Exogenous Gamma and Alpha/Beta Interferon Rescues Human Macrophages from Cell Death Induced by *Bacillus anthracis*

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During the recent bioterrorism-related outbreaks, inhalational anthrax had a 45% mortality in spite of appropriate antimicrobial therapy, underscoring the need for better adjuvant therapies. The variable latency between exposure and development of disease suggests an important role for the host's innate immune response. Alveolar macrophages are likely the first immune cells exposed to inhalational anthrax, and the interferon (IFN) response of these cells comprises an important arm of the host innate immune response to intracellular infection with *Bacillus anthracis*. Furthermore, IFNs have been used as immunoadjuvants for treatment of another intracellular pathogen, *Mycobacterium tuberculosis*. We established a model of *B. anthracis* infection with the Sterne strain (34F₂) which contains lethal toxin (LeTx). 34F₂ was lethal to murine and human macrophages. Treatment with IFNs significantly improved cell viability and reduced the number of germinated intracellular spores. Infection with 34F₂ failed to induce the latent transcription factors signal transducer and activators of transcription 1 (STAT1) and ISGF-3, which are central to the IFN response. Furthermore, 34F₂ reduced STAT1 activation in response to exogenous alpha/beta IFN, suggesting direct inhibition of IFN signaling. Even though 34F₂ has LeTx, there was no mitogen-activated protein kinase kinase 3 cleavage and p38 was normally induced, suggesting that these early effects of *B. anthracis* infection in macrophages are independent of LeTx. These data suggest an important role for both IFNs in the control of *B. anthracis* and the potential benefit of using exogenous IFN as an immunoadjuvant therapy.

Bacillus anthracis, a gram-positive, aerobic, spore-forming bacillus, is found ubiquitously in animals and soil. It causes a wide array of diseases in humans depending on the site of entry (31). When inhaled, *B. anthracis* causes mediastinal hemorrhage, pneumonia, and sepsis, with a high mortality in spite of appropriate therapy (23). Routine vaccination of animals and humans in animal husbandry had virtually eliminated this disease in the United States. Recently, anthrax gained renewed attention as a biowarfare agent. During the terrorist attacks of 2001, 11 people contracted inhalation anthrax, 11 contracted cutaneous anthrax, and hundreds of individuals were exposed to potentially dangerous levels of spores (23). In spite of widespread use of appropriate antibiotics, inhalation anthrax had a 45% mortality rate, underscoring the need for better adjuvant therapies in case of future attacks (23).

Virulence of *B. anthracis* is determined in part by its two megaplasmids, pXO1 and pXO2. pXO1, which is required for virulence, codes for the three components of *B. anthracis* toxins, lethal factor (LF), edema factor, and protective antigen. The majority of information learned about *B. anthracis* lethality has come from studies of lethal toxin (LeTx), a multimer of protective antigen and LF. LeTx is a zinc metallopeptidase which is highly lethal to resident macrophages. A major function of LeTx is proteolytic cleavage of mitogen-activated protein kinase kinase (MKK) family members in vitro and in vivo,

resulting in defective p38 and extracellular signal-regulated kinase phosphorylation (11, 38). One consequence of this inhibition is attenuation of the host innate immune response. Cells treated with sublytic doses of LeTx have attenuated proinflammatory cytokine production in response to bacterial stimuli including LPS and *B. anthracis* cell wall components (12, 38, 40). However, the majority of data regarding LeTx is derived from studies with recombinant proteins administered to murine macrophages in vitro (12, 38, 40). Furthermore, while high levels of LeTx are observed during the late stages of infection, less is known about the extent of activity of LeTx during the early stages of infection with *B. anthracis* spores.

During the initial stages of inhalational anthrax, *B. anthracis* spores are taken up by alveolar macrophages (AM). Spores are able to survive in the phagolysosome and proceed to replicate intracellularly (22). This results in macrophage lysis and release of viable bacteria into the extracellular space. However, the time course for this is highly variable. Humans may not develop systemic disease until 43 days after exposure (30). Furthermore, viable spores have been found in mediastinal lymph nodes of infected monkeys up to 100 days after infection (21). Consequently, there may be numerous mechanisms important for control and destruction of intracellular spores.

The innate immune response is the primary means of pathogen control during the initial stages of infection. The interferon (IFN) system is an important component of innate immunity. There are two broad categories of IFN, alpha/beta IFN (IFN- α/β) and gamma IFN (IFN- γ). All IFNs signal via the janus kinase (JAK) and/or TYK kinases which phosphorylate and activate latent signal transducer and activators of transcription

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(STAT). Specifically, IFN- γ leads to phosphorylation of STAT1 and formation of STAT1 homodimers that translocate to the nucleus and induce transcription by binding to gamma-activated sequences (GAS) in promoters of genes in the IFN response (47). IFN- α/β leads to phosphorylation of STAT1 and STAT2, which then bind with IRF-9 to form ISGF-3 (13, 47). This heterotrimer translocates to the nucleus and binds ISRE sequences in promoters of IFN-responsive genes. Both IFN- α/β and - γ responses induce a large set of genes including a number of genes with antibacterial activity, including the inducible nitric oxide (NO) synthase gene (35). In addition, there are numerous JAK-STAT-independent mechanisms for both IFN signaling and production, including the p38 mitogen-activated protein kinase cascade (16, 28, 50).

IFN- α/β was originally described in viral infections and plays a prominent role in inhibiting viral infection (26, 27, 43). However, IFN- α/β is also involved during the initial stages of infection with intracellular pathogens. Macrophages infected with *Mycobacterium tuberculosis* induce an IFN- α/β response within hours of infection (44, 52). There have been two trials of aerosolized IFN- α as an immunoadjuvant for pulmonary tuberculosis in humans (14). Treated subjects demonstrated earlier resolution of sputum cultures, earlier radiologic improvement, and reductions in cytokine levels in bronchoalveolar lavage fluid. Similar results were obtained in subjects with multidrug-resistant tuberculosis (15, 37).

In contrast, IFN- γ is more prominent in the generation of Th-1 immunity. However, there is significant overlap in the activities of both IFN- α/β and - γ . IFN- γ receptor knockout mice are highly susceptible to mycobacterial infection, as well as infection with other intracellular pathogens, including *M. tuberculosis* and *Listeria monocytogenes* (1, 6, 20, 29, 47). Furthermore, humans with congenital defects in IFN- γ signaling are hypersusceptible to mycobacterial infections (32). As for IFN- α/β , we have previously reported use of aerosolized IFN- γ for the treatment of pulmonary tuberculosis. Treatment results in improved radiologic appearance, faster clearance of sputum cultures and activation of STAT1 in AM from treated subjects (4, 5).

Few data exist on the interaction between *B. anthracis* and the IFN system. Based on work with other pathogens, the intracellular nature of *B. anthracis* suggests a vital role for IFN- α/β and - γ . Furthermore, the ability of *B. anthracis*-derived toxins to modulate the host immune response suggests that *B. anthracis*, similar to other pathogens, may be capable of disrupting IFN signaling. Consequently, we sought to establish a *B. anthracis* infection model in human macrophages to determine whether exogenous IFN could affect cell survival. In addition, we wished to assess the ability of a LeTx⁺ strain of *B. anthracis* to inhibit IFN signaling.

MATERIALS AND METHODS

Cells. THP-1 cells (American Type Culture Collection) at a concentration of 5×10^5 /ml were differentiated with 20 nM 12-*O*-tetradecanoylphorbol 13-acetate (PMA) in Dulbecco's modified Eagle medium (DMEM) (BioWhittaker) with 10% fetal calf serum (FCS) (Life Technologies). Human AM were prepared as previously described (4, 52). Briefly, healthy volunteers underwent bronchoscopy with bronchoalveolar lavage with 100 ml of saline. Fluid was filtered to remove debris. Cells were spun at $800 \times g$ for 5 min and resuspended in DMEM plus 10% fetal calf serum (FCS) at 5×10^5 /ml.

Murine peritoneal macrophages (PM) were obtained as previously described.

Briefly BALB/c mice were injected with 3% Brewer's thioglycolate (Sigma) for 3 to 5 days, after which mice were euthanized with CO₂ and the peritoneal cavity of each mouse was lavaged with 10 ml of sterile saline. This results in >95% macrophages. Cells were washed and resuspended in DMEM-10% FCS at 5×10^5 /ml.

Preparation of *B. anthracis* spores. The Sterne strain of *B. anthracis* spores (34F₂; Colorado Serum Company), 9131 (pXO1⁺ pXO2⁻; Institut Pasteur), and RPL686 (pXO1⁺ pXO2⁻, Δ 686; Institut Pasteur) were prepared as previously described. Briefly, spores were allowed to germinate overnight at 37°C in phage assay broth (8 mg of Difco nutrient broth, 0.15 mg of CaCl₂, 0.2 mg of MgSO₄, 0.05 mg of MnSO₄, 5 mg of NaCl, 10% horse serum). Flasks were then incubated at 30°C for 3 to 5 days. Spores were spun down and washed with sterile H₂O four times. Spores were resuspended in sterile H₂O and heat treated at 65°C for 30 min to kill any vegetative spores. Remaining spores were spun down and washed two times in sterile water and resuspended in sterile H₂O at a concentration of 10^8 /ml. Concentration was determined by quantitative cultures.

Infection and cell viability assays. Aliquots (100 μ l) of macrophage suspension (prepared as described above) were plated in 96-well plates in DMEM plus 10% FCS (antibiotic free). *B. anthracis* spores were added for 30 min at the designated multiplicity of infection (MOI), after which time gentamicin (2.5 μ g/ml; Gibco) was added to each well to kill any vegetative spores as in previously described protocols (10, 18). For experiments involving IFN, human recombinant IFN- γ (Actimmune) or IFN- β (R & D Systems) was added at the time of infection for the specified times and doses. Cell viability was determined by WST-1 assay (Roche) according to the manufacturer's instruction. Each experimental condition was run with five replicates. For trypan blue dye exclusion, cells were stained with 0.2% trypan blue and a total of 200 cells were counted. For quantitative cultures, cells were lysed with 0.1% Triton X-100 (Sigma) and 10- μ l aliquots of lysates in serial dilutions were plated on blood agar plates at 37°C.

Transcription factor analysis. For analysis of transcription factor activity and cytokine production, experiments were repeated in 2.5-cm-diameter culture dishes (Falcon). Supernatant was collected at the designated time points, centrifuged at $800 \times g$ for 10 min to remove cellular debris, and stored at -70°C till analysis. Cells were harvested and lysed as previously described (19, 52). Briefly, cells were harvested by gentle scraping and lysed in NP-40 lysis buffer (0.5% NP-40, 10% glycerol, 0.1 mM EDTA, 20 mM HEPES [pH 7.9], 10 mM NaF, 10 mM NaP_i, 300 mM NaCl, aprotinin [3 μ g/ml], leupeptin [2 μ g/ml], pepstatin [2 μ g/ml], 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na₃VO₄) on ice for 30 min with vigorous agitation. Cells were centrifuged at $10,000 \times g$ for 15 min, and supernatants were removed and stored for future analysis.

Immunoblots. Antibodies for p^{tyr701}-STAT1, STAT1, p-p38, and total p38 were obtained from Cell Signal Technologies. Anti-MKK3 (C terminus) was obtained from Santa Cruz. Antibody for p^{ser727}-STAT1 was graciously provided by David Levy. For p^{ser727}-STAT1 immunoblots, cell lysates were first immunoprecipitated for STAT1. Briefly, equal amounts of protein (30 to 50 μ g) were loaded onto a 10% polyacrylamide gel. After transfer to polyvinylidene difluoride, membranes were blocked in phosphate-buffered saline with 0.1% Tween and 5% milk, followed by overnight incubation at 4°C with primary antibody (anti-phospho-STAT1 or anti-STAT1 [Cell Signal Technology]). Membranes were developed with ECL Plus (Amersham). Images were scanned with a densitometer and bands quantified with ImageQuant software (Molecular Dynamics, Sunnyvale, Calif.).

Electrophoretic mobility shift assay (EMSA) for GAS and ISRE was performed according to the previously described protocol (4, 19, 25, 52). The GAS probe (5'-TACAACAGCCTGATTTCCCGAAATGACGGC-3') and ISRE probe (5'-CTCGGAAAGGGAACCGAAACTGAAGCC-3') (the consensus binding region is indicated in boldface) were labeled with [α -³²P]dCTP using Klenow DNA polymerase fill-in reaction. Full-length oligonucleotides were incubated with 10 μ g of protein, 2.5 μ g of poly(dI:dC), and gel shift buffer and run on a 4% polyacrylamide gel (Bio-Rad). For supershift reactions 1 μ g of anti-STAT1 or anti-STAT2 (Cell Signal Technologies) was added to the mixture. Images were produced by a phosphorimager, and images were quantified with ImageQuant software.

RESULTS

Attenuated strains of *B. anthracis* are lethal to human macrophages. Previous reports demonstrate that the Sterne strain is lethal to murine macrophages (10, 18). We sought to extend these findings using transformed and primary human macro-

FIG. 1. Murine and human macrophages have differential sensitivities to different strains of *B. anthracis*. Murine PM (A) or PMA-differentiated THP-1 cells (B) were incubated with the Sterne strain (34F₂), RPL686, or 9131 at an MOI of 20:1 for 4.5 h. Viability, relative to uninfected controls, was determined by WST-1 assay. Results represent three to five replicates. Error bars, standard errors of the means.

phages. This laboratory has observed that THP-1 cells differentiated with phorbol ester are an accurate model for human AM. Similar to previous investigators, we found that 34F₂ reduced cell viability in murine PM in a dose-dependent manner, with a 30% reduction in viability at 4.5 h at an MOI of 20:1 (Fig. 1A) (10, 18). Furthermore, the Sterne variants RPL686, which has a point mutation in LF (pXO1⁺ pXO2⁻ LF⁻), and 9131, which lacks both virulence plasmids (pXO1⁻ pXO2⁻), had little effect on viability at this MOI (Fig. 1A). PMA-differentiated THP-1 cells had a similar response to infection with 34F₂ (Fig. 1B). However, unlike murine cells, THP-1 cells were also susceptible to RPL686 (pXO1⁺ pXO2⁻ LF⁻) at an MOI of 20:1 (Fig. 1B). Furthermore, there was a 20% reduction in viability in response to 9131 (pXO1⁻, pXO2⁻) compared to controls. Together, these results suggest differences between murine and human models of infections. As a result we conducted all subsequent experiments with human macrophages.

Exogenous IFN- α/β and γ rescue macrophages from *B. anthracis*-induced lethality. Since IFN signaling may be disrupted by *B. anthracis* (38) we sought to determine if exogenous IFN could rescue cells from the lethality of *B. anthracis*. Human AM obtained from healthy volunteers and THP-1 macrophages showed similar mortalities after infection with 34F₂. Treatment with IFN- γ significantly improved cell viability, with a maximal effect at 400 U/ml ($P = 0.009$) (Fig. 2A). Similar results were obtained with IFN- β at 1,000 U/ml ($P = 0.03$) (Fig. 2B). Results were confirmed with trypan blue dye exclusion (data not shown). IFN treatment also reduced mortality in *B. anthracis*-infected human peripheral blood mononuclear cells (data not shown). Together these data suggest that exogenous IFN- α/β and γ improve cell viability during *B. anthracis* infection.

We next sought to determine if IFN affected intracellular *B. anthracis* viability. Treatment with either IFN- γ (140% of control) or IFN- β (88% of control) had no effect on the total number of spores recovered from human AM at 4.5 h compared to untreated controls. Furthermore, neither IFN- α/β nor IFN- γ significantly affected the number of spores recovered at 30 min, indicating that IFN had little effect on phagocytosis (data not shown). The absence of a treatment effect was not

FIG. 2. Exogenous IFN- α/β or γ improves viability in *B. anthracis*-infected macrophages. Human AM were incubated with the Sterne strain (34F₂) with either IFN- β at 1,000 U/ml (A), IFN- γ at 400 U/ml (B), or saline at an MOI of 20:1 for 4.5 h. Viability was determined by WST-1 assay. Results are expressed as changes in viability relative to uninfected controls. Results represent 10 to 15 replicates. Error bars, standard deviations. ★, $P = 0.009$; ♦, $P = 0.03$.

surprising given the resistance of spores to killing by a number of noxious stimuli such as high temperature. Once spores are germinated, however, vegetative bacteria are no longer resistant to killing by heat. Therefore, incubation of infected macrophage lysates at 65°C will kill germinated bacteria but not ungerminated spores. IFN- γ and IFN- β reduced the fraction

FIG. 3. Exogenous IFN- α/β or γ reduces the number of germinated intracellular *B. anthracis* bacteria. Human AM or PMA-differentiated THP-1 macrophages were incubated with the Sterne strain (34F₂) with either IFN- β at 1,000 U/ml, IFN- γ at 400 U/ml, or saline at an MOI of 20:1 for 4.5 h. Cells were lysed, and quantitative cultures were performed on lysate with or without heat treatment (65°C for 30 min). The fraction of germinated spores is the differential between culture results from non-heat-treated (total spores) and heat-treated lysate (ungerminated spores). Data represent three separate experiments. Error bars, standard deviations. *, $P = 0.07$.

FIG. 4. *B. anthracis* inhibits IFN- α/β signaling. Human macrophages were incubated with the Sterne strain (34F₂) with either IFN- β at 1,000 U/ml, IFN- γ at 400 U/ml, or saline at an MOI of 20:1 for 4.5 h. (A) Immunoblot for p^{tyr701}-STAT1 and total STAT1. Infection of human AM with 34F₂ failed to induce p^{tyr701}-STAT1 at 4.5 h (compare lanes 1 and 4) and reduced the degree of STAT1 phosphorylation by IFN- β twofold compared to uninfected IFN-treated controls (compare lanes 3 and 6). 34F₂ had a minimal effect on IFN- γ -induced p^{tyr701}-STAT1 formation (compare lanes 2 and 5). (B) Immunoblot for p^{ser727}-STAT1 and total STAT1 from STAT1-immunoprecipitated cell lysates. Infection of human THP-1 macrophages with 34F₂ failed to induce p^{ser727}-STAT1 at 4.5 h (compare lanes 1 and 4) and reduced the degree of STAT1 phosphorylation by IFN- β twofold compared to uninfected IFN-treated controls (compare lanes 3 and 6). 34F₂ had a minimal effect on IFN- γ -induced p^{ser727}-STAT1 formation (compare lanes 2 and 5). (C) EMSA for ISGF-3. Infection of human AM with 34F₂ failed to induce ISGF-3 DNA binding at 4.5 h (compare lanes 1 and 3) and inhibited IFN- β -induced ISGF-3 production (compare lanes 2 and 4). This band was confirmed to be ISGF-3 in a separate experiment by both cold competition (lane 6) and supershift with antibodies to STAT1, STAT2, and IRF-9 (lanes 7 to 9). (D) EMSA for STAT1 homodimer (GAS binding). IFN- γ induced STAT1 homodimer formation (lane 2), which was unaffected by infection of human AM with 34F₂ (lane 5). This complex was supershifted with STAT1 antibody (lane 3). In all cases lanes were normalized for total protein.

of germinated intracellular bacteria ($P = 0.07$ for IFN- β) (Fig. 3). Similar results were obtained from THP-1 macrophages (data not shown). These data suggest that exogenous IFN are capable of increasing intracellular killing of germinated bacteria.

Effect of *B. anthracis* on IFN signaling. We next wished to determine the effect of *B. anthracis* infection on IFN signaling. We used immunoblots probed with an antibody specific for phosphorylated STAT1 (p^{tyr701}-STAT1) as a marker of IFN activity (47). Unlike *M. tuberculosis*, *B. anthracis* infection failed to induce p^{tyr701}-STAT1 in human AM at 4.5 h (Fig. 4A, compare lanes 1 and 4) (44, 52). In contrast IFN- β and IFN- γ induced significant p^{tyr701}-STAT1 (Fig. 4A, compare lane 1 with lanes 2 and 3). Infection with 34F₂ inhibited the effect of IFN- β on STAT1 phosphorylation in AM twofold (Fig. 4A, compare lanes 3 and 6). However, levels of p^{tyr701}-STAT1 in IFN- β -treated, 34F₂-infected AM were still greater than unstimulated controls. In contrast, 34F₂ had minimal effect on IFN- γ -induced p^{tyr701}-STAT1 formation (Fig. 4A, lanes 2 and 5). The alteration in p^{tyr701}-STAT1 was not due to changes in the amount of total STAT1. Similar results were obtained when probing for serine phosphorylation of STAT1 (p^{ser727}-

STAT1) (Fig. 4B). Similar data were obtained with murine PM (data not shown). These data suggest that *B. anthracis* inhibits IFN- α/β signaling.

To confirm the results of the immunoblot, we used EMSA with an ISRE containing oligonucleotide to determine whether the changes in p^{tyr701}-STAT1 produced alterations in DNA binding activity of ISGF-3, the heterotrimer of STAT1, STAT2, and IRF-9. As predicted by the results of the immunoblot, infection with 34F₂ inhibited ISGF-3 formation in response to IFN- β (Fig. 4C, lanes 2 and 4). Also, infected AM failed to induce ISGF-3 at 4.5 h (Fig. 4C, lane 3) or at 20 h (data not shown). The identity of ISGF-3 was confirmed by both cold competition (Fig. 4C, lane 6) and supershift with antibodies to STAT1, STAT2, and IRF-9 (Fig. 4C, lanes 7 to 9). To assay the IFN- γ effect, EMSA was performed with oligonucleotide containing a GAS sequence. STAT1 homodimer formation in response to IFN- γ was unaffected by infection (Fig. 4D, lanes 2 and 5). Supershift with STAT1 antibody (Fig. 4D, lane 3) and cold competition with GAS oligonucleotide (data not shown) confirmed the identity of this DNA-protein complex. Together, these data suggest that in-

FIG. 5. Infection with *B. anthracis* is not associated with MKK3 cleavage at 4.5 h. Human AM were incubated with the Sterne strain (34F₂) at an MOI of 20:1 for 4.5 h. (A) Immunoblot for MKK3. Infection with 34F₂ is associated with increased MKK3. (B) Immunoblot for phosphorylated and total p38. Infection with 34F₂ is associated with increased p38 phosphorylation. All lanes were normalized for protein.

fection with the Sterne strain is capable of inhibition of IFN- α/β but not IFN- γ signaling.

We next sought to determine whether LeTx was active at this early point in infection. The main target of LeTx in macrophages is the MKK family members including MKK3 (38). LeTx cleaves and inactivates MKK-3. Formation of MKK-3 cleavage products is highly sensitive for LeTx activity (11, 38). Infection of AM with 34F₂ (LF⁺) did not produce MKK3 cleavage. In fact, there was an increase in total MKK3 after infection with 34F₂ (Fig. 5A). Similar results were obtained with murine PM (data not shown). The principal target of MKK3 is phosphorylation of p38 (p-p38). As predicted from the increased MKK-3, infection produced a threefold increase in p38 phosphorylation (Fig. 5B). In all cases lanes were normalized for total protein and there was no change in total p38. Together these data indicate that the metalloproteinase activity of LeTx is not active at these early time points and therefore 34F₂ kills AM and inhibits IFN signaling by other mechanisms.

DISCUSSION

In this study, we demonstrated that the Sterne strain of *B. anthracis* is lethal to human AM. This lethality can be modulated by both exogenous IFN- α/β and IFN- γ , possibly by reducing the number of germinated intracellular bacteria. Infection with *B. anthracis* also fails to induce an IFN- α/β response, as evidenced by defective p^{tyr701}-STAT1 and p^{ser727}-STAT1 induction and subsequent ISGF-3 formation. It is likely that this is in part due to inhibition of IFN- α/β signaling by *B. anthracis*. This inhibition of IFN signaling is not due to LeTx metalloproteinase activity, suggesting a LeTx-independent mechanism for inhibiting the innate immune response in macrophages.

Currently few data exist on the interaction between *B. anthracis* and human AM, the primary target of inhalational anthrax. Our data suggest that human macrophages and mu-

rine PM are equally susceptible to infection with the Sterne strain of *B. anthracis*. The similar responses of primary human AM and THP-1 macrophages reconfirm our previous observation that THP-1 macrophages provide a good model for human AM (52). The lack of susceptibility of murine PM to other attenuated strains of *B. anthracis*, 9131 and RPL686, is in accord with previous investigations (18). Surprisingly, these strains were still lethal to human macrophages, suggesting some interspecies differences in response to *B. anthracis*. This underscores the importance of confirming existing murine data in human macrophages.

The ability of both IFN- α/β and IFN- γ to rescue cells from the lethality of *B. anthracis* is surprising. As viability data were normalized for IFN-treated, noninfected cells, this effect is not due to an independent effect of IFN on viability. While IFN did improve macrophage viability, it is unclear whether this is due to changes in apoptosis, lysis or both. As both *B. anthracis* LeTx and IFN themselves are capable of inducing apoptosis, this needs to be addressed by further studies (2, 9, 10, 17, 41).

While the mechanism by which IFN improves cell viability remains unclear, one possibility is that IFN reduces the intracellular burden of *B. anthracis*. Our data suggest that IFNs are capable of inhibiting intracellular germination of *B. anthracis*. As spore germination is required for production of toxins and other proteins required for cell death, this could certainly explain the improvement in macrophage viability. The mechanism by which IFN impairs germination remains unclear. One possibility is increased activation of the host innate immune response. IFN- α/β and IFN- γ increase production of NO and reactive oxygen species which are essential to the host control of intracellular pathogens including *M. tuberculosis* (3, 8, 35). Conversely, IFN could directly inhibit bacterial germination and/or growth factors. The recent identification of *gerS* and *atxA1* as pivotal for germination and bacterial release, respectively, provides additional targets for investigation (10, 22).

The protective effect of IFN in vitro suggests that *B. anthracis* may modulate the host IFN response to facilitate its own survival. The lack of p^{ser727}- and p^{tyr701}-STAT1 formation and ISGF-3 DNA binding activity in *B. anthracis*-infected cells indicates that *B. anthracis* inhibits the generation of an IFN- α/β response. The induction of IFN- α/β and subsequent signaling in macrophages are observed during infection with numerous intracellular pathogens, including *M. tuberculosis* and *Bacillus subtilis*, a family member of *B. anthracis*, as early as 3 h after infection (36, 44, 52). The lack of IFN- α/β signaling in our study may be the result of impaired IFN- α/β production and/or inhibition of IFN signaling. However, the ability of *B. anthracis* to attenuate signaling by exogenous IFN- α/β suggests that direct inhibition of IFN signaling is partially responsible for this observation. This observation is not unique. Previous investigators reported that *B. anthracis* LeTx inhibition of IFN- γ induced NO production from murine macrophages although they did not describe the means of this inhibition (38). Furthermore, multiple studies document that *M. tuberculosis* also inhibits IFN- α/β signaling, both at the level of STAT1 phosphorylation and transcriptional activation (42, 49).

The mechanism of *B. anthracis* inhibition of IFN- α/β signaling in our study remains unclear. The finding of impaired p^{tyr701}-STAT1 and p^{ser727}-STAT1 formation suggests that multiple pathways may be affected. Inhibition of IFN- α/β -induced

p^{tyr701}-STAT1 formation suggests inhibition of either the IFN- α/β receptor or the downstream kinases JAK1 or Tyk2. This may be either direct inhibition, the result of upregulation of endogenous inhibitors such as suppressors of cytokine signaling, or increased phosphatase activity (7, 24, 39).

Formation of p^{ser727}-STAT1 is a JAK-independent means of STAT1 activation and is essential for nuclear translocation of STAT1-containing transcription factors and subsequent promoter-enhancing activity (53). The mechanism of p^{ser727}-STAT1 inhibition is also unclear. Studies suggest that inhibition of p38 can abolish p^{ser727}-STAT1 formation in response to IFN- α/β (48). However, the lack of MKK3 cleavage and slight increase in p38 phosphorylation during infection of a LeTx-competent strain of *B. anthracis* suggests that this inhibition is independent of the proteolytic effects of LeTx. This is not surprising, as other investigators have demonstrated that cleavage of MKK family members, a highly sensitive indicator of LeTx activity, is not responsible for LeTx cytotoxicity. Macrophages from LeTx-resistant mice manifest levels of MKK cleavage similar to those manifested by LeTx-susceptible strains, suggesting other biologic effects (46, 51). Furthermore, the vast majority of studies investigating the effects of LeTx on intracellular signaling have utilized recombinant proteins. While more specific for LeTx activity, they utilize levels of toxin which may be manyfold higher than what is observed during the early stages of intracellular infection. This implicates the presence of other non-LeTx-mediated mechanisms of cell death during the early stages of infection with live spores. Together our data suggest that inhibition of p^{ser727}-STAT1 formation in this model lies either distal to p38 activation or involves p38-independent signaling intermediates such as IRAK, MYD88, or phosphatidylinositol 3-kinase (33, 34, 45).

In conclusion, this study provides important data regarding the role of *B. anthracis* infection on human AM, the primary target of inhalational anthrax. By demonstrating that *B. anthracis* is capable of altering IFN signaling in vitro and exogenous IFNs are capable of overcoming this inhibition and rescuing infected macrophages, we now suggest a potential role for exogenous IFN as an immunoadjuvant in vivo.

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