Published in final edited form as:

J Immunol. 2013 August 1; 191(3): 1276–1286. doi:10.4049/jimmunol.1300867.

Francisella tularensis* SchuS4 and SchuS4 lipids inhibit IL-12p40 in primary human dendritic cells by inhibition of IRF1 and IRF8

Robin Ireland¹, Rong Wang^{1,**}, Joshua B. Alinger¹, Pamela Small², and Catharine M. Bosio^{1,†}

¹Immunity to Pulmonary Pathogens Section, Laboratory of Intracellular Parasites, Rocky Mountain Laboratories, NIAID, NIH, Hamilton, MT 59840

²Department of Microbiology, University of Tennessee, Knoxville, TN, 37996

Abstract

Induction of innate immunity is essential for host survival of infection. Evasion and inhibition of innate immunity is a strategy used by pathogens, such as the highly virulent bacterium *Francisella tularensis*, to ensure their replication and transmission. The mechanism and bacterial components responsible for this suppression of innate immunity by *F. tularensis* are not defined. Here, we demonstrate that lipids enriched from virulent *F. tularensis* strain SchuS4, but not attenuated Live Vaccine Strain (LVS), inhibit inflammatory responses *in vitro* and *in vivo*. Suppression of inflammatory responses is associated with I κ B α independent inhibition of NF- κ Bp65 activation and selective inhibition of activation of Interferon Regulatory Factors (IRFs). Interference with NF- κ Bp65 and IRFs is also observed following infection with viable SchuS4. Together these data provide novel insight as to how highly virulent bacteria selectively modulate the host to interfere innate immune responses required for survival of infection.

INTRODUCTION

Virulent *Francisella tularensis* ssp *tularensis* is a Gram negative bacterium and the causative agent of the fatal disease coined tularemia. There are four major subspecies of *Francisella* (1). *F. tularensis* ssp. *novicida* and ssp. *mediasiatica* are generally considered attenuated for humans. *F. tularensis* ssp *holarctica* (Type B; *F. holarctica*) causes serious disease in humans but is not typically fatal. *F. tularensis* ssp *tularensis* (Type A; *F. tularensis*) is highly infectious and can cause a lethal infection following inhalation of as few as 10 organisms in both humans and rodent models (2, 3). *F. tularensis* ssp *tularensis* was previously utilized as a biological weapon and, thus presents a viable concern as a potential bioweapon today (4). Although a vaccine generated from serial passage of *F. holarctica*, i.e. Live Vaccine Strain (LVS), was produced in the 1960s it is no longer licensed as a vaccine for use against tularemia (5). Further, treatment of individuals infected with *F. tularensis* with antibiotic (when delivered in a timely manner) does not always result in complete clearance of the bacterium (6). Thus, there is a need for development of both novel vaccines and therapeutics to combat this highly virulent pathogen.

The effectiveness of vaccines and therapeutics is tied to their ability to trigger innate immune responses. Induction of innate immunity is an important component of host defense

*This work was supported by the Intramural Research Program of the National Institutes of Health, National Institute of Allergy and Infectious Diseases

[†]Corresponding Author: Catharine M. Bosio, Ph.D., Immunity to Pulmonary Pathogens Section, Laboratory of Intracellular Parasites, Rocky Mountain Laboratories/NIAID/NIH, Hamilton, MT 59840, Ph: 406-363-9425, bosioc@niaid.nih.gov.

^{**}Current Affiliation: U.S. Meat Animal Research Center, Clay Center, Nebraska

since this response slows replication and dissemination of microorganisms allowing the adaptive response time to develop. Unlike attenuated subspecies and strains, virulent *F. tularensis* is not sensed by host receptors or other detection machinery (7–10). In addition to evading detection by the host, virulent *F. tularensis* also suppresses the ability of host cells to mount inflammatory responses (7, 8, 11). Together the ability of the bacterium to both evade and suppress innate immune responses is a primary mechanism of virulence.

Generation of novel vaccines and therapeutics for treatment of tularemia has been hampered by our lack of understanding of the host pathways associated with innate immunity which are modulated by virulent *F. tularensis*. Similarly, we have not identified the components of the bacterium that facilitate suppression of innate inflammatory responses in the host. Identification of both the host signaling pathways and components uniquely associated with virulent *F. tularensis* that mediate inhibition of inflammation will greatly facilitate development of new therapeutics and vaccines.

In this report we demonstrate that lipids isolated from fully virulent *F. tularensis* strain SchuS4, but not attenuated LVS, inhibit innate immune responses in primary human cells *in vitro* and in the mouse lung *in vivo*. Inhibition of inflammatory responses by SchuS4 lipids was a result of targeting specific host proteins required for transcription of genes necessary for innate immunity. Importantly, these findings were recapitulated following infection with viable SchuS4.

MATERIALS AND METHODS

Reagents

Ultrapure *E. coli* strain K12 LPS, Pam3CSK4, Pam2CSK4, lipoteichoic acid (LTA), ssRNA40/LyoVec and R848 (imidazoquinolone compound) were purchased from Invivogen (San Diego, CA). *E. coli* strain O127:B7 LPS was purchased from Sigma (St. Louis, MO). Recombinant GM-CSF and IL-4 were purchased from Peprotech (Rocky Hill, NJ). Pronase was obtained from Roche Diagnostics (Indianapolis, IN).

Bacteria

Virulent *F. tularensis* ssp *tularensis* strain SchuS4 was kindly provided by Jeannine Peterson, Ph.D. (Centers for Disease Control, Fort Collins, CO). Attenuated *F. tularensis* ssp *holarctica* Live Vaccine Strain (LVS) was originally obtained from Dr. Jean Celli (Rocky Mountain Laboratories, Hamilton, MT). Stock vials of SchuS4 and LVS in broth were generated as previously described (10, 12).

Isolation of Total Membrane Fraction

Total membrane fraction (MF) from LVS and SchuS4 were isolated as previously described (13–15). Briefly, SchuS4 was grown in modified Mueller-Hinton broth as previously described (10, 12, 13). Following overnight culture, bacteria were pelleted by centrifugation for 15 minutes at $8000 \times g$. The resulting pellet was resuspended in the following buffer 50 mM Tris/HCl, 0.6 ug/ml DNase, 0.6 ug/ml RNase, 1 mM EDTA (all from Sigma) and 1 Complete EDTA free protease inhibitor cocktail tablet (Roche) followed by centrifugation and resuspension in the buffer described above. Bacteria were lysed via processing in Fast Prep Lysing Matrix B tubes using a FastPrep24 (MPBio) for 10 cycles of 45 seconds with 2 minute rest periods on ice in between each cycle. The resulting slurry was then centrifuged at 10,000 rpm for 10 minutes. The supernatant was collected and centrifuged twice at $100,000 \times g$ for 4 h. The pellet was resuspended in buffer containing 50 mM Tris/HCl, 1 mM EDTA and dialyzed against PBS using 3000 MW cutoff Slide-A-Lyzer cassettes (Pierce). Protein concentration of MF was determined using a BCA Protein Assay Reagent

Kit according to the manufacturer's instructions. MF was then aliquoted, irradiated to render it sterile, and stored at -80°C . As indicated MF was heated at 56°C for 4 hours or incubated with 2 mg/ml pronase in 0.1M Tris buffer pH 7.0 at 40°C for 3 hours followed by heating at 87°C for 30 minutes to deactivate the pronase prior to use.

Preparation of Francisella lipids

Lipids were isolated from LVS and SchuS4 using the modified Folch method for isolation of bacterial lipids as previously described (16–19). Briefly, 1×10^9 bacteria were thawed and plated onto 8–150 mm petri dishes containing MMH agar. Bacteria were incubated at $37^{\circ}\text{C}/7\%\text{CO}_2$ for 48 hours. Bacteria were collected from the agar plates and added to 100 ml HPLC grade Chloroform:Methanol (2:1) (both from Sigma). The resulting mixture was stirred vigorously for 30 minutes at room temperature. Then, 20 mls of endotoxin free water was added and the mixture was stirred for an additional 10 minutes. The mixture was centrifuged at $4000 \times g$ for 10 minutes at room temperature to separate organic and aqueous phases. Organic and aqueous phases were pipetted into separate containers and dried or concentrated under nitrogen. Dried organic samples were reconstituted in absolute ethanol (Warner-Graham) to 20 mg/ml. Average yield of lipids from Francisella was 80 mg/4 g wet weight of bacteria, representing approximately 2% of wet weight. Thus, $30 \mu\text{g}/\text{ml}$ of lipid is 0.00075% wet weight of bacteria. The aqueous phase was concentrated to 1 ml. Both preparations were stored at 4°C for up to 2 months. Absence of proteins and carbohydrates present in the organic phase was confirmed by analysis of preparations on silver stained SDS-PAGE gels, quantification of protein using Bradford Assay (Sigma) according to manufacturer's instruction, periodate stain of SDS-PAGE gels, and western blotting for Francisella O-antigen and capsule. No evidence of proteins or carbohydrates was observed in organic phase preparations.

Generation and stimulation of human dendritic cells

Primary human dendritic cells (hDC) were differentiated from apheresed monocytes as previously described (7, 10, 11). Human monocytes, enriched by apheresis, were obtained from peripheral blood provided by the Department for Transfusion Medicine and the NIH Clinical Center at the National Institutes of Health (Bethesda, MD) under a protocol approved by the NIH Clinical Center Institutional Review Board. Signed, informed consent was obtained from each donor acknowledging that their donation would be used for research purposes by intramural investigators throughout NIH. Monocytes were further enriched using Ficoll-paque Premium (GE Healthcare) and were differentiated into hDC following culture in RPMI1640 supplemented with 10% heat-inactivated fetal calf serum (FCS), 0.2 mM L-glutamine, 1 mM HEPES buffer and 0.1 mM nonessential amino acids (all from Invitrogen) (complete RPMI [cRPMI]) and 100 ng/ml GM-CSF and 20 ng/ml IL-4 over the course of 4 days. On day 3 of culture 100% of each cytokine per well in 1 ml cRPMI was added and cells were used on day 4 of culture. The resulting differentiated hDC were $>97\%$ CD1a⁺/DC-SIGN⁺ and $<1\%$ CD14⁺. hDC were seeded at 5×10^5 cell/ml in cRPMI. MF or lipid isolated from SchuS4 and LVS was added at the indicated concentrations. Cells treated with EtOH served as vehicle control samples. Twenty-four hours after addition of MF or lipid supernatants were harvested and assessed for cytokine as described below. Viability of hDC following incubation with EtOH and lipid was assessed by trypan blue exclusion assay. Percent of viable cells was calculated using the following formula: # trypan blue negative cells/total cells counted $\times 100$. As indicated, ultrapure *E. coli* LPS or *E. coli* LPS O127:B8 (10 ng/ml), Pam3CSK4 (5–10 $\mu\text{g}/\text{ml}$), Pam2CSK4 (10 $\mu\text{g}/\text{ml}$), LTA (100 $\mu\text{g}/\text{ml}$), R848 (5 $\mu\text{g}/\text{ml}$), or LPS (10 ng/ml) + 100 U/ml IFN- γ (Peprotech) were added 18 hours after MF or lipid treatment and cells were cultured for an additional 20 hours before supernatants were collected for assessment of cytokines.

Mice and *in vivo* assessment of inflammation

Specific-pathogen-free, 6–8 week old male C57Bl/6J (wild type) ($n = 5$ group) were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were housed in sterile microisolator cages in the ABSL-2 facility at the NIAID/Rocky Mountain Laboratories (RML). All mice were provided sterile water and food *ad libitum* and all research involving animals was conducted in accordance with Animal Care and Use guidelines and animal protocols were approved by the Animal Care and Use Committee at RML.

SchuS4 lipids and *E. coli* LPS were administered intranasally. Briefly, lipids were resuspended at 25 $\mu\text{g}/25 \mu\text{l}$ in PBS. Mice were anesthetized by intraperitoneal injection of 100 μl 12.5 mg/ml ketamine+3.8 mg/ml xylazine. Lipids were administered in a volume of 25 μl into a single nare. Mice treated with ethanol served as negative controls. Eighteen hours after exposure to lipids, mice were anesthetized as described above and 200 ng/25 μl of *E. coli* LPS O127:B8 (Sigma) was delivered into a single nare. Five hours after delivery of LPS mice were euthanized and bronchoalveolar lavage (BAL) fluid was collected for assessment of cytokines by ELISA and cellular populations by flow cytometry as previously described (8, 20).

Assessment of Cytokines

Human TNF- α , IL-6, IL-10, IL-12p40, and TGF- β were assessed by commercially available ELISA kits according to manufacturer's instructions (BD Biosciences). Concentrations of mouse TNF- α , IL-6, and IL-12p40 in BAL fluid were assessed by commercially available ELISA kits according to manufacturer's instructions (BD Biosciences).

Flow Cytometry

Influx of inflammatory cells into mouse airways and expression of hDC cell surface receptors was assessed by flow cytometry as previously described (8, 10, 11). Briefly, directly conjugated antibodies for these analyses were purchased from eBiosciences (San Diego, CA). The following antibodies in various combinations were used for flow cytometric analysis: PeCy7 anti-mouse CD11b (clone M1/70), PerCp-Cy5.5 anti-mouse CD11c (clone N418), PE anti-mouse Ly6C (clone HK1.4), FITC anti-mouse Ly6G (clone 1A8), PeCy7 anti-human CD1a (clone HI149), PerCpCy5.5 anti-human HLA-DR (clone LN3), FITC anti-human CD80 (clone 2D10.4), APC anti-human CCR7 (clone 3D12), Alexa Fluor 488 anti-human CD86 (clone IT2.2). Staining with directly conjugated antibodies was done in FACS buffer at 4°C. Then, the cells were washed and fixed in 1% paraformaldehyde for 30 minutes at 4°C. Cells were washed a final time, resuspended in FACS buffer and stored at 4°C until analyzed. Flow cytometry was done using a LSR II flow cytometer (BD Biosciences). Analysis gates were set on viable unstained cells and were designed to include all viable cell populations. Approximately 10,000 events were analyzed for each sample. Isotype control antibodies were included when analyses and panels were first being performed to assure specificity of staining, but were not routinely included with each experiment. Neutrophils were defined as Ly6G⁺ cells. Macrophages were identified as CD11c⁺/CD11b^{+/-} cells. Data was analyzed using FlowJo software (Treestar, Ashland, OR).

Microscopy

hDCs were infected with SchuS4 or treated with PBS, ethanol (vehicle control) or Schu4 lipids for the indicated times, followed by 10 ng/ml *E. coli* strain O127:B7 LPS (Sigma). hDCs were spun onto a glass slide using a Shandon Cytospin 4 and fixed in 3% paraformaldehyde for 20 minutes. hDCs were incubated with 0.3% Triton X-100 in PBS + 10% horse serum for 1 hour at room temperature followed by addition of rabbit anti-NF- κB p65 XP antibody, anti-IRF-1 XP antibody or anti-IRF-3 XP antibody (all from Cell

Signaling Technology, CA) for 1 hour at room temperature. Slides were washed with PBS and incubated with Alexa Fluor 568-conjugated goat anti-rabbit antibody (Cell Signaling Technology) and Alexa Fluor 488-conjugated mouse anti-*F. tularensis* LPS antibodies (US Biological, Swampscott, MA) and counterstained with DAPI to label cell nuclei. Samples were visualized using a Carl Zeiss LSM 710 confocal scanning laser microscope for quantitative analysis and image acquisition. Confocal images were acquired and assembled using Adobe Photoshop CS2 software (Adobe Systems, San Jose, CA). Translocation was quantitated using a Zeiss Axio Imager M1 fluorescence microscope. Starting in the middle of the slide, 3–4 fields in succession were counted for a total of >300 cells/sample. Data was pooled from 3 identical experiments with 3 different donors.

Quantification of NF- κ B translocation

Translocation of NF- κ B in hDCs was quantified using a commercially available TransAM assay as previously described (21). Briefly, hDCs were treated with PBS, EtOH (vehicle control), SchuS4 lipids and/or ultrapure *E. coli* LPS. For assessment of SchuS4 lipid mediated translocation of NF- κ B cells were harvested 3 hours after addition of stimuli or vehicle controls. For assessment of lipid mediated inhibition of NF- κ B translocation cells were incubated with PBS, EtOH or SchuS4 lipids for 24 hours followed by addition of ultrapure *E. coli* LPS. Cells were harvested 3 hours after addition of LPS. Nuclear extracts were obtained using a Nuclear Extract Kit (Active Motif; Carlsbad, CA) according to the manufacturer's directions. Nuclear protein in each sample was quantified using the Bradford assay (Pierce) and adjusted to 5 μ g/sample prior to application in the NF- κ B TransAM transcription factor assay kit (Active Motif).

DNA affinity binding assays

Determination of IRF1 and IRF8 binding to IL-12p40 promoter sequences was performed as previously described with minor modifications (22). hDC were lysed in a non-denaturing lysis buffer (20mM Tris HCl, 150mM NaCl, 10% glycerol and 1% Triton) containing Halt Protease inhibitor cocktail (Thermo Scientific) and Phosphatase Inhibitor Cocktail (Active Motif). Total protein was quantified by BCA assay (Thermo Scientific). M280 Streptavidin-coated Dynabeads (Invitrogen) were prepared per manufacturer's instructions and ligated to 5' biotinylated double stranded DNA (Integrated DNA Technologies) corresponding to the –244bp to –154bp region of the human IL12p40 promoter containing the Ets2 sequence or the –101 to –11 region of the IL-12p40 promoter containing the ISRE sequence (Supplemental Figure 1). Beads were washed in TGED buffer (10mM Tris HCl, 10% glycerol, 0.1mM EDTA and 0.01% Triton X 100) and blocked overnight at 4°C in TGED + 0.5% BSA. Whole hDC lysate (100 μ g) was added to 200 μ g of the DNA coated beads in a total of 500 μ l TGED buffer + 0.1M NaCl and the mixture was incubated with constant mixing at 4°C overnight. Beads were then washed four times with TGED buffer + 0.1M NaCl and protein attached to DNA sequences was eluted in 20 μ l 1x sample buffer + reducing agent (Invitrogen). Presence of IRF1 and IRF8 were assessed by SDS-PAGE and Western blotting using anti-IRF1 XP and anti-IRF8 antibodies (both from Cell Signaling Technologies).

Statistical Analyses

For comparison between three or more groups, analysis was done by one-way ANOVA followed by Tukey's multiple comparisons test or with significance determined at $p < 0.05$. For comparison of two groups, analysis was done by unpaired Student's t-test with significance determined at $p < 0.05$.

RESULTS

Total membrane fraction derived from virulent *F. tularensis* SchuS4 inhibit production of IL-12p40

An important mechanism of virulence associated with virulent subspecies of *F. tularensis* is its ability to evade and suppress multiple inflammatory responses in human cells, including production of IL-12 (7, 9, 10, 23, 24). The absence of IL-12 and the subsequent suppression of this cytokine among human cells is unique to virulent subspecies of *F. tularensis*, e.g. SchuS4 (7). We have previously established that heat stable components of virulent *F. tularensis* SchuS4 secreted or shed from the bacteria have the capacity to inhibit host inflammatory responses (10). Thus, we postulate that the component of *F. tularensis* mediating inhibition of IL-12p40 is a bacterial lipid or carbohydrate. Lipids and carbohydrates are often incorporated and associated with membrane structures in bacteria. Therefore, we tested total membrane fraction (MF) enriched from whole cell lysates of SchuS4 for its ability to stimulate and/or inhibit cytokine production from hDC. As a positive control for stimulation of IL-12 by bacterial components we also tested MF obtained from attenuated *F. tularensis* LVS. Similar to our previous observations using intact bacteria, MF from SchuS4 does not elicit IL-12p40 from hDC (Figure 1A and (7, 10). In contrast, and consistent with our previous observations in hDC infected with LVS both LVS MF and *E. coli* LPS readily induced production of IL-12 from hDC (Figure 1A and (7). We also assessed whether the absence of IL-12 was associated with induction of anti-inflammatory cytokines. However, we do not detect either IL-10 or TGF- β in supernatants of hDC treated with SchuS4 MF (data not shown).

Since SchuS4 MF fails to induce secretion of detectable IL-12 we next determined if SchuS4 MF inhibits the ability of hDC to respond to secondary stimuli, as previously observed following infection with viable SchuS4 (10). hDC pretreated with SchuS4 MF secrete significantly less IL-12p40 in response to LPS compared to cells treated with LPS alone (Figure 1B). We next determined if the “active” component of the SchuS4 MF had characteristics shared with lipid and/or carbohydrate containing compounds, e.g. heat stable and pronase resistant. Similar to our previous observations, the components of SchuS4 MF capable of inhibiting secretion of IL-12 by hDC in response to *E. coli* LPS are heat stable and pronase resistant (Figure 1C and D). These data strongly suggest that anti-inflammatory molecule(s) associated with SchuS4 may be a lipid and/or carbohydrate.

Lipids derived from virulent *F. tularensis* SchuS4 inhibit inflammatory responses

Data generated with the MF of SchuS4 suggest that the components associated with the MF which inhibit inflammatory responses are either lipids or carbohydrates. Therefore, we separated lipid and non-lipid materials from SchuS4 and LVS using a modified Folch method and determined their ability to activate or inhibit inflammatory responses in hDC. Non-lipid components of the bacteria present in the aqueous phase of the SchuS4 or LVS preparations do not stimulate a measurable cytokine response in hDC at the concentrations tested (Supplemental Figure 2). When examined for suppressive activity, non-lipid products present in aqueous preparations from SchuS4 and LVS are capable of inhibiting inflammatory responses in hDC (Supplemental Figure 2). However, the non-stimulatory yet suppressive activity of non-lipid material associated with LVS is inconsistent with the ability of viable LVS and total MF isolated from LVS to provoke inflammatory responses in hDC. Thus, the non-lipid products isolated from both SchuS4 and LVS do not faithfully replicate the differential stimulatory and/or inhibitory activity of SchuS4 and LVS in hDC. Since we are pursuing identification of a heat and pronase resistant molecule that shares the unique non-stimulatory, but inhibitory properties observed among hDC infected with SchuS4, we tested and compared lipid material found in the organic phase of our extractions

of SchuS4 and LVS. Neither lipids isolated from LVS nor SchuS4 induce detectable concentrations of IL-12p40 from hDC following incubation of cells with doses of lipid as high as 30 $\mu\text{g/ml}$ (Figure 2). Lipids isolated from LVS do not inhibit hDC responsiveness to LPS (Figure 2). In contrast, SchuS4 lipids potently suppress secretion of IL-12 by hDC in response to *E. coli* LPS (Figure 2). Similarly to SchuS4 MF, SchuS4 lipids do not induce secretion of the anti-inflammatory cytokines IL-10 or TGF- β (data not shown). Additionally, SchuS4 lipids also inhibit hDC response to other TLR agonists, including Pam3CSK4 (TLR2/1), Pam2CSK4 (TLR2/6), LTA (TLR2), R848 (TLR8) and ssRNA (TLR8) (Supplemental Figure 3). Viability of hDC treated with SchuS4 lipid was 99.1% compared to 98.2% of EtOH treated cells and 99% viability in untreated cells. Thus, suppression of cytokine production observed in hDC treated with SchuS4 lipids was not associated with toxicity of SchuS4 lipid to hDC. Together these data show that lipids isolated from SchuS4 interfere with pro-inflammatory responses activated following engagement of multiple TLRs in hDC.

In addition to production of cytokines, activation of hDC by exposure to TLR ligands is measured by the increases in expression of several cell surface receptors, a process known as phenotypic maturation. Therefore, we also examined changes in CD80, CD86, HLA-DR and CCR7 on the surface of hDC treated with lipids before and after exposure to Pam3CSK4. SchuS4 lipids do not induce increased expression of any cell surface receptor assessed (Figure 3). Interestingly, SchuS4 lipids have no effect on the upregulation of HLA-DR and CCR7 among hDC treated with Pam3Csk4 (Figure 3). However, in correlation with the inhibition of cytokine production, hDC treated with SchuS4 lipids express significantly less CD80 and CD86 on the cell surface following addition of Pam3CSK4 (Figure 3). Thus, SchuS4 lipids also impact the ability of hDC to undergo phenotypic maturation.

SchuS4 lipids inhibit inflammatory responses *in vivo*

We have previously demonstrated that viable SchuS4 inhibits inflammatory responses *in vivo*, as measured by interference of recruitment of neutrophils following intranasal infection (8). Given the ability of SchuS4 lipids to inhibit inflammatory responses *in vitro* (Figure 2) we next determined if these bacterial components could suppress pulmonary inflammation *in vivo*. Mice were given 25 μg of SchuS4 lipid intranasally prior to administration of 200 ng *E. coli* LPS via the same route. Five hours after inoculation with LPS, airway cells and fluid were collected and assessed for the presence of neutrophils and cytokines. Instillation of *E. coli* LPS fails to reproducibly induce production of IL-12p40 in the airways of mice, thus we assessed secretion of TNF- α and IL-6 as representative pro-inflammatory cytokines. Administration of SchuS4 lipids do not elicit recruitment of neutrophils or secretion of TNF- α or IL-6 into the airways of treated mice (data not shown). Rather, SchuS4 lipids significantly inhibit recruitment of neutrophils and production of TNF- α and IL-6 in the airways of mice treated with LPS (Figure 4). Thus, SchuS4 lipids are also capable of inhibiting inflammatory responses *in vivo*.

SchuS4 lipids inhibit translocation of NF- κ B to the nucleus

Production of IL-12p40 is dependent on activation and translocation of specific transcription factors to induce gene expression. One of these factors includes NF- κ B, specifically NF- κ B p65 (25). In a resting cell, p65 is bound to I κ B α in the host cytosol. Upon cellular activation, the kinases in the IKK family phosphorylate I κ B α targeting it for ubiquitination and degradation. This, in turn, results in release the p65 subunits for activation and translocation to the nucleus where they participate in transcription of pro-inflammatory genes. Inhibition of cytokine production by SchuS4 lipids in hDC may be due to interference of activation and/or translocation of NF- κ B transcription factors to the nucleus of the host cell. We first examined the ability of SchuS4 lipids to modulate phosphorylation of I κ B α .

SchuS4 lipids do not independently induce phosphorylation and/or degradation of $\text{I}\kappa\text{B}\alpha$ (Figure 5A), nor do they interfere with activation of $\text{I}\kappa\text{B}\alpha$ following incubation of cells with *E. coli* LPS (Figure 5B).

Data above suggests that SchuS4 lipids are not modulating activation of NF- κ Bp65 through canonical pathways. However, activation of p65 can occur independently of $\text{I}\kappa\text{B}\alpha$ and the absence of an effect on the activation of $\text{I}\kappa\text{B}\alpha$ by SchuS4 lipids does not rule out modulation of NF- κ Bp65 (26, 27). Using a quantitative assay we next determined if SchuS4 lipids had any effect on translocation of p65 to the host nucleus. SchuS4 lipids do not independently induce translocation of NF- κ B family members in hDC, but readily inhibit translocation of p65 in response to LPS (Figure 5C and D). Inhibition of p65 translocation is confirmed by microscopy. In agreement with our quantitative results, approximately 50% of hDC pretreated with SchuS4 lipid do not translocate the NF- κ B p65 to their nucleus in response to LPS (Figure 5E). Together our data suggests that the suppression of cytokine production in hDC by SchuS4 lipid is not mediated through manipulation of canonical pathways upstream of NF- κ B, but rather is affecting activation and translocation of NF- κ B family members either directly or via non- canonical pathways.

SchuS4 lipids inhibit activation of IRFs

In addition to p65, transcription of IL-12p40 is also dependent on Interferon Response Factors (IRF), including IRF1 and IRF8 (28–30). IRF1 is also a critical transcription factor for several IFN- γ regulated genes involved in host defense against intracellular pathogens (31). Thus, we examined the impact SchuS4 lipids had on the activation and translocation of IRF1 in hDC. SchuS4 lipids do not induce translocation of IRF1, but do inhibit translocation of IRF1 among hDC responding to LPS (Figure 6A and B). In addition to IRF1, IRF8 is also essential for transcription of IL-12p40 gene (32). There are no detection methods available to view IRF8 translocation by microscopy. The accepted technique to analyze IRF8 activity within the context of IL-12p40 transcription is examination of its ability to bind specific sequences (Ets2 and ISRE) in the promoter of the gene encoding IL-12p40 (22, 32, 33). IRF1 and IRF8 form a heterocomplex and, thus, both bind to Ets2 and ISRE promoter regions in the IL-12p40 (32). Therefore, we determined if lipid affected the ability of IRF1 and IRF8 bind to the IL-12p40 promoter in primary hDC. Binding of IRF1 and IRF8 is optimal following stimulation of hDC with LPS+IFN- γ . Before assessing the effect SchuS4 lipids had on the ability of IRF1 and IRF8 to bind regions in the IL-12p40 promoter, we first determined if SchuS4 lipids could inhibit production of IL-12p40 in hDC stimulated with LPS+IFN- γ . Similar to hDC stimulated with LPS alone, exposure to SchuS4 lipids results in secretion of significantly less IL-12p40 by hDC treated with with LPS+IFN- γ (Figure 7A). We next examined DNA binding activity of IRF1 and IRF8 in SchuS4 lipid treated cells. SchuS4 lipids have no effect on the ability of IRF1 and IRF8 to bind to the ISRE in the IL-12p40 promoter following stimulation with LPS+IFN- γ (Figure 7B). In contrast, SchuS4 lipids inhibit binding of both IRF1 and IRF8 to the Ets2 sequence in the IL-12p40 promoter of hDC treated with LPS+IFN- γ compared to EtOH treated controls (Figure 7B). To determine if the inhibitory effect SchuS4 lipids had on IRF1 and IRF8 activity is a global phenomenon affecting multiple IRF family members, we also examined the ability of SchuS4 to affect activity of an IRF which is not required for transcription of IL-12p40. Similar to IRF1 SchuS4 lipids do not induce translocation of IRF3 in hDC (Figure 6C and D). However, in contrast to their effect on IRF1 and IRF8, SchuS4 lipids do not inhibit translocation of IRF3 following stimulation of hDC with LPS (Figure 6C and D). Together this data suggest that SchuS4 lipids inhibit production of IL-12p40 by inhibiting translocation of IRF1 and binding of IRF1 and IRF8 to the Ets2 element in the IL-12p40 promoter.

Viable SchuS4 inhibits translocation of NF- κ B and IRF1

It is well documented that virulent *F. tularensis* inhibits production of IL-12p40 in mouse and human cells (7–11, 34). However, the mechanism by which this occurs is not clear. Data presented above indicate that lipids isolated from virulent SchuS4 inhibit IL-12p40 production by interfering with activation of NF- κ B and IRF1. Thus, we next sought to confirm that interference with these transcription factors also occurred among hDC infected with viable *F. tularensis* SchuS4. In agreement with previous reports and activity observed with SchuS4 lipids, infection with viable SchuS4 inhibited the ability of hDC to secrete IL-12p40 (Figure 8A and (7)). Furthermore, similar to data presented above, viable SchuS4 inhibits translocation of both NF- κ B and IRF1 among infected hDC (Figure 8B and C), but has no effect on translocation of IRF3 (Figure 8D). Thus, consistent with suppression mediated by SchuS4 lipids, viable *F. tularensis* SchuS4 also inhibits translocation of the same transcription factors tied to production of IL-12p40 that are affected by SchuS4 lipids.

DISCUSSION

Production of proinflammatory cytokines, including IL-12, is a critical step in the control and eradication of invading microorganisms. Evasion and suppression of this host response is an important feature embodied by highly virulent pathogens. Case in point, in contrast to more attenuated strains and subspecies, virulent *F. tularensis* both evades triggering inflammatory responses and subsequently inhibits the ability of the host to mount effective responses at key, early time points after infection (8–10, 35). However, the bacterial components, and specific host pathways modulated by these components, are largely undefined. Dendritic cells represent a target for the intracellular replication of *F. tularensis* and pulmonary infection is typically considered to be the most dangerous manifestation of tularemia (10, 20, 36, 37). Thus, we utilized model systems incorporating primary human dendritic cells and intranasal instillation in the mouse to identify the components of *F. tularensis* and specific signaling pathways involved in immunosuppression mediated by this organism.

In this report we provide evidence that lipids isolated from virulent, but not attenuated, *F. tularensis* are an important bacterial constituent for the suppression of human and murine innate inflammatory responses. We demonstrate that, while not provoking an inflammatory response, lipids isolated from virulent *F. tularensis* SchuS4 readily inhibited upregulation of select cell surface receptors on hDC associated with hDC activation and also suppressed production of IL-12 by primary human dendritic cells in response to a variety of secondary stimuli. We also found that SchuS4 lipids potently inhibited inflammation *in vivo* using a mouse model of pulmonary inflammation. Our data also suggest that the suppressive lipids associated with SchuS4 are not associated with LPS, i.e. lipid A, since the lipid products described herein are fully capable of suppressing responses in hDC whereas SchuS4 LPS does not inhibit hDC function in any detectable manner (10). We extended our studies to decipher two signaling pathways required for IL-12 transcription that are manipulated by SchuS4 lipids. SchuS4 lipids inhibit translocation of NF- κ Bp65 in a manner independent of I κ B α degradation. SchuS4 lipids also selectively target IRF family members by inhibiting nuclear localization of IRF1, but not IRF3, in LPS stimulated hDC. Finally, our data point toward a physiological role for SchuS4 lipids in mediating the suppression observed in hDC infected with viable SchuS4 by demonstrating that both SchuS4 lipids and viable SchuS4 inhibit both NF- κ B and IRF1 activation following infection of hDC.

This work is an extension of our original observation that heat stable products either secreted or shed from virulent *F. tularensis* SchuS4 were able to interfere with hDC responsiveness to *E. coli* LPS (10). These data strongly suggest that a non-protein component derived from SchuS4 contributes to the inability of hDC to respond to secondary

stimuli. In the study herein, we have found that following biochemical extraction, both non-lipid (present in the aqueous phase) and lipids (present in the organic phase) isolated from SchuS4 are able to modulate the ability of hDC to respond to various secondary stimuli. However, material isolated from the aqueous phase of both attenuated LVS and SchuS4 was capable of inhibiting cytokine secretion in hDC. LVS was derived via serial passage from a virulent Type B subspecies and is capable of causing overt disease in some humans (38). LVS also retains a route and dose dependent virulence in mice (5, 39). Furthermore, the composition of the O-antigen polysaccharide that represents the primary non-lipid species in membranes of *Francisella* is very similar among LVS and Type A isolates (40). Thus, LVS may retain features in its carbohydrate profile that are associated with pathogenicity which may be more prominently featured in fully virulent isolates. Nevertheless, intact LVS and MF isolated from LVS provokes production of pro-inflammatory cytokines from hDC, whereas intact SchuS4 and MF derived from SchuS4 do not (Figure 1 and (10)). Thus, the non-stimulatory, but suppressive activity of non-lipid components present in LVS is not consistent with the activity of the intact organism with human cells.

In contrast to non-lipid material present in the aqueous phase of our extraction, lipid products present in the organic phase of the SchuS4 extractions are unique in their ability to interfere with inflammatory response in hDC. Neither LVS nor SchuS4 lipids provoke cytokine production from hDC. However, only SchuS4 lipids are able to inhibit the ability of hDC to secrete IL-12 in response to multiple TLR agonists, which is consistent with the ability of viable SchuS4 to evade induction of pro-inflammatory cytokines while potently suppressing hDC responsiveness to various TLR ligands (7, 10). This suggests that SchuS4 contains immunosuppressive lipid species that are either unique or more abundant than those found in LVS.

To date information on the lipid composition of virulent *Francisella* is sparse. There are a handful of reports that characterize the lipid species associated with attenuated and virulent strains of *F. tularensis*. Virulent *F. tularensis* has a lipid profile and content that is unusual and unique among Gram negative bacteria. Whereas the membranes from most Gram negative bacteria contain about 10–20% lipid, *F. tularensis* membranes are comprised of nearly 70% lipid (41). Some have suggested that a large portion of this lipid is free lipid A unassociated with O-antigen which would partition to the organic phase of our extraction (42), although this phenomenon has only been documented for *F. novicida* and not the other subspecies of *F. tularensis*. Lipid A is the lipid portion of the large lipopolysaccharide (LPS) present in the cell wall of all Gram negative bacteria. The lipid A of LPS is the portion recognized by TLR4 on host cells. There is a direct association between the number of acyl groups present on the lipid A and the stimulatory activity of this molecule (43). Specifically, lipid A species with 4 or fewer acyl groups do not provoke inflammatory responses from host cells (43). Among attenuated strains, i.e. LVS and *F. novicida*, lipid A is tetraacylated and, thus, is a poor stimulator for cytokine responses (44, 45). Given the presence of such unique lipid A in *Francisella* subspecies, one could postulate that perhaps this is the molecule mediating the suppressive effect in our preparations. However, there are several factors that argue against this hypothesis.

First, although the presence of tetraacylated lipid A has not been confirmed among Type A strains such as SchuS4, purified LPS from SchuS4 also lacks the stimulatory properties observed in lipid A and LPS isolated from LVS and *F. novicida* (10). Moreover, in addition to the weak ability of SchuS4 LPS to stimulate responses in host cells it does not inhibit the ability of cells to respond to secondary stimuli (10). Second, new evidence suggests that recognition of altered lipid A/LPS molecules is not the same among mice and humans. For example, pentaacylated LPS isolated from *Neisseria meningitidis* stimulates weak responses from human cells, but retains its ability to induce cytokine production from mouse cells (as

reviewed, (43). Thus, assuming lipid A is involved, one hypothesis may be that the lack of suppressive activity of LVS lipids in hDC as compared to SchuS4 lipids is attributable to the fact that we were examining human cells. However, we also observed that SchuS4 lipids readily inhibit inflammatory responses in the mouse (Figure 4 and Crane et al, submitted). Therefore, the ability of SchuS4 lipids to modulate inflammatory responses is not dependent on the species of the host, thus arguing against a role for lipid A. Finally, in addition to the major lipid A species found in Francisella, low abundant forms of unique lipid A species have also been described (45). However, the presence and relative abundance of these minor lipid A species were not different among subspecies of *F. tularensis* tested, i.e. virulent Type B *F. tularensis*, *F. novicida* and *F. philomargia*. Together, this suggests that it is unlikely that a unique lipid A species found in SchuS4 is responsible for suppressing inflammatory responses in human cells. Rather, we propose that other lipids found in SchuS4 act to inhibit pro-inflammatory responses in hDC. In support of this hypothesis, preliminary data from our laboratory suggests that fatty acids, which we would propose to be present as free fatty acids rather than those found attached to the lipid A backbone, are the classes of SchuS4 lipids that mediate anti-inflammatory responses observed in hDC (unpublished data).

In addition to identifying the class of molecules found in SchuS4 which inhibit inflammatory responses, we also identify three host signaling molecules required for transcription of genes encoding pro-inflammatory cytokines that are modulated by both SchuS4 lipids and viable SchuS4. NF- κ Bp65 is critical for the transcription of multiple inflammatory cytokines, including IL-12 (25). Others have suggested that *F. tularensis* may inhibit translocation of this host protein as a mechanism of virulence. However, in these studies it was suggested that interference with p65 activation occurs through modulation of canonical signaling pathways in the host cell, specifically via the inhibition of phosphorylation of I κ B α subunits that (in their non-phosphorylated state) retain p65 in the cytosol (46). Our data demonstrates that SchuS4 is capable of preventing p65 translocation independently of activation of I κ B α . Although it is widely accepted that phosphorylation of I κ B α is a critical step in the nuclear translocation of p65, it is not sufficient for the activation of NF- κ B subunits. For example, it has been shown that several kinases are important for phosphorylation of p65 and that interference with their activity can result in poor translocation and DNA binding of p65 (47, 48). Thus, SchuS4 mediated inhibition of p65 translocation may lie in the interference with kinases required for phosphorylation of p65 downstream of I κ B α .

In addition to disruption of the activation of p65, we also show that SchuS4 selectively inhibits activation of members of the IRF family of transcription factors, specifically IRF1 and IRF8. The IRF family consists of 9 different proteins which were originally characterized as transcription factors that regulated gene expression from Type I IFN and IFN inducible genes (49). It is now appreciated that IRFs also play critical roles in cell survival, oncogenesis, and mediating signals through pattern recognition receptors (PRR) for cytokine production including IL-12 (49). Initially our finding that SchuS4 and SchuS4 lipids modulated activation of IRF1 was surprising since we had previously shown SchuS4 induced rapid, transient production of IFN- β and, as described above, IRFs are intimately involved in transcription of Type I IFN (7). However, depending on the receptors engaged, transcription of IFN- β requires specific IRFs. For example, expression of IFN- β stemming from engagement of PRR is dependent on IRF3 (50, 51). Thus, due to its ability to induce IFN- β SchuS4 may not impact IRF3 activation. Indeed, there is not a significant difference in the translocation of IRF3 among cells infected with SchuS4 or treated with SchuS4 lipid. This supports our previous work that SchuS4 is not negatively impacting Type I IFN pathways.

In contrast to IFN- β , transcription of IL-12p40 requires IRF1 and IRF8 (33). Others have shown that microbial toxins, e.g. cholera toxin, inhibits binding of IRF8, but not IRF1, to specific sites in the promoter region of the IL-12p40 gene to suppress transcription of this cytokine (22). We found that SchuS4 lipids inhibit binding of both IRF1 and IRF8 at the Ets2 site within the IL-12p40 promoter. Thus, the inhibition of IRF1 and IRF8 by SchuS4 as a mechanism to interfere with production of IL-12p40 is unique. IRF1 also plays integral roles in the transcription of several genes encoding elements required for control of *F. tularensis*, including iNOS and Caspase-1 (13, 1, 52, 53). Thus, the targeted interference of IRF1 activation by SchuS4 represents a mechanism by which the bacterium broadly incapacitates host innate immune responses.

Regulation of IRF1 activity and its interaction with IRF8 is not well defined. To date Casein Kinase II is the only kinase shown to phosphorylate IRF1 (54). This interaction has not been confirmed in primary cells. IRF1 activity can also be regulated by two other IRFs. IRF2 can act as a repressor of IRF1 via its ability to compete for binding at the same DNA binding sites (55). However, we observe an inhibition of IRF1 translocation to the host nucleus. Further, it is not clear if IRF2 is capable of binding the Ets2 region of the human IL-12p40 promoter. Together this suggests that IRF1 competition with IRF2 is not the mechanism for SchuS4 mediated inhibition of IL-12p40. We also found that binding of IRF8 to the Ets2 site was inhibited by SchuS4 lipid. Heterocomplexes of IRF1 and IRF8 have been shown to be required for transcription of IL-12p40 (32). Thus, the impact SchuS4 lipid has on IRF1 and IRF8 binding to the Ets2 site may be due to poor formation of IRF1-IRF8 heterocomplexes. It is not clear if the heterocomplex of IRF1 and IRF8 that binds to the IL-12p40 promoter must form prior to its entry to the nucleus. It is possible that the formation of this complex is inhibited by SchuS4 lipids in the cytosol. Alternatively, IRF1 has a short half-life and is targeted for ubiquitination and degradation by the host cell (56). It is possible that SchuS4 accelerates the degradation of IRF1, thus reducing the overall pool available for activation and translocation. We are currently investigating all of these scenarios as possible mechanisms behind the SchuS4 mediated inhibition of IRF1 and IRF8 activity.

In conclusion, we show here that lipids derived from SchuS4 suppress innate immune responses *in vitro* and *in vivo* in a manner consistent with that observed during infection with viable bacteria. We also identify three transcription regulators critical for expression of multiple inflammatory genes, NF κ Bp65, IRF1, and IRF8 whose activation/translocation to the host nucleus is inhibited by SchuS4 and SchuS4 lipids. Together, these data provide new targets for vaccines and therapeutics directed against *F. tularensis* and enhances our understanding of pathogen manipulation of innate immune responses in the host. Additionally, given the impressive anti-inflammatory action of SchuS4 lipids *in vivo*, future studies should include testing these novel molecules for their effectiveness at limiting inflammation driven by unrelated conditions such as allergy, autoimmunity, or infection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank Dr. Brian Kelsall and Dr. Frank Gherardini for their thoughtful insights with regard to these studies.

References

1. Oyston PC, Sjøstedt A, Titball RW. Tularemia: bioterrorism defence renews interest in *Francisella tularensis*. Nat Rev Microbiol. 2004; 2:967–978. [PubMed: 15550942]

2. Saslaw S, Eigelsbach HT, Prior JA, Wilson HE, Carhart S. Tularemia vaccine study. II. Respiratory challenge. *Arch Intern Med.* 1961; 107:702–714. [PubMed: 13746667]
3. Wayne Conlan J, Shen H, Kuolee R, Zhao X, Chen W. Aerosol-, but not intradermal-immunization with the live vaccine strain of *Francisella tularensis* protects mice against subsequent aerosol challenge with a highly virulent type A strain of the pathogen by an alphabeta T cell- and interferon gamma- dependent mechanism. *Vaccine.* 2005; 23:2477–2485. [PubMed: 15752834]
4. Dennis DT, Inglesby TV, Henderson DA, Bartlett JG, Ascher MS, Eitzen E, Fine AD, Friedlander AM, Hauer J, Layton M, Lillibridge SR, McDade JE, Osterholm MT, O'Toole T, Parker G, Perl TM, Russell PK, Tonat K. Tularemia as a biological weapon: medical and public health management. *JAMA.* 2001; 285:2763–2773. [PubMed: 11386933]
5. Eigelsbach HT, Downs CM. Prophylactic effectiveness of live and killed tularemia vaccines. I. Production of vaccine and evaluation in the white mouse and guinea pig. *J Immunol.* 1961; 87:415–425. [PubMed: 13889609]
6. Risi GF, Pombo DJ. Relapse of tularemia after aminoglycoside therapy: case report and discussion of therapeutic options. *Clin Infect Dis.* 1995; 20:174–175. [PubMed: 7727647]
7. Bauler TJ, Chase JC, Bosio CM. IFN- β Mediates Suppression of IL-12p40 in Human Dendritic Cells following Infection with Virulent *Francisella tularensis*. *J Immunol.* 2011; 187:1845–1855. [PubMed: 21753150]
8. Bosio CM, Bielefeldt-Ohmann H, Belisle JT. Active suppression of the pulmonary immune response by *Francisella tularensis* Schu4. *J Immunol.* 2007; 178:4538–4547. [PubMed: 17372012]
9. Butchar JP, Cremer TJ, Clay CD, Gavrilin MA, Wewers MD, Marsh CB, Schlesinger LS, Tridandapani S. Microarray analysis of human monocytes infected with *Francisella tularensis* identifies new targets of host response subversion. *PLoS ONE.* 2008; 3:e2924. [PubMed: 18698339]
10. Chase JC, Celli J, Bosio CM. Direct and indirect impairment of human dendritic cell function by virulent *Francisella tularensis* Schu S4. *Infect Immun.* 2009; 77:180–195. [PubMed: 18981246]
11. Chase JC, Bosio CM. The presence of CD14 overcomes evasion of innate immune responses by virulent *Francisella tularensis* in human dendritic cells in vitro and pulmonary cells in vivo. *Infect Immun.* 2010; 78:154–167. [PubMed: 19841074]
12. Elkins KL, Rhinehart-Jones TR, Culkin SJ, Yee D, Winegar RK. Minimal requirements for murine resistance to infection with *Francisella tularensis* LVS. *Infect Immun.* 1996; 64:3288–3293. [PubMed: 8757866]
13. Ireland R, Olivares-Zavaleta N, Warawa JM, Gherardini FC, Jarrett C, Hinnebusch BJ, Belisle JT, Fairman J, Bosio CM. Effective, broad spectrum control of virulent bacterial infections using cationic DNA liposome complexes combined with bacterial antigens. *PLoS Pathog.* 2010; 6:e1000921. [PubMed: 20523903]
14. Sutherland MD, Goodyear AW, Troyer RM, Chandler JC, Dow SW, Belisle JT. Post-exposure immunization against *Francisella tularensis* membrane proteins augments protective efficacy of gentamicin in a mouse model of pneumonic tularemia. *Vaccine.* 2012; 30:4977–4982. [PubMed: 22652404]
15. Huntley JF, Robertson GT, Norgard MV. Method for the isolation of *Francisella tularensis* outer membranes. *Journal of visualized experiments: JoVE.* 2010
16. Folch J, Lees M, Sloane Stanley GH. A simple method for the isolation and purification of total lipides from animal tissues. *J Biol Chem.* 1957; 226:497–509. [PubMed: 13428781]
17. Beatty WL, Rhoades ER, Ullrich HJ, Chatterjee D, Heuser JE, Russell DG. Trafficking and release of mycobacterial lipids from infected macrophages. *Traffic.* 2000; 1:235–247. [PubMed: 11208107]
18. Dunnick JK, O'Leary WM. Correlation of bacteria lipid composition with antibiotic resistance. *J Bacteriol.* 1970; 101:892–900. [PubMed: 4314544]
19. Liu X, Curtiss R 3rd. Thermorecovery of cyanobacterial fatty acids at elevated temperatures. *Journal of biotechnology.* 2012; 161:445–449. [PubMed: 22944207]
20. Bosio CM, Dow SW. *Francisella tularensis* induces aberrant activation of pulmonary dendritic cells. *J Immunol.* 2005; 175:6792–6801. [PubMed: 16272336]

21. Semnani RT, Venugopal PG, Leifer CA, Mostbock S, Sabzevari H, Nutman TB. Inhibition of TLR3 and TLR4 function and expression in human dendritic cells by helminth parasites. *Blood*. 2008; 112:1290–1298. [PubMed: 18541719]
22. la Sala A, He J, Laricchia-Robbio L, Gorini S, Iwasaki A, Braun M, Yap GS, Sher A, Ozato K, Kelsall B. Cholera toxin inhibits IL-12 production and CD8alpha+ dendritic cell differentiation by cAMP-mediated inhibition of IRF8 function. *J Exp Med*. 2009; 206:1227–1235. [PubMed: 19487420]
23. Bublitz DC, Noah CE, Benach JL, Furie MB. *Francisella tularensis* suppresses the proinflammatory response of endothelial cells via the endothelial protein C receptor. *J Immunol*. 2010; 185:1124–1131. [PubMed: 20543103]
24. Cremer TJ, Ravneberg DH, Clay CD, Piper-Hunter MG, Marsh CB, Elton TS, Gunn JS, Amer A, Kanneganti TD, Schlesinger LS, Butchar JP, Tridandapani S. MiR-155 induction by *F. novicida* but not the virulent *F. tularensis* results in SHIP down-regulation and enhanced pro-inflammatory cytokine response. *PLoS ONE*. 2009; 4:e8508. [PubMed: 20041145]
25. Trinchieri G. Interleukin-12 and the regulation of innate resistance and adaptive immunity. *Nat Rev Immunol*. 2003; 3:133–146. [PubMed: 12563297]
26. Bohuslav J, Chen LF, Kwon H, Mu Y, Greene WC. p53 induces NF-kappaB activation by an IkappaB kinase-independent mechanism involving phosphorylation of p65 by ribosomal S6 kinase 1. *J Biol Chem*. 2004; 279:26115–26125. [PubMed: 15073170]
27. Wong BC, Jiang X, Fan XM, Lin MC, Jiang SH, Lam SK, Kung HF. Suppression of RelA/p65 nuclear translocation independent of IkappaB-alpha degradation by cyclooxygenase-2 inhibitor in gastric cancer. *Oncogene*. 2003; 22:1189–1197. [PubMed: 12606945]
28. Taki S, Sato T, Ogasawara K, Fukuda T, Sato M, Hida S, Suzuki G, Mitsuyama M, Shin EH, Kojima S, Taniguchi T, Asano Y. Multistage regulation of Th1-type immune responses by the transcription factor IRF-1. *Immunity*. 1997; 6:673–679. [PubMed: 9208840]
29. Goodridge HS, Harnett W, Liew FY, Harnett MM. Differential regulation of interleukin-12 p40 and p35 induction via Erk mitogen-activated protein kinase- dependent and -independent mechanisms and the implications for bioactive IL-12 and IL-23 responses. *Immunology*. 2003; 109:415–425. [PubMed: 12807488]
30. Maruyama S, Sumita K, Shen H, Kanoh M, Xu X, Sato M, Matsumoto M, Shinomiya H, Asano Y. Identification of IFN regulatory factor-1 binding site in IL-12 p40 gene promoter. *J Immunol*. 2003; 170:997–1001. [PubMed: 12517966]
31. Kamijo R, Harada H, Matsuyama T, Bosland M, Gerecitano J, Shapiro D, Le J, Koh SI, Kimura T, Green SJ, et al. Requirement for transcription factor IRF-1 in NO synthase induction in macrophages. *Science*. 1994; 263:1612–1615. [PubMed: 7510419]
32. Ma X, Neurath M, Gri G, Trinchieri G. Identification and characterization of a novel Ets-2-related nuclear complex implicated in the activation of the human interleukin-12 p40 gene promoter. *J Biol Chem*. 1997; 272:10389–10395. [PubMed: 9099678]
33. Masumi A, Tamaoki S, Wang IM, Ozato K, Komuro K. IRF-8/ICSBP and IRF-1 cooperatively stimulate mouse IL-12 promoter activity in macrophages. *FEBS Lett*. 2002; 531:348–353. [PubMed: 12417340]
34. Parsa KV, Ganesan LP, Rajaram MV, Gavrilin MA, Balagopal A, Mohapatra NP, Wewers MD, Schlesinger LS, Gunn JS, Tridandapani S. Macrophage pro-inflammatory response to *Francisella novicida* infection is regulated by SHIP. *PLoS Pathog*. 2006; 2:e71. [PubMed: 16848641]
35. Crane DD, Scott DP, Bosio CM. Generation of a Convalescent Model of Virulent *Francisella tularensis* Infection for Assessment of Host Requirements for Survival of Tularemia. *PLoS ONE*. 2012; 7:e33349. [PubMed: 22428026]
36. Bar-Haim E, Gat O, Markel G, Cohen H, Shafferman A, Velan B. Interrelationship between dendritic cell trafficking and *Francisella tularensis* dissemination following airway infection. *PLoS Pathog*. 2008; 4:e1000211. [PubMed: 19023422]
37. Dennis DT, Inglesby TV, Henderson DA, Bartlett JG, Ascher MS, Eitzen E, Fine AD, Friedlander AM, Hauer J, Layton M, Lillibridge SR, McDade JE, Osterholm MT, O'Toole T, Parker G, Perl TM, Russell PK, Tonat K. and B. Working Group on Civilian. Tularemia as a biological weapon: medical and public health management. *JAMA*. 2001; 285:2763–2773. [PubMed: 11386933]

38. Tigertt WD. Soviet viable *Pasteurella tularensis* vaccines. A review of selected articles. Bacteriol Rev. 1962; 26:354–373. [PubMed: 13985026]
39. Fortier AH, Slayter MV, Ziemba R, Meltzer MS, Nacy CA. Live vaccine strain of *Francisella tularensis*: infection and immunity in mice. Infect Immun. 1991; 59:2922–2928. [PubMed: 1879918]
40. Apicella MA, Post DM, Fowler AC, Jones BD, Rasmussen JA, Hunt JR, Imagawa S, Choudhury B, Inzana TJ, Maier TM, Frank DW, Zahrt TC, Chaloner K, Jennings MP, McLendon MK, Gibson BW. Identification, characterization and immunogenicity of an O-antigen capsular polysaccharide of *Francisella tularensis*. PLoS ONE. 2010; 5:e11060. [PubMed: 20625403]
41. Hood AM. Virulence factors of *Francisella tularensis*. The Journal of hygiene. 1977; 79:47–60. [PubMed: 267668]
42. Wang X, Ribeiro AA, Guan Z, McGrath SC, Cotter RJ, Raetz CR. Structure and biosynthesis of free lipid A molecules that replace lipopolysaccharide in *Francisella tularensis* subsp. *novicida*. Biochemistry. 2006; 45:14427–14440. [PubMed: 17128982]
43. Maeshima N, Fernandez RC. Recognition of lipid A variants by the TLR4-MD-2 receptor complex. Frontiers in cellular and infection microbiology. 2013; 3:3. [PubMed: 23408095]
44. Beasley AS, Cotter RJ, Vogel SN, Inzana TJ, Qureshi AA, Qureshi N. A variety of novel lipid A structures obtained from *Francisella tularensis* live vaccine strain. Innate immunity. 2012; 18:268–278. [PubMed: 21709054]
45. Schilling B, McLendon MK, Phillips NJ, Apicella MA, Gibson BW. Characterization of lipid A acylation patterns in *Francisella tularensis*, *Francisella novicida*, and *Francisella philomiragia* using multiple-stage mass spectrometry and matrix-assisted laser desorption/ionization on an intermediate vacuum source linear ion trap. Anal Chem. 2007; 79:1034–1042. [PubMed: 17263332]
46. Melillo AA, Bakshi CS, Melendez JA. *Francisella tularensis* antioxidants harness reactive oxygen species to restrict macrophage signaling and cytokine production. J Biol Chem. 2010; 285:27553–27560. [PubMed: 20558723]
47. Naumann M, Scheidereit C. Activation of NF-kappa B in vivo is regulated by multiple phosphorylations. EMBO J. 1994; 13:4597–4607. [PubMed: 7925300]
48. Perkins ND. Post-translational modifications regulating the activity and function of the nuclear factor kappa B pathway. Oncogene. 2006; 25:6717–6730. [PubMed: 17072324]
49. Tamura T, Yanai H, Savitsky D, Taniguchi T. The IRF family transcription factors in immunity and oncogenesis. Annu Rev Immunol. 2008; 26:535–584. [PubMed: 18303999]
50. Sakaguchi S, Negishi H, Asagiri M, Nakajima C, Mizutani T, Takaoka A, Honda K, Taniguchi T. Essential role of IRF-3 in lipopolysaccharide-induced interferon-beta gene expression and endotoxin shock. Biochem Biophys Res Commun. 2003; 306:860–866. [PubMed: 12821121]
51. Sato M, Suemori H, Hata N, Asagiri M, Ogasawara K, Nakao K, Nakaya T, Katsuki M, Noguchi S, Tanaka N, Taniguchi T. Distinct and essential roles of transcription factors IRF-3 and IRF-7 in response to viruses for IFN-alpha/beta gene induction. Immunity. 2000; 13:539–548. [PubMed: 11070172]
52. Henry T, Monack DM. Activation of the inflammasome upon *Francisella tularensis* infection: interplay of innate immune pathways and virulence factors. Cell Microbiol. 2007; 9:2543–2551. [PubMed: 17662071]
53. Tamura T, Ishihara M, Lamphier MS, Tanaka N, Oishi I, Aizawa S, Matsuyama T, Mak TW, Taki S, Taniguchi T. DNA damage-induced apoptosis and Ice gene induction in mitogenically activated T lymphocytes require IRF-1. Leukemia. 1997; 11(Suppl 3):439–440. [PubMed: 9209417]
54. Lin R, Hiscott J. A role for casein kinase II phosphorylation in the regulation of IRF-1 transcriptional activity. Molecular and cellular biochemistry. 1999; 191:169–180. [PubMed: 10094406]
55. Harada H, Fujita T, Miyamoto M, Kimura Y, Maruyama M, Furia A, Miyata T, Taniguchi T. Structurally similar but functionally distinct factors, IRF-1 and IRF-2, bind to the same regulatory elements of IFN and IFN-inducible genes. Cell. 1989; 58:729–739. [PubMed: 2475256]

56. Pion E, Narayan V, Eckert M, Ball KL. Role of the IRF-1 enhancer domain in signalling polyubiquitination and degradation. *Cellular signalling*. 2009; 21:1479–1487. [PubMed: 19450680]

Figure 1. Inhibition of IL-12p40 in hDC is mediated by components in SchuS4 membranes
 (A) SchuS4 hDC were incubated with the indicated concentration of total membrane fraction (MF) isolated from SchuS4 or LVS for 18 hours. Culture supernatants were assessed for IL-12p40 by ELISA. * = significantly greater than untreated or SchuS4 MF treated hDC ($p < 0.05$). (B) hDC were incubated with the indicated concentration SchuS4 MF for 18 hours followed by addition of *E. coli* LPS (10 ng/ml). Twenty hours later supernatants were assessed for IL-12p40 by ELISA. * = significantly less than LPS only treated control ($p < 0.05$). (C and D) hDC were incubated with, MF, MF heat treated for 4 hours at 56°C (C) or MF treated with pronase (D) (all at 10 μg/ml) for 18 hours followed by addition of LPS. Twenty hours later supernatants were assessed for IL-12p40 by ELISA. * = significantly

less than LPS only treated control ($p < 0.05$). In each experiment each condition was tested in triplicate. Error bars represent SEM. Data is representative of three experiments of similar design using different donors.

Figure 2. SchuS4 lipids inhibit secretion of IL-12p40 by hDC

hDC were treated with the indicated concentration of lipids isolated from SchuS4 or LVS for 18 hours followed by addition of LPS for an additional 20 hours. EtOH served as vehicle control. Culture supernatants were assessed for IL-12p40 by ELISA. ns= not significantly different. * = significantly less than EtOH+LPS treated controls ($p < 0.05$). In each experiment each condition was tested in triplicate. Error bars represent SEM. Data is representative of three experiments of similar design using different donors.

Figure 3. SchuS4 lipids partially inhibit phenotypic maturation of hDC

hDC were incubated with SchuS4 lipids (30 μ g/ml) for 18 hours followed by Pam3CSK4 (10 μ g/ml) for an additional 20 hours. Changes in surface expression of the indicated receptors was monitored by flow cytometry. * = significantly less than hDC treated with Pam3CSK4 alone ($p < 0.05$). ns = not significantly different. In each experiment each condition was tested in triplicate. Error bars represent SEM. Data is representative of three experiments of similar design using different donors.

Figure 4. SchuS4 lipids inhibit inflammatory responses *in vivo*

Mice (n=5/group) were given 25 μ g/25 μ l SchuS4 lipids or EtOH (vehicle control) intranasally followed by intranasal instillation of 200 ng/25 μ l *E. coli* LPS 18 hours later. (-) = completely untreated mice. Five hours after administration of LPS, BAL cells and fluid were collected and assessed for neutrophils, TNF- α and IL-6. * = significantly less than EtOH treated controls ($p < 0.05$). BLD = below level of detection. Error bars represent SEM. Data is representative of three experiments of similar design.

Figure 5. SchuS4 lipids inhibit activation of NF-κBp65

(A) hDC were treated with SchuS4 lipids (30 μg/ml), EtOH (vehicle control), PBS, or LPS (10 ng/ml) and cell lysates were collected at the indicated time points. Phosphorylation and degradation of IκBα was assessed by western blot. (B) hDC were treated with 30 μg/ml SchuS4 lipid, EtOH or PBS for 18 hours followed by addition of LPS. At the indicated time points after LPS addition cell lysates were collected and assessed for phosphorylation and degradation of IκBα by western blot. (C) hDC were treated with SchuS4 lipid, EtOH, PBS, or LPS and translocation of NF-κBp65 (p65) to the cell nucleus was assessed 3 hours later using quantitative TransAm assay. ns = not significantly different. * = significantly greater than all other samples ($p < 0.05$). (D and E) hDC were treated with SchuS4 lipid, EtOH, PBS

(-) for 18 hours followed by LPS. Three and 1 hours after addition of LPS translocation of NF- κ Bp65 to the cell nucleus was assessed using quantitative TransAm assay (D) or by microscopy (E), respectively. * = significantly less than all other samples ($p < 0.05$). In each experiment each condition was tested in triplicate. Error bars represent SEM. Graphed data is pooled from three experiments utilizing different donors. Microscopy images are representative of three experiments of similar design using different donors.

Figure 6. SchuS4 lipids inhibit translocation of IRF1 in hDC

hDC were treated with SchuS4 lipid (30 μ g/ml) or EtOH (vehicle control), for 18 hours followed by LPS (10 ng/ml). Ninety minutes after addition of LPS translocation of IRF1 (A and B) and IRF3 (C and D) to the cell nucleus was assessed by microscopy. Translocation of IRF1 and IRF3 was quantitated by scoring greater than 500 cells for presence of nuclear IRF1 or IRF3. ns = not significantly different. * = significantly less than all other samples ($p < 0.05$). In each experiment each condition was tested in duplicate. Error bars represent SEM. Graphed data is pooled from three experiments utilizing different donors. Microscopy images are representative of three experiments of similar design using different donors.

Figure 7. SchuS4 lipids inhibit binding of IRF1 and IRF8 to the human IL-12p40 promoter hDC were treated with SchuS4 lipids or EtOH (vehicle control) followed by LPS+IFN- γ . (A) Supernatants were assessed for IL-12p40 by ELISA. SchuS4 lipids significantly inhibited the ability of hDC to secrete IL-12p40 in response to LPS and IFN- γ . * = $p < 0.05$ compared to EtOH and untreated controls. Error bars represent SEM. Data is pooled from 4 separate experiments using 4 different donors. (B) SchuS4 lipids inhibit binding of IRF1 and IRF8 to the Ets sequence, but not the ISRE sequence, in the IL-12p40 promoter. Whole cell extracts from hDC treated with EtOH, SchuS4 lipids and/or LPS+IFN- γ as indicated were incubated with a biotinylated DNA probe corresponding to the Ets or ISRE sequence conjugated to streptavidin beads. Beads were washed and bound materials eluted for assessment of IRF1 and IRF8 by SDS-PAGE followed by Western blotting. Data is representative of two experiments using two different donors.

Figure 8. Viable *F. tularensis* SchuS4 inhibits translocation of NF-κBp65 and IRF1 in hDC hDC were infected with a MOI = 50 viable SchuS4. As indicated, 24 hours after infection cells were treated with either Pam3CSK4 (5 μg/ml) or LPS (10 ng/ml). Eighteen hours later culture supernatants were assessed for IL-12p40 by ELISA (A) and 90 minutes after addition of Pam3CSK4 cells were assessed for translocation of NF-κBp65 by microscopy (B). Ninety minutes after addition of LPS cells were assessed for translocation of IRF1 and IRF3 by microscopy (C). Translocation of NF-κBp65, IRF1 and IRF3 was quantitated by scoring greater than 500 cells for presence of nuclear NF-κBp65, IRF1 or IRF3. ns = not significantly different. * = significantly less than uninfected cells ($p < 0.05$). In each experiment each condition was tested in duplicate. Error bars represent SEM. Graphed data is pooled from three experiments utilizing different donors. Microscopy images are representative of three experiments of similar design using different donors.