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Selective Reduced Intracellular Proliferation of *Salmonella enterica* Serovar Typhimurium within APCs Limits Antigen Presentation and Development of a Rapid CD8 T Cell Response

Homam Albaghdadi,* Nirmal Robinson,† Brett Finlay,‡ Lakshmi Krishnan,*‡ and Subash Sad1∗∗‡

Ag presentation to CD8+ T cells commences immediately after infection, which facilitates their rapid expansion and control of pathogen. This paradigm is not followed during infection with virulent *Salmonella enterica* serovar Typhimurium (ST), an intracellular bacterium that causes mortality in susceptible C57BL/6J mice within 7 days and a chronic infection in resistant mice (129×1SvJ). Infection of mice with OVA-expressing ST results in the development of a CD8+ T cell response that is detectable only after the second week of infection despite the early detectable bacterial burden. The mechanism behind the delayed CD8+ T cell activation was evaluated, and it was found that dendritic cells/macrophages or mice infected with ST-OVA failed to present Ag to OVA-specific CD8+ T cells. Lack of early Ag presentation was not rescued when mice or dendritic cells/macrophages were infected with an attenuated *aroA* mutant of ST or with mutants having defective *Salmonella* pathogenicity island I/II genes. Although extracellular ST proliferated extensively, the replication of ST was highly muted once inside macrophages. This muted intracellular proliferation of ST resulted in the generation of poor levels of intracellular Ag and peptide-MHC complex on the surface of dendritic cells. Additional experiments revealed that ST did not actively inhibit Ag presentation, rather it inhibited the uptake of another intracellular pathogen, *Listeria monocytogenes*, thereby causing inhibition of Ag presentation against *L. monocytogenes*. Taken together, this study reveals a dichotomy in the proliferation of ST and indicates that selectively reduced intracellular proliferation of virulent pathogens may be an important mechanism of immune evasion. The *Journal of Immunology*, 2009, 183: 3778–3787.

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*Abbreviations used in this paper: LM, *L. monocytogenes*; BHI, brain-heart infusion; MOI, multiplicity of infection; NRAMP, natural resistance-associated macrophage protein; SPI, *Salmonella* pathogenicity island; ST, *Salmonella enterica* serovar Typhimurium; TRITC, tetramethylrhodamine isothiocyanate; WT, wild type.

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We have previously reported that both naive and memory CD8+ T cells respond with delayed kinetics during ST-OVA infection (34). OVA-specific CD8+ T cells that are eventually induced against ST-OVA display a persistently activated phenotype, secrete IFN-γ, and kill targets specifically in vivo, suggesting that the OVA-specific CD8+ T cells generated are functional (34). In this report we examined whether the muted CD8+ T cell priming during infection with ST is due to its poor intracellular proliferation or to its active inhibition of Ag presentation. Using various mutants of ST and various in vitro and in vivo models of Ag presentation, our results indicate that ST displays selectively poor intracellular proliferation that translates into the generation of poor intracellular Ag levels and MHC-peptide complexes and hence results in poor CD8+ T cell priming.

Materials and Methods

Bacterial strains

The gene for OVA was introduced into the virulent SL1344 ST strain and into various mutants (aroA, phoP, ssrA, and invA) of ST. The plasmid pKK-OVA (35, 36) (10–100 ng of DNA) carrying the full-length OVA was electroporated into ST as described previously (34). Expression of OVA by recombinant wild-type (WT) ST-OVA and the various mutants of ST-OVA was determined by an enhanced chemiluminescence detection system as described previously (37). ST-OVA were grown in liquid culture at 37°C under constant shaking in brain-heart infusion (BHI) medium (Difco Laboratories). At mid-log phase (OD600 nm = 0.8), bacteria were harvested and frozen at −80°C (in 20% glycerol). CFU were determined by performing serial dilutions in 0.9% NaCl, which were spread on BHI-streptomycin agar plates.

OVA-expressing LM (LM-OVA), as described previously (38), was grown to an OD600 nm of 0.4. The bacteria were grown in BHI medium (Difco Laboratories) supplemented with 50 μg/ml streptomycin (Sigma-Aldrich). At mid-log phase (OD600 nm = 1.0), bacteria were harvested and frozen in 20% glycerol and stored at −80°C. CFU were determined by performing serial dilutions in 0.9% NaCl, which were spread on BHI-streptomycin agar plates.

Mice and immunizations

C57BL/6 and 129 × 15vl mice were obtained from The Jackson Laboratory. B6129 F1 mice were generated in-house in our experimental animal facility by mating 129 × 15vl female mice with C57BL/6 male mice. CD45.1 45.2 OT-1 TCR transgenic mice were derived in house by matings of donor OT-1 TCR transgenic mice (The Jackson Laboratory) with B6.SIL mice (The Jackson Laboratory). For immunization, frozen stocks of bacteria were thawed and diluted in 0.9% NaCl, which were spread on BHI-streptomycin agar plates.

Assessment of bacterial burden

Single cell suspensions were obtained from the spleens of infected mice in RPMI 1640. An aliquot of the suspension was lysed with water for 30 s and then evaluated for the numbers of viable bacteria. CFU were determined by plating 100-μl aliquots of serial 10-fold dilutions in 0.9% saline on BHI plates as described above.

Quantitative RT-PCR

Quantitative RT-PCR was performed as described in detail previously (6, 37). Expression of OVA mRNA and total bacterial RNA (16S) was evaluated in LM-OVA and ST-OVA growing extracellularly in broth cultures, intracellularly within macrophages in vitro, and within spleens in vivo. For evaluation of expression in infected spleens, 5–10 μg of total RNA was taken for cDNA synthesis. For in vitro cultures, only 50–250 ng of RNA was used for cDNA synthesis and −100 ng of cDNA was used for real-time PCR. To obtain a standard curve for each primer-template set, five different PCRs were performed in parallel by using as a template 10-fold dilutions of known amounts of ST-OVA or LM-OVA chromosomal DNA (0.01, 0.1, 1, 10, and 100 attomol), together with triplicate or duplicate reactions of the uncharacterized samples. PCR conditions were optimized based on the melting curve of each primer and its target. PCR was performed in sealed tubes in a 96-well microtiter plate in an iCycler iQ thermal cycler (Bio-Rad Laboratories). The 26-μl reaction consisted of 12.5 μl of quantitative PCR SYBR Green SuperMix (ABgene), 1.2 μl of each primer, 9.1 μl of DNase/RNase-free, double-distilled H2O, and 1 μl of template. Thermal conditions were as follows: activation at 95°C for 15 min, followed by 40 cycles of denaturation at 95°C for 30 s, and extension at 72°C for 1 min. Melting curve protocol was performed to verify that the products had the expected melting temperature. Fluorescence was measured during the annealing step and plotted against the amplification cycle. Absolute quantitative analysis of the data was extrapolated from the standard curve, and atomole quantities were mathematically converted to numbers of detectable RNA molecules. Primer efficiencies were between 98 and 100%.

Intracellular proliferation

The intracellular proliferation of LM and ST was evaluated on IC-21 macrophages (H-2b) as described previously (22). IC-21 cells were seeded (in RPMI 1640 plus 8% FBS) into 24-well plates (104-105/well) and infected with LM-OVA or ST-OVA. After 15 min, cells were washed and extracellular bacteria removed after incubation in medium containing gentamicin (50 μg/ml). Each incubation step was conducted at 37°C in a CO2 incubator. Two hours after infection, cells were washed with medium containing gentamicin (5 μg/ml) and the numbers of intracellular bacteria were enumerated by lysing macrophages and plating serial dilutions on BHI agar plates. Aliquots of cells were further cultured in RPMI plus 8% FBS medium containing 5 μg/ml gentamicin to allow intracellular, but not extracellular, proliferation of LM or ST. At various time intervals, intracellular bacterial burden was evaluated. Cells were spun down, medium was aspirated, and cells were lysed using cell lysis buffer (1% Triton X-100 plus 0.1% SDS in PBS (pH 7.2)). Appropriate dilutions were made in 0.9% saline, and 100-μl aliquots were plated on BHI plates.

Ag presentation models

Ag presentation was evaluated using in vitro and in vivo models. For evaluation of Ag presentation in vitro, macrophages (IC-21 cells; H-2b) or dendritic cells (JAWS, H-2w) were irradiated (10,000 rad) and seeded (in RPMI 1640 plus 8% FBS) into 24-well plates (104-105/well) and infected with bacteria (various doses). After 15 min, cells were washed and extra- cellular bacteria were removed after incubation in medium containing gentamicin (50 μg/ml). Each incubation step was conducted at 37°C in a CO2 incubator. At 2 h, cells were washed with medium containing gentamicin (5 μg/ml). OT-1 (CD45.1 45.2) T cells were labeled with CFSE (40) and added to the cultures (1 × 105/well). After 4 days of culture, cells were harvested from the wells, stained with anti-CD8 and anti-CD45.1 Abs, and the expression of CFSE on OT-1 cells was evaluated by flow cytometry.

For evaluation of Ag presentation in vivo, CFSE-labeled OT-1 cells were injected (5 × 105) into B6.129 F1 mice i.v. Within 3–4 days, mice were infected with various bacteria through the i.v. route. Five days after infection, spleens were removed from the recipient mice. The presence of donor OT-1 CD8+ T cells (CD45.1+) and the reduction in CFSE intensity of donor cells was evaluated.

Flow cytometry

Aliquots (5 × 105) of spleen cells were incubated in 200 μl of PBS plus 1% BSA (PBS-BSA) with anti-CD16/32 at 4°C. Cells were then incubated on ice for 30 min with anti-CD8 and anti-CD45.1 Abs. Cells were washed and then incubated at room temperature with PE-H-2KbOVA257–264 tetramer. After 30 min, cells were washed with PBS and fixed in 0.5% form- aldeyde and acquired on BD FACSCanTo flow cytometer.

Imaging of LM- and ST-infected cells

Frozen stock of LM was thawed and spun down to remove the medium. The centrifuged bacteria were suspended in 200 μl of PBS containing 0.1 mg/ml tetramethylrhodamine isothiocyanate (TRITC) and 0.01 mg/ml of LM-GFP and LM-TRITC combined at a multiplicity of infection (MOI) of 10. The plates were centrifuged at 1600 rpm for 10 min followed by incubation at 37°C for 30 min. After the pulse, the extracellular bacteria were removed by washing with RPMI 1640 containing 50 μg/ml gentamicin. The intracellular bacteria were further chased for 2 h in RPMI 1640 containing 8% PBS and 50 μg/ml gentamicin. Following the chase, cells were fixed with 4% paraformaldehyde for 20 min at room temperature. The cover slips were then mounted on glass slides using Pro-Long Gold antifade reagent (Invitrogen-Molecular Probes) and observed under a fluorescence microscope (Olympus IX81).
Measurement of cell viability

IC21 cells were seeded at a 2 × 10^4 cells/well in a 96-well, flat-bottom culture plate. Cells were infected at various MOI with ST-OVA, LM-OVA, or a combination of both for 1 h at 37°C. Infection medium was removed and cells were washed and subsequently incubated in RPMI 1640 plus 8% FBS and 50 μg/ml gentamicin for 1 h, after which the cells were incubated in the same medium containing 5 μg/ml gentamicin for 24 h at 37°C. Subsequently, a neutral red dye cytotoxicity kit (Xenometrix) was used to assess cell viability following the manufacturer’s instructions. Briefly, culture medium was removed and the labeling solution containing the Neutral Red dye was added and incubated at 37°C for 3 h. Cells were inspected under a microscope to ensure minimal crystal formation and uptake of the dye by the cells (cells appear red in color). The labeling solution was aspirated and the cells were washed with fixative solution (0.1% CaCl₂ in 0.5% formaldehyde) for 1 min. Finally, the fixative solution was removed and the dye was solubilized using a solubilization solution (1% acetic acid in 50% ethanol). The colorimetric reading was measured in a plate reader at 540 nm with a reference wavelength of 690 nm.

Results

**ST infection of mice does not result in early CD8⁺ T cell activation**

B6.129 F1 mice were infected with 10^3 ST or LM. On day 6 of infection, ~10% of CD8⁺ T cells (irrespective of antigen specificity) in LM-infected mice expressed an activated phenotype (CD44^hi/CD62L^low) (Fig. 1A). In contrast, ST-infected mice did not show any appreciable level of activation in CD8⁺ T cells. Similar results were obtained when the activation status of CD4⁺ T cells was evaluated (our unpublished observations). To evaluate the response in an Ag-specific manner, mice were infected with 10^3 ST-OVA (i.v.). If Ag is presented, then this would result in activation of OT-1 T cells. As expected, when OT-1 cells were transferred on day 1, 7, or 14 of infection did not undergo appreciable expansion (Fig. 3). When OT-1 cells were transferred on days 21, 30, or 60 there was an appreciable increase in the numbers of OT-1 cells indicative of potent Ag presentation during these time periods. On day 120 of ST-OVA infection the transferred OT-1 cells did not increase in number appreciably. In the case of LM-OVA infection, OT-1 cells increased in number massively when transferred on the first day of infection, but failed to do so when transferred on day 7 or beyond.

**Early priming of CD8⁺ T cells is undetectable in ST-OVA-infected mice**

We tested the possibility that the enormous delay in CD8⁺ T cell response in ST-OVA-infected mice despite the high bacterial burden was related to a possible lack of Ag presentation. To test this, we used the in vivo Ag presentation model in which mice are injected with 10^3 bacteria and CFSE-labeled OT-1 CD8⁺ T cells (i.v.). If Ag is presented, then this would result in activation of OT-1 CD8⁺ T cells and the subsequent dilution of CFSE expression. At day 5 after infection, transferred OT-1 CD8⁺ T cells had undergone extensive proliferation in LM-OVA-infected mice (Fig. 2, A–C), whereas ST-OVA infection failed to stimulate the OT-1 CD8⁺ T cells. All of the infected mice had significant bacterial burden at day 5 after infection (Fig. 2D).

We also evaluated the relative expansion in donor OT-1 cells within 7-day intervals during the various stages of ST-OVA infection. OT-1 cells transferred on day 1, 7, or 14 of infection did not undergo appreciable expansion (Fig. 3). When OT-1 cells were transferred on days 21, 30, or 60 there was an appreciable increase in the numbers of OT-1 cells indicative of significant Ag presentation during these time periods. On day 120 of ST-OVA infection the transferred OT-1 cells did not increase in number appreciably. In the case of LM-OVA infection, OT-1 cells increased in number massively when transferred on the first day of infection, but failed to do so when transferred on day 7 or beyond.

**Macrophages and dendritic cells infected with ST-OVA fail to stimulate CD8⁺ T cells**

To further address the mechanism behind the lack of rapid CD8⁺ T cell priming in ST-OVA infected mice, we set up an in vitro model of Ag presentation. First, we determined the sensitivity of this assay and noted that a minimum of 10 pmol of OVA-peptide concentration was needed for CFSE-labeled OT-1 cells to undergo division (Fig. 4A). When dendritic cells or macrophages were infected with LM-OVA, they induced massive cycling of CFSE-labeled OT-1 cells indicative of potent Ag presentation (Fig. 4, B and C). However, infection with ST-OVA failed to induce cycling of OT-1 cells, reiterating the notion that Ag presentation does not occur readily during infection with ST-OVA.

**Virulence factors of ST are not the reason for the lack of Ag presentation**

Because it was possible that some of the virulence factors of ST may be responsible for causing inhibition of Ag presentation, we tested Ag presentation in the in vitro model using the various mutants of ST, including *phoP* (global regulator of virulence), *invA* (Spi-1 mutant), *ssaR* (Spi-2 mutant), and *aroA* (defective for in
vivo replication). Mutants with defective intracellular survival and invasion were tested. We have previously reported that all the mutants express similar levels of OVA compared with WT (37). Macrophages infected with LM-OVA, ST-OVA, and the various mutants of ST displayed similar burdens at 1 h after infection, indicating similar infection (Fig. 5A). None of the mutants of ST rescued Ag presentation (Fig. 5B), suggesting that the tested virulence factors do not inhibit Ag presentation.

It was possible that the in vitro model may have missed the potential Ag presentation ability of some of the mutants of ST. We thus evaluated Ag presentation in the in vivo model. When all of the mice received a uniform dose (10^3; i.v.) of bacteria, only LM-OVA induced the proliferation of transferred CFSE-labeled OT-1 cells (Fig. 6, A and B). As expected, infection of mice with the various mutants of ST resulted in a variable bacterial burden at day 5 postinfection (Fig. 6C). In another set of experiments, we increased the dose of the various mutants to the maximum without causing fatality. Even after infection with high doses of mutants, transferred CFSE-labeled OT-1 cells failed to proliferate (Fig. 6, D and E). Infection with higher doses of mutants resulted in increased bacterial burden relative to WT (Fig. 6F).

**FIGURE 4.** Macrophages and dendritic cells infected with ST-OVA fail to promote Ag presentation. A, CFSE-labeled OT-1 cells were cultured with various concentrations of OVA-peptide. After four days, the proliferation of CD8^+ T cells was evaluated by measuring CFSE dilution by flow cytometry. B and C, Dendritic cells (JAWS, H-2^b) (B) and macrophages (IC-21, H-2b) (C) were seeded in 24-well-plates in RPMI 1640 plus 8% FBS. Bacteria were added at various MOI values and the plates were centrifuged to promote bacterial adsorption and incubated at 37°C for 15 min. Cells were subsequently washed vigorously and incubated with medium containing gentamicin (50 μg/ml) for 2 h. Subsequently, cells were washed and cultured in medium containing gentamicin (5 μg/ml) with CFSE-labeled OT-1 splenocytes. After 4 days, cells were harvested and stained in anti-CD8α and anti-CD45.1 Abs. The degree of CD8^+ T cell proliferation induced by infected dendritic cells (B) and macrophages (C) was evaluated by measuring CFSE dilution using flow cytometry.

**Dichotomy in the intracellular vs extracellular proliferation of ST**

It is well established that ST grows rapidly in culture, faster than LM (41). Based on culture studies, we calculated the relative doubling time for LM-OVA to be 50 min in comparison to 30 min for ST (Fig. 7A). This ∼2-fold difference in doubling becomes large over time. ST grows to numbers that are 10-fold more than those of LM within 4 h. However, for Ag presentation it is more relevant...
to determine the relative intracellular doubling of the pathogen. We first determined the relative infection of dendritic cells with LM and ST. JAWS cells were infected with ST-GFP and TRITC-labeled LM. LM-infected cells had 2- to 4-fold more bacteria 1 h after infection (Fig. 7B). Similarly, when bacterial uptake was determined by measuring CFU at 1 h after infection, LM uptake was 2- to 4-fold more (Fig. 7C). Having seen only a modest difference in the relative uptake of LM vs ST by APCs, we measured the relative intracellular doubling of LM-OVA and ST-OVA in macrophages and dendritic cells. Extracellular growth of the pathogens was controlled by maintaining the cells in medium containing low levels of the antibiotic gentamicin (42). Intracellular proliferation of LM was significantly greater than that of ST (Fig. 7D). Within macrophages, the calculated intracellular doubling time was 1.85 h for LM-OVA and 7 h for ST-OVA respectively. Taken together, these results indicate that the relative uptake of ST is slightly lower than that of LM; however, once inside macrophages and dendritic cells, ST does not appear to proliferate as strongly as LM.

Generation of reduced intracellular Ag levels upon infection with ST-OVA

When LM-OVA and ST-OVA were grown extracellularly, the numbers of OVA mRNA molecules and the total bacterial (16S) RNA molecules generated were similar (Fig. 8A). We have previously reported that LM-OVA and ST-OVA also express similar levels of OVA protein (34). We wished to determine the relative level of OVA generated by the two bacteria when they are growing intracellularly within macrophages. LM-OVA-infected macrophages contained high levels of 16S RNA and OVA mRNA (Fig. 8B). In contrast, macrophages infected with ST-OVA contained low levels of total (16S) and OVA mRNA. The initial infection of macrophages with LM-OVA and ST-OVA was relatively similar 1 h after infection (Fig. 8C), however, LM-OVA proliferated enormously during the subsequent 24 h in contrast to ST-OVA (Fig. 8D), which explains the reduced level of OVA mRNA in ST-OVA-infected macrophages.

ST-OVA infection does not result in detectable MHC-peptide complex levels

One of the key events in Ag presentation is the generation of sufficient MHC-peptide levels on the surface of dendritic cells. We therefore determined the relative levels of H-2Kb-OVA257–264 complex levels on ST-OVA and LM-OVA dendritic cells. For this, we used an Ab that specifically binds to this complex (43). First, we determined the sensitivity of this assay and noted that a minimum of 10 nmol of OVA-peptide levels were needed for detection by this Ab (Fig. 9A). Infection of DCs with LM-OVA, ST-OVA,
and the various mutants of ST-OVA was not appreciably different at 1 h after infection (Fig. 9B). Infection of dendritic cells with LM-OVA, but not LM, resulted in significant augmentation of MHC-peptide levels (Fig. 9C). WT ST-OVA and the various mutants failed to induce any detectable MHC-peptide levels on the surface of dendritic cells. Taken together, these results indicate that although ST induces a productive infection in DCs similar to LM, the inefficient proliferation of ST intracellularly results in the generation of reduced Ag levels that culminates in poor generation of MHC-I-peptide complexes on the surface of dendritic cells.

**Reduced Ag levels in the spleens of ST-OVA-infected mice**

We determined the levels of OVA mRNA and the total bacterial (16S) RNA in the spleens of mice infected with LM-OVA and ST-OVA. In ST-OVA-infected mice, 16S RNA was detectable at all the time intervals; however, the relative level of OVA mRNA fell below the limit of detection (Fig. 10A). In LM-OVA-infected mice, low levels of OVA mRNA could be detected at the peak of infection (day 3). For both 16S as well as OVA mRNA, the levels generated were higher in the spleens of LM-OVA-infected mice. In the long term, ST-OVA infected mice had high bacterial burden in the spleens (Fig. 10C), yet the levels of 16S RNA and OVA were low. The experiments described above were done in mice that were infected with a $10^3$ dose of the bacteria (i.v.). This dose could not be increased due to experimental limitations, as higher doses of ST cause lethality.

**ST-OVA infection does not result in active inhibition of Ag presentation**

We wished to evaluate the alternative possibility that ST infection induces inhibition of Ag presentation, although our results with mutants of ST indicated otherwise. Macrophages pulsed with LM-OVA induced Ag presentation to CFSE-labeled OT-1 cells (Fig. 11). Infection of macrophages with ST-OVA did not stimulate OT-1 cells. Furthermore, coinfection of macrophages with LM-OVA and ST-OVA (10 MOI each) did not result in inhibition of LM-OVA-Ag presentation, indicating that ST-OVA-pulsed macrophages do not inhibit general Ag presentation.
To further evaluate the possible inhibition of Ag presentation, we pulsed macrophages with LM-OVA and coinfected them with increasing doses of ST. Bacterial burden in the macrophages was evaluated at 1 and 24 h after infection. When macrophages were infected with a very high dose of ST, reduced LM-OVA burden could be detected even at 1 h after infection, implying that a high dose of ST impairs the uptake of LM-OVA (Fig. 12A). At 24 h postinfection there was an increase in the burden of LM-OVA in macrophages; however, the absolute count of LM-OVA was less when cells were coinfected with 100 MOI of ST. ST and LM-OVA colonies could be differentially counted based on the difference in colony size, with LM-OVA colonies being relatively smaller in size (Fig. 12B). Interestingly, infection with high doses of LM-OVA did not influence the uptake of ST (Fig. 12C). As before, ST failed to display significant proliferation within macrophages from 1 h until 24 h. We also enumerated the number of macrophages at 1 h after infection. To determine the initial uptake of the bacteria, macrophages were pulsed with 10 MOI of bacteria as described previously, and the intracellular bacteria were enumerated at 1 h postinfection (B). Differences in bacterial uptake (B) were not significant (p = 0.109) as determined by one-way ANOVA. To measure the levels of MHC-peptide complexes during infection (C), dendritic cells were infected at an MOI of 10 as described previously and incubated thereafter for 24 h at 37°C in low Gentamicin medium. Subsequently, cells were harvested and stained with an Ab specific for H-2Kb-OVA257–264 complex (25D1.16, solid line) or an IgG1 isotype control (dotted line). Cells were washed with PBS, reconstituted in 1% PBS-BSA, and stained with a goat-anti-mouse PE-conjugated Ab. Finally, cells were washed with PBS and resuspended in 0.5% fixative.

FIGURE 11. Coinfection of macrophages with LM-OVA and ST-OVA does not result in the inhibition of Ag presentation against LM-OVA. IC-21 dendritic cells were seeded in 24-well-plates. Bacteria were added separately or together at an MOI of 10 and plates centrifuged to promote bacterial adsorption and incubated at 37°C for 30–60 min. Cells were subsequently washed vigorously and incubated with medium containing gentamicin (50 μg/ml) for 1–2 h to eliminate extracellular bacteria. Subsequently, cells were cocultured with CFSE-labeled OT-1 cells for 4 days in medium containing low levels of gentamicin (5 μg/ml). Cells were finally harvested and stained with anti-CD8α Ab. The degree of CD8+ T cell proliferation was evaluated by measuring CFSE dilution using flow cytometry. OVA-pep, OVA-peptide.
An important question that arises is how Ag presentation is eventually induced against ST. Many potential routes of cross-presentation have been suggested (45, 46). Dendritic cells may pick up Ag from dying APCs and present it to CD8\(^+\) T cells (47). ST induces rapid death of macrophages and dendritic cells (48, 49), and it has been shown that cross-presentation of ST Ags occurs through dendritic cells (50). It has also been shown that the genes of the SPI-I induce rapid apoptosis of infected macrophages (48, 51). Furthermore, in the absence of caspase-1 activity or under SPI-2-inducing conditions, macrophage death is greatly delayed (52). Although it is conceivable that the classical Ag-processing pathway operates with a greater efficiency, a direct comparison of the relative speed and efficiency of “cross-priming” vs classical Ag-processing pathway is not clear (53).

It has been previously shown that ST interferes with the acidification of phagosomes, causing an arrest of phagosome-lysosome fusion (54). Furthermore, there are several reports indicating that ST inhibits Ag presentation and that this may be due to the expression of SPI-II-dependent virulence mechanisms (55–58). Most of these studies were done in different in vitro models and involve the measurement of Ag presentation to CD4\(^+\) T cells using ST strains that are not as virulent. In another study it was shown that Ag presentation by ST-infected APCs does not depend on virulence factors of ST (36). Our results, in vitro and in vivo, indicate that a significant problem lies with the poor intracellular proliferation of ST within APCs. Several lines of evidence lead us toward this interpretation. Firstly, if suppression were to occur, then it should manifest strongly after the second week of infection, a time period when ST burden is at its peak. However, CD8\(^+\) T cell response is induced after the second week of infection, precluding the existence of suppressive mechanisms beyond day 15. Secondly, none of the mutants (aroA; SPI-I/II) of ST induced rapid Ag presentation, indicating that the known virulence mechanisms of ST do not inhibit Ag presentation. Thirdly, when mice were infected with LM and ST, the rapid CD8\(^+\) T cell response against LM was not impaired (our unpublished observations). Fourthly, we have previously reported that normal memory CD8\(^+\) T cells respond better when stimulated with Ag-pulsed spleen cells from ST-infected mice in comparison to normal spleen cells, indicating that the splenic environment in ST-infected mice is stimulatory, not suppressive (37). Finally, in another study SM1 CD4\(^+\) T cell transgenic cells failed to respond to low-dose infection with ST but responded efficiently when flagellin-peptide was administered in the same environment (59).

With an intracellular doubling time of 7 h for ST, T cell response should still develop much earlier. It is not clear what the threshold levels are for activating T cells in vivo. ST resides in the phagosomes of infected cells, so the Ags may be presented via the alternate pathway, which may induce further delay. We
have consistently noted a slightly reduced uptake of ST by dendritic cells that, coupled with the poor intracellular replication of ST within APCs, may cause an even greater delay in T cell priming. Incubation of DCs with a 100-fold more ST-OVA in comparison with LM-OVA did not induce Ag presentation, indicating that the relative difference in infection of APCs by itself may not explain the lack of Ag presentation. In comparison with LM, ST induces enormous and persistent inflammatory responses characterized by expression of numerous chemokines and inflammatory cytokines (our unpublished observations). It is possible that some of these events influence the maturation and Ag-presenting ability of DCs (58).

Intracellular proliferation is a prerequisite for proper Ag presentation and T cell activation in case of live intracellular pathogens or vaccines (60, 61). Logically, bacterial proliferation directly affects Ag abundance and, therefore, Ag presentation. CD8 T cell response against ST peaks only after the bacterial burden peaks (second week of infection) (34), supporting the notion that minimal antigenic threshold levels must be achieved before T cell priming proceeds (62). Indeed, when transferred into ST-OVA infected mice on day 21 of infection, OT-1 cells undergo rapid expansion within a 7-day period (Fig. 2), suggesting that at day 21 APCs have acquired the ability to present ST Ags to T cells rapidly. By referring to the loading calibration plot (Fig. 4), it can be inferred that when cells are infected with 1 MOI of LM-OVA, the levels of OVA-peptide generated are more than the EC50 of 0.005 nmol. By contrast, even at 100 MOI of ST-OVA the levels of OVA-peptide generated are lower than the EC50 and, therefore, not enough to prime CD8 T cells. The fact that ST-OVA infection of dendritic cells does not result in detectable surface MHC-peptide complex expression supports this notion. The sensitivity of our MHC-peptide titration curve remarkably overlaps with the one generated by Germain’s group (43).

There have been an increasing number of reports emphasizing the slow proliferation rate of ST in the intracellular compartment (63–67). Because ST is not an obligate intracellular pathogen, it appears that at any given time point ~50% of ST in the spleen are not cell associated (our unpublished observations). A revised view thus emerges regarding proliferation of ST in vivo, namely that despite the extensively perceived proliferation of ST, intracellular numbers within macrophages remain low, suggesting that the growth of ST occurs in fits and starts, with extracellular bacteria amplifying net numbers and intracellular bacteria contributing to chronicity and persistence. ST proliferates within epithelial cells (22), and this could also contribute toward an increase in the overall bacterial numbers. The reduced intracellular replication rates in APCs may be a deliberate pathogenic strategy of Salmonella to avoid immune responses, relying on extracellular replication to increase bacterial load.

Susceptible mice, including C57BL/6J, succumb to ST infection within 7 days. In contrast, resistant 129 × 1SJ mice harbor a chronic infection lasting >60 days. Because ST resides in the phagosomes of infected cells, this has been attributed to the function of the NRAMP1 gene (68), which codes for an ion transporter that helps deplete phagosomes of critical ions, such as manganese and iron. Furthermore, the phagosomes of infected cells pose a hostile environment characterized by poor nutrient content, low pH, antibacterial peptides, and lysosomal enzymes (28). Although ST appears to have evolved strategies to adapt to these conditions (69, 70), it is conceivable that ST merely persists, rather than proliferates, in such an environment.

All of the mutants that we used in this study expressed similar levels of OVA as determined by Western blotting and quantitative RT-PCR analysis (37), which indicates that the lack of Ag presentation by APCs infected with mutants could not be due to the disproportionate expression of OVA by mutants. We have previously reported that the SPI-I and SPI-II mutants of ST induce poor CD8 T cell response, leading to the conclusion that the magnitude of the CD8 T cell response is governed by pathogen virulence (37).

Previously, we reported that even conventional memory CD8 T cells, when transferred into naive hosts and challenged with ST-OVA, respond with delayed kinetics similar to those of naive CD8 T cells (34). How can vaccine-induced memory CD8 T cells facilitate protection against a pathogen whose Ags are not readily processed and presented? In such a scenario, irrespective of the number of memory T cells generated, they will not be able to expand into effectors rapidly. Considering that the susceptible mice die within 7 days of infection, a delay in T cell priming can be viewed as catastrophic. Relative to CD8 T cells, CD4 T cells have been shown to be intrinsically slow in responding to pathogens in general (71). Therefore, the immune system may have to rely on the innate immune compartment to curb ST burden for the first few weeks of infection. It may then be envisaged that down-regulation of innate immunity due to aging, pregnancy (72), or other reasons can have catastrophic outcomes. In this report we have highlighted that there is a dichotomy in the proliferation of ST. Rapid extracellular proliferation makes ST a highly virulent pathogen that needs to be controlled immediately for host survival. At the same time, the muted intracellular proliferation of ST within APCs compromises the development of acquired immune response and facilitates immune evasion.

Disclosures
The authors have no financial conflict of interest.

References


