coalesce in the laboratory, they have allowed careful elucidation of previously anecdotal evidence of host-pathogen interactions. Here, we present an application of transcriptome analysis to the human response to the LVS vaccine. Using highly vigorous controls and statistical methods, we present a gene-level view of host-pathogen interactions that may be predictive of the formation of long-lasting, protective immune responses.

The primary obstacle to transcriptome analysis of vaccine response in humans is compensating for human-to-human variability. The wide range of results from anti-*F. tularensis* microagglutination titers, the current clinical standard for assessment of LVS response, underscore the variability that is expected in human studies (Table 1). Thus, in the search for early predictors of vaccine outcomes, any new standard must endure an enormous range of human genetic diversity and remain consistent. Surprisingly, all statistical analysis of this study showed the LVS vaccine was able to elicit an extremely consistent response (correlation > 0.88) across all genes, at all timepoints, in all volunteers (Figure 2 and Figure 3A). In addressing the need for universal biomarkers predictive of vaccine outcome, these results are more than reassuring.

It must further be noted that while we sought to examine changes in gene regulation post-vaccination, it is more than reasonable to assume that expression of genes that may be instrumental to vaccine efficacy were not changed in a two-fold-manner and therefore not considered here. Second, our analysis of PBMCs can only be thought of as a surrogate for measuring immune responses taking place in the skin, at the site of vaccination.

While prior reports suggested that human LVS responses are not measurable until day ten post-challenge, largely relied on use of *in vitro* re-stimulation assays to reach their conclusions (Karttunen *et al.*, 1991). Since then, a more clear understanding of the importance of innate responses to the development of adaptive immune response has emerged (Elkins *et al.*, 2003). It follows that the most effective vaccines elicit strong innate immune responses that aid in the development of potent adaptive recall responses. Indeed, as we have reported for humans, and others have shown for mice, early responses within the first 48 h of LVS vaccination influence development of immunity (Telepnev *et al.*, 2005; Isherwood *et al.*, 2005; Fuller *et al.*, 2006; Elkins *et al.*, 2003). Transcriptome analysis distinguishes the first 48 h post vaccination as being the timeframe in which most immune system genes and most genes overall are modulated (Figure 3B, 4, and 5). The first 48 h are so robust that gene expression at later timepoints (>48 h) is more similar to un-immunized volunteers than to any early point (<48 h) after vaccination (Figure 3A). It is worthy of note that recent murine transcriptional analyses following aerosol challenge with LVS showed a lack of inflammation-related gene modulation until day 4 (Andersson *et al.*, 2006). Whether or not the mode of infection (intranasal vs. intradermal), mechanism of measurement (lung lavage vs. PBMC), inoculum (20 CFU vs. 1.2×10^9), or obvious differences between human and murine susceptibility to LVS can account for these disparities is unclear. Still, our results clearly have statistically significance responses and uniquely quantify human *in vivo* responses, further encouraging the pursuit of transcriptome analysis as a vigorous alternative to current correlates of immunity for LVS and other vaccines.

At this time, global analysis of gene expression on the scale reported here, is too time-consuming and expensive for the clinical laboratory. Several validated biomarkers would greatly increase the feasibility of transcriptome analysis for vaccine development. Immune-related genes mirrored the overall kinetics of modulated genes following vaccination (Figure 5). Namely, immune gene regulation was most prevalent at early (<48 h) timepoints. Examination of specific immune genes revealed a patterned response in which innate effector- and pro-inflammatory- related genes dominated early transcripts and were largely upregulated (Table 2–Table 5). While studies were performed on total PBMC populations, cell-specific activation markers revealed that macrophages, DCs, neutrophils, NK cells, and mast cells were among the cell types that rapidly responded to LVS vaccination by modulating effector-related genes. A similar bias towards innate immune system cells was reported in our previous studies in which human PBMCs were immunophenotyped post-LVS vaccination (18). Recent murine studies in which innate and pro-inflammatory immune responses are demonstrated to be vital

to protection against lethal LVS infection are in agreement with the data presented here (16, 20, 28, 39–42).

Not only are responding genes related in their kinetics, examination of specific markers related to response by effector cells revealed the existence of a concerted anti-LVS response (Figure 6 and Figure 7). Components of the TLR recognition and signaling pathways were highly represented in kinetic and fold-change analyses (Table 2–Table 5 and Figure 7). While murine data also emphasized the importance of TLRs in LVS responses, knockout mouse studies implicate TLR 2 as being vital to response (25, 28). In contrast, TLR4 and 8 appeared to be the most upregulated TLRs at early timepoints (< 48 h) (Figure 7). TLR4 is noted for its ability to induce T_H1-biasing, and thus cell-mediated, responses in DCs, lending further support to our hypothesis that cell-mediated, and specifically innate, responses are important to human LVS vaccination (30, 43).

Reports from other groups have implicated the inflammasome pathway as essential to murine response to *F. tularensis*, specifically IL-1 β , IL-18, ASC, and caspase-1 (41, 44). Our data did not reveal any pattern or any statistically significant changes in the aforementioned genes except for IL-18, which did meet cut-off criteria for “up early” (Table 5). Of the caspase genes examined, (CASP1/4/6/8), none made our criteria for being included with the observed kinetic patterns, nor did any show statistically significant changes over the course of the study. Our data involved transcript upregulation but not protein expression, therefore it is difficult to ascertain if differences observed are due to experimental issues (mRNA vs. protein), model system (human vs. murine), or kinetics (+18, +48, +192, +336 h vs. +6 d). Experiments to address these differences are underway in our laboratory.

The pivotal role of DCs in development of acquired immune responses is highlighted by our data. Again, the transcriptome analysis presented herein was performed on total PBMC populations, not isolated cells. However, the strong upregulation of genes related to antigen processing and presentation by professional antigen presenting cells (Figure 6) along with supportive murine and human studies make inferences regarding DC activation justified (18, 28). Studies in our laboratory regarding isolated human DC responses to LVS also suggest DC activation and priming of cell mediated immunity does occur *in vitro* (*manuscript in preparation*).

Transcriptome analysis of human immune events has the potential to revolutionize our understanding of drug development, immunology, and pathogenesis. Results from transcriptome analysis could be used to compare efficacy of different lots of the LVS vaccine and serve as a benchmark with which to compare new anti-*F. tularensis* vaccines. As the need to test anti-*F. tularensis* vaccines in challenge models emerges, transcriptome analysis will allow for a better assessment of how murine or nonhuman primate models compare to human responses to the vaccine. This would allow vaccine evaluation in an animal model that most closely emulates human LVS response and thus better predicts human anti-pathogenic *F. tularensis* strain responses. As with all transcriptional analysis, the correlation with protein expression and/or activity must be addressed. However, it is encouraging to designate a subset of proteins of interest, as well as timepoints in which to examine them. Experiments to examine the correlation between the gene regulation reported here and protein expression and function are currently underway in our laboratory.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

LVS, Live vaccine strain; PCA, principal components analysis.

Figure 1.

Study design yielded data confirming commonality of LVS vaccine response. **(A)** Five consenting volunteers donated PBMCs six days before LVS vaccination, then 18, 48, 192, and 336 h post-vaccination. PBMCs were processed for RNA isolation as described in the Methods section. **(B)** Photographic documentation of vaccine site pustule formation and ulceration of the volar surface of the arm at the indicated timepoints, post-vaccination. Photographs are representative of reactions seen in all volunteers.

Figure 2.

Heatmap visualization of correlation matrix for all five volunteers (22, 23, 24, 27, and 32) at all five time points (**a**, -6 d; **b**, 18 h; **c**, 48 h; **d**, 192 h; **e**, 336 h) all using 54000+ probe sets. The plot indicated high correlation across all samples and that no sample-to-sample comparison showed less than a 0.89 correlation. Further, no sample showed an average correlation to all other samples of less than 0.92.

Figure 3.

Covariance principal component analysis and fold change assessment. **(A)** PCA analysis revealed the lack of severe outliers and the presence of three principal groupings (indicated by circles): pre-vaccination, -144 h; early post-vaccination, 18 and 48 h; and late post-vaccination, 192 and 336 h. **(B)** Pairwise comparison of number of genes changing more than twofold (up or down) relative to the earlier timepoint. A paired Student's t-test indicated $p < 0.01$ for all comparisons.

Figure 4.

K-means clustering of genes reveals ten prevalent patterns. (A) Mean normalized signal intensities were examined for changes in regulation across all five time points. ANOVA-based pattern-matching analysis revealed the ten most highly observed kinetic patterns. Lines represent individual gene patterns allowing assessment of “noise” in each group. Patterns with F-scores >10 were considered significant. (B) Quantification of genes that made the statistical cutoff (Pearson’s correlation of ± 0.95) to be included in a given cluster group represented in 4A. The two combined statistical filters yielded tightly observable clusters of gene regulation across time.

Figure 5.

Categorization of clustered genes reveals strong bias towards immune and biosynthesis/metabolism-related genes. Genes clustered in the four most prevalent patterns were sorted by function. Genes were categorized based on relatedness to host defense (immune), cell cycle regulation and proliferation, cell death and apoptosis, general protein/lipid/carbohydrate synthesis or metabolism, unknown function, or function that were common to too many pathways to allow obvious categorization. Not that some genes fell into more than one category.

Figure 6. Heatmap visualization of DC and antigen-presenting associated gene modulation was either up- or downregulated at early times post-vaccination. The heatmap represents mean fold-change differences in intensity as compared to the pre-vaccination group for five donors at four post-vaccination timepoints for the indicated antigen presenting- and processing-associated genes. Multiple listings of a single gene indicate that gene is represented by more than one gene fragment in the array.

Figure 7. Heatmap visualization reveal selective upregulation of TLR/NOD-associated genes post-vaccination. The heatmap represents mean fold-change differences in intensity as compared to the pre-vaccination group for five donors at four post-vaccination timepoints for the indicated TLR/NOD-associated genes. Multiple listings of a single gene indicate that gene is represented by more than one gene fragment in the array.

Table 1

<i>Volunteer</i>	<i>Pre-vaccination titer</i>	<i>Post-vaccination titer</i>
[[22]]	[[0]]	[[160]]
[[23]]	[[0]]	[[20]]
[[24]]	[[0]]	[[320]]
[[27]]	[[0]]	[[40]]
[[32]]	[[0]]	[[1280]]

Table 2

[[Gene Symbol]]	[[Gene Name]]	[[Biological Process]]	[[GeneID]]
[[LRP1]]	low density lipoprotein-related protein 1 (alpha-2-macroglobulin receptor)	cell proliferation, endo/phagocytosis of apoptotic cells, lipid metabolism, cell adhesion, interacts with NOS1AP, C3, furin, lactotransferrin,	[[4035]]
[[M6PR]]	mannose-6-phosphate receptor (cation dependent)	transport of phosphorylated lysosomal enzymes from the golgi complex and the cell surface to lysosomes, receptor mediated endocytosis	[[4074]]
[[TXNIP]]	[[thioredoxin interacting protein]]	novel regulators of tumor necrosis factor signaling and inflammation in endothelial cells required for platelet granule secretion	[[10628]]
[[VAMP8]]	vesicle-associated membrane protein 8 (endobrevin)		[[8673]]
[[MAPKAPK3]]	mitogen-activated protein kinase-activated protein kinase 3	response to stress, signal transduction, associated with IL1alpha and beta regulation	[[7867]]
[[SPN]]	sialophorin (gpL115, leukosialin, CD43)	cellular defense response, chemotaxis, associated with CD44, FYN, WASP, P-selectin, ICAM2	[[6693]]
[[CMRF-35H/Irp60]]	leukocyte membrane antigen (CD300a)	inhibitor of IgE induced mast cell degranulation, NK cell cytotoxicity inhibitor	[[11314]]
[[ARRB1]]	[[arrestin, beta 1]]	sensory perception, signal transduction, regulating receptor-mediated immune functions	[[408]]
[[DKFZP564J0863]]	[[DKFZP564J0863 protein/ATL3]]	[[immune response]]	[[25923]]

Table 3

[[Gene Symbol]]	[[Gene Name]]	[[Biological Process]]	[[GeneID]]
[[CD96]]	[[CD96 antigen (TACTILE)]]	cell adhesion, immune response (NK and T cells), antigen presentation	[[10225]]
[[NAP1L1]]	nucleosome assembly protein 1-like 1	DNA replication, positive regulation of cell proliferation	[[4673]]
[[RORA]]	RAR-related orphan receptor A	regulation of transcription, signal transduction, reduces VCAM-1/ICAM-1 expression	[[6095]]
[[CCL5]]	chemokine (C-C motif) ligand 5 (RANTES)	cell adhesion, cellular defense response, chemotaxis, exocytosis, inflammatory response, response to oxidative stress, induced by TNFalpha and IL1alpha	[[574178]]
[[PTPRCAP]]	protein tyrosine phosphatase, receptor type, C-associated protein (CD45 associated protein)	defense response, lymphocyte signaling	[[5790]]
[[TNFRSF25]]	tumor necrosis factor receptor superfamily, member 25	apoptosis, signal transduction, lymphocyte proliferation	[[8718]]
[[TRBV19]]	T cell receptor beta variable 19	T-cell receptor, signal transduction, activation	[[28568]]
[[CD3D]]	CD3D antigen, delta polypeptide (TiT3 complex)	T-cell receptor complex, signal transduction, activation	[[915]]
[[ZAP70]]	zeta-chain (TCR) associated protein kinase 70kDa	protein amino acid phosphorylation, lymphocyte protein kinase cascade	[[7535]]
[[NUMA1]]	nuclear mitotic apparatus protein 1	mitotic anaphase, cytoskeletal rearrangement, caspase activation	[[4926]]
[[NOSIP]]	nitric oxide synthase interacting protein	modulator of eNOS activity, interacts with NOS1/3	[[51070]]
[[PRKCH]]	protein kinase C, eta	intracellular signaling cascade (Akt and mTOR)	[[5583]]
[[KLF2]]	[[Kruppel-like factor 2]]	negative regulation of gamma globulin genes, induction of eNOS, apoptosis, IL-2 promoter activation	[[10365]]
[[NACA]]	nascent-polypeptide-associated complex alpha polypeptide	protein biosynthesis, co-activator of Jun transcription factors and FADD signaling	[[4666]]
[[STMN3]]	[[stathmin-like 3]]	intracellular signaling cascade, microtubule-destabilizing phosphoproteins	[[50861]]
[[GSPT1]]	G1 to S phase transition 1	G1/S transition of mitotic cell cycle, mRNA catabolism, JNK1 interaction, apoptosis block	[[2950]]
[[F11R]]	F11 receptor/ Junction adhesion molecule (JAM1)/CD321	cell motility, inflammatory response, NFkappaB activation, negative regulation by TNF alpha and IFN gamma, associated with CD3D	[[50848]]
[[PAR1/UBE3A]]	Prader-Willi syndrome chromosome region 1, GMCSFRalpha precursor, IL3Ralpha precursor (CD123)	brain development, proteolysis and peptidolysis, regulation of transcription, DNA-dependent, ubiquitin cycle, ubiquitin-dependent protein catabolism, associates with Cbl and BLK	[[7337]]
[[RHOH]]	ras homolog gene family, member H	T-cell differentiation, negative regulation of I-kappaB kinase/NF-kappaB cascade, regulation of transcription	[[399]]
[[PPP2R5C]]	protein phosphatase 2, regulatory subunit B (B56), gamma isoform	signal transduction, paxillin and PKC delta associated	[[5527]]
[[GA17/PCID1/hfl-B5]]	dendritic cell protein/ PCI domain containing 1	[[herpesvirus entry mediator]]	[[10480]]

Table 4

[[Gene Symbol]]	[[Gene Name]]	[[Biological Process]]	[[GeneID]]
[[LCK]]	Lymphocyte cell-specific protein tyrosine kinase	intracellular signaling cascade, protein phosphorylation, associated with CBL, CTLA4, ZAP70, CD4, CBL, JAK3, CD2, SYK, CD8, CD45	[[3932]]

Table 5

[[Gene Symbol]]	[[Gene Name]]	[[Biological Process]]	[[GeneID]]
[[CREG1]]	cellular repressor of E1A-stimulated genes 1	antagonizes transcriptional activation and cellular transformation by E1A	[[8804]]
[[LAMP2]]	lysosomal-associated membrane protein 2 (CD107b)	protection, maintenance, and adhesion of the lysosome, promoting immunological recognition and antigen presentation, cytolytic granule exocytosis marker, cathepsin, CD63 associated	[[3920]]
[[FCGR2A]]	Fc fragment of IgG, low affinity IIa, receptor for (CD32)	immune response, lymphocyte activation: CRP, BLK, LAT, HCK, PI3K, SHC1	[[2212]]
[[DCL-1]]	type I transmembrane C-type lectin receptor DCL-1 (CD302)- DEC205 ligand	Mono/mac and DC (expressed on migrating DC) receptor, associated with CD8, CD40L, CD80, CD86, Nkp30 (CD337), IL4, IL-12Ralpha, IL15, GMCSF	[[9936]]
[[GLIPR1]]	GLI pathogenesis-related 1 (glioma)	CD58 (LFA3) and CD2 associated, costimulation	[[11010]]
[[CSF2RB]]	colony stimulating factor 2 receptor, beta, low-affinity (granulocyte-macrophage) – IL3/IL5 receptor low-affinity	antimicrobial humoral response, cytokine and chemokine mediated signaling pathway: JAK2, signal transduction: LYN SHC1, PKC beta	[[1439]]
[[QPCT]]	glutaminyl-peptide cyclotransferase (glutaminyl cyclase)	protein modification, proteolysis, MICA and HLA-B associated	[[25797]]
[[RNASE2]]	ribonuclease, RNase A family, 2 (liver, eosinophil-derived neurotoxin) EDN/RNS2	RNA catabolism, selectively chemotactic for dendritic cells, proinflammatory-DCs, neutrophils, and granulocytes	[[6036]]
[[IL18]]	interleukin 18 (interferon-gamma-inducing factor)	pro-inflammatory immune response, chemokine production, apoptosis, cytokine production: GMCSF, IFN-gamma, TNF, IL1alpha, IL2 IL13, IL2, positive regulation of activated T-cell proliferation, adhesion: ICAM1, VCAM1	[[3606]]
[[CD36]]	CD36 antigen (collagen type I receptor, thrombospondin receptor)	blood coagulation, cell adhesion ICAM1, phagocytosis, CD9-associated, LYN signaling	[[948]]
[[LY96/MD-2]]	lymphocyte antigen 96/ TLR4 associated	antibacterial humoral response, TLR4 (LPS) linked signal transduction: MYD88, IRAK1, TLR2, TLR9, TLR10, CD14, PI3K, NFkappaB, cellular defense response, inflammatory response: IL8, TNF	[[23643]]
[[LILRA6 (ILT8/ LILRB6)]]	(leukocyte immunoglobulin-like receptor, subfamily B (with TM and ITIM domains), member 2, leukocyte immunoglobulin-like receptor, subfamily B (with TM and ITIM domains), member 3, leukocyte immunoglobulin-like receptor, subfamily B (with TM and ITIM domains), member 6)	inhibitory immunoreceptors expressed on monocytes, B cells, dendritic cells and natural killer (NK) cells, associated with TLR2, DC-SIGN, IL4, SLAMF1, KLRA1, ITLN1	[[79168]]
[[CD39/ ENTPD1]]	ectonucleoside triphosphate diphosphohydrolase 1 (Lymphoid cell activation antigen)	antimicrobial humoral response, blood coagulation, cell adhesion, cell-cell signaling, expressed primarily on activated lymphoid cells, marks differentiation of granulocytic and monocytic cells	[[953]]
[[NCF2]]	neutrophil cytosolic factor 2	cellular defense response, superoxide metabolism	[[4688]]
[[CD116/ CSF2RA]]	colony stimulating factor 2 receptor, alpha, low-affinity (granulocyte-macrophage)	controls the production, differentiation, and function of granulocytes and macrophages, associated with CXCR3, JAK2, c-fos, gamma IP10, c-Kit, IL3R	[[1438]]
[[IL13RA1]]	interleukin 13 receptor, alpha 1	binds IL13 with a low affinity, alternate accessory protein common cytokine receptor gamma chain for IL4 signaling, associated with TYK2, JAK1, STAT3, STAT6, IFNgamma, IL5, IL7, IL13, IL19	[[3597]]
[[SCAP2]]	src family associated phosphoprotein 2	protein complex assembly, signal transduction, associated with FYB, FYN, HCK, LYN, PTPRH and PRAM1	[[8935]]
[[IRAK3]]	interleukin-1 receptor-associated kinase 3	cytokine and chemokine mediated signaling pathway, inhibits dissociation of IRAK1 and IRAK4 from the toll-like receptor signaling complex by either inhibiting the phosphorylation of IRAK1 and IRAK4 or stabilizing the receptor complex	[[11213]]
[[CLEC4A/ CLECSF6]]	C-type (calcium dependent, carbohydrate-recognition domain) lectin, superfamily member 6	antimicrobial humoral response, cell adhesion, cell surface receptor linked signal transduction, inflammatory immune response, linked to CD99 and other CTL/CTLD superfamily members in the natural killer gene complex region	[[50856]]