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Distinct Innate Responses are Induced by Attenuated *Salmonella enterica* serovar Typhimurium Mutants

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Abstract

Upon bacterial infection the host cells generate a wide variety of cytokines. Genetic attenuation of bacterial physiological pathogens can be accomplished not only by disruption of normal bacterial processes, but also by the loss of the ability to redirect the host immune system. We examined nine attenuated *Salmonella* Typhimurium mutants for their ability to replicate as well as the cytokines produced after infection of Bone Marrow Derived Macrophages (BMDM). Infection of BMDM with attenuated *Salmonella* mutants led to host cytokine patterns distinct from those that followed WT infection. Surprisingly, each bacterial mutant had a unique cytokine signature. Because some of the mutants induced an IL-10 response not seen in WT, we examined the role of IL-10 on *Salmonella* replication. Surprisingly, addition of IL-10 before or concurrent with infection restricted growth of WT *Salmonella* in BMDM. Bacterial attenuation is not a single process and results in attenuated host responses, which result in unique patterns for each attenuated mutants.

Introduction

Salmonella enterica serovar Typhimurium is a Gram-negative rod shaped bacterium that causes severe gastroenteritis. *Salmonella* infections impose a large global health burden with approximately 200 million to 1 billion cases annually [1]. *Salmonella* exposure poses a significant public health threat and can come from a wide variety of sources including raw eggs, raw meat, and pet feces.

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We are delighted to provide this contribution to the Ray D. Owen issue of Cellular Immunology. We have chosen this contribution since it uses one of Owen's favorite scientific strategies, the use of genetics to probe important immunological questions. Here we combine host genetics with the genetics of *Salmonella* to ask how bacteria and hosts interact and how loss of function mutants in bacteria can result in novel cytokine responses from the host.

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The infectious cycle of *Salmonella* has been well-characterized in mice as well as human cell lines [2-4]. *Salmonella* initially enters the cell using genes encoded on Salmonella Pathogenicity Island-I (SPI-1). Proteins encoded by SPI-1 are recognized by host cells and cellular responses lead to the recruitment of PMNs and activation of host NF κ B [5-8]. *Salmonella* is phagocytosed by the host mucosal macrophages (M cells). The bacteria resides in an acidic membrane enclosed vacuole, the Salmonella Containing Vacuole (SCV). In response to the acidic environment *Salmonella* upregulates genes on the second pathogenicity island (SPI-2) which allow the bacterium to exit the SCV to the cytosol where it replicates [9-13]. Counterintuitively, engagement of host TLRs are critical in upregulation of SPI-2 genes and bacterial replication [14].

While the virulence factors carried on these two pathogenicity islands have been well-characterized, we sought to discover other *Salmonella* encoded genes that are able to actively reshape the host immune responses. This differs from simple immune avoidance mechanisms used by pathogens to avoid destruction such as *Streptococcus pneumoniae* masking antibody targets of the host response by creating a thick polysaccharide coat or *Neisseria* under going phase variation. Here we focus on specific host immune responses that are actively stimulated or blocked by various attenuated *Salmonella* Typhimurium mutants. Attenuation of bacterial pathogens leads to a unique a varied cytokine profiles, not simply more cytokine than wild type infection.

Materials and Methods

Bacterial Strains and Growth Conditions

The strains of *Salmonella* Typhimurium used in this study and their sources are listed in Table 1. *pabA/B*, *hisG/crp*, *phoP/hisG/rpsL*, *sifA*, and *recA* are standard genetic deletions. The *phoP/Q*⁻, *fur*⁻, *crp*⁻, and *recA*⁻ were designed as delayed attenuation mutants where the native promoter has been replaced with an arabinose inducible promoter [15-19]. All strains were grown in either Luria Broth (LB) or Purple Broth (PB) (Sigma-Aldrich) with arabinose as appropriate. Log phase *Salmonella* bacterial cultures were used to infect cell lines. 1mL of log phase culture was measured for OD₆₀₀ and compared to empirically determined standards for each strain. Final inoculation counts were determined by serial dilution and plating on LB agar. For the arabinose controlled promoters *Salmonella* and target cells were grown in the presence or absence of 0.2% arabinose to control gene expression.

Mice

C57BL/6J (B6), C57BL/10ScNJ (TLR4^{-/-}), and B6.129P2-*Il10*^{tm1Cgn}/J (IL-10^{-/-}) mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and then bred in-house. All mice were housed under specific-pathogen-free conditions at the University of Arizona and used in accordance with the Institutional Animal Care and Use Committees.

Cell Lines and Bone Marrow Derived Macrophage Production

J774.1 cells were obtained from ATCC (Manassas, VA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Atlas), L-

glutamine (HyClone), sodium pyruvate (HyClone), and penicillin-streptomycin (Life Technologies). Medium was replaced with antibiotic-free medium 24 h prior to inoculation with *Salmonella*. For production of primary macrophages bone marrow cells were flushed from mouse femurs and incubated for seven days on non-tissue culture-treated 15-cm² dishes with L cell-conditioned medium containing GM-CSF. Following differentiation, non-adherent cells were removed by multiple washes with PBS and bone marrow-derived macrophages were removed from plates by scraping. Cells were then washed and resuspended in Complete DMEM without antibiotics for infection.

***In vitro* growth assays**

A total of 2×10^5 cells/well (BMDM or J774) were seeded into a 96-well plate for *Salmonella* intracellular growth assays and incubated two hours to allow adherence to the plate. In some experiments recombinant murine interleukin-10 (Peprotech) (rIL-10) was added either two hours before or at the time of infection. Cells were inoculated at a range (100-3.125) of multiplicities of infection (MOI) the ratio of bacteria to eucaryotic cells. Infection was facilitated by centrifugation at $300 \times g$ for 5 min. Cells were incubated for one hour with bacteria, and the medium was then removed. Fresh medium containing 50 µg/mL gentamicin (Sigma) was added to kill extracellular bacteria. One hour after gentamicin addition, medium was removed, and the cells were washed twice before the addition of fresh antibiotic free medium. All culture medium used in the arabinose inducible promoter system experiments contain 0.2% arabinose as indicated in the figures. To determine intracellular growth, medium was removed at indicated time points post-infection, and 1 mL of PBS was added to the cells. Cells were removed from the plate by vigorous pipetting. Cells were lysed by vortexing at maximal speed for one minute. Serial 1:10 dilutions of the lysate were made and plated onto LB agar. The resulting colonies were counted 24 hours later.

Luminex Analysis

Cytokines/chemokines in culture supernatant were measured using a 20-analyte Luminex bead-based approach according to the manufacturer's protocol (Invitrogen), using a BioPlex 100/200 array reader (Bio-Rad Laboratories). Using integrated cytokine/chemokine standard curves, the assay reports the following mouse analytes in pg/mL: fibroblast growth factor (FGF) basic, granulocyte-macrophage colony-stimulating factor (GM-CSF), IFN- γ , IL-1 α , IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-10, IL-12 (p40/p70), IL-13, IL-17, IP-10, KC, monocyte chemoattractant protein 1 (MCP-1), monokine induced by IFN- γ (MIG), macrophage inflammatory protein 1 α (MIP-1 α), tumor necrosis factor alpha (TNF- α), and vascular endothelial growth factor. A five-parameter nonlinear logistic regression model was used to establish a standard curve and to estimate the probability of occurrence of a concentration at a given point. Upper and lower levels of quantification were determined by use of BioPlex Manager software based on goodness of fit and percent recovery. Calculated pg/mL for experimental specimens were multiplied by the inherent assay dilution factor (df = 2) and reported as final observed pg/mL.

Statistical Analysis

Statistical analysis was carried out using Graph Pad Prism Version 6 (La Jolla, CA). The statistical test used for each experiment is described in the figure legends.

Results

Kinetics of *Salmonella* Invasion and Replication

We chose the *Salmonella* mutants were chosen for this study because they were all attenuated in mouse infection models and imparted varying levels of protective immunity against later lethal challenge, however their behavior in cells has been poorly characterized [15-19]. First we sought to determine if each mutant was able to invade and replicate in Bone Marrow Derived Macrophages (BMDM), as described above. At two and six hours post infection the cells were lysed and bacterial burdens were determined by serial dilution and plating. Interestingly, 8/9 mutants had significantly lower counts at two hours indicating that these strains are deficient in invasion as compared to the WT strain (Figure 1). The one strain that showed similar invasion to WT was the *hisG/rpsL/phoP* mutant. We then compared the growth of each strain from two to six hours. Two strains, the *hisG/crp* mutant and the *phoP/Q*⁻ mutant failed to grow. All other mutant strains showed 2-3 fold growth, while the WT strain showed about 5 fold growth, indicating that all of the mutants grew more slowly in BMDM than WT.

Host Cytokine Patterns Induced Upon *Salmonella* Infection Are Strain Specific

We determined if the host innate immune system was differentially activated by these mutant *Salmonella* strains as opposed to simply inducing more of the same cytokines. We infected BMDM in triplicate with WT or mutant *Salmonella* at a range of different MOI. *Salmonella* were allowed to invade host BMDM as above. At six hours post infection the supernatant was harvested and frozen at -80°C until analyzed for cytokine/chemokine levels. A broad panel of cytokines/chemokines was chosen to allow for unbiased examination of host response.

Of the 20 cytokines tested 11 (FGF-Basic, GM-CSF, IFN- γ , IL-2, IL-4, IL-5, IL-13, IL-17, KC, MIG and VEGF) were not detected at any MOI and are not shown. Importantly, each mutant shows a unique pattern of cytokine expression (Table 2). IL-1 α was only observed in WT infection at the highest MOI of 100, it was not detected upon infection with most of the attenuated mutant strains, although two strains (*phoP/Q*⁻ and *fur*⁻) showed some IL-1 α in one replicate at the highest MOI (Figure S1). It is not clear if this is produced by new synthesis or increased processing of existing IL-1 α precursors.

In contrast, IL-1 β was found only after infection with the *phoP/Q*⁻ and *recA* mutant at the intermediate MOIs, 25-6.25 and only in some of the triplicate wells. IL-1 β was also detected following infection with the *fur*⁻ mutant infection at MOIs of 100 and 12.5. Interestingly, the *recA*⁻ mutant showed no detectable IL-1 β at any MOI indicating that the mechanism of the bacterial mutation (deletion vs promoter swap) alters the host response (Figure S2). The inflammatory cytokine IL-6 showed an inverse dose-dependent response in the WT strain. After WT infection, no cytokine was detected at the highest MOIs tested 100, 50, 25

whereas robust IL-6 production was detected at the lower MOIs. IL-6 was not detected at any MOI upon infection with the *pabAB*, *hisG/crp*, or *phoP/rpsL/hisG* mutants. The remaining mutants showed an increased production of IL-6 at the higher MOIs as compared to WT infection. At the lower MOIs WT infection led to more IL-6 production than any of the mutants (Figure S3). Among other cytokines showing an inverse dose-dependent response are MCP-1 (Figure S4) and TNF- α (Figure S5). They are higher in all of the mutants than WT at the higher MOIs at lower MOIs the WT is higher or equal to all of the mutant strains. MIP-1 α showed a similar dose dependence in all of the mutants, except the *recA*⁻ mutant that induced higher levels at most MOIs tested (Figure S6). One other cytokine showing an interesting pattern among mutants was Interferon- γ Induced Protein 10 (IP-10). The mutant *Salmonella* infections showed a variable production of IP-10 as compared to WT. Infection with *phoP/Q*⁻ and *fur*⁻ mutants showed relatively similar levels to WT infection. The *crp*⁻, *pabA/B*, *hisG/crp*, *phoP/hisG/rpsL*, and *sifA* mutants induced slightly higher IP-10 than WT at the higher MOI and but lagged behind WT at the lower MOI. Again with the *recA* mutants the mechanism of attenuation effects the cytokine pattern. The arabinose promoter *recA*⁻ strain in arabinose free medium induced IP-10 at lower levels than WT at lower MOIs, and higher levels than WT at higher MOIs. The gene deletion strain *recA* consistently induced IP-10 at higher or equal levels than WT at all MOIs tested (Figure S7).

Two cytokines, IL-10 and IL-12p70, showed consistent changes in some mutant strains as compared to WT. IL-10 was not detected upon WT infection at any MOI. In contrast, infection with the *phoP/Q*⁻ mutant and the *recA*⁻ mutants induced IL-10 production at most of the MOIs tested (Figure S8). IL-10 was also detected at some MOIs in *phoP/hisG/crp* strain. IL-12p70 was detected after infection with most mutant *Salmonella* strains, the exceptions were the *pabA/B* and *hisG/crp* strains that showed no detectable levels of IL-12p70 at any MOI tested (Figure S9).

Salmonella is primarily a gastrointestinal pathogen. Since TLR4 is expressed at very low levels in the GI tract we repeated these cytokine experiments in TLR4^{-/-} BMDM to mimic these low TLR conditions in the gut [20-22]. Similar to infection of WT BMDM, FGF Basic, GM-CSF, IL-2, IL-5, and IL-17 were not detected at any MOI following infection with any of the strains tested. The inflammatory cytokines IL-6 and MCP-1 are completely dependent on TLR4. IL-1 α showed a similar profile to the WT BMDM infections where it is only found after infection with the highest doses of WT *Salmonella* (Figure S10) suggesting TLR4 is not required. Unlike in WT BMDM where IL-1 β was only detected in a few mutants and MOIs, most mutants induced high levels of IL-1 β at every MOI tested (Figure S11). Similar “TLR4^{-/-} dependent” responses were seen in IL-4 (Figure S12), IL-13 (Figure S13), KC (Figure S14) and VEGF (Figure S15). These cytokines that are only seen in the absence of TLR4 highlight a need to explore inapparent innate responses. MIP-1 α was detected in all the infections, but all of the mutants showed lower levels compared to WT (Figure S16). IL-10 (Figure S17) and IL-12p70 (Figure S18) were detected after infection with most of the mutant *Salmonella* strains, although it is important to note the uninfected production of IL-10 was higher in the TLR4^{-/-} BMDM.

IL-10 rescues *phoPQ* mutant *Salmonella in-vitro* growth

IL-10 is often considered to be an anti-inflammatory cytokine. We were surprised to see it upregulated in attenuated mutant infections. IL-10 was detected after infection with the *phoP/Q*⁻ and *recA*⁻ mutants at all MOIs tested. Host inflammation plays a complex role in *Salmonella* growth and pathogenesis. Hosts that are deficient for IL-12 or IFN- γ are particularly sensitive to *Salmonella* infection so it might be thought that IL-10 production would mimic that seen in hosts deficient for these inflammatory cytokines. The *phoP/Q*⁻ mutant, which induced the highest levels of IL-10 also fails to replicate in host macrophages suggesting a possible protective role for host IL-10. With this in mind we sought to determine if the *phoP/Q*⁻ mutant proliferation could be rescued in IL-10^{-/-} BMDM. To this end WT or IL-10^{-/-} BMDM were infected with either WT or *phoP/Q*⁻ *Salmonella* at an MOI of 5, additionally the *hisG/crp* mutant that fails to grow in BMDM, but also fails to induce host IL-10 was included. Two and six hours post infection BMDMs were lysed and bacterial levels were determined by serial dilution and plating. The WT *Salmonella* showed similar invasion and replication numbers regardless of the BMDM type infected (Figure 2). The *phoP/Q*⁻ replication was rescued in the absence of host IL-10. There was no difference in invasion of either WT or IL-10^{-/-} BMDM. The *hisG/crp* mutant failed to grow in either BMDM line indicating that its attenuation was independent of host IL-10 production, consistent with the lack of IL-10 detected by Luminex-based cytokine profiling.

IL-10 Restricts WT *Salmonella in-vitro* growth

To determine if IL-10 can restrict WT *Salmonella*, BMDM were treated with a range of concentrations of rIL-10 either four hours before or at the time of infection with either WT or *phoP/Q*⁻ *Salmonella*. *Salmonella* infection was performed as above and bacteria harvested two and six hours post infection. As seen previously the *phoP/Q*⁻ mutant showed no significant growth regardless of the amount of IL-10 added. Interestingly, the addition of IL-10 either before or at the time of infection restricted the growth of WT *Salmonella* as compared to infections in the absence of IL-10 ($p < 0.001$) (Figure 3), indicating a host protective role for IL-10 in the infection of BMDM.

Discussion

Comparing the host response in various mutants provide important insights into host responses to attenuated pathogens. Much of vaccine development is predicated on the assumption that attenuated organisms produce an immune response equivalent to the pathogen. Here we show that the attenuated mutants have distinct cytokine signatures. The finding that these presumptive vaccine candidates differ not only in the amount but types of cytokine induced is critical information. All of these mutants fail to cause disease in mice [8, 15-19, 23-26]. When carrying out *in vivo* infections there is little discernable difference amongst the mutant strains. Examining the host immune pathways activated by individual attenuated mutants highlights that the host responds to each of the attenuated mutants with a unique cytokine signature. None of the nine mutants had identical production of cytokines compared to WT *Salmonella* infection or one another (Table 2). The same is true in the TLR4^{-/-} BMDM (Table 3). Additionally, the mechanism of genetic manipulation of the bacteria also had an effect on the cytokines produced. The genetic deletion *recA* gave a

different cytokine profile than an arabinose promoter *recA*⁻ strain in the absence of arabinose. Thus the idea that attenuated mutants produce a similar response as WT only lower in magnitude is incorrect.

Our experiments showed very little IL-1 α being produced by BMDM after *Salmonella* infection, protein was only detected in WT infection at the highest MOI (100) tested. IL-1 α has been previously shown to be produced by human monocytes upon incubation with high levels (~1 μ g/mL) of porin protein from *Salmonella* [27, 28]. This dependency on high levels of porin coupled with the WT *Salmonella* invading at a higher efficiency than the mutants, and thus having more porin per target cell, may explain why no other conditions resulted in IL-1 α production.

One surprising result was the lack of IL-1 β detected in our assay system. Other groups have previously shown *Salmonella* induced high levels of IL-1 β [29, 30]. This IL-1 β production has been shown to be important for both *Salmonella* pathogenesis and redirection of the host immune system. Perkins *et al.* showed high levels of transcript at four and six hours post infection in peritoneal macrophages, whereas Franchi *et al.* showed moderate levels of protein at 16 hours from BMDM, but much higher levels from intestinal mononuclear phagocytes (iMPs). Perhaps the IL-1 β mRNA in our system is not translated six hours post infection or the protein is produced at low levels and signaling in an autocrine manner and so consumed. Testing of alternate time points as well as RT-PCR can be carried out in the future. Of note, while we did detect IL-1 β in the WT infection of TLR4^{-/-} BMDM, almost every mutant induced detectable levels of IL-1 β , suggesting that in this system TLR4 signaling can suppress IL-1 β .

Three cytokines showed a complete dependence of TLR4, IL-6, TNF- α and MCP-1. These proinflammatory cytokines, hallmarks of LPS stimulation of TLR4, were absent in the TLR4^{-/-} infections. Interestingly the mutants tested showed similar dose-dependent responses as compared to WT infection. At higher MOIs most of the mutants showed increased cytokine, while at lower MOIs the WT *Salmonella* induced more IL-6, TNF- α and MCP-1. This was obviously dependent on LPS structure. The only mutants in the tested panel that have a direct effect on lipid A structure are the *phoP/Q*⁻ and *hisG/crp/phoP* mutants. In response to low pH, Fe⁺³, Mg⁺² or oxygen the two component system *phoPQ* regulates genes in the lipid A modification pathway, namely the *pagL* and *pagP* (Pho Activated Genes) a deacylase and acylase respectively, and the *pmrAB* two component system which controls addition of ethanolamine and aminoarabinose to the terminal phosphates [31-33]. Under normal media growth conditions in LB or Purple Broth the PhoPQ system is inactive, thus the lipid A structure of all the strains tested should be identical. All mutants strains having the same lipid A structure but many inducing much higher levels of TLR4 dependent cytokines at the higher MOIs indicate a possible downregulation of TLR4 induced inflammation by WT *Salmonella*.

MIP-1 α secretion by Peyer's Patch cells has been shown to lead to immune damage. Blocking of MIP-1 α secretion leads to increased CD4⁺ CD25⁺ Regulatory T Cells and decreased immunopathology [34]. In WT BMDM model we once again see a dose-dependent production of MIP-1 α in the mutants where at high MOI most mutant infections

show increased MIP-1 α secretion compared to WT but at lower MOI we see the reverse. This seems to fit with the TLR4 dependent pattern we see in the MCP-1 and IL-6. MIP-1 α is generally driven by LPS stimulation of TLR4. Unlike IL-6 and MCP-1 we see MIP-1 α secretion in the TLR4^{-/-} BMDM infection model, indicating that there is a TLR4 independent pathway that also induces production of MIP-1 α . Also of note, in the TLR4^{-/-} BMDM infection almost all of the mutants showed lowered MIP-1 α production at all but the highest MOI. Recent work has indicated the B subunits of a AB5 toxin ArtAB (ADP- ribosylating toxin) in *Salmonella* triggering host production of MIP-1 α [35]. The decreased bacterial load and replication may explain the lowered MIP-1 α production in the TLR4^{-/-} BMDM.

Humans deficient for IL-12R are extremely sensitive to *Salmonella* infections, indicating an important role for IL-12 in host defense against *Salmonella* [36, 37]. Most of our mutants showed an increase in IL-12 production compared to WT infection. Of note, the metabolism mutants, *pabA/B* and *hisG/crp*, failed to induce greater IL-12 production. These mutants have a much lower cytokine profile compared to the other mutants.

Some cytokines (IL-4, IL-13, KC and VEGF) were only induced in the absence of TLR4, suggesting an inhibitory effect of TLR signaling. All of these cytokines are signatures of a Th2 like response. Inflammatory signals through TLR4 potentiate Th1 responses. In the absence of TLR4, it makes sense that we could see a stronger Th2 response. However none of Th2 skewing cytokines are produced in the WT *Salmonella* infection of TLR4^{-/-} BMDM. This lack of production in WT infection indicates it is not simply a lack of TLR4 that allows for IL-4, IL-13, KC and VEGF, but particular bacterial phenotypes in the absence of TLR4.

Addition of arabinose to the arabinose inducible promoter strains rescued IL-10, IL-12, IL-12p40/70 and VEGF production. Some cytokine production from mutant *Salmonella* infection was not rescued by addition of arabinose. In the TLR4^{-/-} BMDM IL-1 β , IL-4, and KC showed nearly equal levels regardless of arabinose additions. This may be due to differential regulation of the genes under the new promoter or a possible second site mutation in these strains.

Continuing experiments removing the dominant signal, like TLR4 in *Salmonella* or TLR2 in *Francisella*, allow for the discovery of possible other immune responses that are manipulated by pathogens [38]. These subdominant signals, while not likely directly involved in pathogenesis and survival, can allow insight into mechanisms of host subversion that might be applicable to other pathogens of interest.

The IL-10 restriction of *Salmonella* replication in BMDM was surprising. Many groups have shown data that IL-10 allows for rapid *Salmonella* growth and spread in animal models [39-43]. Our experiments carried out in BMDMs seem to fit with the need for *Salmonella* to use inflammation from the host to upregulate SPI-2 genes in order to replicate in the cytoplasm. Further work will be needed to determine if IL-10, like the loss of TLR2/4/9, can inhibit SPI-2 transcription and translation.

Overall these results support a revised model of the immune response to an attenuated pathogen. Infection with attenuated strains of similar phenotypes, like lowered invasion or

slower growth, does not result in the same immune response as WT bacteria, only of a lower magnitude. Instead, it means that a loss of function mutant can result in both new cytokine responses not seen in WT infection and loss of other cytokine responses. Both of these changes give clues to how the bacteria are modifying host responses. It is easy to see how a loss of function in a bacterial gene that suppresses the immune response would result in a new cytokine secretion of one that was previously suppressed. This is the classic loss of a suppressor. It is more difficult to see how a loss of function mutant would result in the loss of a cytokine response. In our case loss of *phoPQ* and *fur* result in the loss of MIP-1 α at all of the MOIs tested. The simplest explanation would be either the direct gene products (or a molecule regulated by them) results in the stimulation of MIP-1 α . However that is difficult to reconcile with the observation that many different mutations result in the downregulation of the same cytokine, such as IL-1 α . Altogether, our data suggests that we need to rethink the impact of attenuation on the immune response, and develop more nuanced models of how attenuation impacts immunity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Nine attenuated *Salmonella* mutants were assayed for cellular invasion and host cytokine production.
- Every mutant examined gave a distinct cytokine fingerprint compared to WT infection.
- IL-10 restricted growth of WT *Salmonella* in Bone Marrow Derived Macrophages.

Figure 1. Invasion and Replication Kinetics of *Salmonella* in BMDM

C57BL/6 BMDM were infected with the indicated *Salmonella* strain at a MOI of 5. Wells were lysed at 2 (open bars) and 6 (filled bars) and enumerated by serial dilution. Values are represented as mean and SEM for 6 wells per condition. Data is from one representative of 3 experiments. All mutant strains are significantly different from WT in growth ($P < 0.001$). Stars indicate ****, $p < 0.0001$, ***, $p < 0.001$, **, $p < 0.01$, bars show the significance of growth of individual strain between 2 and 6 hours. Student's *t* test corrected for multiple comparisons.

Figure 2. IL-10 dampens *Salmonella* replication in BMDM

C57BL/6 BMDM were infected with the WT (A) or *phoP/Q* *Salmonella* at a MOI of 5. rIL-10 was added at the indicated concentration either 3 hours before (Pre) or at the time of infection. Wells were lysed at 2 (open bars) and 6 (filled bars) and enumerated by serial dilution. Values are represented as mean and SEM for 3 wells per condition. Data is from one representative of 2 experiments. Student's *t* test corrected for multiple comparisons.

Figure 3. Loss of IL-10 rescues growth defects of *phoP/Q* *Salmonella* in BMDM

C57BL/6 (WT) or IL-10^{-/-} BMDM were infected with the indicated *Salmonella* strain at a MOI of 5. Wells were lysed at 2 (open bars) and 6 (filled bars) and enumerated by serial dilution. Values are represented as mean and SEM for 3 wells per condition. Data is from one representative of 2 experiments. ****, $p < 0.0001$, ***, $p < 0.001$, **, $p < 0.01$ relative to 2 hour timepoint. (Student's unpaired *t* test)

Table 1
Strains used in this study

Strain Number	Description	Function	Source	Strain Designation
$\chi 3761$	WT <i>Salmonella</i>	-	UK-1	WT
$\chi 8442$	<i>pabA1516 pabB232</i>	Aminobenzoate auxotroph	UK-1	<i>pabA/B</i>
$\chi 8499$	<i>hisG crp28</i>	Histidine synthesis, sugar transport	SL-1344	<i>hisG/crp</i>
$\chi 8770$	<i>phoP233 hisG rpsL</i>	Two-component kinase, histidine synthesis, Strep resistance	SL-1344	<i>phoP/hisG/rpsL</i>
$\chi 8926$	<i>sifA26</i>	SPI-2 secreted effector	UK-1	<i>sifA</i>
$\chi 9199$	<i>P_{phoPQ107}::TTaraCP_{BAD}phoPQ araBAD23</i>	Two-component kinase and sensor	UK-1	<i>phoP/Q</i>
$\chi 9201$	<i>P_{fur33}::TTaraCP_{BAD}fur araBAD23</i>	Ferric uptake regulator	UK-1	<i>fur</i>
$\chi 9202$	<i>P_{crp527}::TTaraCP_{BAD}crp araBAD23</i>	Sugar transport	UK-1	<i>crp</i>
$\chi 9833$	<i>recA62</i>	Homologous recombination/DNA repair	UK-1	<i>recA</i>
$\chi 11144$	<i>P_{recA74}::TTaraCP_{BAD}recA araBAD23</i>	Homologous recombination/DNA repair	UK-1	<i>recA</i>

Table 2
Fold change in cytokines compared to WT *Salmonella* infection in WT BMDM

MOI 100 WT BMDM	IL-1 α	IL-1 β	IL-6	MCP-1	TNF- α	MIP-1 α	IL-10	IL-12p40/70	MOI 50 TLR4 $^{-/-}$ BMDM	IL-1 β	IL-6	MCP-1	TNF- α	MIP-1 α	IL-10	IL-12p40/70
χ 8442 <i>pabA/B</i>									χ 8442 <i>pabA/B</i>							
χ 8499 <i>hisG/crp</i>									χ 8499 <i>hisG/crp</i>							
χ 8770 <i>phoP/hisG/rpsL</i>									χ 8770 <i>phoP/hisG/rpsL</i>							
χ 8926 <i>sifA</i>									χ 8926 <i>sifA</i>							
χ 9199 <i>phoP/Q</i>									χ 9199 <i>phoP/Q</i>							
χ 9201 <i>fur</i>									χ 9201 <i>fur</i>							
χ 9202 <i>crp</i>									χ 9202 <i>crp</i>							
χ 9833 <i>recA</i>									χ 9833 <i>recA</i>							
χ 11144 <i>recA</i> $^{-}$									χ 11144 <i>recA</i> $^{-}$							
MOI 25 WT BMDM	IL-1 α	IL-1 β	IL-6	MCP-1	TNF- α	MIP-1 α	IL-10	IL-12p40/70	MOI 12.5 TLR4 $^{-/-}$ BMDM	IL-1 β	IL-6	MCP-1	TNF- α	MIP-1 α	IL-10	IL-12p40/70
χ 8442 <i>pabA/B</i>									χ 8442 <i>pabA/B</i>							
χ 8499 <i>hisG/crp</i>									χ 8499 <i>hisG/crp</i>							
χ 8770 <i>phoP/hisG/rpsL</i>									χ 8770 <i>phoP/hisG/rpsL</i>							
χ 8926 <i>sifA</i>									χ 8926 <i>sifA</i>							
χ 9199 <i>phoP/Q</i>									χ 9199 <i>phoP/Q</i>							
χ 9201 <i>fur</i>									χ 9201 <i>fur</i>							
χ 9202 <i>crp</i>									χ 9202 <i>crp</i>							
χ 9833 <i>recA</i>									χ 9833 <i>recA</i>							
χ 11144 <i>recA</i> $^{-}$									χ 11144 <i>recA</i> $^{-}$							
MOI 6.25 WT BMDM	IL-1 α	IL-1 β	IL-6	MCP-1	TNF- α	MIP-1 α	IL-10	IL-12p40/70	MOI 3.125 TLR4 $^{-/-}$ BMDM	IL-1 β	IL-6	MCP-1	TNF- α	MIP-1 α	IL-10	IL-12p40/70
χ 8442 <i>pabA/B</i>									χ 8442 <i>pabA/B</i>							
χ 8499 <i>hisG/crp</i>									χ 8499 <i>hisG/crp</i>							
χ 8770 <i>phoP/hisG/rpsL</i>									χ 8770 <i>phoP/hisG/rpsL</i>							
χ 8926 <i>sifA</i>									χ 8926 <i>sifA</i>							

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MOI 100 WT BMDM	IL-1 α	IL-1 β	IL-6	MCP-1	TNF- α	MIP-1 α	IL-10	IL-12p40/70	MOI 50 TLR4 ^{-/-} BMDM	IL-1 β	IL-6	MCP-1	TNF- α	MIP-1 α	IL-10	IL-12p40/70
χ^9199 <i>phoP/Q</i> ⁻									χ^9199 <i>phoP/Q</i> ⁻							
χ^9201 <i>fur</i> ⁻									χ^9201 <i>fur</i> ⁻							
χ^9202 <i>crp</i> ⁻									χ^9202 <i>crp</i> ⁻							
χ^9833 <i>recA</i>									χ^9833 <i>recA</i>							
χ^11144 <i>recA</i> ⁻									χ^11144 <i>recA</i> ⁻							
Fold Change to WT																
<0.75																
2.00-4.99																
5.00-9.99																
>10.00																

Table 3
Fold change in cytokines compared to WT *Salmonella* infection in TLR4^{-/-} BMDM

MOI 100 TLR4 ^{-/-} BMDM	IL-1α	IL-1β	IL-4	IL-13	KC	VEGF	MIP-1α	IL-10	IL-12p40/70	MOI 50 TLR4 ^{-/-} BMDM	IL-1α	IL-1β	IL-4	IL-13	KC	VEGF	MIP-1α
χ8442 <i>paba/B</i>	Blue						Blue			χ8442 <i>paba/B</i>						Blue	
χ8499 <i>hisG/erp</i>	Blue						Blue	Blue		χ8499 <i>hisG/erp</i>							
χ8770 <i>phoP/hisG/rpsL</i>	Blue						Blue			χ8770 <i>phoP/hisG/rpsL</i>							
χ8926 <i>sifA</i>	Blue	Blue					Blue			χ8926 <i>sifA</i>		Blue				Blue	
χ9199 <i>phoP/Q</i>	Blue	Orange					Blue			χ9199 <i>phoP/Q</i>		Blue				Blue	
χ9201 <i>fur</i>	Blue	Orange					Blue			χ9201 <i>fur</i>		Blue				Blue	
χ9202 <i>erp</i>	Blue	Orange			Orange		Blue		Orange	χ9202 <i>erp</i>		Blue			Orange		Blue
χ9833 <i>recA</i>	Blue						Blue			χ9833 <i>recA</i>							Blue
χ11144 <i>recA</i>	Blue						Blue			χ11144 <i>recA</i>							Blue
MOI 25 TLR4 ^{-/-} BMDM	IL-1α	IL-1β	IL-4	IL-13	KC	VEGF	MIP-1α	IL-10	IL-12p40/70	MOI 12.5 TLR4 ^{-/-} BMDM	IL-1α	IL-1β	IL-4	IL-13	KC	VEGF	MIP-1α
χ8442 <i>paba/B</i>							Blue			χ8442 <i>paba/B</i>							
χ8499 <i>hisG/erp</i>							Blue	Blue		χ8499 <i>hisG/erp</i>							
χ8770 <i>phoP/hisG/rpsL</i>							Blue			χ8770 <i>phoP/hisG/rpsL</i>						Blue	
χ8926 <i>sifA</i>							Blue			χ8926 <i>sifA</i>							
χ9199 <i>phoP/Q</i>					Orange					χ9199 <i>phoP/Q</i>							
χ9201 <i>fur</i>							Blue			χ9201 <i>fur</i>							Blue
χ9202 <i>erp</i>					Orange				Orange	χ9202 <i>erp</i>							
χ9833 <i>recA</i>							Blue			χ9833 <i>recA</i>							Blue
χ11144 <i>recA</i>							Blue			χ11144 <i>recA</i>							Blue
MOI 6.25 TLR4 ^{-/-} BMDM	IL-1α	IL-1β	IL-4	IL-13	KC	VEGF	MIP-1α	IL-10	IL-12p40/70	MOI 3.125 TLR4 ^{-/-} BMDM	IL-1α	IL-1β	IL-4	IL-13	KC	VEGF	MIP-1α
χ8442 <i>paba/B</i>							Blue			χ8442 <i>paba/B</i>							
χ8499 <i>hisG/erp</i>							Blue	Blue		χ8499 <i>hisG/erp</i>							
χ8770 <i>phoP/hisG/rpsL</i>							Blue			χ8770 <i>phoP/hisG/rpsL</i>						Blue	
χ8926 <i>sifA</i>							Blue			χ8926 <i>sifA</i>							

MOI 100 TLR4 ^{-/-} BMDM	IL-1α	IL-1β	IL-4	IL-13	KC	VEGF	MIP-1α	IL-10	IL-12p40/70	MOI 50 TLR4 ^{-/-} BMDM	IL-1α	IL-1β	IL-4	IL-13	KC	VEGF	MIP-1α
<i>χ⁹199 phoP/Q⁻</i>										<i>χ⁹199 phoP/Q⁻</i>							
<i>χ⁹201 fur⁻</i>										<i>χ⁹201 fur⁻</i>							
<i>χ⁹202 crp⁻</i>										<i>χ⁹202 crp⁻</i>							
<i>χ⁹833 recA</i>										<i>χ⁹833 recA</i>							
<i>χ¹1144 recA⁻</i>										<i>χ¹1144 recA⁻</i>							
Fold Change to WT																	
<0.75																	
2.00-4.99																	
5.00-9.99																	
>10.00																	