The Journal of Immunology

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J Immunol 2011; 187:1845-1855; Prepublished online 13 July 2011;
doi: 10.4049/jimmunol.1100377
http://www.jimmunol.org/content/187/4/1845

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

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

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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. ***The Journal of Immunology*****, 2011, 187: 1845–1855.**

Modulation of the host immune response is a common strategy used by microorganisms to successfully colonize, replicate, and spread from host to host. Many pathogens have been noted to manipulate the host immune system by either exacerbating or suppressing innate and adaptive immune responses. For example, *Pseudomonas aeruginosa* and *Salmonella* species cause severe inflammatory responses as part of their pathogenesis (1, 2). In contrast, Ebola virus, *Brucella abortus*, and *Francisella tularensis* dampen early inflammatory responses, allowing these pathogens to replicate in an unrestricted fashion, culminating in a lethal infection (3–5). In the case of infections with virulent *F. tularensis*, there is evidence that this organism interferes with innate immunity, as well as adaptive responses, as evidenced by the lack of protection from reinfection observed in humans who survived primary type A infection (4, 6, 7). Thus, suppression of the host immune response is a key component of pathogenesis, and dissecting the mechanism by which pathogens accomplish this suppression is essential for the development of novel vaccines and therapeutics.

F. tularensis is a Gram-negative, facultative intracellular bacterium and is the causative agent of tularemia. There are four subspecies of *F. tularensis* that are genetically similar but display remarkable differences in virulence in the human host (reviewed in Ref. 8). *F. tularensis* ssp. *mediasiatica* and *novicida* are attenuated in humans, whereas subspecies *holarctica* (type B) typically causes a mild disease. Further attenuation of a type B strain resulted in live vaccine strain (LVS), which was briefly used in the United States as a vaccine for tularemia (9). In contrast, *F. tularensis* ssp. *tularensis* (type A) is highly virulent in humans, as evidenced by its ability to cause severe lethal disease following exposure to ≤15 bacteria (10). It is important to note that although subspecies *novicida* and *holarctica* and strain LVS are all relatively attenuated in humans, they have retained virulence for laboratory mice and can be used under Biosafety Level 2 laboratory conditions. However, because these strains lack virulence in humans, it is not clear whether the mechanisms of pathogenicity observed in the mouse model will hold true for humans.

One potential difference between attenuated and virulent strains of *F. tularensis* is their ability to induce inflammatory responses in human cells. It was shown that, following opsonization with normal serum, attenuated strains of *F. tularensis* induced secretion of proinflammatory cytokines, whereas virulent *F. tularensis* SchuS4 failed to provoke similar responses in human cells (6, 11, 12). The goal of our study was to identify host components activated by virulent *F. tularensis* SchuS4, but not attenuated LVS, that contribute to the interference of inflammatory responses following infection of human dendritic cells (hDC).

We demonstrated in this study that there are important differences in the interaction of attenuated LVS and virulent *F. tularensis* strain SchuS4 with hDC. Specifically, we showed that only SchuS4 induced IFN-β following infection of hDC. However, in contrast to previous observations in mouse cells, induction of IFN-β was not associated with propagation of proinflammatory responses and was not correlated with activation of the host inflammasome in resting hDC (13–15). Further, unlike cells infected with LVS, preexposure to IFN-β did not enable hDC to control SchuS4 replication. Finally, we demonstrated that IFN-β induced by SchuS4 infection of hDC acts as an anti-inflammatory cytokine via selective suppression of IL-12p40 in these important host cells.

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Received for publication February 4, 2011. Accepted for publication June 4, 2011.

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

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The online version of this article contains supplemental material.

Abbreviations used in this article: BAF, bafilomycin; cRPMI, complete RPMI; DC, dendritic cell; hDC, human dendritic cell; LVS, live vaccine strain; PFA, paraformaldehyde; qRT-PCR, quantitative RT-PCR; rh, recombinant human.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1100377

Materials and Methods

Generation of human monocyte-derived dendritic cells

Human monocyte-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 rhIL-4 (both from PeproTech, Rocky Hill, NJ). On day 3 of culture, cells were replenished with 100 ng/ml rhGM-CSF and 20 ng/ml rhIL-4. All cells were used on day 4 of culture. As indicated, some hDC were pretreated with 1000 U/ml rhIFN-β (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, SchuS4Δ0369c, and SchuS4Δ0369c+p0369 were provided by Dr. Jean Celli (Rocky Mountain Laboratories, Hamilton, MT). *F. tularensis* strain SchuS4 was provided by Dr. Jeannine 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 × 10^7/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 7% CO_2 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 × 10^5 cells/ml in reserved DC medium, and plated at 1 or 0.5 ml/well in 24- or 48-well tissue culture plates, respectively. Intracellular bacteria were enumerated following lysing of hDC with H_2O and plating serial dilutions of cell lysate onto modified Mueller–Hinton broth agar plates. Agar plates were incubated at 37°C/7% CO_2 for 48 h, and individual colonies were enumerated.

Where indicated, cells were treated with rhIFN-β (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-β Ab, 3 μg/ml monoclonal mouse anti–IFN-β Ab (both from PBL IFN Source), or normal rabbit IgG or mouse IgG1 (R&D Systems) as a nonspecific Ab controls, 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 supernatants was quantified using commercially available ELISA, according to the manufacturer's instructions (R&D Systems, Minneapolis, MN). The presence of IFN-β in culture supernatants was quantified using the Verikine 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 4–12% 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 (Thermofisher, 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-human IFN-β Abs (PBL IFN Source), the percentage of cytokine secretion 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% CO_2 for 5 h. After incubation, cells were washed, fixed in 2% PFA, permeabilized with 0.25% saponin, and incubated with anti-human IL-12/IL-23p40/70 (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 resuspended in FACS buffer (eBioscience). Cells were acquired using a CyFlow ML flow cytometer (Partec, Swedesboro, 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 neutralizing 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 RT^2 qPCR-Grade RNA Isolation Kit; cDNA was generated using an RT^2 First Strand Kit and quantified using RT^2 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 purified 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 Biosystems). Input RNA was normalized to GAPDH, and fold change in the indicated genes was quantified 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. 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 7× working stock of Green FLICA reagent was added to each well. Cells were incubated at 37°C/7% CO_2 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 cytometer (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, 2 mM $MgCl_2$ [pH 7.3]), and the plasma membrane was selectively 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 permeabilized in 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-*Francisella* 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-α, 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. 1*A*). 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. 1*A*). In agreement with our and others' previous observations, SchuS4 failed to stimulate secretion of TNF-α, 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. 1*B*) (6). Significantly more TNF-α 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. 1*B*). 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-α, 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-α, IL-6, and IL-12p40 compared with uninfected cells in response to *E. coli* LPS (Fig. 1*C*) (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-β 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-α and IL-1β (Fig. 2*A*). Furthermore, SchuS4 also failed to induce expression of genes traditionally associated with suppression of host responses, such as IL-10 and TOLLIP (Fig. 2*A*). In contrast, SchuS4 infection of hDC resulted in increased expression of IFN-β within 8 h of infection (Fig. 2*A*). SchuS4 failed to induce expression of IFN-α and IFN-γ. Therefore, induction of IFN-β was not due to general activation of types I and II IFN genes (Fig. 2*A*). The initiation of IFN-β 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-β 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-β in hDC over time. In contrast to the virulent SchuS4 strain, LVS failed to induce transcription of IFN-β within 8 h of infection (Fig. 2*B*). Furthermore, induction of IFN-β 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-β protein. Twelve and sixteen hours postinfection, SchuS4-infected hDC secreted significantly more IFN-β compared with LVS-infected hDC and uninfected controls (Fig. 2*C*, data not shown). IRF7 is a gene whose expression is dependent on IFN-β (18). Thus, to determine whether the transcription and secretion of IFN-β had functional consequences, we assessed changes in gene transcription of IRF7. SchuS4-infected hDC had significantly increased transcription of IRF7 compared with LVS-infected cells (Supplemental Fig. 1). These data demonstrated that production of IFN-β 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-β

We next set out to determine the cellular location and mechanism by which virulent *F. tularensis* strain SchuS4 induced IFN-β. *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-β expression following binding to TLR2 (20). Thus, it was possible that *F. tularensis* initiated early expression of IFN-β 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-β gene expression. Consistent with previous reports in cell lines, inhibition of phagocytosis (using cytochalasin D) inhibited induction of IFN-β by *E. coli* LPS in hDC by 78.6% compared with untreated controls (Fig. 3*A*) (21). hDC treated with cytochalasin D expressed significantly less IFN-β following infection with SchuS4 (a 98.6% reduction) compared with untreated controls (Fig. 3*A*). Therefore, phagocytosis is required for induction of IFN-β following SchuS4 infection of hDC.

Endosomal acidification and viable bacteria are required for induction of IFN-β

F. tularensis is a facultative intracellular bacterium that transiently passes through a phagosome/endosome 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-β (23). Because *F. tularensis* can reside in both of these cellular compartments, the signal to produce IFN-β 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-β gene expression by

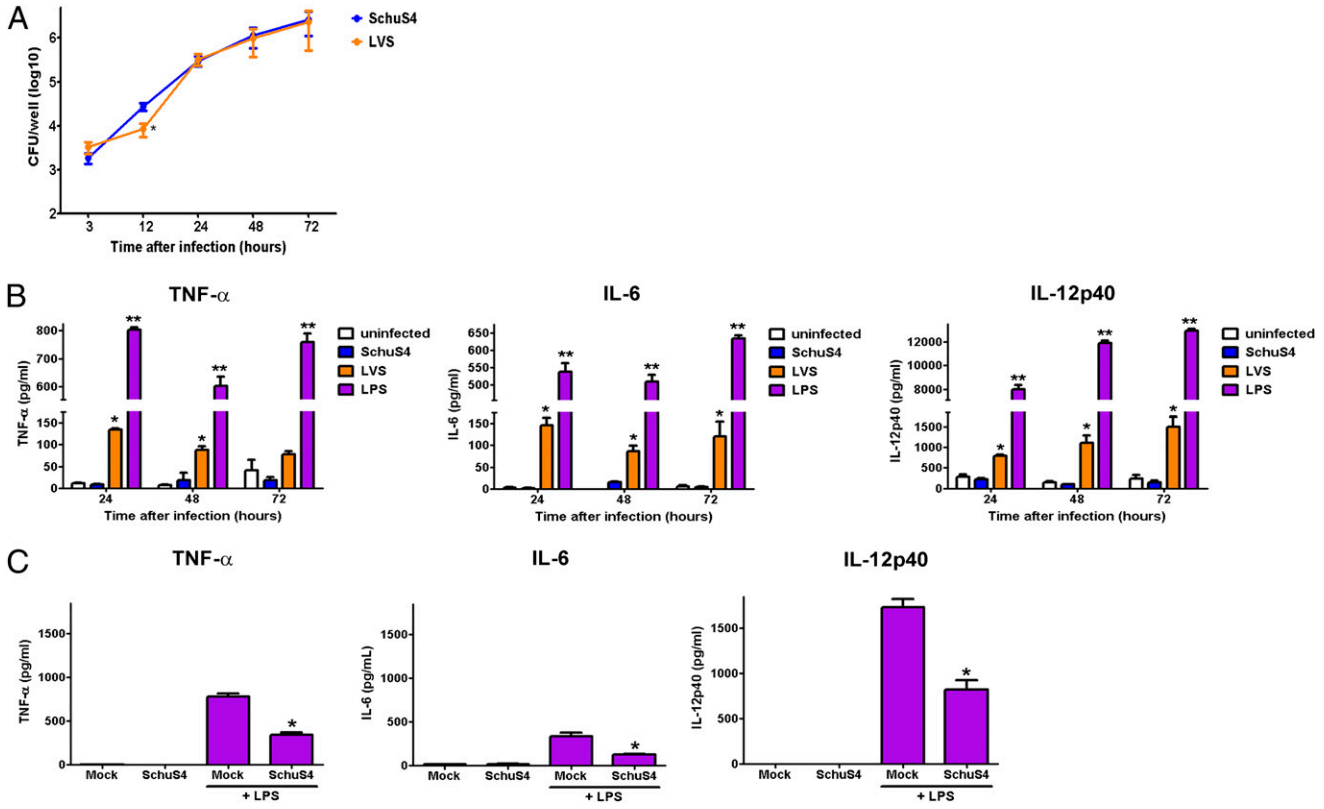

FIGURE 1. Differential induction of proinflammatory cytokines by attenuated and virulent strains of *F. tularensis*. Primary hDC were infected at a multiplicity of infection of 50 with the indicated strains of *F. tularensis. A*, Intracellular bacteria were enumerated at the indicated times postinfection. *$p < 0.01$, compared with SchuS4-infected hDC. *B*, Supernatants were harvested from uninfected or *F. tularensis*-infected cultures at the indicated 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.

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. 3*B*). 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 (data not shown). Therefore, it is unlikely that a lack of iron significantly contributed to the inability of BAF-treated SchuS4-infected hDC 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. 3*C*). 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. 4*A*, 4*B*, 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. 4*A*). 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. 4*C*). We also determined whether SchuS4Δ0369c induced IFN-β at later time

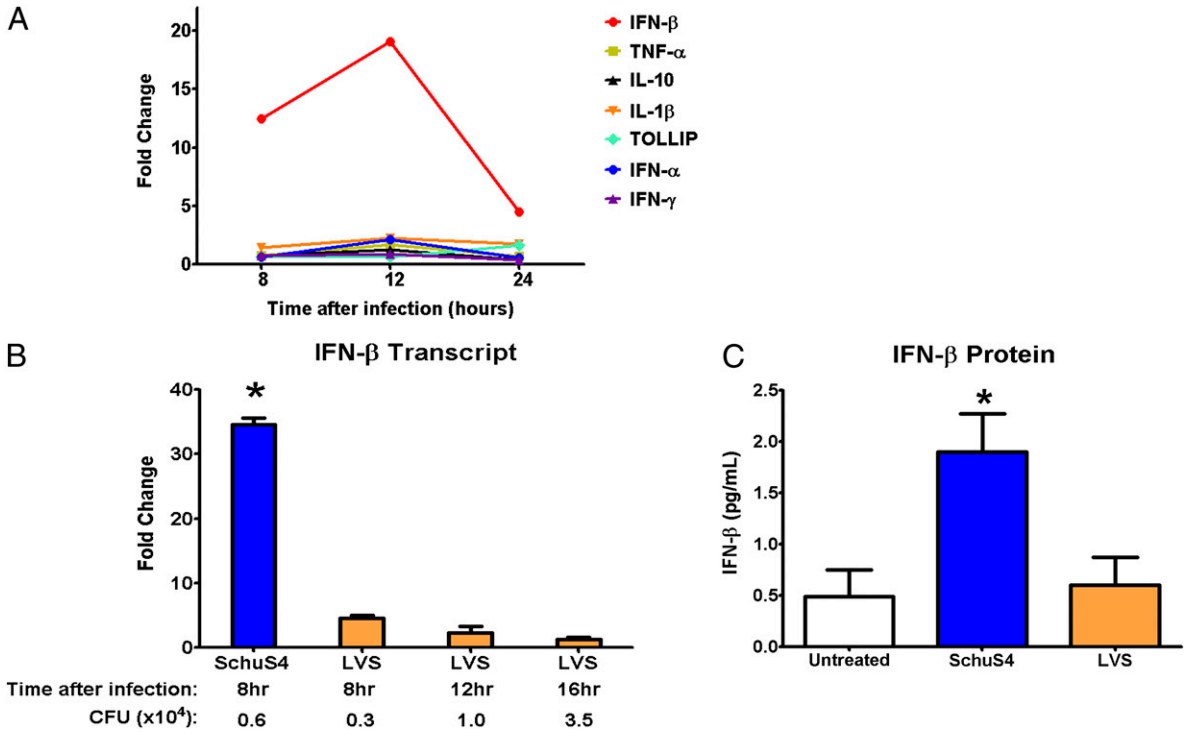

FIGURE 2. Induction of IFN-β by virulent *F. tularensis*. *A*, hDC were infected with SchuS4. RNA was harvested at the indicated time points postinfection for analysis of host genes by qRT-PCR. The fold change of TNF-α, IL-1β, IL-10, TOLLIP, IFN-α, IFN-β, and IFN-γ, normalized to uninfected samples, are depicted. *B*, RNA from hDC infected with *F. tularensis* strain SchuS4 or LVS was harvested at the indicated time points postinfection for analysis of IFN-β by qRT-PCR. IFN-β transcript levels were normalized to those from uninfected hDC. The intracellular bacterial loads at the indicated time points are shown. $*p < 0.01$, compared with LVS-infected cells at each time point. *C*, hDC were infected with SchuS4 or LVS, and culture supernatants were assessed for IFN-β by ELISA 12 h postinfection. Uninfected hDC served as negative controls. $*p < 0.05$, compared with LVS and uninfected samples. Error bars represent SEM. Each data point represents the mean of triplicate samples. Data 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 (within 8 h) replication are not sufficient for induction of IFN-β. Furthermore, the presence of viable, but nonreplicating, SchuS4Δ0369c are not sufficient to induce IFN-β at later time points in hDC.

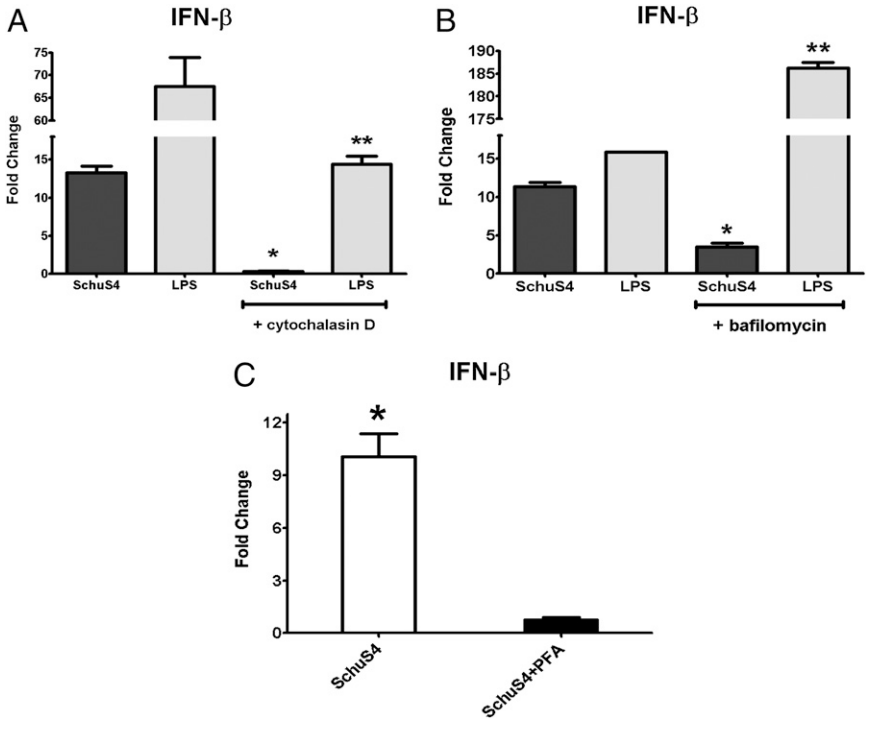

FIGURE 3. Requirements for SchuS4-mediated IFN-β production in hDC. One hour prior to infection, hDC were treated with cytochalasin D to inhibit phagocytosis (*A*) or BAF A to inhibit endosomal acidification (*B*). Eight hours postinfection, RNA was extracted for analysis by qRT-PCR. RNA collected from hDC treated with LPS served as a positive control. IFN-β transcript levels were normalized to those from mock-infected cells. $*p < 0.01$, compared with untreated, SchuS4-infected hDC; $**p < 0.01$, compared with hDC treated with LPS in the absence of inhibitors. *C*, hDC were exposed to SchuS4 that had been killed with 2% PFA (SchuS4+PFA). Eight hours postinfection, RNA was extracted for analysis of IFN-β transcripts by qRT-PCR. $*p < 0.01$, compared with PFA-killed SchuS4. Error bars represent SEM. Each data point represents the mean of triplicate samples. Data are representative of three experiments of similar design.

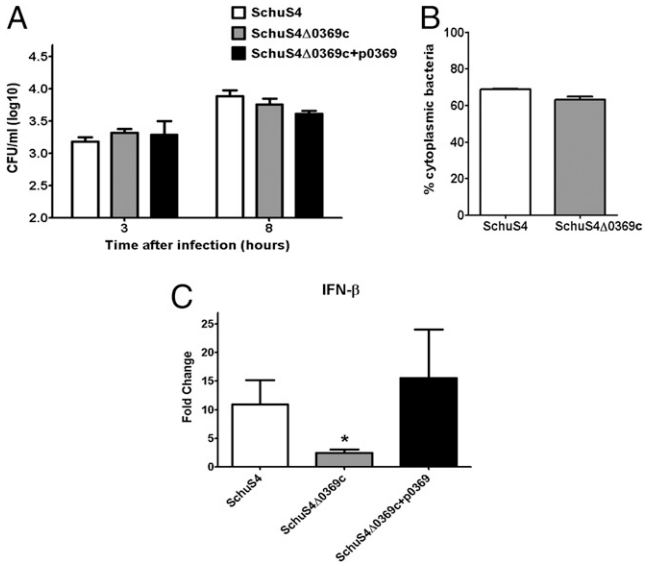

FIGURE 4. Endosomal escape and early cytosolic replication are not sufficient for SchuS4-mediated induction of IFN-β. hDC were infected with the indicated SchuS4 strains. *A*, Intracellular bacteria were enumerated at the indicated times points postinfection. *B*, Cytoplasmic bacteria were identified 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.

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. 5*A*) (16). LVS also failed to induce secretion of IL-1β among infected hDC (Fig. 5*A*). However, hDC primed with IFN-β, followed by stimulation with *E. coli* LPS, secreted IL-1β (Fig. 5*A*). 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. 5*B*). 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. 5*B*). 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

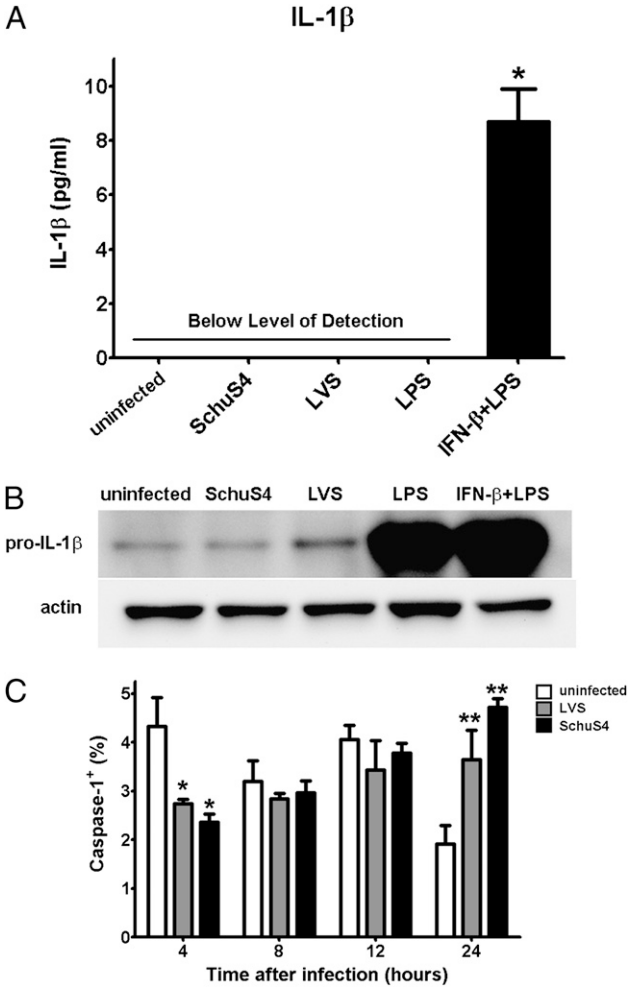

FIGURE 5. IFN-β is not correlated with activation of the inflammasome following *F. tularensis* infection of hDC. *A* and *B*, hDC were mock infected or infected with the indicated strains of *F. tularensis* or treated with *E. coli* LPS with or without pretreatment with rhIFN-β. *A*, Forty-eight hours 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.

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 over time 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. 5*C*). Neither SchuS4 nor LVS induced activation of caspase-1 during the first 12 h of infection (Fig. 5*C*). 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. 5*C*). However, the percentages of cells positive for activated caspase-1 in SchuS4- and LVS-infected

cultures were not significantly different from each other. Similarly, analysis for cleaved caspase-1 by Western blot did not reveal an increased presence of cleaved caspase-1 in LVS- or SchuS4-infected hDC compared with uninfected controls (data not shown). Thus, production of IFN-β did not correlate with strong activation of the inflammasome in *F. tularensis*-infected hDC.

SchuS4-induced IFN-β selectively inhibits IL-12p40

IFN-β is a pleiotropic cytokine that can provoke both pro- and anti-inflammatory responses (31). In humans and hDC, IFN-β can inhibit production of IL-12p40 and TNF-α (32, 33). In our previous publication, we observed that control of replication of SchuS4 in the extracellular space inhibited suppression of TNF-α but not IL-12p40 (6). This suggested that inhibition of production of these two cytokines by SchuS4 may occur through different mechanisms. Thus, it is possible that IFN-β induced during SchuS4 infection may suppress induction of IL-12p40 and/or TNF-α. To assess this, we first determined whether rhIFN-β could inhibit production of IL-12p40 and TNF-α by hDC in response to LPS. In agreement with previous publications, we found that exposure of hDC to as little as 10 U of rhIFN-β significantly hampered the ability of these cells to produce IL-12p40 (Fig. 6*A*) (32, 34, 35). 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. 6*A*) (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. 6*B*, 6*C*). However, neutralization of IFN-β failed to restore the ability of SchuS4-infected hDC to increase TNF-α either intracellularly or in culture

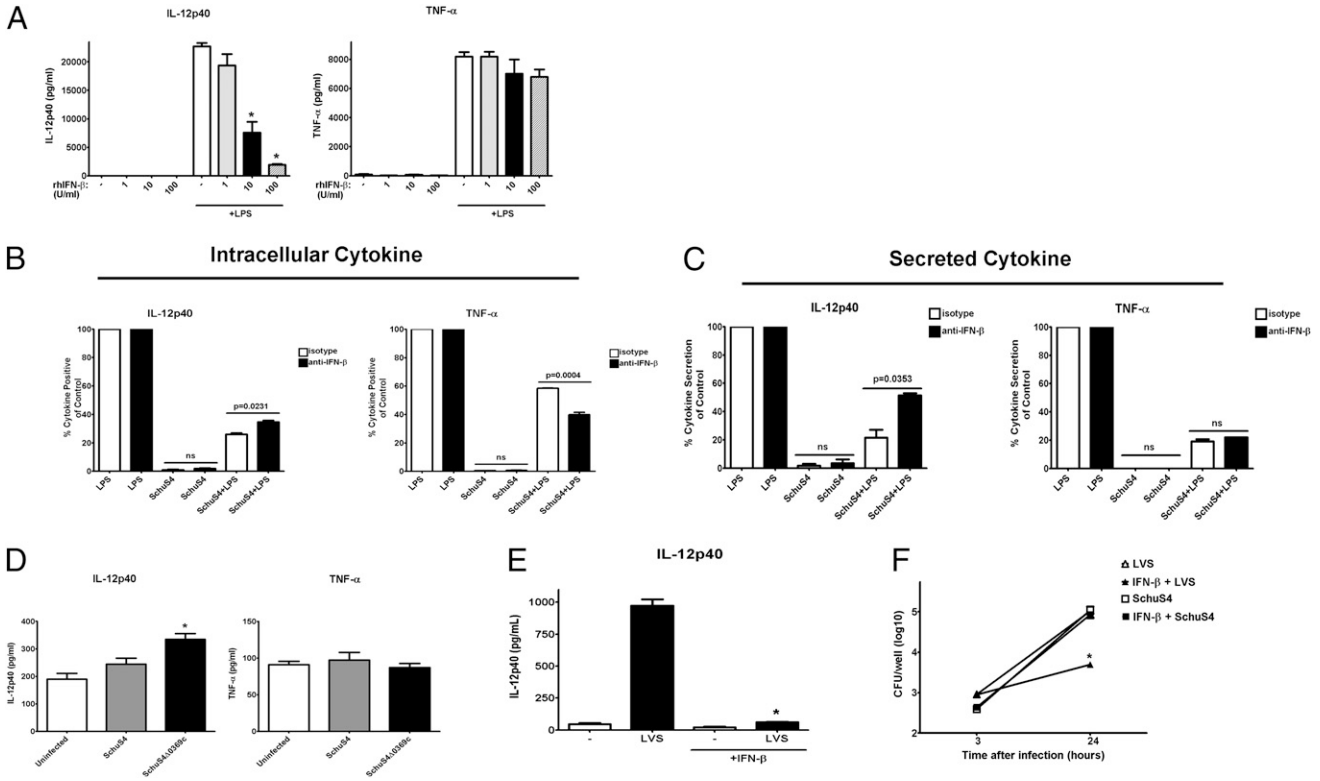

FIGURE 6. SchuS4-induced IFN-β selectively inhibits IL-12p40. *A*, hDC were treated with PBS (−) or the indicated concentration of rhIFN-β for 16 h prior to exposure to *E. coli* LPS. Supernatants were harvested 24 h later and examined for IL-12p40 and TNF-α by ELISA. $*p < 0.01$, compared with cells 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. 6*B*, 6*C*). 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. 6*B*, 6*C*). 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 SchuS4Δ0369c failed to induce IFN-β in hDC, we hypothesized that this strain may induce IL-12p40 following infection of these host cells. Indeed, SchuS4Δ0369c 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. 6*D*). 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. 6*E*). 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. 6*F*). In contrast, a similar control of SchuS4 replication in hDC treated with rhIFN-β was not observed (Fig. 6*F*). Therefore, the ability of rIFN-β to interfere with hDC production of IL-12p40 is not restricted to infection with specific *Francisella* species. However, the mechanism by which rIFN-β mediates interference of 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 correlation with 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. 2*C*).

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. 6*B*, 6*C*). 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. 1*B*, 1*C*) (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 pneumonic 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 rIFN-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 (SchuS4Δ0369c), 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 SchuS4Δ0369c 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 FTL1306 is conserved among *F. tularensis* ssp. *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.

Acknowledgments

We thank Dr. Audrey Chong for guidance with the phagosomal integrity assays and Dr. Shelly Robertson for help with qRT-PCR.

Disclosures

The authors have no financial conflicts of interest.

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