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# Development of tolerogenic dendritic cells and regulatory T cells favors exponential bacterial growth and survival during early respiratory tularemia

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## ABSTRACT

**Tularemia is a vector-borne zoonosis caused by *Ft*, a Gram-negative, facultative intracellular bacterium. *Ft* exists in two clinically relevant forms, the European biovar B (*holarctica*), which produces acute, although mild, self-limiting infections, and the more virulent United States biovar A (*tularensis*), which is often associated with pneumonic tularemia and more severe disease. In a mouse model of tularemia, respiratory infection with the virulence-attenuated Type B (LVS) or highly virulent Type A (SchuS4) strain engenders peribronchiolar and perivascular inflammation. Paradoxically, despite an intense neutrophilic infiltrate and high bacterial burden, $T_H1$-type proinflammatory cytokines (e.g., TNF, IL-1$\beta$, IL-6, and IL-12) are absent within the first ~72 h of pulmonary infection. It has been suggested that the bacterium has the capacity to actively suppress or block NF-$\kappa$B signaling, thus causing an initial delay in up-regulation of inflammatory mediators. However, our previously published findings and those presented herein contradict this paradigm and instead, strongly support an alternative hypothesis. Rather than blocking NF-$\kappa$B, *Ft* actually triggers TLR2-dependent NF-$\kappa$B signaling, resulting in the development and activation of tDCs and the release of anti-inflammatory cytokines (e.g., IL-10 and TGF-$\beta$). In turn, these cytokines stimulate development and proliferation of $T_{regs}$ that may restrain $T_H1$-type proinflammatory cytokine release early during tularemic infection. The highly regulated and overall anti-inflammatory milieu established in the lung is permissive for unfettered growth and survival of *Ft*. The capacity of *Ft* to evoke such a response represents an important immune-evasive strategy. *J. Leukoc. Biol.* 90: 493–507; 2011.**

## Introduction

Recognition of most bacterial pathogens triggers release of proinflammatory cytokines leading to activation of myeloid lineage cells (e.g., MΦ and DCs) and priming of antigen-specific type 1 $CD4^+$ $T_H1$ and $CD8^+$ cytotoxic T cells. Typically, activation of MΦ and DCs results in release of $T_H1$-type proinflammatory cytokines (e.g., TNF, IL-1$\beta$, IL-6, and IL-12). These immunomodulators enhance pathogen uptake and direct antimicrobial activities against them. One of the principal innate immune cells rapidly recruited to inflammatory foci and responsible for bacterial killing and clearance is the neutrophil. Neutrophils have an antimicrobial armamentarium that includes ROS, reactive nitrogen species, cationic peptides, and lytic enzymes. Another proinflammatory cytokine produced at sites of bacterial colonization is IL-17A, which synergizes with TNF, IL-1$\beta$, and IL-6 to further activate neutrophils and enhance their bactericidal activities [1].

In stark contrast to this classical $T_H1$-type, proinflammatory response, the early phase (<72 h) of respiratory infection with *Ft* spp. *holarctica* (from which the attenuated LVS was derived) and spp. *tularensis* (e.g., SchuS4 strain) is typified by a lack of TNF, IL-1$\beta$, IL-6, and IL-12 [2–9]. However, the inflammatory milieu in the *Ft*-infected lung at this time is characterized by: the presence of active MMP9 [10]; degradation of ECM, which generates tripeptide fragments of Pro-Gly-Pro that are chemot-

Abbreviations: APC=allophycocyanin, BDIS=BD Immunocytometry Systems, BHI=brain heart infusion, BLP=bacterial lipoprotein, BMDM=bone marrow-derived monocyte, CR2=complement receptor 2, ELAM=endothelial cell leukocyte adhesion molecule, FoxP3=forkhead box P3, *Ft*=*Francisella tularensis*, HAd=host-adapted, ICCS=intracellular cytokine staining, i.n.=intranasal, LVS=live vaccine strain, MΦ=macrophage, MHB=Mueller-Hinton broth, MMP9=matrix metalloproteinase 9, naMΦ=nonalveolar macrophage, Pam3CSK=palmitoyl-3-cysteine-serine-lysine, PI=postinfection, PTL=Parthenolide, qPCR=quantitative PCR, rFlt3L=recombinant fetal liver tyrosine kinse 3 ligand, rTul4=recombinant *Francisella tularensis* Tul4, SOCS=suppressor of cytokine signaling, SodB=SOD B gene, tDC=tolerogenic DC, $T_{reg}$=regulatory T cell

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actic for neutrophils [10]; an intense neutrophilic infiltrate [10]; and the production of IL-17A [5, 7, 11]. In addition, it has been reported that *Ft* induces $PGE_2$ [5] and activation of DCs, resulting in release of IL-10 and TGF-$\beta$ [2]. Despite the fact that all of the aforementioned immune responses are NF-$\kappa$B-dependent, some studies suggest the bacterium has the capacity to actively block NF-$\kappa$B signaling [12–16].

Telepnev et al. [12] proposed that infection with *Ft* blocks phosphorylation of I$\kappa$B-$\alpha$ and p38-MAPK, thereby inhibiting TNF, IL-1$\beta$, and IL-12 production by mouse and human MΦ in response to the TLR4 agonist LPS. An extension of this work suggests that *Ft* initially triggers NF-$\kappa$B signaling, which then is subsequently down-regulated, as bacteria escape into and replicate within the cytosol of MΦ [13]. Butchar et al. [14] suggest that *Ft* can subvert host responses and block cytokine production via induction of SOCS, specifically the family members SOCS1 and SOCS3, which can inhibit the NF-$\kappa$B pathway. Shirey et al. [17] propose that *Ft* initially triggers a classical activation program in MΦ and then redirects their differentiation such that the cells become alternatively activated, typified by expression of arginase 1 and TGF-$\beta$ rather than iNOS and TNF. Melillo et al. [15] suggest the basis for host cell suppression of proinflammatory cytokines is the capacity of *Ft* antioxidant enzymes to scavenge host-derived ROS. Such enzyme activity is thought to block signals required for MΦ cytokine production, including activation of PI3K and Akt phosphorylation, I$\kappa$B-$\alpha$ degradation, and nuclear localization of NF-$\kappa$B. Most recently, although contrary to what Melillo et al. [15] propose, Medina and coworkers [16] postulate that *Ft* restrains TLR2-triggered, proinflammatory responses via simultaneous activation of PI3K and downstream enhancement of MKP-1. In this scenario, the action of PI3K is thought to inhibit p38-MAPK-dependent, proinflammatory signals.

Clearly, a complete understanding of tularemia pathogenesis, particularly the mechanism whereby host cells respond to *Ft* in vitro, remains elusive. One fundamental caveat associated with the aforementioned studies is that due deference is not paid to the seminal finding by Hazlett et al. [18] and others [19–21]—that in vitro growth conditions have a profound qualitative and quantitative effect on the in vitro and in vivo host response to *Ft* LVS and SchuS4. *Ft*, cultivated under conditions that preclude HAd (e.g., growth in modified MHB, Thayer-Martin-based broth, or agar media) versus those that facilitate HAd (e.g., growth in BHI broth, Chamberlain's defined medium, or replication within isolated MΦ or infected tissues), differs substantially; the former growth conditions impose upon the bacterium a proinflammatory phenotype that *Ft* fails to exhibit in vivo during natural infection. As the studies described above [12–16] were conducted with *Ft* grown under conditions that engender an aberrant, proinflammatory phenotype, the physiological relevance of the findings to tularemia pathogenesis and the interpretation of results with respect to host cell signaling events warrant re-evaluation.

Given that a broader understanding of tularemia pathogenesis can only be achieved once the basic immune processes, which underlie early disease development, are revealed, the present study had two objectives. First, we sought to clarify whether *Ft* actively blocks NF-$\kappa$B signaling and if so, by what mechanism(s). Second, we sought to test an alternative hypothesis to explain the lack of TNF, IL-1$\beta$, IL-6, and IL-12 early during tularemic infection. Instead of blocking NF-$\kappa$B signaling, we postulate that *Ft* triggers NF-$\kappa$B-dependent development and activation of tDCs and $T_{regs}$ to restrain $T_H1$-type, proinflammatory cytokine release through elaboration of anti-inflammatory cytokines. The results presented herein detail the mechanism whereby *Ft* "side-steps" host cellular defenses to facilitate its nearly unfettered proliferation. We demonstrate that *Ft* has the capacity to drive the development and activation of tDCs and $T_{regs}$, thereby eliciting a predominantly anti-inflammatory host response following colonization of the pulmonary system. These findings should stimulate re-evaluation of the current paradigm regarding *Ft*-host response and provide a conceptual foundation for development of rational and effective immunotherapeutic strategies to combat this and potentially other bacterial respiratory pathogens.

## MATERIALS AND METHODS

### Bacteria

*Ft* LVS (ATCC 29684; American Type Culture Collection, Manassas, VA, USA) was kindly provided by Dr. Karen Elkins (U.S. Food and Drug Administration, Bethesda, MD, USA). *Ft* SchuS4, originally isolated from a human case of tularemia, was obtained from the U.S. Army Medical Research Institute for Infectious Diseases (Frederick, MD, USA). All experiments using SchuS4 were conducted within a Centers for Disease Control-certified Animal Biosafety Level-3/Biosafety Level-3 facility at Albany Medical College (Albany, NY, USA). The bacteria were cultured in modified MHB or BHI broth. A single colony picked from a MHB-agar plate was used to initiate a 5-ml MHB or BHI culture that was maintained for 12 h at 37°C while shaking at 220 RPM. These "starter" cultures were then used to inoculate (1:200) a 100-ml MHB or BHI culture, which was maintained for 12–16 h. Bacteria were harvested when cultures achieved an absorbance of 260 nm OD of 0.2, at which point, CFU/ml were determined by serial dilution and colony plating, as described elsewhere [3]. In addition, *Ft* LVS was recovered from culture supernatant following 24-h coincubation with mouse BM-derived MΦ (500 MOI). These MΦ-grown bacteria were used in comparative, in vitro cell-based studies along with their MHB- and BHI-grown counterparts.

### Mice

WT C57BL/6 mice (purchased from National Cancer Institute, Bethesda, MD, USA) and congenic $TLR2^{-/-}$ animals were housed in the Animal Resources Facility at Albany Medical College. Food and water were provided ad libitum. All animal procedures conformed to the Institutional Animal Care and Use Committee guidelines. All experiments were conducted using equal numbers of male and female mice of 6–8 weeks of age.

### In vitro cell culture and infection

Mouse RAW264.7 MΦ-like cells were cultured in DMEM, supplemented with 10% FBS, 2 mM Glutamax, 1 mM sodium pyruvate, and 25 mM HEPES in 5% $CO_2$ at 37°C. Unless otherwise indicated, these standard culture conditions were used for all experiments. The RAW264.7 cells were stably transfected with the human ELAM (E-selectin) promoter (–760 to +60), driving expression of destabilized eGFP. Cells ($5\times10^5$/ml) were unstimulated or stimulated for 6 h with *Ft* LVS (MOI of 100), LPS (100 ng), Pam3CSK (100 ng; a synthetic analog of a BLP), or purified rTul4 lipoprotein (100 ng). Following stimulation, cells were analyzed by flow cytometry for eGFP expression. To confirm that induced eGFP expression was strictly dependent on NF-$\kappa$B activity, one set of cells was stimulated in the pres-

ence of 30 μM of the IKK inhibitor PTL. In another series of experiments, the capacity of RAW264.7 cells to be infected by and support the replication of *Ft* was evaluated as described previously [3].

## Isolation and infection of BMDM and BM-derived DCs

BM cells were isolated from the femurs and tibias of 6- to 8-week-old mice and processed as described previously to enrich for BMDM [22]. For tolerance experiments, BMDM ($5\times10^5$ cells/ml) were exposed to BHI-grown *Ft* (MOI of 10), LPS (100 ng/ml), or Pam3CSK (100 ng/ml) for 4 h. Cells then were washed twice with sterile PBS, received medium alone or were re-exposed to BHI-grown *Ft* (MOI of 100), LPS (500 ng/ml), or Pam3CSK (500 ng/ml), and incubated for an additional 24 h. Culture supernatants then were recovered and TNF levels measured by commercial ELISA (eBioscience, San Diego, CA, USA). For transcript analysis, BMDMs ($5\times10^5$ cells/ml) were infected with MHB-, BHI-, and MΦ-grown *Ft* (MOI of 100 for each) for different periods of time, and total RNA was recovered and subjected to quantitative analysis of transcripts (i.e., *socs1, socs3, tnf,* and *inos*), as described elsewhere [22, 23]. Transcription of the *arg1* gene was analyzed using the following oligonucleotide forward and reverse primers: 5′-ACCACGGGGACCTGGCCTTT-3′ and 5′-CCTGGCGTGGCCAGAGATGC-3′, respectively. All of the qPCR reactions were run in triplicate with no-template controls, and mean comparative threshold values were used for all of the calculations using 18S rRNA as an internal normalization control. Transcript levels for infected groups are presented as a fold change over their corresponding uninfected control group. A greater than twofold change, with respect to mock control, was considered significant.

DCs were generated by incubating BM precursor cells with mouse rFlt3L (50 ng/ml; R&D Systems, Minneapolis, MN, USA) for 9 days. Cells received fresh rFlt3L-containing medium every 3rd day. At the end of the culture period, nonadherent cells representing DCs were harvested and used in experiments. The dendritic phenotype of these cells was confirmed by flow cytometry-based examination of surface expression for CD11c, CD11b, CD8a, B220, and CR2 using commercial fluorochrome-conjugated antibodies (eBioscience). The DC population was >93% $CD11c^+$ and expressed high levels of B220 but very low levels of CD8a, CD11b, and CR2. DCs ($5\times10^5$ cells/ml) from WT and $TLR2^{-/-}$ mice were infected with MHB-, BHI-, and MΦ-grown *Ft* LVS or MHB- and BHI-grown *Ft* SchuS4 (MOI of 100 for each) for 24 h, and culture supernatants were analyzed for proinflammatory cytokines (i.e., TNF, IL-1β, and IL-6) by CBA (BD PharMingen, San Diego, CA, USA). Flow cytometric analysis was performed using a FACSArray flow cytometer (BDIS, San Jose, CA, USA). Data were acquired and analyzed using BD FACSArray software and FCAP Array software, version 1.0 (BDIS), respectively. Anti-inflammatory cytokines (i.e., IL-10 and TGF-β) were measured using commercial ELISA (eBioscience).

## Measurement of NF-κB activation by TransAM NF-κB ELISA

Activation of NF-κB in BMDMs and DCs following in vitro infection with *Ft* grown in different media was assessed using a TransAM NF-κB ELISA kit (Active Motif, Carlsbad, CA, USA). Nuclear extracts were collected using a nuclear extraction kit (Active Motif) from cells stimulated with LPS (100 ng/ml) or *Ft* (MOI of 100), with and without prior addition of 30 μM PTL. Binding of the p65 subunit of NF-κB to a consensus-binding sequence (5′-GGGACTTTCC-3′) within target oligonucleotide-coating 96-well microtiter plates was detected by incubation for 1 h with primary antibody, followed by incubation with anti-IgG HRP conjugate and developing solution. The amount of p65 was quantified colorimetrically at 450 nm with a reference wavelength of 655 nm.

## Western blot analysis of SOCS expression

Antibodies directed against SOCS1 and SOCS3 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and antibodies against β-actin were obtained from Bethyl Laboratories (Montgomery, TX, USA). Protein samples (25–100 μg, depending on the target) were resolved by SDS-PAGE, and Western blotting was performed as described elsewhere [22]. Specific signal was developed using the SuperSignal West Dura chemiluminescent substrate (Pierce Endogen, Rockford, IL, USA).

## Infection of mice

All infection experiments used groups of three to five mice. Prior to i.n. inoculation, animals were deeply anesthetized via i.p. injection of a cocktail of Ketamine (20 mg/ml) and Xylazine (1 mg/ml). Following dilution in sterile PBS, $1\times10^3$ CFU of BHI-grown *Ft* LVS in a volume of 20 μl were instilled i.n. (10 μl/nare); actual dosages were confirmed by colony plating. Sham-inoculated controls received an equal volume of uninoculated BHI broth diluted in PBS. Killed mice were necropsied at various times PI, and lungs were perfused with PBS and excised aseptically. The smaller lobe of the lung was used for preparation of lung homogenate for bacterial counting and/or cytokine measurements as described previously [3], and the remainder was used for isolation of single cell suspensions for flow cytometry analysis or for histological evaluation. Tissues were processed using standard histological methods to obtain 5-μm-thick paraffin sections that were stained with H&E as described previously [3].

## Bacterial burden and cytokine measurements

Portions of lung (20 mg) were suspended in 0.5 ml PBS containing protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN, USA) and were homogenized using a mechanical Mini Bead Beater (Biospec Products, Bartlesville, OK, USA) and sterile, inert Zirconia beads. Homogenates were processed, and quantification of bacterial numbers was performed as described previously [3]. Results are expressed as $\log_{10}$ CFU/ml. Lung homogenates were also assayed for the presence of proinflammatory and anti-inflammatory cytokines as described above. In addition, measurement of IL-17A was performed using a commercial ELISA kit (eBioscience).

## Antibodies

For flow cytometry, mouse FITC-anti-CD4 (clone GK1.5), PE-anti-CD3 (clone 17A2), APC-anti-CD25 (PC61), PE-anti-FoxP3 (clone 150D), PE-anti-IgG1 isotype control (clone MOPC-21), APC-anti-IL-17A (clone TC11-18H10.1), APC-anti-rat IgG1 isotype control (clone RTK2071), FITC-anti-CD11b (clone M1/70), APC-anti-IL-10 (clone JES5-16E3), PerCP/Cy5.5-anti-Gr-1 (clone RB6-8C5), PerCP/Cy5.5-anti-CD49 pan NK cell (clone DX5), PE/Cy7-anti-CD11c (clone N418), PE-anti-F4/80 (clone BM8), PE/Cy7-rat IgG2b isotype control, and Pacific blue-anti-Gr-1 (clone RB6-8C5) were purchased from BioLegend (San Diego, CA, USA). Mouse FITC-anti-γδ TCR (clone GL-3), APC-anti-IL-17A (clone 17B7), APC-anti-TNF (clone MP6-XT22), APC-anti-rat IgG2a isotype control (clone 17-4321), PE-anti-CCR9 (CD199; clone CW-1.2), FITC-anti-CD103 (clone 2E7), and PE-anti-CD103 (clone 2E7) were purchased from eBioscience. APC-anti-mouse TGF-β1 (latency-associated peptide; clone 27232) was purchased from R&D Systems.

## Flow cytometry

Single cell suspensions were prepared from the lungs of control and infected mice. Briefly, the lungs were cut into small pieces and suspended in 1 ml digestion buffer containing collagenase type I (Worthington Biochemical, Lakewood, NJ, USA) and rDNase I (Roche, Mannheim, Germany). Following digestion for 30 min at 37°C, the lung cells were passed through a cell strainer, collected by centrifugation (250 *g*, for 10 min), and resuspended in fluorescent assay buffer. For ICCS, lung cells were incubated with surface marker-specific antibodies for 30 min and placed in Fixation buffer (eBioscience). The fixed cells were then permeabilized with Permeabilization buffer (eBioscience) and incubated with cytokine-specific or isotype control antibodies for 40 min. The cells were gated on the basis of forward- and side-scatter characteristics and with respect to specific surface marker and/or cytokine expression. Specific cell populations are

graphed as a percentage of the total cells recovered from uninfected lungs or infected lungs at various times. In addition to presenting the percentage of specific cell types observed, the total number of cells was calculated.

To characterize putative tDCs, $CD11c^{high}CD11b^{low}$ cells were analyzed for surface expression of CD103 and CCR9. To identify $T_{regs}$, lung cells first were incubated with antibodies directed against CD4 and CD25, followed by intracellular staining with anti-FoxP3 or isotype control antibody. Multiparameter FACS analysis was performed on a LSRII instrument (Becton Dickinson, Franklin Lakes, NJ, USA), and data were compensated and analyzed using FlowJo software, version 7.6.1 (Tree Star, Ashland, OR, USA).

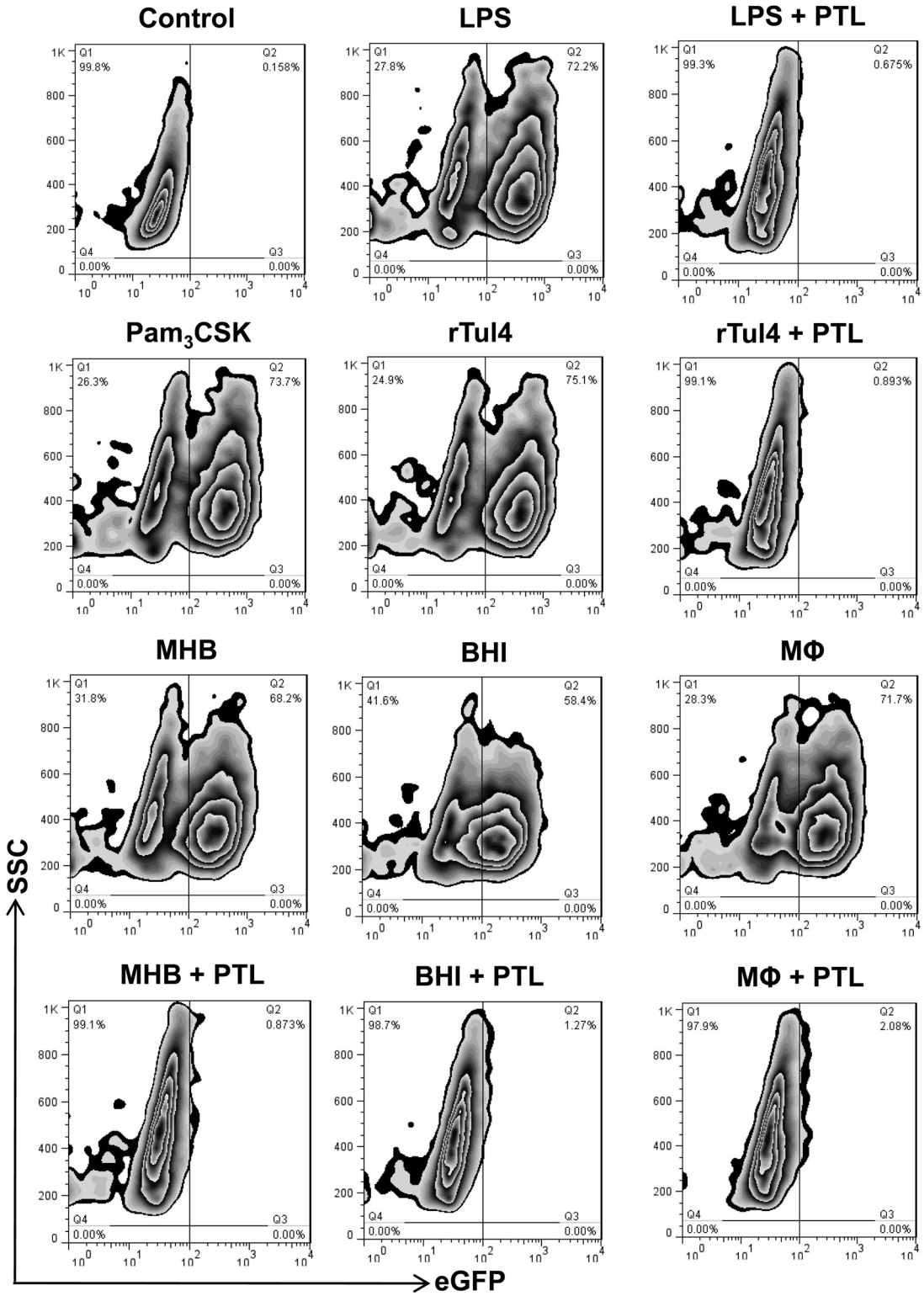


**Figure 1. *Ft* induces activation of NF-κB signaling.** RAW264.7 cells ($5\times10^5$/ml) were unstimulated or stimulated for 6 h with *Ft* LVS (MOI of 100), LPS (100 ng), Pam3CSK (100 ng), or purified rTul4 lipoprotein (100 ng). *Ft* was grown in liquid culture (i.e., MHB or BHI broth) or isolated from infected MΦ, as described in Materials and Methods. Following stimulation, cells were analyzed by flow cytometry for eGFP expression. To confirm activation of NF-κB (as reflected in induced GFP expression), one set of cells was stimulated in the presence of the IKK inhibitor PTL (30 μM). FACS results are representative of three independent experiments. SSC, Side-scatter.

### Statistical analysis

Where applicable, all results were expressed as mean ± SEM from two or more independent experiments. Depending on the distribution of the dataset, comparisons between groups were made using a parametric ANOVA test with Tukey's post-test or a nonparametric Kruskal-Wallis test with Dunn's post-test. Differences between control and experimental groups were considered significant at $\alpha = 0.05$ level. The relationship among the level of TGF-$\beta$, total number of $T_{regs}$, and the log of bacterial burden in the lungs was determined by linear regression analysis. The correlations for these measures in individual animals are presented for Days 0, 1, and 3 (during the exponential growth phase of the bacteria).

## RESULTS

### *Ft* triggers rather than blocks NF-κB-dependent signaling

A number of studies have attributed the lack of $T_H1$-type proinflammatory cytokines during early-phase tularemia to blockade of NF-κB signaling [12, 13, 15, 16]. However, this notion is discrepant with a number of immunopathogenic responses to the bacterium that are NF-κB-dependent, including activation of MMP9 and the production of IL-10, IL-17, TGF-$\beta$, and $PGE_2$ [2, 5, 7, 10, 11]. To clarify whether *Ft* LVS has the capacity to activate or ablate NF-κB activity, a well-accepted and validated RAW264.7 reporter cell line was used. These cells, stably transfected with the human ELAM (i.e., E-selectin) promoter, driving expression of eGFP, were used to monitor the response to infection with MHB (i.e., non-HAd)- and BHI- and MΦ-grown (i.e., HAd) *Ft.* eGFP expression by this reporter cell line is strictly NF-κB-dependent and cannot occur if the signaling pathway is blocked. Following incubation with known TLR4 (i.e., LPS) and TLR2 (i.e., Pam3CSK and rTul4) agonists, the majority of RAW264.7 cells expressed eGFP (**Fig. 1** and Supplemental Fig. 1A). Similarly, infection of cells with *Ft* LVS (regardless

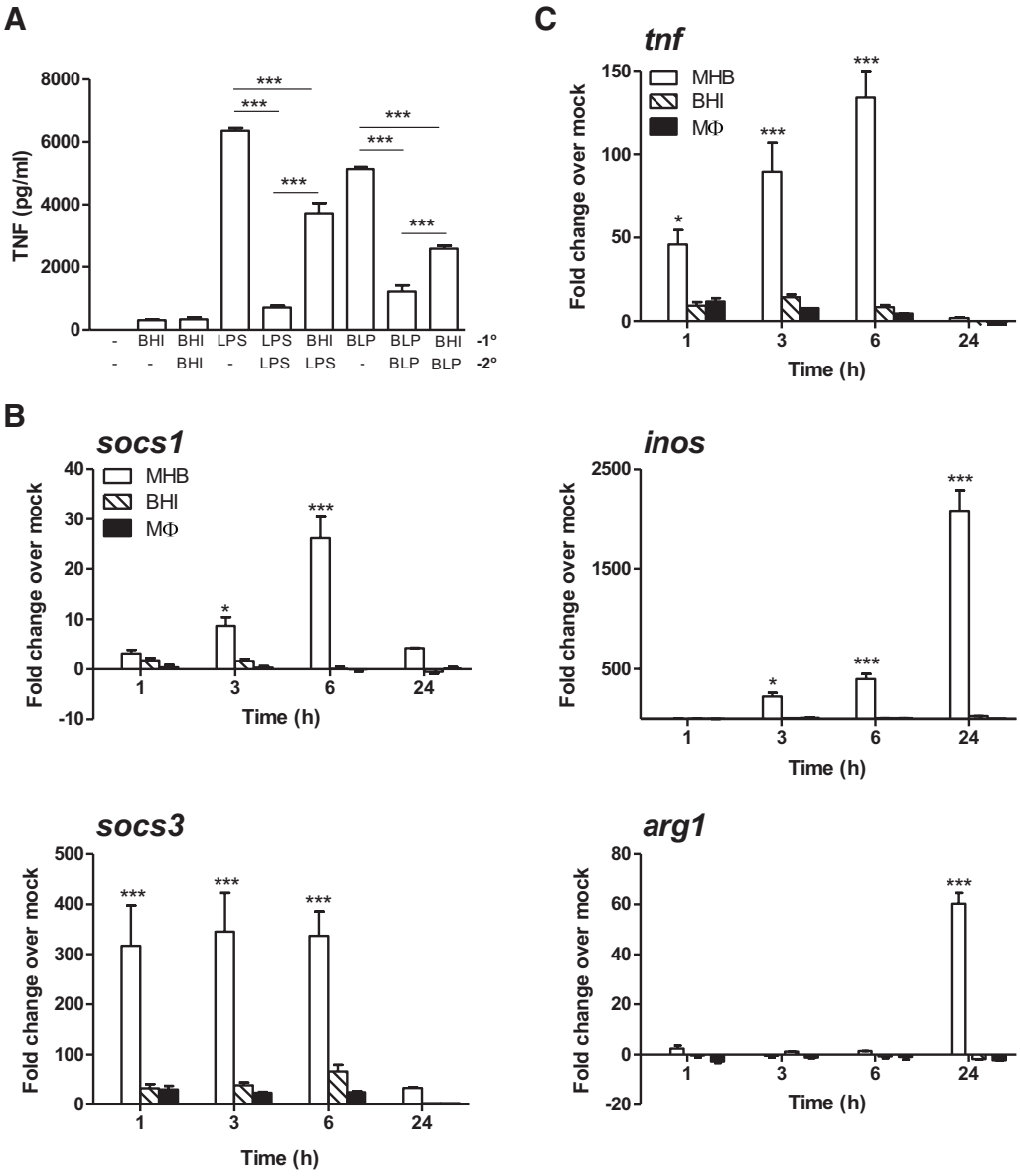


**Figure 2. The inability of *Ft* to elicit TNF production does not reflect induction of tolerance, SOCS activity, or alternative activation of MΦ.** (A) BMDMs ($5\times10^5$ cells/ml) were exposed to a low-dose primary (1°) stimulus of BHI-grown *Ft* LVS (MOI of 10), LPS (100 ng/ml), or BLP (100 ng/ml) for 4 h at 37°C. Cells were then washed twice and re-exposed to a higher homologous or heterologous secondary (2°) stimulus, and TNF levels were measured after 24 h. The values are expressed as mean ± SEM from two independent experiments. (B and C) BMDMs ($5\times10^5$ cells/ml) were infected with non-HAd (i.e., MHB-gown) or HAd (i.e., BHI- or MΦ-grown) *Ft* LVS (MOI of 100), and total RNA was recovered from cells after 1, 3, 6, and 24 h incubation. qPCR was used to determine transcript levels for *sosc1, socs3, tnf, inos,* and *arg1.* The values are expressed as mean ± SEM from three independent experiments. $^*P < 0.05$; $^{***}P < 0.001$.

of means of cultivation) significantly increased NF-κB-dependent eGFP expression within just 6 h (the earliest time-point studied), and the percentage of positive cells was even greater at 24 h (data not shown). To confirm that expression of eGFP by *Ft*-infected cells was in fact driven by nuclear translocation of NF-κB, some cells were stimulated with TLR agonists or infected with bacteria in the presence of the IKK inhibitor PTL, which prevents release of NF-κB p50/p65 heterodimers from its IκB-α inhibitor, thus trapping it in the cytosol. In the presence of PTL, eGFP expression triggered by purified TLR agonists or *Ft* LVS was ablated completely (Fig. 1 and Supplemental Fig. 1A). To extend this finding to primary cells, the TransAM NF-κB p65 DNA-binding ELISA was performed using nuclear extracts from BMDMs incubated for 1 h with non-HAd- and HAd-*Ft* LVS at a MOI of 100. As seen in Supplemental Fig. 1B, regardless of growth conditions, *Ft* LVS stimulated the nuclear translocation of NF-κB. Similarly, activation of NF-κB was observed in *Ft*-infected DCs, irrespective of HAd status (data not shown). Furthermore, as in RAW264.7 cells, cellular activation of NF-κB in response to *Ft* was ablated completely in BMDMs (Supplemental Fig. 1B) and DCs by inclusion of PTL. As a control, the potential effect of different growth conditions on the capacity of *Ft* LVS to invade and replicate within cells was evaluated. HAd status failed to significantly influence the replication of bacteria within RAW264.7 cells or BMDMs (Supplemental Fig. 1C).

### Neither tolerance nor alternative activation of MΦ explains the inability of *Ft* to elicit TNF production

Induction of tolerance (i.e., blockade of NF-κB signaling) has been invoked as a possible explanation for the inability of *Ft* to stimulate TNF, IL-1β, IL-6, and IL-12 following in vitro infection of host cells or during early respiratory infection of mice [12–16]. Therefore, using a classic experimental design [22], we examined whether *Ft* has the capacity to tolerize cells against subsequent re-exposure and response to a homologous or heterologous TLR stimulus. BMDMs were exposed to a low-dose primary stimulus of BHI-grown *Ft* (MOI of 10), LPS (100 ng/ml), or BLP (100 ng/ml) for 4 h at 37°C. Cells were then washed twice and re-exposed to a higher homologous or heterologous stimulus, and TNF levels were measured after 24 h. LPS and BLP served as controls for canonical TLR4- and TLR2-tolerizing agonists, respectively. Cells whose primary and homologous stimuli were LPS or BLP exhibited an 89% and 76% reduction in TNF levels, respectively (**Fig. 2A**). In contrast, neither primary exposure alone nor primary plus re-exposure of cells to BHI-grown *Ft* elicited a TNF response above or below unstimulated controls. More importantly, primary exposure to *Ft*, followed by re-exposure to LPS or BLP, failed to ablate TNF release. The response of infected cells to secondary stimulation with the TLR4 or TLR2 agonist only diminished TNF levels by 41% and 50%, respectively. This reduction in TNF did, however, suggest that HAd-*Ft* could at least temper proinflammatory cytokine responses to TLR agonists. As such, we explored whether *Ft* activated SOCS, which target the NF-κB signaling cascade as a means of tolerizing MΦ to continued bacterial stimulation [22]. BMDMs were infected with MHB-, BHI-, or MΦ-grown bacteria, and total RNA was recovered from cells at different time-points. MHB-grown *Ft* induced an ~30- and ~300-fold increase in *socs1* and *socs3* transcript levels above baseline by

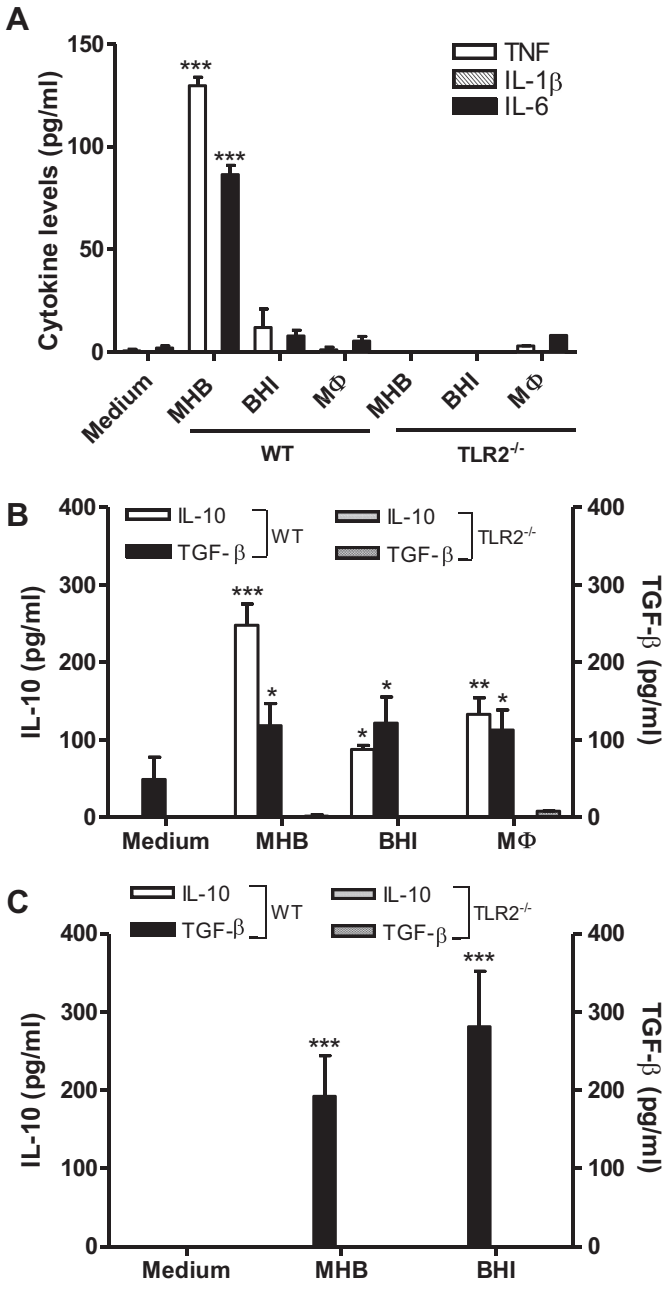


**Figure 3. TLR2 is required for *Ft* LVS- and SchuS4-induced anti-inflammatory cytokine release from DCs.** DCs ($5\times10^5$ cells/ml) from WT and TLR2$^{-/-}$ mice were infected with MHB-, BHI-, and MΦ-grown *Ft* LVS (A and B) or MHB- and BHI-grown SchuS4 (C; MOI of 100 for each) for 24 h; culture supernatants were analyzed for proinflammatory cytokines by CBA; and anti-inflammatory cytokines were measured using commercial ELISA. The values are expressed as mean ± SEM from three independent experiments. *$P < 0.05$; **$P < 0.01$; and ***$P < 0.001$.

6 h PI, respectively (Fig. 2B). When cells were infected in the presence of the proteosome inhibitor MG132 and total cellular lysate analyzed by Western blot, this pattern of response was mirrored at the protein level (Supplemental Fig. 2). In contrast, HAd-*Ft* induced significantly lower transcription and translation of either of these negative regulators of NF-κB signaling. Nevertheless, the small amount of SOCS1 and SOCS3 protein produced may account for the HAd-*Ft*-induced reduction in TNF response to purified TLR agonists observed in Fig. 2A.

Next, we tested whether HAd-*Ft* exhibited a greater capacity to induce an alternative rather than classical activation program in MΦ compared with their non-HAd counterparts. Following infection with MHB-grown bacteria, transcript levels for *tnf*, *inos*, and *arg1* were elevated significantly above baseline over the course of 24 h (Fig. 2C). By comparison, BHI- and MΦ-grown organisms failed to induce transcription of these genes. Curiously, by 24 h of coincubation, the MHB-grown *Ft* simultaneously induced transcription of *inos* and *arg1* as if BMDMs were receiving "mixed signals" from the bacterium as to whether a classical or alternative program should be initiated. Finally, another possibility to consider is whether HAd bacteria might block proinflammatory cytokine production through alteration of PI3K-AKT-p38-MAPK signaling, as suggested by other groups [15, 16]. In preliminary experiments, BMDMs were incubated with a PI3K inhibitor (LY294002), a general MAPK inhibitor (arctigenin), or a specific p38-MAPK inhibitor (SB202190) in a dose-escalation manner prior to infection with BHI- or MΦ-grown *Ft*. None of the inhibitors tested augmented or derepressed TNF production in response to HAd bacteria (data not shown). Thus, no evidence currently exists to implicate the PI3K-AKT-p38-MAPK pathway in modulating proinflammatory cytokine responses to HAd-*Ft* in vitro or during natural infection.

## *Ft* LVS and SchuS4 elicit anti-inflammatory cytokines from DCs and do so in a TLR2-dependent manner

A number of studies have identified DCs as an initial target for infection by *Ft* and have implicated them in the process of bacterial dissemination as well [4, 24, 25]. It also is appreciated that TLR2 plays a critical role in tularemia pathogenesis [3, 26]. As such, it was of interest to determine to what extent TLR2 regulates cytokine production by DCs. Following in vitro infection with MHB-grown *Ft* LVS, DCs produced proinflammatory (i.e., TNF and IL-6 but not IL-1β) and anti-inflammatory (e.g., IL-10 and TGF-β) cytokines and did so in a TLR2-dependent manner (**Fig. 3A** and **B**). In contrast, BHI- and MΦ-grown LVS failed to elicit proinflammatory cytokines but did stimulate the release of IL-10 and TGF-β (Fig. 3B). These studies were then extended to characterize the anti-inflammatory cytokine response to *Ft* SchuS4 grown in MHB and BHI broth. As with LVS, this highly virulent Type A strain induced TGF-β in a strictly TLR2-dependent manner, irrespective of the medium used for its cultivation (Fig. 3C). However, MHB- and BHI-grown SchuS4 was unable to stimulate significant production of IL-10 by DCs. To what extent SchuS4-induced pro- and anti-inflammatory cytokine production by an

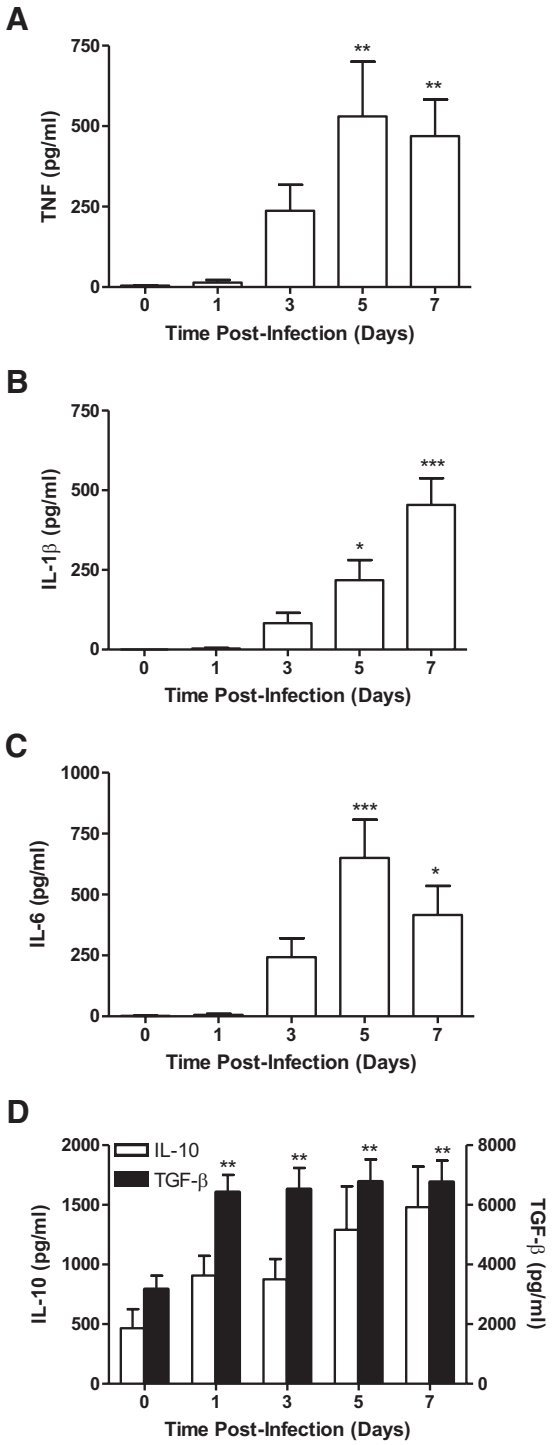


**Figure 4. *Ft* induces primarily anti-inflammatory cytokines during early respiratory infection.** Four groups of C57BL/6 mice were i.n.-infected with $1 \times 10^3$ CFU of BHI-grown *Ft* LVS and were killed at Days 1, 3, 5, and 7 PI. One group of sham-inoculated mice served as a control. The lung homogenates prepared from the uninfected lungs and infected lungs were analyzed for proinflammatory (A–C) and anti-inflammatory (D) cytokines. The values are expressed as mean ± SEM from four independent experiments ($n$=14 total mice/group). $^*P < 0.05$; $^{**}P < 0.01$; and $^{***}P < 0.001$.

isolated cell type(s) recapitulates host cell responses during early-phase respiratory tularemia is currently under investigation.

### Infection with *Ft* results in temporally regulated production of pro- and anti-inflammatory cytokines

Despite the ability of *Ft* to trigger NF-κB signaling, as reported previously by us [3] and other groups [2, 4, 9, 27, 28], pulmonary infection fails to elicit early production (~72 h) of classical $T_H1$-type, proinflammatory cytokines such as TNF, IL-1β, IL-6, and IL-12. Appreciating the profound effect that HAd has on the cytokine-stimulatory capacity of *Ft* and the course of disease [18], mice were infected with BHI-grown *Ft* LVS, and the in vivo kinetics of cytokine production were evaluated. With respect to proinflammatory cytokine production, following i.n. inoculation of $1 \times 10^3$ CFU of HAd-*Ft* LVS, TNF, IL-1β, and IL-6 were only observed at 5 and 7 days PI (**Fig. 4A–C**). In contrast, TGF-β levels were elevated significantly above baseline within 24 h and continued to rise during the course of infection. IL-10 levels trended higher over the same period; however, they were not significantly different from uninfected controls (Fig. 4D).

Next, we sought to determine whether the observed temporal disparity in proinflammatory cytokine production was reflected in tissue pathology. Accordingly, lungs recovered at early (Day 2) and late (Day 6) time-points PI were examined for gross and his-

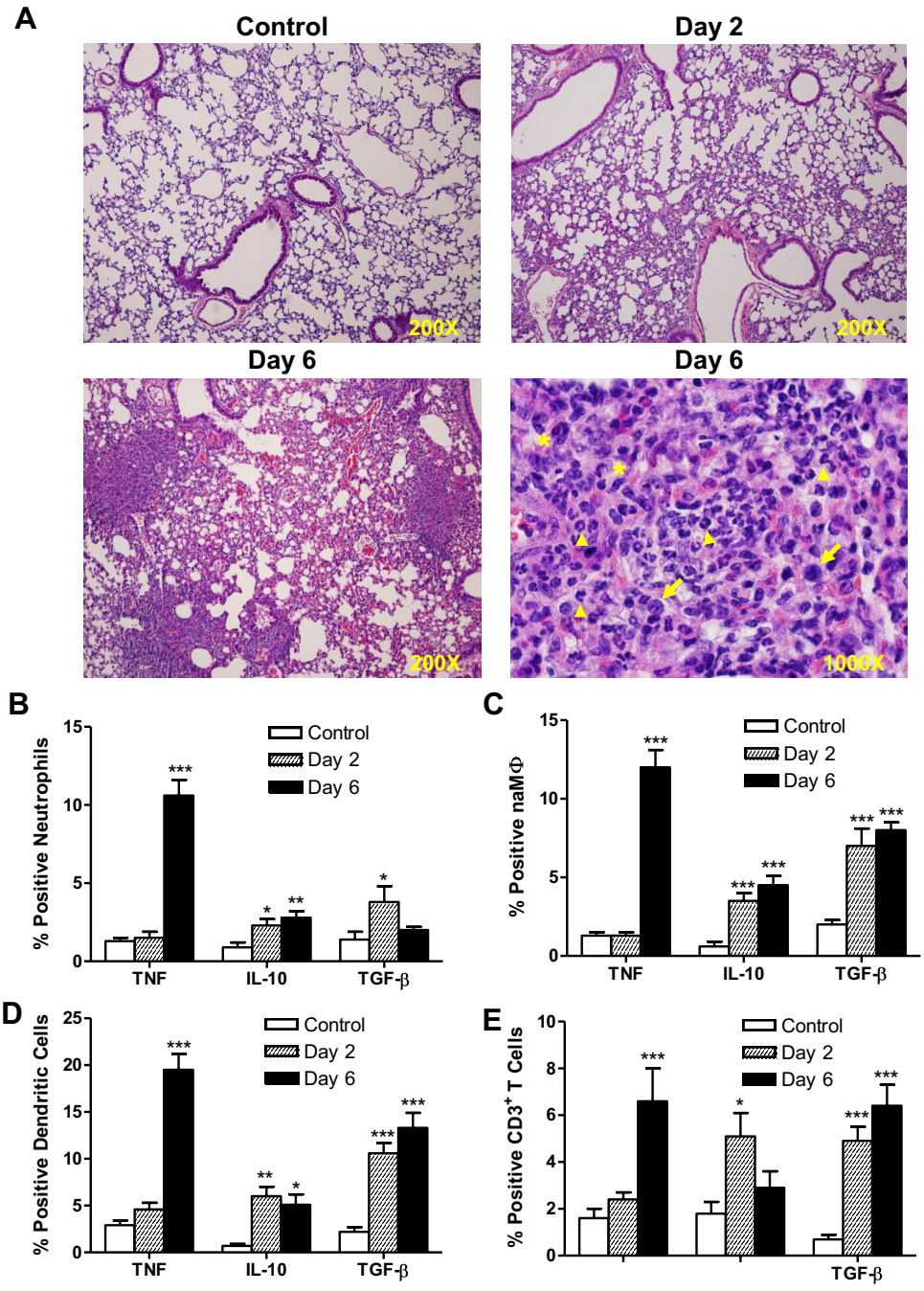


**Figure 5. Myeloid and lymphoid cells are a source of anti-inflammatory cytokines during early respiratory infection.** Two groups of C57BL/6 mice were i.n.-infected with $1 \times 10^3$ CFU of BHI-grown *Ft* LVS and killed on Days 2 and 6 PI. One group of sham-inoculated mice served as a control. (A) Histological evaluation of the lungs at Days 2 and 6 PI. Original magnification is ×200 or ×1000, oil immersion. Arrowheads, Polymorphonuclear cells; arrows, mononuclear cells; and *, multinucleated giant cells. (B–E) Lung cells were analyzed for ICCS by flow cytometry. The percentages neutrophils, naMΦ, DCs, and $CD3^+$ T cells expressing TNF, IL-10, or TGF-β are shown. The values are expressed as mean ± SEM from two independent experiments ($n$=11 total mice/group). $^*P < 0.05$; $^{**}P < 0.01$; and $^{***}P < 0.001$.

tological changes. Upon gross examination, the lungs of mice killed at Day 2 PI had no lesions, whereas lungs from mice infected for 6 days showed congestion and consolidation. Histological evaluation of H&E-stained lung sections revealed that infiltration of the parenchyma by polymorphonuclear cells and mononuclear cells is quite limited at Day 2 when compared with uninfected controls (**Fig. 5A**). At this time-point, cellular infiltrates are restricted to the basement membrane immediately beneath alveolar epithelial cells. Bronchiolar lumen also contained mild inflammatory exudates. However, on Day 6, focal areas of stellate necrosis and massive cellular infiltration were observed in the lungs. The inflammatory foci consisted of polymorphonuclear and mononuclear cells and occasional multinucleated giant cells (a pathologic indicator of severe intracellular parasitism). The necrotic areas were surrounded by zones of mixed cellular infiltrates, and thrombi were observed within small blood vessels.

Having broadly characterized the pattern of cytokine production in lung tissue during the course of infection, we next wanted to identify specific cell types responsible for their release. To compare and contrast early- and late-phase intracellular cytokine profiles, mice were infected with $1 \times 10^3$ CFU of BHI-grown *Ft*, and animals were killed on Days 2 and 6 PI. Multiparameter flow cytometry was used to analyze lung cells that were stained for myeloid lineage markers (CD11b, CD11c, Gr1-1, and F4/80) or a lymphoid marker (CD3) in association with cytokine antibodies (TNF, IL-10, or TGF-$\beta$) and gated on specific phenotypic surface markers. Based on the expression of phenotypic markers, myeloid cells in the lungs were identified as neutrophils ($CD11b^{high}GR1^{high}F4/80^{low}CD11c^{low}$), naMΦ ($CD11b^{high}GR1^{low}F4/80^{high}CD11c^{low}$), or DCs ($CD11b^{low}GR1^{low}F4/80^{low}CD11c^{high}$). As shown in Fig. 5, neutrophils (Fig. 5B), naMΦ (Fig. 5C), DCs (Fig. 5D), and $CD3^+$ T cells (Fig. 5E) are generally a source of IL-10 and TGF-$\beta$ during early- and late-phase disease (at Days 2 and 6, respectively), whereas production of TNF by these same cells was only observed at the later stage (Day 6). Among all of the inflammatory cells within the lung, DCs were the main source of IL-10 and TGF-$\beta$ at Days 2 and 6, whereas later during infection, they also became the principal producer of TNF (Fig. 5D). Not only were increases in the percentage of cells expressing specific cytokines observed, but the total numbers of cytokine-expressing cells also increased significantly during the course of disease (**Table 1**).

## *Ft* rapidly stimulates a variety of cells to produce IL-17A in the lung

IL-17A is a member of the IL-17 family of proinflammatory cytokines produced within hours following epithelial cell injury or activation of PRRs [1]. Interestingly, microbial infection of mice results in early (within 4–8 h) production of IL-17A, which enhances neutrophil migration and production of IL-6 and other chemokines [29]. Although respiratory tularemia is characterized by a lack of $T_H1$-type proinflammatory cytokines during the first 3 days, the induction of IL-17A, a precursor to development of $T_H1$-biased immunity, has been observed as early as 2 days PI with *Ft* LVS [7]. To confirm and extend this observation, mice were infected with $1 \times 10^3$ CFU of *Ft* LVS, and IL-17A levels were found to be elevated nearly threefold above uninfected controls as early 1 day PI. Levels of IL-17A remained higher than baseline for the duration of the experiment (**Fig. 6A**). Next, it was determined what subsets of immune cells were responsible for producing IL-17A. Significant numbers of $CD4^+$ (Fig. 6B) and $CD4^-$ (Fig. 6C) cells contained cytosolic IL-17A as early as 24 h PI. The percentage of cells expressing IL-17A is highest at 24 h PI and then wanes (as a result of infiltration of the lungs by additional inflammatory cells) over the 1st week of infection. In contrast, the total number of $CD4^+$ and $CD4^-$ IL-$17A^+$ cells in the lung steadily climbs throughout the course of disease. Among the $CD4^-$ cells expressing IL-17A, a significant percentage and number were γδ T cells (Fig. 6D), neutrophils (Fig. 6E), and $Dx5^+$ NK cells (Fig. 6F). Interestingly, MΦ were not found to be a source of IL-17A in the *Ft*-infected lung (data not shown).

## *Ft* induces the development and activation of tDCs and $T_{regs}$ in the lung

It is their differential and exclusive capacity to produce anti-inflammatory but not proinflammatory cytokines in response to HAd-*Ft* LVS and ShuS4, which suggests that the DCs activated during early-phase respiratory tularemia may be tolerogenic in nature [30]. To explore this possibility, multiparameter flow cytometry was used to characterize the $CD11c^{high}CD11b^{low}$ cells recovered from uninfected mice and those infected with $1 \times 10^3$ CFU of *Ft* LVS for 2 or 6 days. At both time-points studied, there was a significant increase in the percentage of $CD11c^{high}CD11b^{low}$ cells expressing CD103 or CCR9 (**Fig. 7A** and **Table 2**), two well-characterized tDC markers [31, 32]. Interestingly, dual staining for CD103 and CCR9 revealed that all

**TABLE 1. Total Number of Cells from Whole Lung Expressing Pro- and/or Anti-Inflammatory Cytokines during Early- and Late-Phase Respiratory Tularemia**

| | TNF | | | IL-10 | | | TGF-$\beta$ | | |
|---|---|---|---|---|---|---|---|---|---|
| Cells | Control | Day 2 | Day 6 | Control | Day 2 | Day 6 | Control | Day 2 | Day 6 |
| Neutrophils | 2087 ± 340 | 2295 ± 711 | 231,141 ± 36,087[a] | 2162 ± 682 | 8180 ± 4370 | 68,120 ± 5956[a] | 3536 ± 1326 | 13,210 ± 6771 | 37,273 ± 5968[a] |
| naMΦ | 2904 ± 526 | 5019 ± 1210 | 382,143 ± 47,106[a] | 972 ± 377 | 17,528 ± 4143[b] | 143,938 ± 13,339[a] | 4123 ± 786 | 19,119 ± 3526[b] | 195,646 ± 16,722[a] |
| DCs | 3540 ± 684 | 10,581 ± 2436 | 171,924 ± 15,549[a] | 844 ± 227 | 4872 ± 1040[b] | 44,992 ± 6895[a] | 2862 ± 393 | 10,103 ± 2011[b] | 149,390 ± 21,525[a] |
| $CD3^+$ T cells | 3003 ± 1363 | 8029 ± 745 | 35,340 ± 7921[a] | 3139 ± 567 | 17,802 ± 2044[a] | 3797 ± 679 | 765 ± 270 | 18,935 ± 5168[a] | 17,009 ± 2878[a] |

[a] $P < 0.001$; [b] $P < 0.05$.

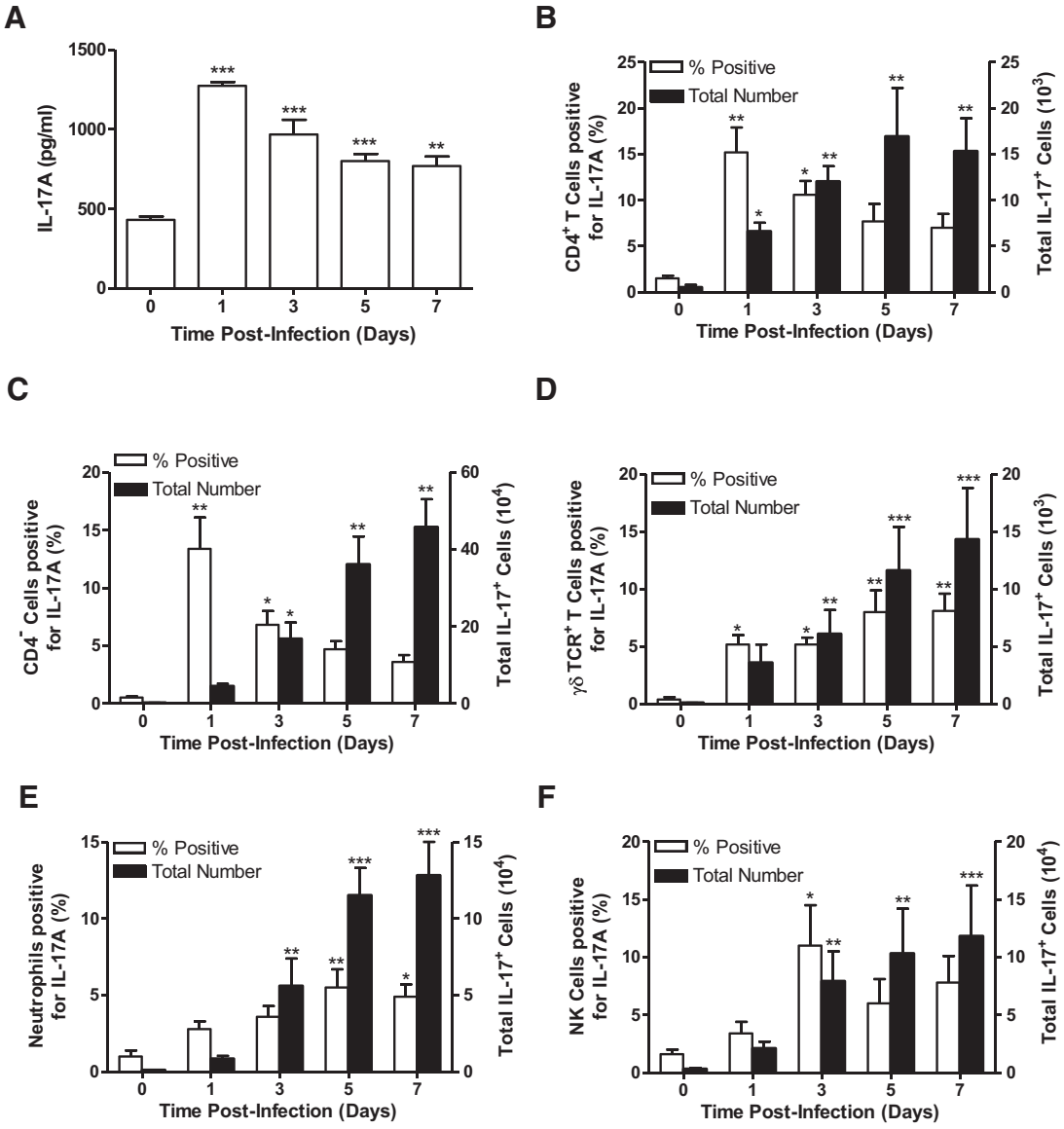


**Figure 6. IL-17-producing cells were activated rapidly in the lungs of *Ft*-infected mice.** Four groups of C57BL/6 mice were i.n.-infected with $1 \times 10^3$ CFU of BHI-grown *Ft* LVS and killed at Days 1, 3, 5, and 7 PI. One group of sham-inoculated mice served as a control. (A) Levels of IL-17A were measured in the lung homogenates by ELISA. (B–F) Lung cells were analyzed for ICCS of IL-17A by flow cytometry. The percentages and total numbers of different cell types producing IL-17A are shown. The values are expressed as mean ± SEM from four independent experiments ($n$=14 total mice/group). $^*P < 0.05$; $^{**}P < 0.01$; and $^{***}P < 0.001$.

$CD103^+$ cells express CCR9, but the reverse was not true (data not shown). A statistically significant, albeit small, subset of $CD11c^{high}CD11b^{low}CD103^+$ cells (Fig. 7B and Table 2) and $CD11c^{high}CCR9^+$ cells (Fig. 7C) expressed IL-10 and TGF-$\beta$. With regard to total cell numbers, $CCR9^+$ tDCs expressed significantly more IL-10 and TGF-$\beta$ at Days 2 and 6 PI than did cells from uninfected lungs (Table 2). Also, although not at Day 2, $CD103^+$ tDCs did show a dramatic increase in expression of these anti-inflammatory cytokines by Day 6. The juxtaposition of tDCs, TGF-$\beta$, and IL-10 early during respiratory tularemia led to the hypothesis that naïve $CD4^+$ T cells in the lung might acquire the phenotypic characteristics of $T_{regs}$ within this anti-inflammatory milieu [30]. To test this notion, lung cells were recovered from uninfected and infected mice at multiple time-points and were analyzed for the presence of FoxP3. As seen in Fig. 7D, within the first 24 h of infection, the percentage of $CD4^+CD25^+$ cells expressing FoxP3 rises tenfold above uninfected control, as does the total number of $Foxp3^+$ cells (sevenfold above uninfected control). Notably, the total number of $T_{regs}$ resident in the lung increases substantially during the course of disease, such that by Day 6, there is a 23-fold increase in their total numbers. Increases in the percentage of $FoxP3^+$ cells were also observed in the mediastinal LN and spleen of infected mice (data not shown).

## Development of tDCs and $T_{regs}$ favors exponential bacterial growth and survival during early respiratory tularemia

Given the necessity for TNF, IL-1$\beta$, and IL-6 to enhance the activation and increase the bactericidal activities of neutrophils, we postulated that the presence of TGF-$\beta$ and $T_{regs}$ would not only block the production of such proinflammatory cytokines but also favor proliferation of *Ft*. The first 72 h of infection is typified by a four-log increase in bacterial burden within the lung of mice (**Fig. 8A**); however, whether a direct correlation between TGF-$\beta$ and $T_{regs}$ and the accumulation of *Ft* in tissues exists was unknown. Accordingly, for data collected during the bacterium's exponential growth phase, for

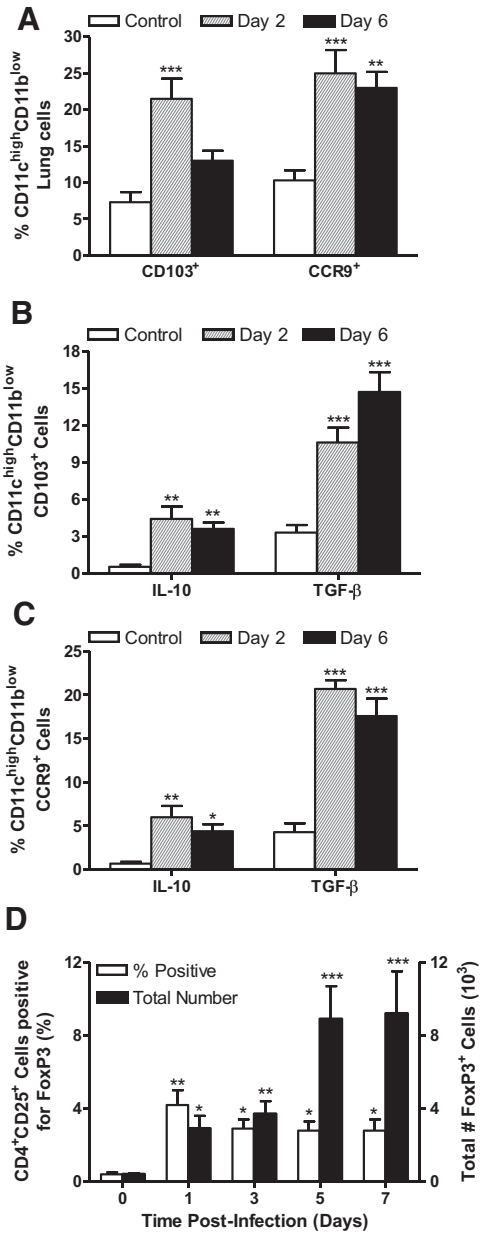


**Figure 7. *Ft* induces the development of pulmonary tDCs and $T_{regs}$.** Two groups of C57BL/6 mice were infected with $1 \times 10^3$ CFU of BHI-grown *Ft* LVS and killed at Days 2 and 6 PI. One group of sham-inoculated mice served as a control. (A) $CD11c^{high}$ lung cells were evaluated by flow cytometry for surface expression of the tDC markers, CD103 and CCR9. $CD11c^{high}CD103^+$ cells (B) and $CD11c^{high}CCR9^+$ cells (C) were analyzed for IL-10 and TGF-β cytokine production by flow cytometry. (D) $T_{regs}$ were identified on the basis of FoxP3 expression. Gating on $CD4^+$ cells, the percentages, and total numbers of $CD25^+FoxP3^+$ cells were calculated. The values are expressed as mean ± SEM from four independent experiments ($n$=14 total mice/group). *$P < 0.05$; **$P < 0.01$; and ***$P < 0.001$.

each animal, we analyzed by regression the association among TGF-β, $T_{regs}$, and bacterial burden. The TGF-β concentrations ($r^2$=0.67, and $r^2$=0.77 for Days 1 and 3, respectively) and numbers of $T_{regs}$ ($r^2$=0.65, and $r^2$=0.71 for Days 1 and 3, respectively) were significantly associated with variations in bacterial burden (Fig. 8B and C). That is, the animals with higher TGF-β levels and/or greater numbers of $T_{regs}$ had higher bacterial burdens. This suggests a cause and effect relationship, wherein the anti-inflammatory effects of TGF-β and/or $T_{regs}$ are regulating the extent of bacterial growth and survival. However, as TGF-β levels and the numbers of $T_{regs}$ also share a linear relationship (Fig. 8D), this experiment cannot determine whether one or both of these factors are responsible for repressing control of *Ft* growth.

## DISCUSSION

The two principal objectives of the present study were to clarify whether *Ft* blocks activation of NF-κB as a means of ablating proinflammatory cytokine production and if not, to test an alternative hypothesis to explain the lack of TNF, IL-1β, IL-6, and IL-12 early during tularemic infection. With regard to the first point, we present evidence that instead of ablating NF-κB signaling, infection of a RAW264.7 cell line and/or isolated primary BMDMs and DCs and mice by HAd-*Ft* results in the TLR2- and NF-κB-dependent activation of an anti-inflammatory response that likely restricts the early production of $T_H1$-type proinflammatory cytokines. On the second point, substantive evidence is provided, suggesting that tDCs and $T_{regs}$ sit at the "center" of this anti-inflammatory program and that IL-10 and TGF-β, not blockade of NF-κB signaling, facilitate bacterial growth and survival through diminution of neutrophil and MΦ antimicrobial effector functions.

*Ft* LVS and SchuS4 are capable of inducing NF-κB signaling, which directly stimulates the release of IL-10 and TGF-β in vitro and/or in vivo and does so in a strictly TLR2-dependent manner. A variety of cell types (i.e., neutrophils, MΦ, DCs, and $CD3^+$ T cells) are induced to express these anti-inflammatory cytokines during the course of infection, whereas proinflammatory cytokines, such as TNF, IL-β, and IL-6, are only produced during late-phase disease or in vitro in response to MHB-grown (i.e., non-HAd) *Ft*. The restriction on secretion of $T_H1$-type proinflammatory cytokines is a result of adaptation of *Ft* LVS and SchuS4 (data not shown) to its mammalian environment and perhaps the bacterium's capacity to evoke the effector functions of tDCs and $T_{regs}$. *Ft*-activated $CD11c^{high}CD11b^{low}$ cells, observed during early- and late-phase disease, express CD103 and/or CCR9 (markers associated with tDCs [31, 32]) and are positive by intracytoplasmic staining for IL-10 and TGF-β. Although the exact origin and nature of these tDCs remain unknown, $CD103^+$ [32] and $CCR9^+$ [31] subsets have a demonstrated capacity to promote development of $T_{regs}$. To our knowledge, this is the first study to identify tDCs within an infectious disease model and to implicate their release of IL-10 and/or TGF-β in facilitating bacterial growth and survival.

A number of studies have reported that tDCs influence the expansion of $T_{regs}$ through secretion of TGF-β and/or engagement of inhibitory receptors [33–36]. During early tularemic infection of the lung, given the juxtaposition of

**TABLE 2. Total Number of Cells from Whole Lung Expressing Pro- and/or Anti-Inflammatory Cytokines during Early- and Late-Phase Respiratory Tularemia**

| | Surface marker | | | IL-10 | | | TGF-β | | |
|---|---|---|---|---|---|---|---|---|---|
| Cells | Control | Day 2 | Day 6 | Control | Day 2 | Day 6 | Control | Day 2 | Day 6 |
| $CD11c^{high}$ $CD11b^{low}$ $CD103^{+}$ | 7182 ± 1897 | 41,777 ± 3243[a] | 216,990 ± 15,645[b] | 185 ± 64 | 3250 ± 980 | 20,885 ± 3641[b] | 821 ± 178 | 6949 ± 1184 | 119,650 ± 14,815[b] |
| $CD11c^{high}$ $CD11b^{low}$ $CCR9^{+}$ | 11,765 ± 1535 | 43,656 ± 4994[a] | 209,001 ± 15,285[b] | 231 ± 70 | 9927 ± 4102[a] | 44,011 ± 5582[b] | 1719 ± 395 | 23,069 ± 4692[a] | 103,137 ± 13,570[b] |

[a] $P < 0.05$; [b] $P < 0.001$.

elevated levels of TGF-β and an increased frequency of tDCs, it is possible that resident, naïve $CD4^{+}$ T cells are driven to acquire phenotypic characteristics of $T_{regs}$. Alternatively, the $T_{regs}$ observed in the lung may have been generated "off-site" and then recruited to the inflammatory focus, a possibility that currently is under investigation. Regardless of their origin, $T_{regs}$ are found in the lungs of *Ft*-infected mice as early as 24 h PI, where they persist and actually increase in total number during the course of disease. Although not tested in the present study, evidence in the literature shows that $T_{regs}$ are also an important source of IL-10 and TGF-β [37]. Relevant to *Ft* burden in tissues and early tularemia pathogenesis, it is important to note that a wide range of pathogens, including bacteria [35, 38], viruses [39, 40], and helminthic parasites [41], promotes their own survival by activating DCs, which facilitates induction of $T_{regs}$ and limits production of proinflammatory cytokines [42]. In so doing, the anti-inflammatory milieu, within which pathogens can proliferate, is augmented and perpetuated.

Despite the generally anti-inflammatory character of the lung for the first 72 h, recent studies report that by the 3rd day, IL-17A is detectable in the BAL fluid of *Ft* LVS-infected mice [6, 7]. IL-17A plays a role in host defense against *Ft* [6, 7], as well as other microbial pathogens [43]. Unlike with *Klebsiella pneumoniae* infection, wherein IL-17A not only enhances neutrophil migration but also production of IL-6 [44], the rapid production of IL-17A (within 24 h) in response to *Ft* reported herein is not associated with induction of IL-6. In fact, the lack of IL-6 (along with TNF and IL-1β) is what may undermine the effectiveness of innate immunity to *Ft*. This also likely explains why IL-17A does not play a profound role in controlling the growth of *Ft* until Day 4 PI [6] (when $T_H1$-type proinflammatory cytokines are produced), and its inhibition decreases cumulative survival but does not shorten the mean time-to-death of mice [7].

As examples of how the findings presented herein might facilitate evaluation and in some cases, re-evaluation of the existing literature, it is instructive to focus attention on the consequences of using non-HAd *Ft* for in vitro cell-based studies [12, 13, 15, 16], mutation of *sodB* [45] and required for intracellular proliferation factor A (*ripA*) in *Ft* [8, 46], and antibody-based inhibition of TGF-β during infection with *Ft* SchuS4 [4]. To begin, irrespective of growth conditions (i.e., use of MHB or BHI broth or replication within MΦ), *Ft* LVS and/or SchuS4 have the capacity to trigger TLR2-dependent, NF-κB-mediated cytokine production. What distinguishes the cellular response is whether a combination of pro- and anti-inflammatory cytokines (as is the case with non-HAd bacteria) or only anti-inflammatory cytokines (as is the case with HAd bacteria) is produced. Contrary to evidence provided by Melillo et al. [15] and Medina et al. [16], using non-HAd-*Ft*, BHI- and MΦ-grown bacteria neither inhibit nor enhance PI3K signaling as a means of ablating cytokine release from host cells. The observation that NK-κB-dependent IL-10 and/or TGF-β are produced in vitro and in vivo following infection lends support to this notion. Thus, signaling cascades, purportedly initiated or blocked by non-HAd-*Ft*, appear not to be physiologically relevant to tularemia pathogenesis. Similarly, despite the ability of BHI-grown *Ft* to temper the release of TNF from MΦ in response to purified TLR4 and TLR2 agonists (an effect whose physiological relevance needs to be determined), HAd organisms exhibited a minimal capacity to induce SOCS1 and SOCS3 (well-established negative regulators of NF-κB signaling). HAd-*Ft* also appeared not to mediate a cytokine-modulatory effect through the PI3K/AKT/p38-MAPK cascade or induction of an alternative activation program in MΦ. Again, published literature offering tolerization, modulation of PI3K activity, or alternative activation as an explanation for the complete absence of $T_H1$-type proinflammatory cytokines in in vitro cell-based assays and early during tularemic infection is based on use of non-HAd-*Ft*.

When considering the course of infection initiated by inoculation of mice with *Ft* genetically deficient for SodB, these mutants elicit a strong proinflammatory cytokine response from mouse and/or human MΦ (data not shown), replicate to lower numbers in infected tissues, and display a highly attenuated virulence phenotype in mouse survival studies [45]. Likewise, mutants lacking the ability to express RipA stimulate production of TNF, IL-1β, and IL-18 in vitro and in vivo and are limited in their capacity to replicate within infected tissues [8]. In light of the present study's findings, imbuing WT *Ft* with the ability to block NF-κB signaling through the action of SodB and/or RipA is less well-

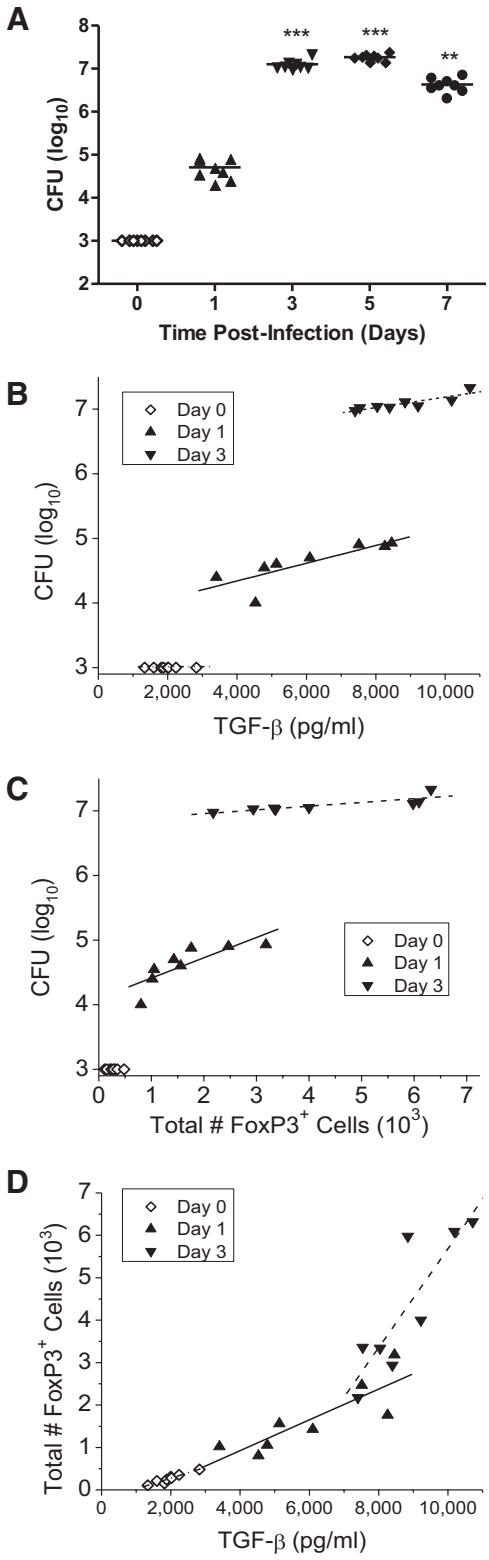


**Figure 8. Levels of TGF-$\beta$, numbers of pulmonary $T_{regs}$, and *Ft* burden are interrelated features of early tularemia pathogenesis.** (A) Four groups of C57BL/6 mice were i.n.-infected with $1 \times 10^3$ CFU of BHI-grown *Ft* LVS and killed at Days 1, 3, 5, and 7 PI. One group of sham-inoculated mice served as a control. Bacterial burden was determined by colony plating and presented as $\log_{10}$ CFU. (B–D) Points represent individual animals, and lines are linear regression fits to data for each day. (B) The bacterial burden is positively correlated with TGF-$\beta$ levels on Days 1 [$r^2=0.67$; $P=0.013$; $\log(CFUs)=3.79+137(10^{-6})\times$TGF-$\beta$] and 3 [$r^2=0.77$; $P<0.01$; $\log(CFUs)=6.37+82(10^{-6})\times$TGF-$\beta$]. (C) The bacterial burden is positively correlated with numbers of $T_{regs}$ on Days 1 [$r^2=0.65$; $P=0.02$; $\log(CFUs)=4.10+314(10^{-6})\times T_{regs}$] and 3 [$r^2=0.71$; $P<0.01$; $\log(CFUs)=6.84+58(10^{-6})\times T_{regs}$]. (D) Numbers of $T_{regs}$ are positively correlated with TGF-$\beta$ levels at Days 0 ($r^2=0.91$; $P<0.001$; $T_{regs}=-231+0.254\times$TGF-$\beta$), 1 ($r^2=0.71$; $P<0.01$; $T_{regs}=-524+0.362\times$TGF-$\beta$), and 3 ($r^2=0.75$; $P<0.01$; $T_{regs}=-5982+1.17\times$TGF-$\beta$).

supported by evidence than the interpretation that these mutants are less able to establish an anti-inflammatory environment (i.e., typified by the presence of tDCs and $T_{regs}$ and secretion of IL-10 and TGF-$\beta$) than their WT counterparts. In fact, a variety of other *Ft* mutants (i.e., *katG* [15], *iglC* [13], *pyrF* [47], *tolC* [48], *flmF2* and *flmK* [49], and *mglA* [18]) are known to be more proinflammatory than their WT counterparts; it is unlikely that all of these genes encode immune-suppressive products that block NF-$\kappa$B signaling. In many instances, the proteins encoded are internal components of the bacterium, so how they can impede signaling events in the host cell's cytosol remains to be explained and demonstrated in a well-controlled manner.

Finally, an elegant study performed by Bosio and coworkers [4] showed that respiratory infection of mice with 50 CFU of SchuS4 stimulated local and systemic release of TGF-$\beta$ and no production of TNF and was associated with a dramatic four-log increase in bacterial growth within the first 72 h of infection (as was reported here using LVS). Upon treatment of infected mice with anti-TGF-$\beta$ antibodies, an increase in TNF levels was inversely associated with decreased bacterial burden in the lung [4]. This relationship between pro- and anti-inflammatory cytokine production and bacterial growth and survival of SchuS4 is entirely consistent with the linear relationship among TGF-$\beta$, $T_{regs}$, and the tissue burden of *Ft* LVS, demonstrated in the present study. In fact, preliminary evidence from our lab suggests that administration of anti-TGF-$\beta$ antibodies to HAd-*Ft* LVS-infected mice also significantly lowers the burden of bacteria in the lung (data not shown).

Collectively, the findings described above lend considerable support to the hypothesis that instead of blocking NF-$\kappa$B signaling, *Ft* triggers NF-$\kappa$B-dependent development and activation of tDCs and $T_{regs}$ as a potential means of restraining $T_H1$-type proinflammatory cytokine production early in the infectious disease process. By doing so, *Ft* establishes an anti-inflammatory milieu, in which to replicate unencumbered by potent antimicrobial innate immune responses. This body of work should stimulate re-evaluation of the field's understanding of mechanisms underlying *Ft*-host cell interactions as they relate to tularemia pathogenesis.

## AUTHORSHIP

S.P., A.S., B.S., G.H.P., P.F.J., and T.R., performed research and analyzed data. S.P., A.S., B.S., E.J.G., and T.J.S. designed the research. S.P. and T.J.S. wrote the paper.

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