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IFN-β Mediates Suppression of IL-12p40 in Human Dendritic Cells following Infection with Virulent Francisella tularensis

Timothy J. Bauler, Jennifer C. Chase, and Catharine M. Bosio

Active suppression of inflammation is a strategy used by many viral and bacterial pathogens, including virulent strains of the bacterium Francisella tularensis, to enable colonization and infection in susceptible hosts. In this study, we demonstrated that virulent F. tularensis strain SchuS4 selectively inhibits production of IL-12p40 in primary human cells via induction of IFN-β. In contrast to the attenuated live vaccine strain, infection of human dendritic cells with virulent SchuS4 failed to induce production of many cytokines associated with inflammation (e.g., TNF-α and IL-12p40). Furthermore, SchuS4 actively suppressed secretion of these cytokines. Assessment of changes in the expression of host genes associated with suppression of inflammatory responses revealed that SchuS4, but not live vaccine strain, induced IFN-β following infection of human dendritic cells. Phagocytosis of SchuS4 and endosomal acidification were required for induction of IFN-β. Further, using a defined mutant of SchuS4, we demonstrated that the presence of bacteria in the cytosol was required, but not sufficient, for induction of IFN-β. Surprisingly, unlike previous reports, induction of IFN-β by F. tularensis was not required for activation of the inflammasome, was not associated with exacerbation of inflammatory responses, and did not control SchuS4 replication when added exogenously. Rather, IFN-β selectively suppressed the ability of SchuS4-infected dendritic cells to produce IL-12p40. Together, these data demonstrated a novel mechanism by which virulent bacteria, in contrast to attenuated strains, modulate human cells to cause disease.

Materials and Methods

Generation of human monocye-derived dendritic cells

Human monocye-derived dendritic cells (DC) were generated from apheresed monocytes, as previously described (6). Briefly, monocytes were enriched by apheresis and negative selection using Dynabeads MyPure Monocytes Kit for untouched human cells, per the manufacturer’s instructions (Invitrogen, Carlsbad, CA). Cells were differentiated upon culture in RPMI 1640 (Invitrogen) supplemented with 10% heat-inactivated FCS, 0.2 mM l-glutamine, 1 mM HEPES buffer, and 0.1 mM nonessential amino acids (all from Invitrogen) [complete RPMI (cRPMI)]; 100 ng/ml recombinant human (rh) GM-CSF; and 20 ng/ml rHL-4 (both from PeproTech, Rocky Hill, NJ). On day 3 of culture, cells were replensed with 100 ng/ml rhGM-CSF and 20 ng/ml rHL-4. All cells were used on day 4 of culture. As indicated, some hDC were pretreated with 1000 U/ml IFN-α (PBL IFN Source, Piscataway, NJ) 16 h prior to infection or 1–100 U/ml prior to stimulation with Escherichia coli LPS.

Bacteria

*F. tularensis* strain LVS, SchuS4Δ369c, and SchuS4Δ369pcp369 were provided by Dr. Jean Celli (Rocky Mountain Laboratories, Hamilton, MT). *F. tularensis* strain SchuS4 was provided by Dr. Jeanne Peterson (Centers for Disease Control and Prevention, Fort Collins, CO). As previously described, bacterial stocks were generated by growing strains overnight in modified Mueller–Hinton broth, aliquoted into 1-ml samples, and frozen at −80°C (4, 6, 16). Immediately prior to infection, bacterial stocks were thawed, pelleted by centrifugation, and resuspended in cRPMI. Frozen stocks were titered by enumerating viable bacteria from serial dilutions plated on modified Mueller–Hinton agar, as previously described (4, 6, 16). The number of viable bacteria in frozen stock vials varied by <1% over a 10-mo period. Where indicated, SchuS4 was killed by incubation in 2% paraformaldehyde (PFA) for 30 min at 37°C, washed extensively in PBS, and resuspended in cRPMI before addition to hDC cultures.

Infection of hDC

hDC were infected at a multiplicity of infection of 50 with *F. tularensis*, as previously described (6). Briefly, hDC were removed from their original cultures, centrifuged, and adjusted to 1–2 × 10^6/ml in reserved DC medium. Cells treated with medium alone served as negative controls. Bacteria were added, and cells were incubated at 37°C in 5% CO2 for 2 h, washed once, and then incubated with 50 μg/ml gentamicin (Invitrogen) for 45 min to kill extracellular bacteria. Then cells were washed extensively, adjusted to 5 × 10^6 cells/ml in reserved DC medium, and plated at 1–0.5 ml/well in 24- or 48-well tissue culture plates, respectively. Intracellular bacteria were enumerated following lysing of hDC with H2O and plating serial dilutions of cell lysate onto modified Mueller–Hinton broth agar plates. Agar plates were incubated at 37°C/7% CO2 for 48 h, and individual colonies were enumerated.

Where indicated, cells were treated with rhIFN-γ (PBL IFN Source) 16 h prior to stimulation with 10 ng/ml ultrapure *E. coli LPS* (InvivoGen, San Diego, CA). In other experiments, cells were treated with 10 ng/ml ultrapure *E. coli LPS* at the same time that other cells were infected or 24 h postinfection. In some experiments, 14 μg/ml polyclonal rabbit anti–IFN-α Ab, 3 μg/ml monoclonal mouse anti–IFN-γ Ab (both from PBL IFN Source), or normal rabbit IgG or mouse IgG1 (R&D Systems) as a non-specific Ab control, respectively, were added immediately postinfection. To inhibit phagocytosis or endosomal acidification, hDC were pretreated 1 h prior to infection with 10 μg/ml cytochalasin D (Sigma, St. Louis, MO) or 100 nM bafilomycin (BAF) A1 (AG Scientific, San Diego, CA), respectively. In additional experiments, hDC were treated for 1 h prior to infection with 50 μg/ml ferric pyrophosphate (Sigma). Inhibitors and/or ferric pyrophosphate were maintained in the culture media throughout the course of the infection.

Cytokine quantification

The presence of TNF-α, IL-12p40, IL-6, and IL-1β in culture superna
tants was quantitated using commercially available ELISA, according to the manufacturer’s instructions (R&D Systems, Minneapolis, MN). The presence of IFN-β in culture supernatants was quantitated using the Veri
kine High Sensitivity Human IFN-β ELISA kit (PBL IFN Source). Intracellular stores of pro–IL-1β were assessed by Western blot. Briefly, hDC were lysed using 1% Triton X-100. Resulting lysates were run on 10% SDS-PAGE gels. Proteins were transferred to polyvinylidene difluoride membranes, and pro–IL-1β was detected using anti–IL-1β Ab (R&D Systems, Minneapolis, MN), followed by HRP-conjugated anti-
mouse IgG (Jackson ImmunoResearch, West Pike, PA). Bound Abs were detected using SuperSignal West Pico Chemiluminescence Kit (Thermo
tisher, Rockford, IL) and Biospectrum imager (UVP, Upland, CA). Blots were stripped and reprobed with an anti-actin Ab (Cell Signal Technology, Danvers, MA) to confirm equivalent loading. In experiments measuring secretion of cytokines by SchuS4-infected hDC in the presence of either mouse IgG (isotype, R&D Systems) or neutralizing monoclonal anti-
mouse IFN-β Abs (PBL IFN Source), the percentage of cytokine secre
tion of control (uninfected, LPS-treated) cells was calculated using the following equation (concentration of cytokine present in SchuS4-infected culture/average cytokine concentration in LPS-treated, uninfected culture) × 100.

Intracellular cytokine staining

Intracellular cytokines were detected by flow cytometry, as previously described (6, 16). At the indicated time points and 30 min after the addition of ultrapure LPS (10 ng/ml), 5 μg/ml brefeldin A (Invitrogen) was added to DC cultures. Cells were then incubated at 37°C/7% CO2 for 5 h. After incubation, cells were washed, fixed, and permeabilized with 0.25% saponin, and incubated with anti-human IL-12/23-p40/70 (PE; clone C8.6) or mouse IgG1 (PE) as an isotype control (both from eBioscience, San Diego, CA). Cells were washed again, fixed in 1% PFA, and resus
pended in FACS buffer (eBioscience). Cells were acquired using a CyFlow ML flow cytometer (Partec, Swedenboro, NJ) and analyzed with FlowJo Software (Treestar, Ashland, OR). The percentage of responding cells treated with either normal rabbit IgG (isotype; R&D Systems) or neu
tralizing polyclonal anti–IFN-β Abs (PBL IFN Source) were calculated using the following equation: (percentage cytokine positive from SchuS4-infected culture/average percentage cytokine positive from uninfected culture) × 100.

Quantification of transcripts

At the indicated time points, RNA was extracted from infected and mock-infected DC using an RT² qPCR-Grade RNA Isolation Kit; cDNA was generated using an RT² First Strand Kit and quantified using RT² Profiler Human TLR PCR Arrays (all from SA Biosciences, Frederick, MD), according to the manufacturer’s instructions. Fold change was quantified as ΔΔCT and calculated for infected cells normalized to mock-infected samples harvested at the same time point using the SA Biosciences Data Analysis Web Portal (http://www.sabiosciences.com/pcr/arrayanalysis.php). In additional experiments, at the indicated time point, RNA was puri
died from hDC using RNeasy kits (Qiagen, Valencia, CA), and real-time quantitative PCR was run using primer/probe sets for human IFN-β, IRF7, and GAPDH on a 7900HT Fast Real Time PCR System (Applied Bio
systems). Input RNA was normalized to GAPDH, and fold change in the indicated genes was quantitated as ΔΔCT for infected cells normalized to mock-infected samples.

Detection of intracellular activated caspase-1

Intracellular activated caspase-1 was detected using Green FLICA Caspase-1 assay kit (Immunochemistry Technologies, Bloomington, MN), according to the manufacturer’s instructions (17). Briefly, hDC were infected with SchuS4 or LVS, as described above. At the indicated time points, cells were harvested by centrifugation, resuspended in 90 μl cRPMI, and plated into a 96-well plate. Ten microliters of 7X working stock of Green FLICA reagent was added to each well. Cells were incubated at 37°C/7% CO2 for 1 h. Cells were washed three times in 1× FLICA wash buffer, followed by fixation in 1× FLICA fix for 1 h at 4°C. Cells were analyzed for the presence of active caspase-1 by flow cytometry using a CyFlow ML cytomter (Partec) and FlowJo Analysis software (Treestar).

Phagosomal-integrity assay

To differentiate between vacuolar and cytosolic bacteria, phagosomal-
integrity assays were performed, as previously described (17), with the following modifications. Three hours postinfection, hDC were harvested and washed twice with KHM buffer (110 mM potassium acetate, 20 mM HEPES, 1 mM MgCl2 [pH 7.4]) and the phagosomal-integrity assay was performed. Briefly, hDC were infected with SchuS4 or LVS, and permeabilized for 1 min using 50 μg/ml digitonin (Sigma). Cells were washed with KHM buffer and stained for 12 min at 37°C with rabbit anti
calnexin (Stressgen Biotechnologies, Victoria, BC, Canada) and Alexa Fluor 546-anti-*F. tularensis* LPS Abs (US Biological, Swampscott, MA). Then cells were washed with PBS and adhered to glass slides using a Shandon Cytospin 4. Cells were fixed with 3% PFA for 30 min at 37°C and further stained with 0.2% saponin/10% horse serum in PBS for 30 min. Rabbit anti-calnexin Abs were detected using cyanin 5-conjugated anti-rabbit Abs (Invitrogen), and all intracellular bacteria were labeled using Alexa Fluor 488-conjugated anti-Fluorescein Abs. Cells were washed
twice with 0.2% saponin in PBS and then mounted in FluorSave Reagent (Calbiochem, Gibbstown, NJ). Samples were visualized using a Carl Zeiss LSM 510 confocal laser-scanning microscope.

Statistical analysis
For comparison among three or more groups, analysis was done by one-way ANOVA, followed by the Tukey multiple comparisons test, or two-way ANOVA, followed by the Bonferroni or multiple comparisons test, with significance determined at \( p < 0.05 \). For comparison of two groups, analysis was done using an unpaired Student \( t \) test, with significance determined at \( p < 0.05 \).

Results
Secretion of proinflammatory cytokines by hDC following infection with F. tularensis
It was suggested that one difference between virulent and attenuated strains of \( F. \) tularensis is the ability of virulent strains to evade induction of proinflammatory responses. Thus, we first compared bacterial replication and secretion of TNF-\( \alpha \), IL-6, and IL-12p40 into culture supernatants following infection of hDC with either virulent \( F. \) tularensis strain SchuS4 or attenuated \( F. \) tularensis strain LVS. Similar numbers of SchuS4 and LVS were phagocytosed by hDC (Fig. 1A). However, SchuS4 replicated more quickly over the first 12 h of infection compared with LVS. Similar numbers of LVS and SchuS4 were recovered from hDC at 24, 48, and 72 h postinfection (Fig. 1A). In agreement with our and others’ previous observations, SchuS4 failed to stimulate secretion of TNF-\( \alpha \), IL-6, and IL-12p40 in concentrations that were significantly different from uninfected hDC, whereas LVS induced significantly more IL-12p40 and IL-6 compared with uninfected and SchuS4-infected cells at each time point tested (Fig. 1B) (6). Significantly more TNF-\( \alpha \) was also observed in supernatants from LVS-infected hDC 24 and 48 h postinfection compared with samples collected from uninfected and SchuS4-infected cells (Fig. 1B). Together, these data supported the hypothesis that one difference between SchuS4 and LVS is the ability of SchuS4 to undergo intracellular replication without provoking inflammatory cytokines.

SchuS4 actively interferes with secretion of proinflammatory cytokines
We next confirmed that SchuS4 was actively inhibiting secretion of proinflammatory cytokines in hDC following infection. Twenty-four hours postinfection, \( E. \) coli LPS was added to hDC cultures, and secretion of TNF-\( \alpha \), IL-6, and IL-12p40 was measured 24 h later. Consistent with our previous observations using intracellular cytokine staining, hDC infected with SchuS4 produced significantly less TNF-\( \alpha \), IL-6, and IL-12p40 compared with uninfected cells in response to \( E. \) coli LPS (Fig. 1C) (6). Thus, SchuS4 fails to induce inflammatory responses in hDC and actively inhibits their ability to secrete proinflammatory cytokines in response to secondary microbial stimuli.

Production of IFN-\( \beta \) in hDC infected with virulent \( F. \) tularensis
To determine the mechanism by which \( F. \) tularensis inhibits production of proinflammatory cytokines, we performed targeted, quantitative RT-PCR (qRT-PCR) microarray analysis of changes in expression of host genes associated with suppression of inflammation following infection with SchuS4. As expected, SchuS4 failed to induce transcription of genes associated with strong inflammatory responses, including TNF-\( \alpha \) and IL-1\( \beta \) (Fig. 2A). Furthermore, SchuS4 also failed to induce expression of genes traditionally associated with suppression of host responses, such as IL-10 and TOLLIP (Fig. 2A). In contrast, SchuS4 infection of hDC resulted in increased expression of IFN-\( \beta \) within 8 h of infection (Fig. 2A). SchuS4 failed to induce expression of IFN-\( \alpha \) and IFN-\( \gamma \). Therefore, induction of IFN-\( \beta \) was not due to general activation of types I and II IFN genes (Fig. 2A). The induction of IFN-\( \beta \) transcription at early time points postinfection of hDC with SchuS4 suggested that expression of these genes may contribute to the virulence of this bacterium. To determine whether induction of IFN-\( \beta \) was associated with infection of hDC with virulent \( F. \) tularensis and not infection with a more attenuated strain, we examined the ability of LVS to induce IFN-\( \beta \) in hDC over time. In contrast to the virulent SchuS4 strain, LVS failed to induce transcription of IFN-\( \beta \) within 8 h of infection (Fig. 2B). Furthermore, induction of IFN-\( \beta \) was not detected at later time points when hDC harbored similar numbers of LVS compared with SchuS4 (e.g., 24 h postinfection). We also examined culture supernatants for the presence of IFN-\( \beta \) protein. Twelve and sixteen hours postinfection, SchuS4-infected hDC secreted significantly more IFN-\( \beta \) compared with LVS-infected hDC and uninfected controls (Fig. 2C, data not shown). IFRF7 is a gene whose expression is dependent on IFN-\( \beta \) (18). Thus, to determine whether the transcription and secretion of IFN-\( \beta \) had functional consequences, we assessed changes in gene transcription of IFRF7. SchuS4-infected hDC had significantly increased transcription of IFRF7 compared with LVS-infected cells (Supplemental Fig. 1). These data demonstrated that production of IFN-\( \beta \) in human cells is correlated with infection of cells with virulent, but not attenuated, \( F. \) tularensis.

Phagocytosis is required for SchuS4-mediated induction of IFN-\( \beta \)
We next set out to determine the cellular location and mechanism by which virulent \( F. \) tularensis strain SchuS4 induced IFN-\( \beta \). \( F. \) tularensis interacts with a number of receptors present on the surface of the host cell, including mannose receptor, CR3, CR4, CD14, and TLR2 (11, 16, 19). Recently, it was shown that viral ligands can induce IFN-\( \beta \) expression following binding to TLR2 (20). Thus, it was possible that \( F. \) tularensis initiated early expression of IFN-\( \beta \) following interaction with host surface receptors prior to phagocytosis of the bacterium. Using cytochalasin D, we examined what effect inhibition of phagocytosis of SchuS4 had on the induction of IFN-\( \beta \) gene expression. Consistent with previous reports in cell lines, inhibition of phagocytosis (using cytochalasin D) inhibited induction of IFN-\( \beta \) by \( E. \) coli LPS in hDC by 78.6% compared with untreated controls (Fig. 3A) (21). hDC treated with cytochalasin D expressed significantly less IFN-\( \beta \)-induced infection with SchuS4 (a 98.6% reduction) compared with untreated controls (Fig. 3A). Therefore, phagocytosis is required for induction of IFN-\( \beta \) following SchuS4 infection of hDC.

Endosomal acidification and viable bacteria are required for induction of IFN-\( \beta \)
\( F. \) tularensis is a facultative intracellular bacterium that transiently passes through a phagosome/endoosome before escaping into the host cytoplasm where it undergoes replication (22). Host endosomes and cytosol each possess receptors capable of recognizing pathogen-associated molecular patterns that initiate production of IFN-\( \beta \) (23). Because \( F. \) tularensis can reside in both of these cellular compartments, the signal to produce IFN-\( \beta \) could have been emanating from either location. Induction of type I IFN from the endosome often requires endosomal acidification (24, 25). Similarly, endosomal acidification contributes to efficient escape of \( F. \) tularensis into the host cytosol (17). Endosomal-acidification inhibitors, such as BAF, slow and/or block each of these processes. To determine whether induction of IFN-\( \beta \) gene expression by
SchuS4 occurred from the host endosome, we assessed induction of IFN-β in SchuS4-infected hDC that were incubated in BAF. The presence of BAF significantly inhibited the induction of IFN-β in SchuS4-infected hDC (Fig. 3B). It was reported that inhibition of endosomal acidification also interferes with the ability of F. tularensis to acquire iron and ultimately impairs intracellular replication (26, 27). Thus, the effect of BAF on induction of IFN-β could have been a result of inefficient iron acquisition by the bacteria. However, supplementation of BAF-treated hDC with exogenous iron did not restore the ability of SchuS4 to induce IFN-β gene expression. We next determined whether viable SchuS4 bacteria were necessary for the induction of IFN-β. In contrast to live bacteria, organisms fixed in PFA were not able to induce significant expression of IFN-β (Fig. 3C). Although it is possible that PFA fixation altered a structure on the surface of the bacterium required for induction of IFN-β, together these data suggested that endosomal acidification and viable bacteria are required for efficient induction of IFN-β.

Endosomal escape and early cytosolic replication are not sufficient for SchuS4 induction of IFN-β

As discussed above, although acidification of endosomes is a required step for induction of IFN-β via host receptor signaling from that compartment, endosomal acidification is also necessary for efficient escape of F. tularensis into the cytosol (17). A previous report using attenuated strains of F. tularensis in mouse cells showed that endosomal escape was required for induction of IFN-β (13). However, it was not determined whether the mere presence of the bacterium in the cytosol was sufficient for induction of IFN-β or whether replication of the bacterium in this compartment was also required to induce this host protein. To determine whether the presence and/or early replication of SchuS4 in the cytosol was sufficient for induction of IFN-β, we assessed changes in expression of the IFN-β gene in hDC following infection of the cells with an attenuated mutant strain of SchuS4 (SchuS4Δ0369c) that displays similar kinetics of endosomal escape and early replication as does wild-type SchuS4 (28). We first confirmed that the phagosomal escape and growth of SchuS4Δ0369c previously observed in mouse macrophages were similar in hDC. Similar numbers of wild-type and SchuS4Δ0369c were phagocytosed by hDC, and both strains escaped the endosome with comparable efficiency (Fig. 4A, 4B, Supplemental Fig. 2). Furthermore, as observed in mouse macrophages, SchuS4Δ0369c replicated in an analogous fashion to wild-type bacteria during the first 8 h of infection (Fig. 4A). Despite the similarities in uptake and early replication between wild-type SchuS4 and SchuS4Δ0369c, SchuS4Δ0369c induced significantly less IFN-β within 8 h of infection than did either wild-type SchuS4 or the complemented SchuS4Δ0369c mutant, SchuS4Δ0369c+p0369 (Fig. 4C). We also determined whether SchuS4Δ0369c induced IFN-β at later time points postinfection and analyzed for TNF-α, IL-6, and IL-12p40 by ELISA. hDC stimulated 24 h prior to harvest with ultrapure E. coli LPS (10 ng/ml) served as positive controls. *p < 0.01, compared with uninfected and SchuS4-infected hDC; **p < 0.001, compared with all samples. C, SchuS4- or mock-infected hDC cultures were stimulated 24 h postinfection with ultrapure E. coli LPS. Concentrations of TNF-α, IL-6, and IL-12p40 in culture supernatants were determined an additional 24 h after LPS treatment. *p < 0.01, compared with uninfected, LPS-treated samples. Error bars represent SEM. Each data point represents the mean of triplicate samples. Data in A are the mean of eight experiments; data in B and C are representative of three experiments of similar design.
points in infection. However, we did not observe induction of IFN-β by SchuS4Δ0369c over the course of 24 h at levels above that observed at 8 h postinfection (Supplemental Fig. 2). Together, these data demonstrated that endosomal escape and early (with-
IFN-β is not correlated with inflammasome activation following F. tularensis infection of hDC

Following infection with intracellular bacteria, one previously described function of IFN-β is to prime the host cell inflammasome (14, 15, 29, 30). Therefore, we next determined whether SchuS4 infection activated the inflammasome in primary hDC. The inflammasome can cleave pro–IL-1β into its active, mature form, allowing secretion of mature IL-1β into culture supernatant. In agreement with our previous report, SchuS4 failed to induce secretion of IL-1β from hDC (Fig. 5A) (16). LVS also failed to induce secretion of IL-1β among infected hDC (Fig. 5A). However, hDC primed with IFN-β, followed by stimulation with E. coli LPS, secreted IL-1β (Fig. 5A). Thus, the lack of IL-1β secretion among F. tularensis-infected cultures was not due to an inability of hDC to secrete this cytokine. The presence of pro–IL-1β in the intracellular compartment is required for generation of the cleaved, mature form of this protein. Because it was possible that the absence of mature IL-1β was due to an absence of pro–IL-1β in hDC, we next examined whether SchuS4 infection resulted in production of intracellular pro–IL-1β. Uninfected and SchuS4-infected hDC had similar levels of intracellular pro–IL-1β, whereas LVS-infected hDC had modestly more pro–IL-1β compared with uninfected and SchuS4-infected hDC (Fig. 5B). Stimulation of hDC with LPS resulted in increased production of pro–IL-1β. Similarly, hDC primed with IFN-β and stimulated with LPS also had high levels of pro–IL-1β (Fig. 5B). Therefore, the minimal induction of pro–IL-1β observed in LVS-infected cells was not due to a global defect in the ability of hDC to produce this protein.

Although SchuS4 infection failed to induce the production of IL-1β, it was still possible that the inflammasome was being activated. There are two additional methods to assess inflammasome activation: cleavage and secretion of IL-18 and activation of caspase-1. We first examined hDC culture supernatants for IL-18 and did not detect IL-18 in supernatants of LVS- or SchuS4-infected hDC (data not shown). We next determined whether F. tularensis infection resulted in cleavage of mature caspase-1 into its active form. Within the first 4 h of infection, cultures of both SchuS4- and LVS-infected hDC had significantly fewer cells positive for active caspase-1 compared with uninfected controls (Fig. 5C). Neither SchuS4 nor LVS induced activation of caspase-1 during the first 12 h of infection (Fig. 5C). Twenty-four hours postinfection, both LVS- and SchuS4-infected cultures had a small percentage of hDC positive for active caspase-1 compared with uninfected controls (Fig. 5C). However, the percentages of cells positive for activated caspase-1 in SchuS4- and LVS-infected

FIGURE 4. Endosomal escape and early cytosolic replication are not sufficient for SchuS4-mediated induction of IFN-β. hDC were infected with the indicated SchuS4 strains. A, Intracellular bacteria were enumerated at the indicated times postinfection. B, Cytoplasmic bacteria were identified 3 h postinfection using a phagosomal-integrity assay and were enumerated by microscopy. C, RNA was harvested 8 h postinfection for assessment of IFN-β transcript by qRT-PCR. IFN-β transcript levels were normalized to those from uninfected hDC. *p < 0.05, compared with all other samples. Error bars represent SEM. Each data point represents the mean of triplicate samples. A and C represent the mean of five independent experiments, and data in B are representative of three experiments of similar design.

FIGURE 5. IFN-β is not correlated with activation of the inflammasome following F. tularensis infection of hDC. A and B, hDC were mock infected or infected with the indicated strains of F. tularensis or treated with E. coli LPS with or without pretreatment with rhIFN-β. A, Forty-eight hours postinfection, supernatants were collected and analyzed for IL-1β by ELISA. *p < 0.0001, compared with all other samples. B, Eight hours postinfection, intracellular pro–IL-1β was detected in hDC lysates by Western blotting. Blots were stripped and reprobed with anti–β-actin to demonstrate equal loading. C, hDC were mock infected or infected with F. tularensis SchuS4 or LVS. At the indicated time points postinfection, the number of hDC positive for activated caspase-1 was detected by flow cytometry using Green FLICA Caspase-1 assay kit. *p < 0.05, compared with all other samples; **p < 0.01, compared with all other samples. Error bars represent SEM. Each data point represents the mean of triplicate samples. Data are representative of three experiments of similar design.
Intracellular bacteria were enumerated at 3 and 24 h postinfection. Infected with LVS. Twenty-four hours postinfection, supernatants were assessed for IL-12p40 by ELISA. Uninfected hDC served as negative controls. 0.01, compared with untreated, LVS-infected hDC.

Triplicate samples. Error bars represent SEM. Data are representative of three experiments of similar design. ns, not significant.

Infected or mock-infected hDC were treated with ultrapure C100%. Flow cytometry. Data were normalized by defining the percentage of cells in an uninfected culture of hDC that respond to LPS by production of cytokine as 100%. 100%.

Hampered the ability of these cells to produce IL-12p40 (Fig. 6A) (32, 33). In contrast to an earlier publication, we did not observe a significant difference in secretion of TNF-α in response to LPS between untreated hDC and those first exposed to rhIFN-β (Fig. 6A) (32). Therefore, in our culture setting, rhIFN-β selectively interfered with the ability of hDC to produce IL-12p40, but not TNF-α, in response to LPS. We next determined whether IFN-β produced in cultures of SchuS4-infected cells was capable of modulating production of IL-12p40 and/or TNF-α in hDC similarly to that observed with rhIFN-β. All neutralizing Abs tested had an off-target effect of reducing the amount of IL-12p40 and TNF-α produced by uninfected hDC in response to LPS. Therefore, to account for this unexpected effect, we normalized the data to uninfected hDC treated with LPS in the presence of either isotype control or neutralizing anti–IFN-β Abs. The number of cells positive for cytokine by intracellular staining, or the amount of cytokine secreted into culture medium, in these uninfected samples was designated as 100% responding cells or cytokine production. Addition of neutralizing Abs directed against IFN-β partially restored the ability of SchuS4-infected hDC cultures to produce IL-12p40 in response to LPS, as detected by an increase in the percentage of cells producing cytokine and cytokine detectable in culture supernatants (Fig. 6B, 6C). However, neutralization of IFN-β failed to restore the ability of SchuS4-infected hDC to increase TNF-α either intracellularly or in culture.

**FIGURE 6.** SchuS4-induced IFN-β selectively inhibits IL-12p40. A, hDC were treated with PBS (−) or the indicated concentration of rhIFN-β for 16 h prior to exposure to E. coli LPS. Supernatants were harvested 24 h later and examined for IL-12p40 and TNF-α by ELISA. *p < 0.01, compared with cells treated with LPS alone. B, hDC were infected with SchuS4 in the presence of neutralizing polyclonal anti–IFN-β Ab or polyclonal rabbit IgG (isotype) control. Sixteen hours later, infected or mock-infected hDC were treated with ultrapure E. coli LPS and assessed for intracellular IL-12p40 and TNF-α by flow cytometry. Data were normalized by defining the percentage of cells in an uninfected culture of hDC that respond to LPS by production of cytokine as 100%. C, hDC were infected with SchuS4 in the presence of neutralizing monoclonal anti–IFN-β Ab or mouse IgG1 (isotype) control. Sixteen hours later, infected or mock-infected hDC were treated with ultrapure E. coli LPS. Sixteen hours later, culture supernatants were assessed for IL-12p40 and TNF-α by ELISA. Data were normalized by defining the concentration of cytokine secretion in an uninfected culture of hDC that respond to LPS by production of cytokine as 100%. D, hDC were infected with the indicated SchuS4 strains. Uninfected hDC served as negative controls. Supernatants were harvested 48 h postinfection and analyzed for IL-12p40 and TNF-α by ELISA. *p < 0.05, compared with all other samples. E, hDC were treated with rhIFN-β and infected with LVS. Twenty-four hours postinfection, supernatants were assessed for IL-12p40 by ELISA. Uninfected hDC served as negative controls. *p < 0.01, compared with untreated, LVS-infected hDC. F, hDC were primed with rhIFN-β, followed by infection with the designated strains of F. tularensis. Intracellular bacteria were enumerated at 3 and 24 h postinfection. *p < 0.001, compared with all other samples. Each data point represents the mean of triplicate samples. Error bars represent SEM. Data are representative of three experiments of similar design. ns, not significant.
supernatants in response to *E. coli* LPS (Fig. 6B, 6C). Interestingly, neutralization of IFN-β had no effect on production of IL-12p40 or TNF-α in SchuS4-infected cells that were not exposed to *E. coli* LPS (Fig. 6B, 6C). We next assessed the ability of a SchuS4 mutant that did not induce IFN-β in hDC to evoke production of IL-12p40 and TNF-α. Because SchuS4Δ369c failed to induce IFN-β in hDC, we hypothesized that this strain may induce IL-12p40 following infection of these host cells. Indeed, SchuS4Δ369c induced secretion of a small, but significantly higher, amount of IL-12p40 compared with uninfected and SchuS4-infected hDC, whereas neither strain induced secretion of TNF-α at levels that were significantly different from uninfected controls (Fig. 6D). Thus, one function of SchuS4-induced IFN-β in hDC is to selectively suppress production of IL-12p40.

We next determined whether the addition of rhIFN-β would inhibit LVS-induced secretion of IL-12p40 in hDC. hDC infected with LVS and exposed to rhIFN-β secreted significantly less IL-12p40 compared with untreated LVS-infected hDC controls (Fig. 6E). However, it was reported that treatment of cells with IFN-β controls replication of LVS (13). Thus, the reduction of IL-12p40 secretion could be due to killing of bacteria in hDC cultures. Indeed, in agreement with reported observations in mouse cells, hDC exposed to rhIFN-β readily controlled replication of LVS (Fig. 6F). In contrast, a similar control of SchuS4 replication in hDC treated with rhIFN-β was not observed (Fig. 6F). Therefore, the ability of rhIFN-β to interfere with hDC production of IL-12p40 is not restricted to infection with specific *Francisella* species. However, the mechanism by which rhIFN-β mediates interference with the production of IL-12p40 in LVS- and SchuS4-infected hDC may be different.

**Discussion**

Tularemia is a disease that can be divided into phases. The early, critical stages of infection are marked by a striking absence of inflammatory responses, despite exponentially replicating bacteria (36). Further, virulent *F. tularensis* evades early detection in the host and actively suppresses inflammation within the first few days of disease (4). During the last phase of infection, the host rapidly transitions from a quiescent inflammatory response to sepsis paired with massive inflammation and cell death (36). In contrast to these in vivo observations, *F. tularensis* was shown to both suppress and exacerbate inflammatory responses among cells infected in vitro (6, 37–39). Generation of these contradictory outcomes seems to depend on the subspecies and strain of *F. tularensis* used, the cell type analyzed, the species from which the cell was derived, and the activation status of the cell. Given the dichotomy of host responses during different stages of tularemia and the cell types targeted by the bacterium during these stages, the success of novel therapeutics to treat this disease may greatly depend on the phase of infection and type of cell harboring bacteria. Thus, it is critical to clarify and understand virulence mechanisms used by *F. tularensis* to cause disease in the context of the cell type at different stages of infection.

Resting, immature DC serve as sentinels of the immune system, capable of activating both innate and adaptive immune responses (40, 41). Thus, successful modulation of this cell population by virulent microbes can be an essential component in mediating microbial pathogenesis. Previous reports demonstrated that pulmonary DC and alveolar macrophages represent the primary, initial targets of *F. tularensis* during lung infections (42, 43). Identification of how *F. tularensis* suppresses the function of these cells may lead to important insights into the pathogenesis of tularemia and other pulmonary pathogens.

In this report, we provided evidence of at least one mechanism of virulence used by virulent SchuS4, but not attenuated LVS, following infection of human cells. We confirmed that one disparity between LVS and SchuS4 is their ability to induce inflammatory responses in hDC and defined one mechanism by which virulent *F. tularensis* suppresses the function of immature hDC. Specifically, we found that induction of IFN-β by SchuS4 played a central role in the early suppression of a critical aspect of hDC activation. We also observed that induction and secretion of IFN-β were restricted to infection of hDC with virulent *F. tularensis* strain SchuS4. We extended these observations by demonstrating that internalization of viable SchuS4 and endosomal acidification were required for induction of IFN-β. Surprisingly, induction of IFN-β was not associated with propagation of host inflammatory responses during SchuS4 infection. IFN-β was not correlated with secretion of IL-1β in *F. tularensis*-infected hDC nor was it correlated with strong activation of caspase-1 in hDC infected with *F. tularensis*. Furthermore, unlike IFN-β–primed hDC infected with LVS, treatment of hDC with rIFN-β failed to restrict replication of SchuS4. Finally, rather than acting as a signal for inflammation, SchuS4-induced IFN-β suppressed production of IL-12p40 in hDC.

We first confirmed and extended previous observations that described differences in the ability of LVS and SchuS4 to induce secretion of inflammatory cytokines in hDC. In agreement with published data examining secretion of proinflammatory cytokines in hDC by serum-opsonized LVS, we observed consistent production of proinflammatory cytokines following infection of hDC with attenuated, nonopsonized LVS (11). The production of proinflammatory cytokines following LVS infection of hDC correlated well with the fact that LVS is an attenuated vaccine strain in humans. Indeed, in the setting of vaccination, a modest inflammatory response would serve to aid in controlling growth of the vaccine strain, as well as to promote development of effective adaptive immunity. In contrast to LVS, we observed that virulent SchuS4 failed to induce secretion of proinflammatory cytokines and actively suppressed responsiveness of infected cultures to other stimuli (Fig. 1). Together, these data supported the hypothesis that one primary mechanism of virulence used by fully virulent strains of *F. tularensis* in humans is the evasion and inhibition of inflammation.

In contrast to this study, we previously reported that SchuS4 failed to induce IFN-β in hDC 24, 48, and 72 h postinfection (6). However, at that time, we did not appreciate the extreme potency of this protein or the sensitivity of this cytokine to denaturation. Human IFN-β can exert antiviral activity when present in quantities as small as 5 U/ml, which, depending on the source of IFN-β, represents as little as 20 pg/ml of human IFN-β (44). This concentration is well below the level of detection of most ELISA kits designed to detect free IFN-β. Additionally, the data presented in this article suggested that increased gene expression of IFN-β occurs very early postinfection with SchuS4 (i.e., within 8–12 h). Thus, it is possible that our failure to detect IFN-β in cultures of SchuS4-infected hDC in our earlier report was due to both poor sensitivity of human IFN-β ELISAs and the time point at which we examined culture supernatants for IFN-β. Recently, an ELISA with vastly improved sensitivity for human IFN-β was developed. Indeed, when we used this highly sensitive ELISA for detection of IFN-β at earlier time points postinfection (e.g., 12 h), we routinely detected small, but significant, amounts of IFN-β in culture supernatants of SchuS4-infected hDC (Fig. 2C).

To elucidate whether there was a contribution of IFN-β in our system, we neutralized the activity of this cytokine using polyclonal and monoclonal neutralizing Abs directed against this
protein. We found that neutralization of IFN-β partially restored the ability of hDC infected with SchuS4 to produce IL-12p40, but not TNF-α, in response to inflammatory stimuli (Fig. 6B, 6C). Addition of polyclonal Abs or mAbs directed against human IFN-β partially restored the responsiveness of SchuS4-infected hDC to LPS and had no effect on the induction of IL-12p40 in response to SchuS4 alone. The lack of complete restoration of the IL-12p40 response may be attributable to additional, undefined mechanisms by which SchuS4 interferes with host cell cytokine production. Similarly, the absence of IL-12p40 production in response to SchuS4 alone in the presence of neutralizing anti–IFN-β Abs may also suggest that there are multiple mechanisms by which SchuS4 modulates production of IL-12p40. Alternatively, the absence of IL-12p40 in SchuS4-infected hDC may also be attributable to the fact that wild-type SchuS4 may not possess ligands that are capable of inducing inflammatory responses on their own. For example, SchuS4 does not seem to effectively stimulate proinflammatory responses in hDC upon engagement of the host cell in the absence of specific coreceptors or throughout the infection (Fig. 1B, 1C) (6, 16, 45). Additionally, SchuS4 does not provoke production of inflammatory cytokines in mice during the first 3 d of infection (4). Regardless of the inability of SchuS4 to provoke an inflammatory response on its own, our data clearly showed that, in the context of SchuS4 infection of hDC, IFN-β acts as an anti-inflammatory cytokine by selectively targeting production of IL-12p40.

Initially, this anti-inflammatory role for IFN-β in F. tularensis infections was unexpected. Several recent reports showed that activation of the host inflammasome by attenuated strains of F. tularensis, as indicated by release of IL-1β and cell death, was dependent on production of IFN-β following infection of mouse macrophages with attenuated F. tularensis (14, 15, 29). Importantly, IFN-β–dependent activation of the inflammasome in these studies was directly correlated with control of bacterial replication. Therefore, it was proposed that IFN-β might represent an attractive therapeutic for treatment of pneumatic tularemia (21). Alternatively, in other settings, type I IFNs acted as a potent anti-inflammatory in human cells capable of suppressing the production of cytokines, such as IL-12p40 (46, 47). Given these important and contrasting implications, we examined the possibility that SchuS4–mediated IFN-β might be facilitating activation of the inflammasome and the suppression of IL-12p40 in hDC.

To determine whether IFN-β induced during SchuS4 infection contributed to activation of the inflammasome, we examined secretion of mature IL-1β, as well as the presence of active caspase-1. We did not detect mature IL-1β in supernatants of hDC infected with either SchuS4 or LVS, which suggested that the inflammasome was not active in these cells. However, we observed minimal amounts of pro–IL-1β in cell lysates of LVS-infected hDC. Because pro–IL-1β is required to generate mature, cleaved IL-1β, and we failed to detect large quantities of intracellular pro–IL-1β, we could not use secretion of this cytokine as a reliable read-out for inflammasome activation. Thus, we directly assessed activation of caspase-1 in hDC. Using this technique, we found that activated caspase-1 was present in minimal numbers of cells and only at late time points in infection among LVS- and SchuS4–infected cultures. However, because LVS failed to induce IFN-β in hDC, the modest induction of active caspase-1 in hDC infected with F. tularensis was not dependent on IFN-β.

As discussed above, other laboratories demonstrated a protective role for IFN-β in which the addition of rhIFN-β resulted in control of LVS replication in mouse cells (13). Thus, we tested whether addition of rhIFN-β had an effect on replication of LVS and SchuS4 in hDC. Similar to previous studies in mouse cells, addition of rhIFN-β to hDC cultures resulted in control of LVS replication. However, a similar effect of IFN-β on the replication of SchuS4 in hDC was not observed. There are a number of possibilities that might explain why IFN-β was unable to control SchuS4 infection. First, IFN-β has been tied to induction of the inflammasome, resulting in both secretion of IL-1β and cell death (48). Either of these inflammasome-mediated activities may aid in the control of bacterial replication. Thus, it is possible that following treatment with rhIFN-β, SchuS4 either failed to activate the inflammasome or that the bacterium interfered with inflammasome activity. A second possibility for the inability of IFN-β to contribute to control of SchuS4 may lie in suboptimal activation of reactive oxygen and reactive nitrogen species. We previously demonstrated that both reactive oxygen and reactive nitrogen are required for control of SchuS4 in human cells (49). Although IFN-β can activate these pathways, optimal induction of the oxidative burst can be dependent on the presence of IFN-β in combination with other proinflammatory cytokines (50). Unlike LVS, SchuS4 does not induce secretion of these proinflammatory cytokines following infection of hDC (Fig. 1). Therefore, it is possible that IFN-β failed to optimally activate specific antimicrobial pathways in hDC that contribute to control of bacterial replication. The specific mechanism by which SchuS4 evades IFN-β–mediated control of bacterial replication is being examined by our laboratory.

In the present study, we used a combination of chemical compounds and SchuS4 mutants to explore the mechanism by which SchuS4 induced IFN-β. Using these approaches, we demonstrated that induction of IFN-β did not occur following engagement of the bacteria with receptors at the host cell surface. Rather, active phagocytosis of SchuS4, followed by endosomal acidification, was required for induction of IFN-β. F. tularensis briefly transits through a host endosome before escaping into the cytosol where the bacterium undergoes replication. It was suggested that escape of attenuated F. tularensis into the cytosol is sufficient for induction of IFN-β (21). However, experiments conducted with a defined mutant of SchuS4 (SchuS4ΔO369c), which displays similar kinetics for endosomal escape and early replication, clearly demonstrated that endosomal escape and replication of the bacterium during the first 8 h of infection were not sufficient to induce IFN-β in human cells (Fig. 4).

FTT0369c is a protein unique to F. tularensis species and is required for virulence of SchuS4 both in vitro and in vivo (28). The specific function of FTT0369c in F. tularensis physiology has not been identified. A homolog of FTT0369c is present in LVS and is designated FTL1306. Given the dramatic difference in the ability of SchuS4 and LVS to induce IFN-β in hDC and the apparent contribution of FTT0369c toward induction of this cytokine, it was initially surprising that LVS failed to provoke IFN-β in hDC. However, comparison of FTT0369c and FTL1306 sequences revealed 4 aa differences between these two proteins. Thus, a possible explanation for the difference between the ability of SchuS4 and LVS to induce IFN-β in hDC is that these amino acid substitutions lie in areas that are important for the structure and, by extension, specific function of FTL1306 in LVS.

Additionally, FTT0369c may act to regulate expression of other genes essential for virulence in SchuS4. In LVS, FTL1306 contributes to the expression of RipA (T. Kawula, personal communication). Similar to FTT0369c in SchuS4, RipA in LVS was required for both intracellular replication and suppression of proinflammatory responses in mouse macrophages (51, 52). SchuS4 possesses a homolog of RipA, but neither the function of this protein nor the contribution that FTT0369c makes toward its expression has been thoroughly explored. Thus, it is possible that
the failure of SchuS4A0369c to induce IFN-β and suppress IL-12p40 may not be attributed solely to the absence of FTT0369c but rather is an indirect effect via the downregulation of RipA. Finally, because the sequence of FT1L306 is conserved among *F. tularensis* spp. *holarctica*, it is possible that the contribution that this protein makes toward induction of IFN-β reflects both the heightened attenuation of LVS as a vaccine strain and the moderate virulence observed in the *holarctica* subspecies (8).

Type I IFNs, and specifically IFN-β, are cytokines with pleiotropic activity. Type I IFN can enhance antiviral immunity and promote strong inflammatory responses. This inflammation can lead to both resolution and exacerbation of infection. Conversely, IFN-β has been strongly associated with suppressing inflammatory responses in humans. For example, the anti-inflammatory action of IFN-β is believed to be a critical element in the resolution of inflammation in multiple sclerosis and lupus (53, 54). Data from this study showed that, in the context of infection with virulent *F. tularensis* in resting hDC, IFN-β acts as an anti-inflammatory cytokine to suppress IL-12p40 production. IL-12p40 is essential for control of in vivo replication of attenuated strains of *F. tularensis* (55). Furthermore, recent work in our laboratory demonstrated an absolute requirement for IL-12p40 in the survival of intranasal SchuS4 infection (C.M. Bosio, unpublished observations).

Therefore, the ability of SchuS4-induced IFN-β to negatively modulate production of IL-12p40 brings to light an important mechanism of virulence used by these bacteria. Further characterization of the specific host and bacterial components that participate in IFN-β–mediated suppression following *F. tularensis* infection will provide critical information for development of novel vaccines and therapeutics directed against this pathogen, as well as contribute to the understanding of how successful, highly virulent, intracellular bacteria modulate human cells to cause lethal disease.

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