Francisella tularensis SchuS4 and SchuS4 Lipids Inhibit IL-12p40 in Primary Human Dendritic Cells by Inhibition of IRF1 and IRF8

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Francisella tularensis SchuS4 and SchuS4 Lipids Inhibit IL-12p40 in Primary Human Dendritic Cells by Inhibition of IRF1 and IRF8

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Induction of innate immunity is essential for host survival of infection. Evasion and inhibition of innate immunity constitute 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. In this article, we demonstrate that lipids enriched from virulent *F. tularensis* strain SchuS4, but not attenuated live vaccine strain, inhibit inflammatory responses in vitro and in vivo. Suppression of inflammatory responses is associated with IκB- and IRF-independent inhibition of NF-κBp65 activation and selective inhibition of activation of IFN regulatory factors. Interference with NF-κBp65 and IFN regulatory factors is also observed following infection with viable SchuS4. Together these data provide novel insight into how highly virulent bacteria selectively modulate the host to interfere with innate immune responses required for survival of infection. The Journal of Immunology, 2013, 191: 1276–1286.

*V*irulent *Francisella tularensis* ssp. *tularensis* is a Gram-negative bacterium and the causative agent of the fatal disease coined tularemia. *Francisella* has four major subspecies (1): *F. tularensis* ssp. novicida and ssp. mediastatica 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 used as a biological weapon and thus presents a viable concern as a potential bioweapon today (4). Although a vaccine generated from *F. tularensis*—that is, 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, a need has arisen 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 because 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). 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 that are modulated by virulent *F. tularensis*. Similarly, we have not identified those 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 article, 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/Lyoc, and R848 (imidazoquinolone compound) were purchased from Invivogen (San Diego, CA). *E. coli* strain O127:B7 LPS was purchased from Sigma-Aldrich (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 Jeanine Peterson, Ph.D. (Centers for Disease Control, Fort Collins, CO). Attenuated *F. tularensis* ssp. *holarctica* 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 described earlier (10, 12, 13). Following overnight culture, bacteria were pelleted by centrifugation for 15 min at 8000 × g. The resulting pellet was resuspended in 50 mM Tris-HCl, 0.6 µg/ml DNase, 0.6 µg/ml RNase, 1 mM EDTA (all from Sigma-Aldrich), 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 FastPrep Lysing Matrix B tubes using a FastPrep-24 (MPBiomedicals) for 10 cycles of 45 s with 2-min rest periods on ice between each cycle. The resulting slurry was then centrifuged at 10,000 rpm for 10 min. The supernatant was collected and centrifuged twice at 100,000 × g for 4 h. The pellet was resuspended in containing 50 mM Tris/HC1, 1 mM EDTA, then dialyzed against PBS using 3000 m.w. 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 h or incubated with 2 mg/ml pronase in 0.1 M Tris buffer, pH 7.0, at 40°C for 3 h, followed by heating at 87°C for 30 min 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 × 109 bacteria were thawed and plated onto eight 150-mm petri dishes containing MMH agar. Bacteria were incubated at 37°C 7% CO2 for 48 h. Bacteria were collected from the agar plates and added to 100 ml HPLC grade chloroform:methanol (2:1) (both from Sigma-Aldrich). The resulting mixture was stirred vigorously for 30 min at room temperature. Then, 20 ml endotoxin-free water was added, and the mixture was stirred for an additional 10 min. The mixture was centrifuged at 4000 × g for 10 min 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 sample was 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 ∼2% of wet weight. Thus, 30 µg/ml 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 mo. 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 the Bradford assay (Sigma-Aldrich) according to the manufacturer’s instruction, periodate stain of SDS-PAGE gels, and Western blotting for Francisella O-Ag 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 (hDCs) 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 National Institutes of Health Clinical Center at the National Institutes of Health (Bethesda, MD) under a protocol approved by the National Institutes of Health Clinical Center Institutional Review Board. Signed, informed consent was obtained from each donor, acknowledging that his or her donation would be used for research purposes by intramural investigators throughout the National Institutes of Health. Monocytes were further enriched using Ficoll-Paque PREMIUM (GE Healthcare) and were differentiated into MF according to the manufacturer’s instructions (BD Biosciences). Monocytes were cultured for 3 days in medium supplemented with 10% fetal bovine serum and 50 U/ml GM-CSF and 20 ng/ml IL-4 over the course of 4 d. On day 3 of culture, bacteria were pelleted by centrifugation for 15 min at 8000 × g. The resulting pellet was resuspended in the following buffer: 50 mM Tris/HCl, 1 mM EDTA, then dialyzed against PBS containing 50 mM Tris/HC1, 0.6 µg/ml DNase, 0.6 µg/ml RNase, 1 mM EDTA (all from Sigma-Aldrich), 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 FastPrep Lysing Matrix B tubes using a FastPrep-24 (MPBiomedicals) for 10 cycles of 45 s with 2-min rest periods on ice between each cycle. The resulting slurry was then centrifuged at 10,000 rpm for 10 min. The supernatant was collected and centrifuged twice at 100,000 × g for 4 h. The pellet was resuspended in containing 50 mM Tris/HC1, 1 mM EDTA, then dialyzed against PBS using 3000 m.w. 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 h or incubated with 2 mg/ml pronase in 0.1 M Tris buffer, pH 7.0, at 40°C for 3 h, followed by heating at 87°C for 30 min to deactivate the pronase prior to use.

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Assessment of cytokines

Human TNF-α, IL-6, IL-10, IL-12p40, and TGF-β were assessed by commercially available ELISA kits according to the 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 the manufacturer’s instructions (BD Biosciences).

Flow cytometry

Influx of inflammatory cells into mouse airways and expression of hDC cell surface receptors were assessed by flow cytometry, as previously described (8, 10, 11). Briefly, directly conjugated Abs for these analyses were purchased from BioLegend (San Diego, CA). The following Abs 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), and Alex Fluor 488 anti-human CD86 (clone IT2.2). Staining with directly conjugated Abs was done in FACS buffer at 4°C. Then, the cells were washed and fixed in 1% paraformaldehyde for 30 min 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 an LSR II flow cytometer (BD Biosciences). Analysis gates were set on viable un-stained cells and were designed to include all viable cell populations. Approximately 10,000 events were analyzed for each sample. Isotype control Abs were included when analyses and panels were first being performed to ensure 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 were analyzed using FlowJo software (TreeStar, Ashland, OR).

Microscopy

The hDCs were infected with SchuS4 or treated with PBS, ethanol (vehicle control), or Pam2CSK4 (100 µg/ml) and were fixed in 4% paraformaldehyde for 20 min. The hDCs were incubated with 0.3% Triton X-100 in PBS + 10% horse serum for 1 h at room temperature, followed by addition of rabbit anti-NF-κB p65 (Santa Cruz Biotechnology, Santa Cruz, CA) rabbit anti-p65, anti–IFN regulatory factor (IRF)-1 (X-1 Ap, or anti-IRF3 (X-1 Ap (all from Cell Signaling Technology, Beverly, MA) for 1 h at room temperature. Slides were washed with PBS and incubated with Alexa Fluor 568–conjugated goat anti-rabbit Ab (Cell Signaling Technology) and Alexa Fluor 488–conjugated mouse anti-F. tularensis LPS Abs (United States Biological, Swampsco, 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). A minimum of eight to ten confocal images were taken at ×400. Translocation was quantitated using a Zeiss Axio Imager M1 fluorescence microscope. Starting in the middle of the slide, three to four fields in succession were counted, for a total number of trypan blue negative cells/total cells counted × 100. As indicated, ultrapure E. coli LPS or E. coli LPS O127:B8 (10 µg/ml), Pan3CSK4 (5–10 µg/ml), Pan2CSK4 (10 µg/ml), LTA (100 µg/ml), R848 (5 µg/ml), or LPS (10 ng/ml) + 100 U/ml IFN-γ (PeproTech) was added 1 h after MF or lipid treatment, and cells were assessed for culture with an additional 20 h before supernatants were collected for assessment of cytokines.
of >300 cells per sample. Data were pooled from three identical experiments with three different donors.

Quantification of NF-κB translocation

Translocation of NF-κB 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-κB, cells were harvested 3 h after addition of stimuli or vehicle controls. For assessment of lipid-mediated inhibition of NF-κB translocation, cells were incubated with PBS, EtOH, or SchuS4 lipids for 24 h, followed by addition of ultrapure E. coli LPS. Cells were harvested 3 h 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 μg per sample prior to application in the NF-κB 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). The hDCs were lysed in a non-denaturing lysis buffer (20 mM Tris HCl, 150 mM NaCl, 10% glycerol, and 1% Triton) containing RNase Inhibitor mixture (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 the manufacturer’s instructions and ligated to 5’ biotinylated dsDNA (Integrated DNA Technologies) corresponding to the −244 bp to −154 bp region of the human IL-12p40 promoter containing the Ets2 sequence or the −101 to −11 region of the IL-12p40 promoter containing the ISRE sequence (Supplemental Fig. 1). Beads were washed in TGED buffer (10 mM Tris HCl, 10% glycerol, 0.1 mM EDTA, and 0.01% Triton × 100) and blocked overnight at 4°C in TGED + 0.5% BSA. Whole hDC lysate (100 μg) was added to 200 μg DNA-coated beads in a total of 500 μl TGED buffer + 0.1 M NaCl, and the mixture was incubated with constant mixing at 4°C overnight. Beads were then washed four times with TGED buffer + 0.1 M NaCl, and protein attached to DNA sequences was eluted in 20 μl 1× sample buffer + reducing agent (Invitrogen). The presence of IRF1 and IRF8 was assessed by SDS-PAGE and Western blotting using anti-IRF1 XP and anti-IRF8 Abs (both from Cell Signaling Technology).

Statistical analyses

For comparison between three or more groups, analysis was done by one-way ANOVA, followed by a Tukey 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 MF derived from virulent F. tularensis SchuS4 inhibits 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 are unique to virulent subspecies of F. tularensis, such as 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 MF enriched from whole-cell lysates of SchuS4 for its ability to stimulate and/or inhibit cytokine production from hDCs. As a positive control for stimulation of IL-12p40 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 hDCs (Fig. 1A and Refs 7, 10). In contrast, and consistent with our previous observations in hDCs infected with LVS, both LVS MF and E. coli LPS readily induced production of IL-12p40 from hDCs (Fig. 1A and Ref. 7).

![FIGURE 1. Inhibition of IL-12p40 in hDCs is mediated by components in SchuS4 membranes. (A) hDCs were incubated with the indicated concentration of total MF isolated from SchuS4 or LVS for 18 h. Culture supernatants were assessed for IL-12p40 by ELISA. *Significantly greater than untreated or SchuS4 MF–treated hDCs (p < 0.05). (B) hDCs were incubated with the indicated concentration of SchuS4 MF for 18 h, followed by addition of E. coli LPS (10 ng/ml). At 20 h later, supernatants were assessed for IL-12p40 by ELISA. *Significantly less than LPS only–treated control (p < 0.05). (C and D) hDCs were incubated with MF, MF heat treated for 4 h at 56°C (C), or MF treated with pronase (D) (all at 10 μg/ml) for 18 h, followed by addition of LPS. At 20 h 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 are representative of three experiments of similar design, using different donors.](http://www.jimmunol.org/Download)
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 that inhibit inflammatory responses are either lipids or carbohydrates. Therefore, we separated lipid and nonlipid materials from SchuS4 and LVS, using a modified Folch method, and determined their ability to activate or inhibit inflammatory responses in hDCs. Nonlipid components of the bacteria present in the aqueous phase of the SchuS4 or LVS preparations do not stimulate a measurable cytokine response in hDCs at the concentrations tested (Supplemental Fig. 2). When examined for suppressive activity, nonlipid products present in aqueous preparations from SchuS4 and LVS are capable of inhibiting inflammatory responses in hDCs (Supplemental Fig. 2). However, the nonstimulatory yet suppressive activity of nonlipid material associated with LVS is inconsistent with the ability of viable LVS and total MF isolated from LVS to provoke inflammatory responses in hDCs. Thus, the nonlipid products isolated from both SchuS4 and LVS do not faithfully replicate the differential stimulatory and/or inhibitory activity of SchuS4 and LVS in hDCs. Because we are pursuing identification of a heat- and pronase-resistant molecule that shares the unique nonstimulatory but inhibitory properties observed among hDCs 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 those from SchuS4 induce detectable concentrations of IL-12p40 from hDCs following incubation of cells with doses of lipid as high as 30 μg/ml (Fig. 2). Lipids isolated from LVS do not inhibit hDC responsiveness to LPS (Fig. 2). In contrast, SchuS4 lipids potentiate suppression of IL-12p40 by hDCs in response to E. coli LPS (Fig. 2). Similarly to SchuS4 MF, SchuS4 lipids do not induce secretion of the anti-inflammatory cytokines IL-10 or TGF-β (data not shown). In addition, SchuS4 lipids also inhibit the hDC response to other TLR agonists, including Pam3CSK4 (TLR2/1), Pam2CSK4 (TLR2/6), LTA (TLR2), R848 (TLR8), and ssRNA (TLR8) (Supplemental Fig. 3). Viability of hDCs treated with SchuS4 lipid was 99.1% compared with 98.2% of EtOH-treated cells and 99% viability in untreated cells. Thus, suppression of cytokine production observed in hDCs treated with SchuS4 lipids was not associated with toxicity of SchuS4 lipid to hDCs. Together these data show that lipids isolated from SchuS4 interfere with proinflammatory responses activated following engagement of multiple TLRs in hDCs.

In addition to production of cytokines, activation of hDCs 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 hDCs treated with lipids before and after exposure to Pam3CSK4. SchuS4 lipids do not induce increased expression of any cell surface receptor assessed (Fig. 3). Interestingly, SchuS4 lipids have no effect on the upregulation of HLA-DR and CCR7 among hDCs treated with Pam3CSk4 (Fig. 3). However, in correlation with the inhibition of cytokine production, hDCs treated with SchuS4 lipids express significantly less CD80 and CD86 on the cell surface following addition of Pam3CSK4 (Fig. 3). Thus, SchuS4 lipids also affect the ability of hDCs 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 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 hDCs treated with lipids before and after exposure to Pam3CSK4. SchuS4 lipids do not induce increased expression of any cell surface receptor assessed (Fig. 3). Interestingly, SchuS4 lipids have no effect on the upregulation of HLA-DR and CCR7 among hDCs treated with Pam3CSk4 (Fig. 3). However, in correlation with the inhibition of cytokine production, hDCs treated with SchuS4 lipids express significantly less CD80 and CD86 on the cell surface following addition of Pam3CSK4 (Fig. 3). Thus, SchuS4 lipids also affect the ability of hDCs to undergo phenotypic maturation.
in vitro (Fig. 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. At 5 h 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 proinflammatory cytokines. Administration of SchuS4 lipids does 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 (Fig. 4). Thus, SchuS4 lipids are also capable of inhibiting inflammatory responses in vivo.

**SchuS4 lipids inhibit translocation of NF-κB 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-κB, specifically NF-κB p65 (25). In a resting cell, p65 is bound to IκBα in the host cytosol. Upon cellular activation, the kinases in the IKK family phosphorylate IκBα, targeting it for ubiquitination and degradation. This activity, in turn, results in release of the p65 subunit for activation and translocation to the nucleus, where they participate in transcription of proinflammatory genes. Inhibition of cytokine production by SchuS4 lipids in hDCs may be due to interference of activation and/or translocation of NF-κB transcription factors to the nucleus of the host cell. We first examined the ability of SchuS4 lipids to modulate phosphorylation of IκBα. SchuS4 lipids do not independently induce phosphorylation and/or degradation of IκBα (Fig. 5A), nor do they interfere with activation of IκBα following incubation of cells with E. coli LPS (Fig. 5B).

Data above suggest that SchuS4 lipids are not modulating activation of NF-κBp65 through canonical pathways. However, activation of p65 can occur independently of IκBα, and the absence of an effect on the activation of IκBα by SchuS4 lipids does not rule out modulation of NF-κBp65 (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-κB family members in hDCs, but readily inhibit translocation of p65 in response to LPS (Fig. 5C, 5D). Inhibition of p65 translocation is confirmed by microscopy. In agreement with our quantitative results, ~50% of hDCs pretreated with SchuS4 lipid do not translocate the NF-κB p65 to their nucleus in response to LPS (Fig. 5E). Overall, our data suggest that the suppression of cytokine production in hDCs by SchuS4 lipid is not mediated through manipulation of canonical pathways upstream of NF-κB, but rather is affecting activation and translocation of NF-κB family members either directly or via noncanonical pathways.

**SchuS4 lipids inhibit activation of IRFs**

In addition to p65, transcription of IL-12p40 is also dependent on IRFs, 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 hDCs. SchuS4 lipids do not induce translocation of IRF1, but do inhibit translocation of IRF1 among hDCs responding to LPS (Fig. 6A, 6B). In addition to IRF1, IRF8 is also essential for transcription of the IL-12p40 gene (32). No detection methods are available to view IRF8 translocation by microscopy. The accepted technique for analyzing 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 IL-12p40 (32). Therefore, we determined whether lipid affected the ability of IRF1 and IRF8 to bind to the IL-12p40 promoter in primary hDCs. Binding of IRF1 and IRF8 is optimal following stimulation of hDCs with LPS + IFN-γ. Before assessing the effect SchuS4 lipids had on the ability of IRF1 and IRF8 to bind to the IL-12p40 promoter in primary hDCs, we first ascertained whether SchuS4 lipids could inhibit production of IL-12p40 in hDCs stimulated with LPS + IFN-γ. Similar to hDCs stimulated with LPS alone, exposure to SchuS4 lipids results in secretion of

**FIGURE 4.** SchuS4 lipids inhibit inflammatory responses in vivo. Mice (n = 5 per group) were given 25 μg/25 μl SchuS4 lipids or EtOH (vehicle control) intranasally, followed by intranasal instillation of 200 ng/25 μl E. coli LPS 18 h later. At 5 h 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). Error bars represent SEM. Data are representative of three experiments of similar design. –, Completely untreated mice; BLD, below level of detection.
Viable SchuS4 inhibits translocation of NF-κB 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-κB and IRF1. Thus, we next sought to confirm that interference with these transcription factors also occurred among hDCs 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 hDCs to secrete IL-12p40 (Fig. 8A and Ref. 7). Furthermore, similar to data presented above, viable SchuS4 inhibits translocation of both NF-κB and IRF1 among infected hDCs (Fig. 8B, 8C), but has no effect on translocation of IRF3 following stimulation of hDCs with LPS (Fig. 6C, 6D). Together, these 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.

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 are important features embodied by highly virulent pathogens. As a case in point, in contrast to more attenuated strains and subtypes, 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 post 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 the most dangerous manifestation of tularemia (4, 10, 20, 36). Thus, we used model systems incorporating primary hDCs and intranasal instillation in the mouse to identify the components of *F. tularensis* and specific signaling pathways involved in immunosuppression mediated by this organism.

FIGURE 5. SchuS4 lipids inhibit activation of NF-κBp65. (A) hDCs 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 were assessed by Western blot. (B) hDCs were treated with 30 μg/ml SchuS4 lipid, EtOH, or PBS for 18 h, 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) hDCs were treated with SchuS4 lipid, EtOH, PBS, or LPS, and translocation of NF-κBp65 (p65) to the cell nucleus was assessed 3 h later, using quantitative TransAM assay. *Significantly greater than all other samples (p < 0.05). (D and E) hDCs were treated with SchuS4 lipid, EtOH, PBS (−) for 18 h, followed by LPS. At 3 and 1 h after addition of LPS, translocation of NF-κBp65 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 are pooled from three experiments using different donors. Microscopy images are representative of three experiments of similar design, using different donors.
In this article, we provide evidence that lipids isolated from virulent, but not attenuated, <i>F. tularensis</i> are an important bacterial constituent for the suppression of human and murine innate inflammatory responses. We demonstrate that, although not provoking an inflammatory response, lipids isolated from virulent <i>F. tularensis</i> SchuS4 readily inhibited upregulation of select cell surface receptors on hDCs associated with hDC activation and also suppressed production of IL-12p40 by primary hDCs in response to a variety of secondary stimuli. Using a mouse model of pulmonary inflammation, we also found that SchuS4 lipids potentially inhibited inflammation in vivo. Our data also suggest that the suppressive lipids associated with SchuS4 are not associated with LPS—that is, lipid A—because the lipid products described in this article are fully capable of suppressing responses in hDCs, 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-κBp65 in a manner independent of IκB degradation. SchuS4 lipids also selectively target IRF family members by inhibiting nuclear localization of IRF1, but not IRF3, in LPS-stimulated hDCs. Finally, our data point toward a physiological role for SchuS4 lipids in mediating the suppression observed in hDCs infected with viable SchuS4 by demonstrating that both SchuS4 lipids and viable SchuS4 inhibit NF-κB and IRF1 activation following infection of hDCs.

This work is an extension of our original observation that heat-stable products either secreted or shed from virulent <i>F. tularensis</i> SchuS4 were able to interfere with hDC responsiveness to <i>E. coli</i> LPS (10). These data strongly suggest that a nonprotein component derived from SchuS4 contributes to the inability of hDCs to respond to secondary stimuli. In this study, we have found that following biochemical extraction, both nonlipid (present in the aqueous phase) and lipids (present in the organic phase) isolated from SchuS4 are able to modulate the ability of hDCs 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 hDCs. LVS was derived via serial passage from a virulent Type B subspecies and is capable of causing overt disease in some humans (37). LVS also retains a route- and dose-dependent virulence in mice (5, 38). Furthermore, the composition of the O-Ag polysaccharide that represents the primary nonlipid species in membranes of <i>Francisella</i> is very similar among LVS and Type A isolates (39). Thus, LVS may retain features in its carbohydrate profile that are associated with pathogenicity that may be more prominently featured in fully virulent isolates. Nevertheless, intact LVS and MF isolated from LVS provoke production of proinflammatory cytokines from hDCs, whereas intact SchuS4 and MF derived from SchuS4 do not (Fig. 1 and Ref. 10). Thus, the nonstimulatory, but suppressive, activity of nonlipid components present in LVS is not consistent with the activity of the intact organism with human cells.
In contrast to nonlipid 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 hDCs. Neither LVS nor SchuS4 lipids provoke cytokine production from hDCs. However, only SchuS4 lipids are able to inhibit the ability of hDCs to secrete IL-12 in response to multiple TLR agonists, which is consistent with the ability of viable SchuS4 to evade induction of proinflammatory cytokines while potently suppressing hDC responsiveness to various TLR ligands (7, 10). This finding 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. A handful of reports characterize the lipid species associated with attenuated and virulent strains of F. tularensis. Virulent F. tularensis has a lipid profile and content that are unusual and unique among Gram-negative bacteria. Whereas the membranes from most Gram-negative bacteria contain ~10–20%...
lipid, *F. tularensis* membranes are composed of nearly 70% lipid (40). Some have suggested that a large portion of this lipid is free lipid A unassociated with O-Ag, which would partition to the organic phase of our extraction (41), although this phenomenon has been documented only for *F. novicida* and not the other subspecies of *F. tularensis*. Lipid A is the lipid portion of the large 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. A direct association exists between the number of acyl groups present on the lipid A and the stimulatory activity of this molecule (42). Specifically, lipid A species with four or fewer acyl groups do not provoke inflammatory responses from host cells (42). Among attenuated strains—LVS and *F. novicida*—lipid A is tetraacylated and thus is a poor stimulator for cytokine responses (43, 44). 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, several factors 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, pentacylated LPS isolated from *Neisseria meningitidis* stimulates weak responses from human cells but retains its ability to induce cytokine production from mouse cells (as reviewed in Ref. 42). Thus, assuming lipid A is involved, one hypothesis may be that the lack of suppressive activity of LVS lipids in hDCs, compared with 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 (Fig. 4 and D. Crane, R. Ireland, J. Alinger, P. Small, and C. Bosio, submitted for publication). 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 (44). However, the presence and relative abundance of these minor lipid A species were not different among subspecies of *F. tularensis* tested—that is, virulent Type B *F. tularensis*, *F. novicida*, and *F. philomargia*. Together, these findings suggest 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 proinflammatory responses in hDCs. In support of this hypothesis, preliminary data from our laboratory suggest 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 hDCs (R. Ireland and C. Bosio, unpublished observations).

In addition to identifying the class of molecules found in SchuS4 that inhibit inflammatory responses, we also identify three host signaling molecules required for transcription of genes encoding proinflammatory cytokines that are modulated by both SchuS4 lipids and viable SchuS4. NF-κBp65 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 IkBα subunits that (in their nonphosphorylated state) retain p65 in the cytosol (45). Our data demonstrate that SchuS4 is capable of preventing p65 translocation independently of activation of IkBα. Although it is widely accepted that phosphorylation of IkBα is a critical step in the nuclear translocation of p65, it is not sufficient for the activation of NF-κB 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 (46, 47). Thus, SchuS4-mediated inhibition of p65 translocation may lie in the interference with kinases required for phosphorylation of p65 downstream of IkBα.

In addition to disrupting 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 nine different proteins originally characterized as transcription factors that regulated gene expression from Type I IFN and IFN-inducible genes (48). It is now appreciated that IRFs also play critical roles in cell survival, oncogenesis, and mediation of signals through pattern recognition receptors for cytokine production, including IL-12 (48). Initially, our finding that SchuS4 and SchuS4 lipids modulated activation of IRF1 was surprising because 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 pattern recognition receptors is dependent on IRF3 (49, 50). Thus, owing to its ability to induce IFN-β, SchuS4 may not influence IRF3 activation. Indeed, no significant difference can be noted in the translocation of IRF3 among cells infected with SchuS4 or treated with SchuS4 lipid. This finding supports our previous work that SchuS4 is not negatively affecting Type I IFN pathways.

In contrast to IFN-β, transcription of IL-12p40 requires IRF1 and IRF8 (33). Others have shown that microbial toxins, such as 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 inducible NO synthase and caspase-1 (13, 31, 51, 52). 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 are not well defined. To date, casein kinase II is the only kinase shown to phosphorylate IRF1 (53). 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 (54). 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. Considered together, this information 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 of SchuS4 lipid 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 Schu4 lipids in the cytosol. Alternatively, IRF1 has a short half-life and is targeted for ubiquitination and degradation by the host cell (55). It is possible that Schu4 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 Schu4-mediated inhibition of IRF1 and IRF8 activity.

In conclusion, we show in this article that lipids derived from Schu4 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-κBp65, IRF1, and IRF8—whose activation/translocation to the host nucleus is inhibited by Schu4 and Schu4 lipids. Together, these data provide new targets for vaccines and therapeutics directed against Francisella tularensis and enhance our understanding of pathogen manipulation of innate immune responses in the host. In addition, given the impressive anti-inflammatory activity of Schu4 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.

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Disclosures

The authors have no financial conflicts of interests.

References

SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure 1. DNA probes containing Ets2 and ISRE sequences utilized for immunoprecipitation. Schematic of the -244 to -154 sequence of the IL-12p40 promoter with the Ets2 region underlined and in bold. Schematic of the -101 to -11 sequence of the IL-12p40 promoter with the ISRE region underlined and in bold.

Supplemental Figure 2. Non-lipid molecules from Francisella inhibit secretion of IL-12p40 by hDC. hDC were treated with the non-lipid molecules present in the aqueous phase of preparations from LVS and SchuS4 diluted 1:100, 1:500 and 1:1000 in cRPMI for 18 hours followed by addition of LPS for an additional 20 hours. Culture supernatants were assessed for IL-12p40 by ELISA. ns= not significantly different. * = significantly less than LPS alone treated controls (p<0.05). Triangles represent increasing dilutions of aqueous phase. 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.

Supplemental Figure 3. SchuS4 lipids inhibit secretion of IL-12p40 by hDC following addition of multiple TLR agonists. hDC were treated with 30 μg/ml SchuS4 lipids for 18 hours followed by addition of Pam3CSK4 (10 μg/ml), Pam2CSK4 (10 μg/ml), LTA (100 μg/ml), R848 (2 ng/ml) or ssRNA40/LysoVec (ssRNA40) (0.25 μg/ml) 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+TLR agonist 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.
Ets2 -244 to -154
CCATCTCCTCCTTTATATCCCCACCCAAAAG<ins>TCATTTCCCTC</ins>
TTAGTTTCATTACCTGGGATTTTGATGTCATGTTCCCTCCTCCTC
GTTATTGA

ISRE -101 to -11
AGGTTTTTGAGAGAGTTGTTTTTCAATGTGGCAACAAAGTC
<ins>AGTTTCTAGGTTAAGGTTTCCC</ins>ATCAGAAAGGGAGTAGAGTAGATAT
ATAAGTTCCAGTA

Ireland R et al, Supplemental Figure 1