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Modulation of Antigenic Location Converts Chronic into Acute Infection by Forcing CD8$^+$ T Cell Recognition

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SUMMARY

Pathogens that reside in the phagosomes of infected cells persist despite the presence of potent T cell responses. We addressed the mechanism of immune evasion by using a mouse model of *Salmonella typhimurium* (ST). Recombinants of ST were generated that translocated antigen to the cytosol or phagosomes of infected cells. We find that the kinetics of antigen presentation and CD8$^+$ T cell priming is accelerated by cytosolic antigen delivery, although the magnitude of CD8$^+$ T cell response is not influenced by antigenic location. More importantly, only those targets that readily display antigen on the cell surface, owing to antigenic translocation to the cytosol, are recognized and killed by CD8$^+$ T cells. Thus, vaccination approaches developed to control phagosomal pathogens should incorporate methods for modulating antigen presentation such that infected target cells can be readily recognized by CD8$^+$ T cells.

INTRODUCTION

After antigen presentation, naive CD8$^+$ T cells undergo differentiation into effectors that eliminate infected cells (Prlic et al., 2007). CD8$^+$ T cells are activated rapidly during infection with intracellular pathogens such as *lymphocytic choriomeningitis virus* (LCMV) or *Listeria monocytogenes* (LM), which facilitates pathogen control (van Stipdonk et al., 2001; Kaech and Ahmed, 2001). However, in cases of pathogens that reside in phagosomes, such as *Salmonella serovar typhimurium* (ST) and mycobacteria, activation of CD8$^+$ T cells is delayed (Luu et al., 2006; van Faassen et al., 2004; Vidric et al., 2006; Srinivasan et al., 2004b), suggesting a correlation to chronic infection. Activation of CD8$^+$ T cells by alternative routes of cross-presentation have been reported in the case of phagosomal pathogens (Schaible et al., 2003; Houde et al., 2003; Yrlid and Wick, 2000).

ST survives within the phagosomes of host cells, and its intracellular replication is essential for virulence (Leung and Finlay, 1991). ST induces a systemic typhoid-like disease in mice and gastroenteritis in humans (Jones and Falkow, 1996). Susceptibility to ST has been associated with a mutation in the natural resistance-associated macrophage protein (NRAMP) gene in macrophages (Bellamy, 1999). 129X1SvJ or B6.129F1 mice (which have a normal NRAMP gene) are resistant to infection, but ST is not completely eliminated and a chronic infection ensues (Luu et al., 2006). The mechanisms behind the persistence of ST despite induction of potent T cell response are not clear (Srinivasan et al., 2004b; Luu et al., 2006).

CD4$^+$ T cells recognize antigens on the major histocompatibility complex (MHC) class II$^+$ targets that are rare. Yet CD4$^+$ T cells are the predominant T cell subset that limits phagosomal pathogens (Lo et al., 1999; Hess et al., 1996; Flynn et al., 1992). However, phagosomal pathogens are slowly controlled, and are never completely eliminated (Monack et al., 2004; Scanga et al., 2000). On the other hand, CD8$^+$ T cells recognize antigens on MHC class I$^+$ cells (almost every cell), yet they appear to play only a secondary role against phagosomal pathogens. Phagosomal pathogens present a dilemma because they are considered highly immunosuppressive (VanHeyningen et al., 1997; Tobar et al., 2006; Halici et al., 2008; van der Velden et al., 2005; Johanns et al., 2010), yet they induce potent CD4$^+$ and CD8$^+$ T cell responses (Dudani et al., 2002; Luu et al., 2006; Serbina et al., 2000; Hone et al., 1992).

In this study, we addressed whether the location of antigen within an infected cell influences bacterial persistence. We used recombinants of ST that reside in the phagosomes but display differential antigenic trafficking (cytosol versus phagosome). Our results indicate that ST persists despite the generation of a potent CD8$^+$ T cell response due to poor recognition of infected cells by CD8$^+$ T cells.

RESULTS

Antigen Translocation Does Not Affect Bacterial Replication or Their Ability to Infect Cells

We have previously reported that infection of mice with recombinant ST, expressing ovalbumin (OVA), does not induce a detectable CD8$^+$ T cell response within the first week of infection (Luu et al., 2006; Albaghdadi et al., 2009), and this is due to delayed

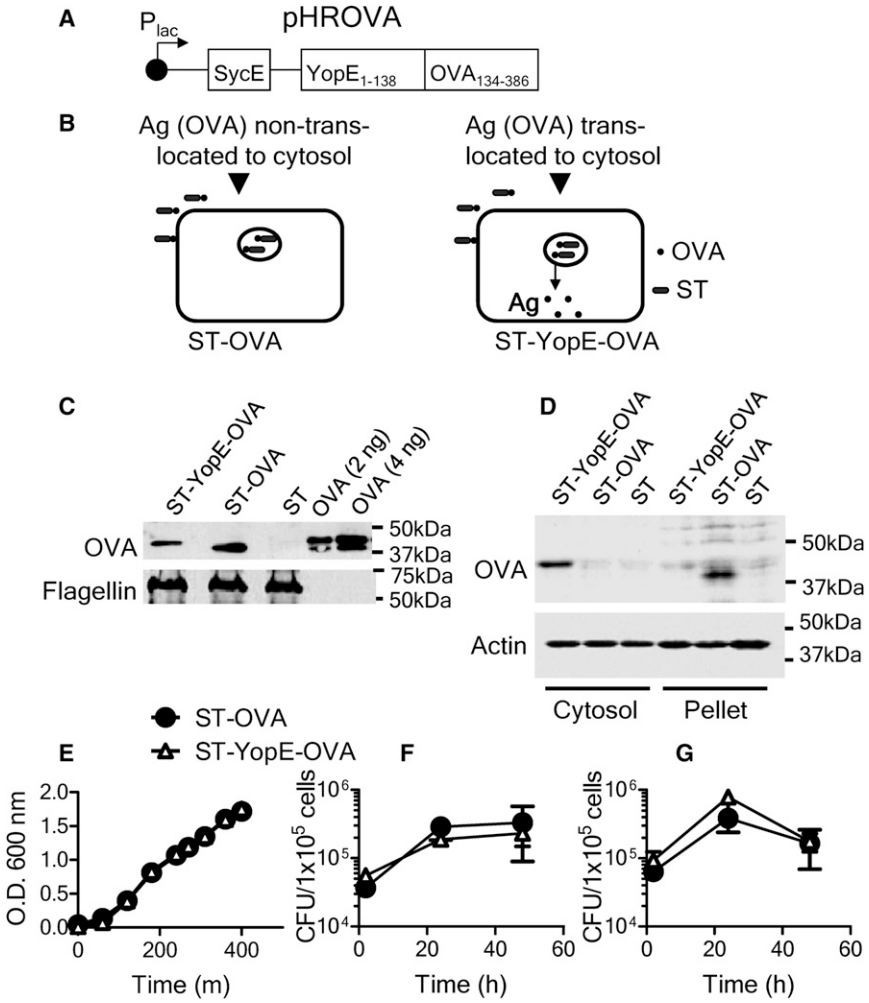

Figure 1. Antigenic Translocation Does Not Influence Proliferation or Phagocytosis of ST

(A and B) Truncated OVA was incorporated into a plasmid coding for the translocation domains of YopE. The translocation of the resulting chimeric protein YopE-OVA to the cytosol by the type III secretion system of ST is mediated by the YopE-specific chaperone SycE also contained in the plasmid.

(C) Expression of OVA, using flagellin as a loading control, was measured in the bacterial pellet by western blotting. Samples were normalized to 1×10^7 cells.

(D) Expression of OVA and actin in the cytosolic and noncytosolic fraction of infected spleens (10^7, i.v. at 24 hr postinfection) was measured by western blotting.

(E) Liquid cultures of bacteria were set up in flasks, and the bacterial growth was measured by OD_{600nm} at various time intervals.

(F) IC-21 macrophages ($H\text{-}2^b$) (5×10^4/well) were infected with bacteria (10 multiplicity of infection [MOI]) for 30 min. After removal of extracellular bacteria, cells were lysed at various time intervals and bacterial burden in the macrophages determined.

(G) Bacterial burden in HeLa cells was evaluated as described in (F).

Results represent the mean of three to six replicates ± SD per group and are representative of three independent experiments. See also Figure S1.

antigen presentation to $CD8^+$ T cells (Luu et al., 2006; Albaghdadi et al., 2009). We generated recombinants of ST that deliver antigen to the phagosomes or cytosol of infected cells while the pathogen still remains confined to the phagosomes. We incorporated OVA into a plasmid coding for the translocation domains of the carrier molecule YopE (Yersinia outer-membrane protein E) (Rüssmann et al., 2001) to generate ST-YopE-OVA. The translocation of the resulting chimeric protein YopE-OVA to the cytosol by the type III secretion system of ST is mediated by the YopE-specific chaperone SycE also contained in the plasmid (Figures 1A and 1B). The recombinant ST that does not translocate OVA to the cytosol (ST-OVA) was previously constructed and characterized (Luu et al., 2006; Albaghdadi et al., 2009). ST-OVA and ST-YopE-OVA had similar levels of OVA expression. Intensity of OVA expression by densitometry was 143 for ST-YopE-OVA, 187 for ST-OVA, 17 for ST, 163 for 2 ng OVA, and 199 for 4 ng OVA. The expression of OVA in the cytosolic fraction of spleen cells was detectable only from ST-YopE-OVA-infected mice (Figure 1D). There was no difference in the doubling time of ST-YopE-OVA versus ST-OVA when grown extracellularly in vitro (Figure 1E). The uptake and the intracellular replication of ST within macrophages (Figure 1F) and epithelial cells (Figure 1G) were also similar for ST-YopE-OVA and ST-OVA. Lack of early T cell activation based on expression of pan-T cell activation markers in ST-infected mice is evident in susceptible (C57BL/6J) and resistant (129X1/SvJ) mice (Figures S1A–S1C). These results indicate that YopE does indeed mediate the translocation of passenger antigens to the cytosol of infected cells, and this does not influence the uptake or intracellular proliferation of ST.

Antigen Translocation to Cytosol Induces Rapid Antigen Presentation

We then ascertained the kinetics of antigen presentation by evaluating the expression of MHC I-OVA-peptide complex after infection of dendritic cells with ST-OVA or ST-YopE-OVA. Infection with ST-YopE-OVA, but not ST-OVA, resulted in increased induction of MHC I-peptide complex (Figure 2A). Furthermore, infection of cells with ST-OVA did not result in any detectable proliferation of OT-1 cells, indicating lack of rapid antigen presentation to $CD8^+$ T cells (Figure 2B). In contrast, infection with ST-YopE-OVA resulted in proliferation of OT-1 cells, indicative of rapid antigen presentation in vitro (Figure 2B). As expected, infection with LM-OVA, which replicates in the cytosol of infected cells, induced potent proliferation of OT-1 cells. Both ST-OVA and ST-YopE-OVA induced similar antigen presentation to

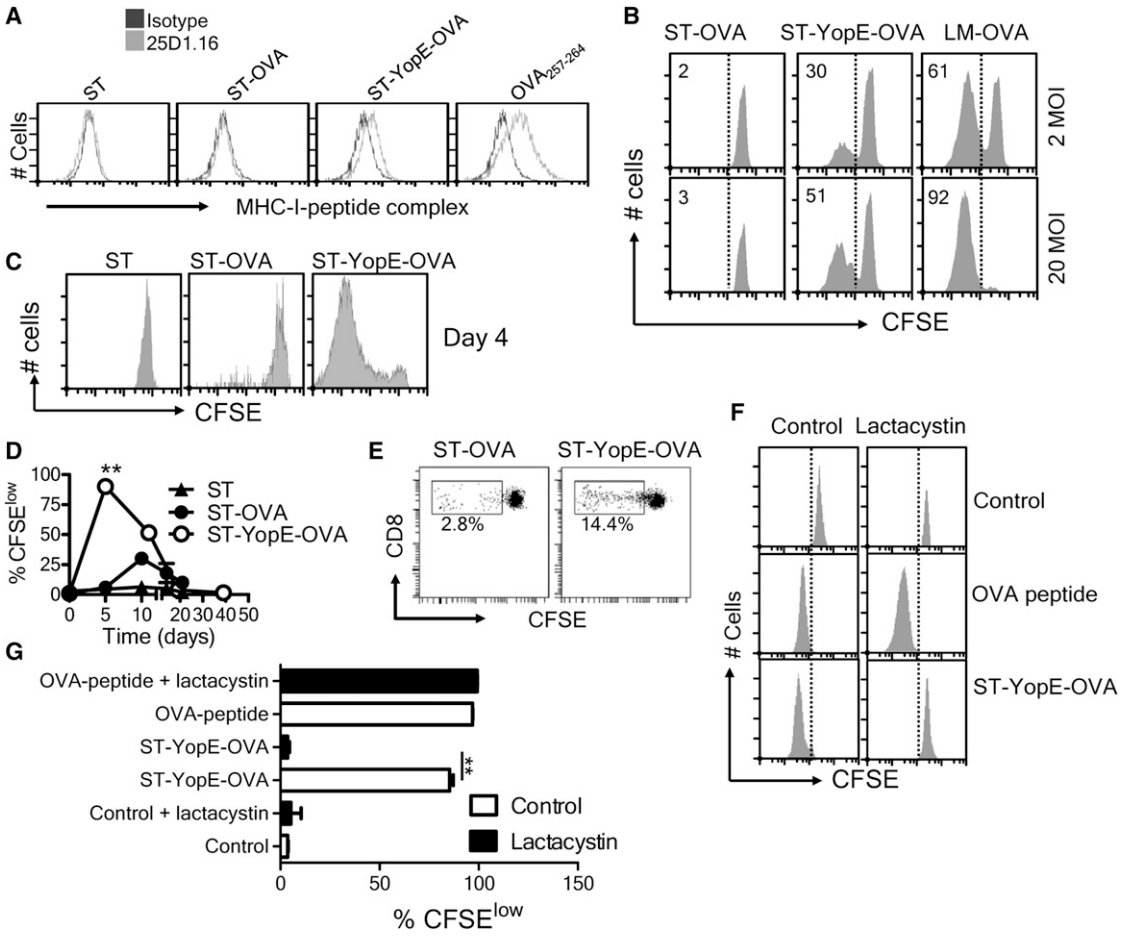

Figure 2. ST-YopE-OVA Induces Rapid Antigen Presentation

(A) Dendritic cells (JAWS) were infected with 50 MOI bacteria for 2 hr, followed by washing and culture in media containing 5 μg/ml gentamicin. At 6 hr, cells were retrieved and the expression of MHC I-peptide complex evaluated by flow cytometry after staining with isotype or 25D1.16 antibody.

(B) IC-21 macrophages (H-2^b) (10^5/well) were infected with bacteria for 30 min. Extracellular bacteria were removed and cells were incubated with CFSE-labeled OT-1 TCR transgenic cells (10^6/well). After 3 days of culture, cells were harvested, stained with anti-CD8 antibody, and the reduction in CFSE intensity of OT-1 $CD8^+$ T cells was evaluated by flow cytometry.

(C and D) B6.129F1 mice were infected (10^3, i.v.) with bacteria. At various time intervals, CFSE-labeled OT-1 cells were injected (5×10^6, i.v.). Four days after the transfer of OT-1 cells, spleens were isolated from recipient mice and spleen cells were stained with OVA-tetramer and anti-CD8 antibody. Reduction of CFSE expression was evaluated in OT-1 cells. Results represent the mean of three mice ± SD per group, and are representative of two or three independent experiments (**$p < 0.01$).

(E) B6.129F1 mice were infected as in (C). At day 3, DCs were isolated using CD11c magnetic beads and cultured (10^5/well) with CFSE-labeled OT-1 cells (10^5/well) for 72 hr in vitro. Reduction in CFSE expression was evaluated by flow cytometry after staining cells with anti-CD8 antibody and OVA tetramers.

(F and G) IC-21 macrophages (5×10^5/well) were cultured for 1 hr with lactacystin (80 μM) at 37°C. Cells were infected with ST-YopE-OVA (10 MOI). After 30 min, cells were washed and kept in medium containing gentamicin (10 μg/ml) with or without lactacystin (40 μM) at 37°C. After overnight incubation, cells were fixed in 0.5% paraformaldehyde followed by incubation with polylysine (2.5 mg/ml) to neutralize residual paraformaldehyde. Purified $CD8^+$ T cells from OT-1 mice were labeled with CFSE and added to the cultures (2×10^5/well). After 2 days of culture, cells were harvested, stained with anti-CD8 antibody, and reduction in CFSE intensity of $CD8^+$ T cells was evaluated. Results represent the mean ± SD per group, and are representative of two independent experiments (**$p < 0.01$).

See also Figure S2.

$CD4^+$ T cells in vitro (Figure S2). We then evaluated whether antigen translocation induces rapid antigen presentation in vivo. At day 4 postinfection, the majority of OT-1 cells proliferated in mice that had been infected with ST-YopE-OVA, indicating rapid antigen presentation (Figure 2C). In contrast, OT-1 cells proliferated poorly in ST-OVA-infected mice. We observed that OT-1 cells underwent activation in ST-OVA-infected mice only at day 11 postinfection (Figure 2 D), confirming delayed antigen presentation. Furthermore, the magnitude of peak antigen presentation by ST-YopE-OVA was substantially higher. Antigen presentation that was induced early on in ST-YopE-OVA-infected mice was reduced to baseline levels at 3 weeks postinfection, implying lack of antigen persistence, a situation that is similar to LM infection (van Faassen et al., 2004). Additionally, dendritic cells isolated from ST-YopE-OVA-infected mice at day 3 postinfection induced stronger proliferation of carboxyfluorescein succinimidyl

ester (CFSE)-labeled OT-1 cells in vitro in comparison to dendritic cells isolated from ST-OVA-infected mice (Figure 2E). These results indicate that lack of early antigen presentation during ST-OVA infection is related to poor antigen processing rather than the development of suppressive dendritic cells.

Cytosolic Antigen Processing Induced by ST-YopE-OVA

Various mechanisms of antigen processing involving cytosolic and noncytosolic (or vacuolar) pathways have been described for the MHC class I pathway (Amigorena and Savina, 2010; Shen and Rock, 2006). Proteasomal degradation of proteins plays a key role in classical cytosolic antigen processing and is specifically inhibited by lactacystin (Craiu et al., 1997). We therefore evaluated antigen presentation with ST-YopE-OVA-infected antigen-presenting cells (APCs) in the presence or absence of lactacystin. While ST-YopE-OVA-infected APCs induced antigen presentation to CFSE-labeled OT-1 $CD8^+$ T cells, lactacystin treatment resulted in complete inhibition of this response (Figures 2F and 2G). As expected, lactacystin treatment had no effect on the proliferation of OT-1 cells induced by $OVA_{257-264}$ peptide that is directly loaded onto MHC I. These results indicate that antigen presentation against ST-YopE-OVA was induced by the classical cytosolic antigen-processing pathway, and a lack of cytosolic antigen processing in ST-OVA-infected cells leads to poor $CD8^+$ T cell activation initially.

Cytosolic Antigen Presentation Results in Pathogen Control and Induction of Early $CD8^+$ T Cell Response

We infected B6.129F1-resistant mice with ST-OVA and ST-YopE-OVA. At day 3 postinfection, mice infected with ST-YopE-OVA or ST-OVA had similar bacterial burdens (Figure 3A). Subsequently, the burden of ST-YopE-OVA was controlled and reduced to nondetectable levels by day 15. In contrast, ST-OVA burden was maintained at high levels, as expected, for prolonged periods (Figure 3A). Similar results were noted in the liver (data not shown). Furthermore, mice infected with ST-YopE-OVA had lower spleen size (Figure 3B). Infection of mice with ST-YopE-OVA resulted in the development of a rapid and potent endogenous OVA-specific $CD8^+$ T cell response, which peaked at day 7 (Figures 3C and S3A). As expected, infection of mice with ST-OVA resulted in a delayed $CD8^+$ T cell response that was nondetectable at day 7 and peaked around day 20. At day 21 postinfection, ST-OVA-infected mice had high numbers of OVA-specific $CD8^+$ T cells (Figure 3C), but this did not result in rapid clearance of ST-OVA subsequently (Figure 3A).

We observed that infection with ST-YopE-OVA, but not ST-OVA, resulted in rapid downregulation of CD62L—as early as day 5—and rapid progression to the memory state (Figures 3D–3F and S3B). Thus, early cytosolic antigen processing and presentation, in the context of ST, accelerates the kinetics and increases $CD8^+$ T cell differentiation and memory development.

We also measured the frequency of OVA-specific $CD8^+$ T cells by ELISPOT assay ex vivo. Again, ST-YopE-OVA-infected mice displayed a rapid generation of OVA-specific, interferon γ (IFN-γ)-secreting $CD8^+$ T cells (Figure 3G). This was followed by a rapid contraction of the response and maintenance of a stable pool of IFN-γ-secreting, OVA-specific $CD8^+$ T cells. Mice infected with ST-OVA, on the other hand, displayed a delayed response that increased progressively and even surpassed the response observed in ST-YopE-OVA-infected mice (Figure 3G). We also measured cytolytic activity of OVA-specific $CD8^+$ T cells in vivo. ST-YopE-OVA-infected mice generated a rapid and potent cytolytic response (Figure 3H) that declined progressively, commensurate with reduction in the frequency of OVA-specific $CD8^+$ T cells. On the other hand, mice infected with ST-OVA generated minimal cytolytic response early on, which increased progressively to high levels by day 30 postinfection (Figure 3H).

We addressed whether the delivery of other antigens through the type III secretion system of ST would also evoke efficient control of infection. Therefore, similar to ST-OVA and ST-YopE-OVA, recombinants of ST were generated to express an immunodominant epitope-containing fragment of the LCMV nucleoprotein (NP) that fused to YopE (ST-YopE-NP) or not (ST-NP). Both the ST-NP and ST-YopE-NP constructs expressed similar levels of NP (data not shown). A similar bacterial burden was noted in the spleens of mice infected with ST-NP and ST-YopE-NP at day 1 and 3 postinfection (Figure 3 I). At later time intervals, while the ST-NP burden remained at high levels, the burden of ST-YopE-NP was controlled. The kinetics of $CD8^+$ T cell response was rapid for ST-YopE-NP and delayed (as expected) for ST-NP (Figure 3J). Furthermore, ST-NP-infected mice displayed a massive increase in the number of spleen cells (Figure 3K). Thus, these results indicate that the phenomenon described herein is observed with other antigens.

Control of Pathogen Burden and Development of Rapid $CD8^+$ T Cell Response in Susceptible Mice

C57BL/6J mice are highly susceptible to ST and die within the first week of infection (Albaghdadi et al., 2009). At day 1 and day 3 postinfection, similar bacterial burdens were noted in ST-OVA- infected and ST-YopE-OVA-infected C57BL/6J mice (Figure 4A). Subsequently, the bacterial burden in ST-OVA-infected mice continued to increase exponentially to lethal levels ($>10^8$), whereas the burden in ST-YopE-OVA-infected mice was rapidly controlled ($<10^3$) and became undetectable at day 14. Abridgment of chronicity in ST-YopE-OVA-infected mice correlated to the early emergence of potent OVA-specific $CD8^+$ T cell response (Figure 4B). We evaluated the long-term survival of ST-YopE-OVA- infected versus ST-OVA-infected C57BL/6J mice. Whereas all of the ST-OVA-infected mice died within the first week of infection, ST-YopE-OVA-infected mice displayed a significant increase in survival, with >50% surviving beyond day 90 postinfection (Figure 4C). Similar results were obtained with ST-YopE-NP (Figure 4D). We measured the colony-forming units (cfu) in the spleens of moribund ST-YopE-OVA-infected mice between days 20 and 30 postinfection, and noted that the bacteria in these mice had lost the plasmid as revealed by total absence of cfu in Ampicillin plates (Figure 4E). These results indicate that potent $CD8^+$ T cell response selects for antigenic loss variants in susceptible hosts. We evaluated the survival of Rag-1-deficient C57BL/6J mice infected with ST-OVA or ST-YopE-OVA. Both ST-OVA-infected and ST-YopE-OVA-infected mice were moribund between days 10 and 15 postinfection (Figure 4F), suggesting that YopE expression by ST does not attenuate the bacterium. Infection with a 100-fold

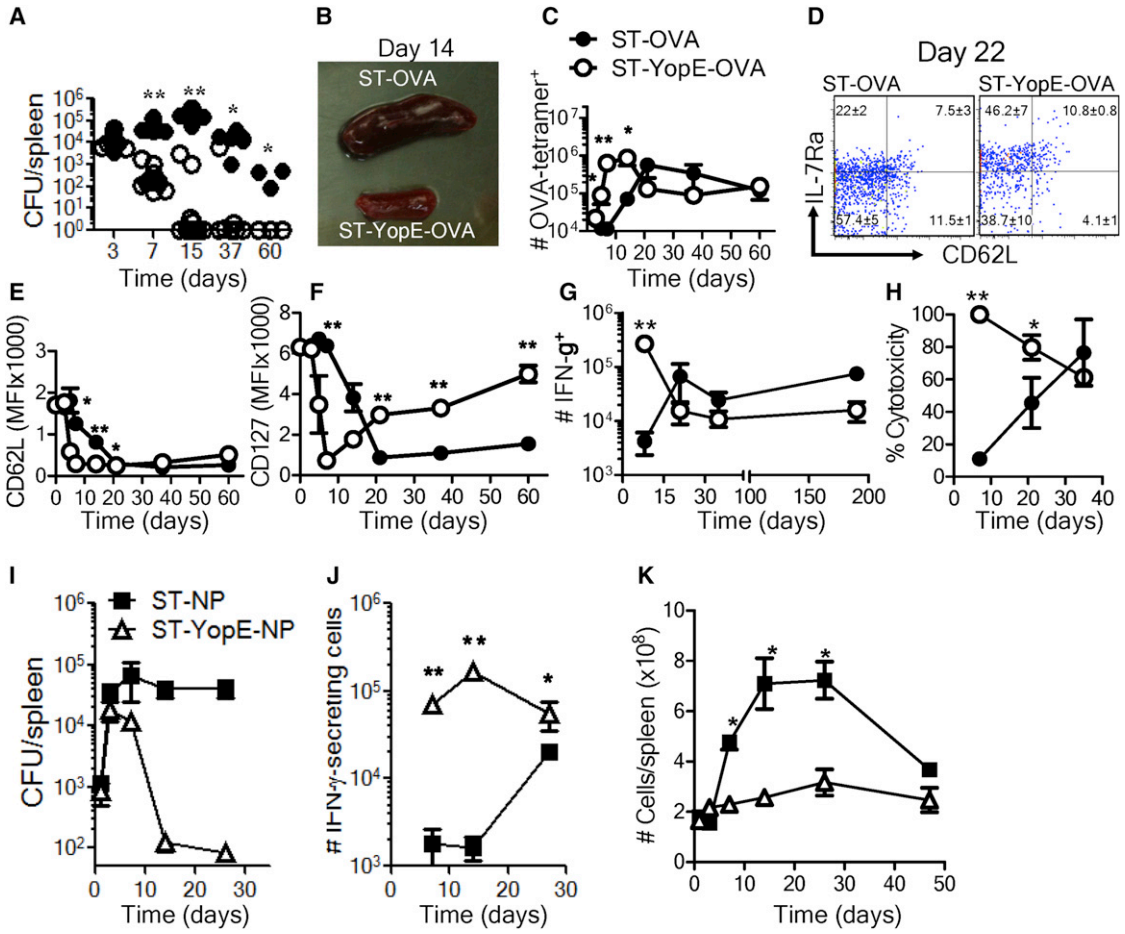

Figure 3. Antigen Translocation Leads to Rapid Control of ST

(A–H) B6.129F1 mice were infected (10^3, i.v.) with bacteria. At various time intervals, spleen cells were removed and bacterial burden evaluated (A). Difference in spleen size in ST-OVA-infected versus ST-YopE-OVA-infected mice (B). Spleen cells were stained with OVA-tetramer and anti-CD8 antibody, and the numbers of OVA-specific $CD8^+$ T cells evaluated (C). Cells were also stained with antibodies against CD62L and CD127 (D). The expression (mean fluorescence intensity) of CD62L (D and E) and CD127 (D and F) on OVA-tetramer$^+$$CD8^+$ T cells was evaluated. Frequency of OVA-specific $CD8^+$ T cells per spleen was also evaluated by ELISPOT assay at various time intervals postinfection (G). In the absence of peptide stimulation, the number of spots was less than 500 per spleen. In vivo cytolytic activity of OVA-specific $CD8^+$ T cells was evaluated by injecting CFSE-labeled, OVA-pulsed, and control spleen cells from naive B6.129F1mice into naive or infected mice. Fate of injected targets was evaluated 24 hr later (H).

(I–K) B6.129F1 mice were infected (10^3, i.v.) with ST-NP and ST-YopE-NP. At various time intervals, spleens were removed from infected mice. Bacterial burdens were evaluated by plating serial dilutions on BHI-agar plates (I). Frequency of NP-specific $CD8^+$ T cells was evaluated by ELISPOT assay (J). The number of spleen cells was evaluated at various time intervals in the two groups of mice (K).

Results represent the mean of three to five mice ± SD per group (C, E–K) and are representative of three independent experiments (**$p < 0.01$ and *$p < 0.05$). See also Figure S3.

higher dose of ST-OVA in comparison to ST-YopE-OVA did not influence the kinetics of $CD8^+$ T cell response or ST-OVA control (Figures S4A and S4B). Taken together, these results underscore the importance of this paradigm wherein the deposition of antigen into the appropriate intracellular compartment can modulate host survival profoundly.

$CD8^+$ T Cells Control ST-YopE-OVA

We next evaluated whether the control of ST-YopE-OVA was indeed mediated by $CD8^+$ T cells. B6.129F1 mice were depleted of $CD4^+$ or $CD8^+$ T cells and infected with ST-YopE-OVA. At day 7 postinfection, mice depleted of $CD8^+$ T cells, but not $CD4^+$ T cells, had >100-fold higher ST-YopE-OVA burden than controls, demonstrating that $CD8^+$ T cells controlled ST-YopE-OVA (Figure 5A). Interestingly, after depletion of $CD8^+$ T cells, the bacterial burden in the spleens of ST-YopE-OVA-infected mice at day 7 was ~10^5, which was similar to the burden noted in mice infected with ST-OVA (Figure 3A). In mice infected with ST-OVA, depletion of $CD4^+$ or $CD8^+$ T cells had no effect on the bacterial burden (Figure 5B). We further confirmed the importance of $CD8^+$ T cells in controlling ST-YopE-OVA burden by infecting wild-type, MHC I-deficient, or MHC II-deficient C57BL/6J mice with ST-YopE-OVA. MHC class I-deficient mice were moribund and harbored very high bacterial loads (~10^8) (Figure 5C),

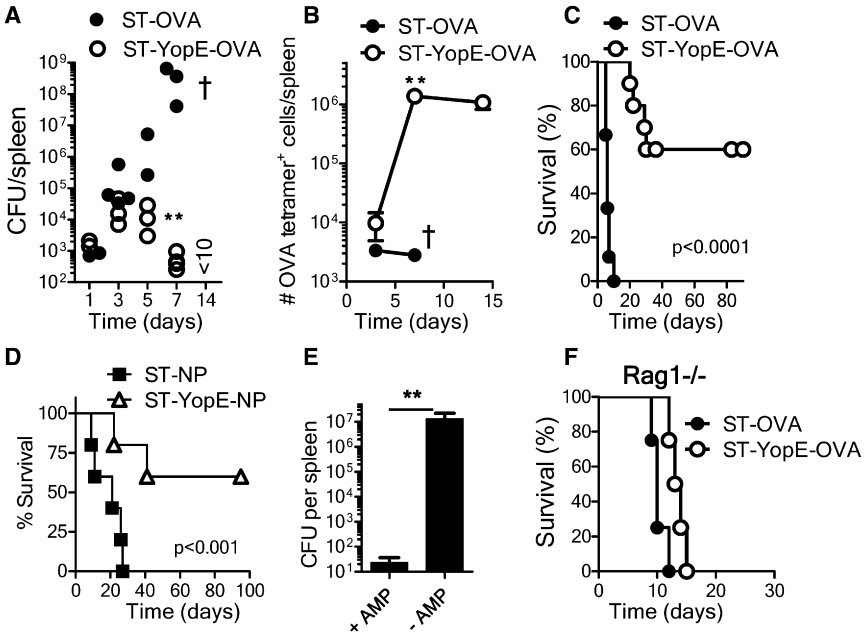

Figure 4. ST-YopE-OVA Infection Results in Rapid CD8⁺ T Cell Response and Pathogen Control in Susceptible Mice

(A–C) C57BL/6J mice were infected (10^3, i.v.) with bacteria. At various time intervals spleens were removed and the bacterial burdens enumerated. Spleen cells were stained with OVA-tetramer and anti-CD8 antibody and the numbers of OVA-tetramer⁺ CD8⁺ T cells per spleen at various time intervals enumerated. Survival was assessed by monitoring the weights and clinical signs of mice.

(D) C57BL/6J mice were infected (10^3, i.v.) with ST-NP or ST-YopE-NP. Survival was assessed by monitoring the weights and clinical signs of mice.

(E) C57BL/6J mice were infected (10^3, i.v.) with ST-YopE-OVA. Between days 20 and 30, the spleens of moribund mice were removed and bacterial burden evaluated on ampicillin-coated or control BHI-agar plates.

(F) B6.Rag1-deficient mice were infected (10^3, i.v.) with bacteria, and survival of mice was monitored. Results represent the mean ± SD per group (B, E) and are representative of two independent experiments (**$p < 0.01$).

See also Figure S4.

whereas MHC class II-deficient mice had very low bacterial burden that was not statistically different from control wild-type hosts. Further experiments indicated that even if the CD8⁺ T cell response is forcibly delayed, complete elimination of ST-YopE-OVA by CD8⁺ T cells still occurs in resistant mice, indicating that the timing of CD8⁺ T cell activation (early or delayed) does not influence the control of ST-YopE (Figure S5). Thus, these results indicate that deposition of antigen into the cytosol of infected cells induces CD8⁺ T cell-dependent control of an otherwise uncontrollable infection.

Target Cell Recognition by CD8⁺ T Cells Is a Critical Factor in Control of ST-YopE

We addressed whether target cell recognition by CD8⁺ T cells is the main reason that facilitates efficient control of ST-YopE-OVA. We generated activated OVA-specific CD8⁺ T cells by stimulating OT-1 cells in vitro with LM-OVA. These primed CD8⁺ T cells were then tested for their ability to specifically kill infected macrophages (Figure 6A). Activated OVA-specific CD8⁺ T cells killed macrophages only when they were infected with ST-YopE-OVA, but not ST-OVA or ST (Figure 6B). We also generated OVA-specific memory CD8⁺ T cells in C57BL/6J mice after infecting them with LM-OVA. At day 41, mice were challenged with ST-OVA or ST-YopE-OVA (Figure 6C). Challenge of immunized mice with ST-YopE-OVA resulted in expansion of OVA-specific CD8⁺ T cells in the blood (data not shown) and spleen at day 3 (Figure 6E) and day 5 (Figures 6D and 6E) postchallenge. In contrast, challenge with ST-OVA failed to induce the expansion of OVA-specific memory CD8⁺ T cell response. Bacterial burden in the spleen of immunized mice challenged with ST-YopE-OVA was undetectable (Figure 6F). In contrast, priming with LM-OVA had no significant effect on the burden of ST-OVA (Figure 6F). Thus, these results indicate that ST-OVA infection of host cells results in a lack of target cell recognition by primed CD8⁺ T cells, which leads to pathogen chronicity.

Bacterial Chronicity Promotes Inflammation and Immune-Evasive Mechanisms

Because chronic intracellular bacteria are associated with numerous immune-evasive mechanisms, we were interested in determining whether these mechanisms are modulated by YopE-mediated antigenic delivery into the cytosol of infected cells. We evaluated cytokine/chemokine expression in the serum of infected mice using a cytokine array. At day 4 postinfection, there was similar induction of cytokines/chemokines in mice infected with ST-OVA and ST-YopE-OVA (Figures 7A and 7B), commensurate with similar bacterial burden in the spleen (Figure 7C). Interestingly, at day 14, when the bacterial burden was controlled in ST-YopE-OVA-infected mice, the expression of cytokines/chemokines was reduced to baseline levels in ST-YopE-OVA-infected mice in comparison to ST-OVA-infected mice (Figure 7B). The expression of both inflammatory and anti-inflammatory cytokines was exacerbated by bacterial chronicity. These results indicate that pathogen chronicity is not due to a selective induction of anti-inflammatory mechanisms.

We also evaluated the induction of CD4⁺CD25⁺FoxP3⁺ (T regulatory cells [T-regs]) in mice infected with ST-OVA versus ST-YopE-OVA. During the initial stages of infection, there was a similar increase in the numbers of T-regs in the two groups of mice (Figure S6A). Subsequently, the numbers of T-regs continued to increase in ST-OVA-infected mice, whereas the numbers of T-regs decreased precipitously at day 14 in ST-YopE-OVA-infected mice (Figure S6B). Depletion of T-regs in ST-OVA-infected mice did not alter the numbers of OVA-specific CD8⁺ T cells significantly (Figures S6C and S6D). Thus, these results indicate that immune-suppressive mechanisms are not the reason for delayed kinetics of CD8⁺ T cell priming against ST.

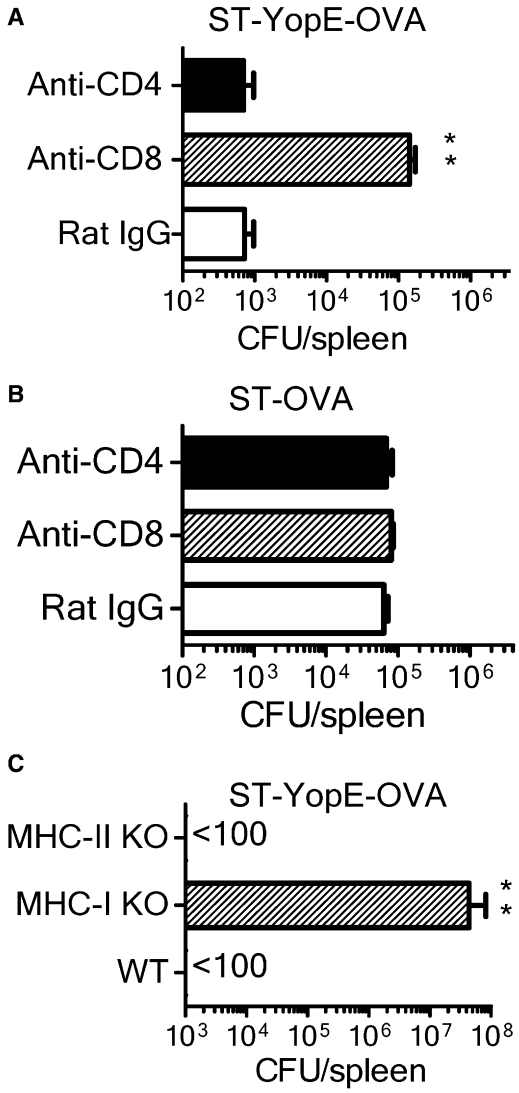

Figure 5. $CD8^+$ T Cells Control ST-YopE-OVA Burden in Susceptible Mice

C57BL/6J mice were injected intraperitoneally with (100 μg/injection) anti-CD4 (clone GK1.5), anti-CD8 (clone 2.43), or Rat IgG isotype antibodies on days −3, 0, and 3 after infection with 10^3 ST-YopE-OVA or 10^3 ST-OVA. At day 7 postinfection, spleens were harvested and the bacterial burden evaluated (A, B). Results represent the mean of three to four mice ± SD per group. Wild-type, MHC I-deficient, or MHC II-deficient mice (all on B6 background) were infected with 10^3 ST-YopE-OVA (C). At day 20 postinfection, spleens were harvested and the bacterial burden evaluated (C). Results represent the mean of five mice ± SD per group (**$p < 0.01$). Results are representative of two independent experiments.

DISCUSSION

Intracellular bacteria that reside in the phagosomes of infected cells, such as ST and *Mycobacterium tuberculosis*, present a dilemma, because they are considered immune evasive yet highly immunogenic at the same time (Jones and Falkow, 1996; Flynn and Chan, 2001). They induce potent $CD4^+$ and $CD8^+$ T cell responses (Srinivasan et al., 2004a; Serbina and Flynn, 2001; Stover et al., 1991; Dudani et al., 2002), but the pathogens are never completely eliminated (Monack et al., 2004; Scanga et al., 2000). It is thus not clear how these pathogens persist despite the generation of potent T cell responses. We addressed the possibility that poor recognition of infected cells, specifically by $CD8^+$ T cells, may be the key mechanism of immune evasion.

Although $CD8^+$ T cells efficiently eliminate LCMV and LM (Kägi et al., 1994; Harty and Bevan, 1992), they appear to play only a secondary role in controlling ST (Lo et al., 1999; Hess et al., 1996) despite induction of a potent $CD8^+$ T cell response in the long-term (Luu et al., 2006). $CD4^+$ T cells appear to be the predominant immune cell that limits ST in resistant mice (Lo et al., 1999; Dougan et al., 2011). However, ST is never completely eliminated (Monack et al., 2004), because it takes >90 days to bring the bacterial burden to undetectable levels in resistant mice (Luu et al., 2006). Our results indicate that when $CD8^+$ T cells are made to recognize infected cells appropriately, they become the most important and protective cell type that profoundly modulates the duration of infection. These results imply that $CD8^+$ T cells may be more efficient at mediating immune surveillance than $CD4^+$ T cells. A possible explanation for this may be related to the ubiquitous expression of MHC I on all cells in comparison to the selective expression of MHC II only on some cells.

Cross-presentation of noncytosolic antigens has been revealed as an alternate pathway that results in the induction of $CD8^+$ T cell response (den Haan and Bevan, 2001; Wei et al., 2010; Hirosue et al., 2010; Basta et al., 2002; Goldszmid et al., 2009). Because ST-OVA/NP resides predominantly in the phagosome of infected cells, it is likely that $CD8^+$ T cells are activated mainly through cross-presentation. Various antigen-processing pathways can be considered against ST-OVA/NP, such as phagosomal-cytosolic pathway, endoplasmic reticulum-phagosome fusion, vacuolar pathway, endosome-to-endoplasmic reticulum pathway and endoplasmic reticulum-associated degradation pathway (Houde et al., 2003; Shen and Rock, 2006; Yrlid and Wick, 2000; Winau et al., 2006). Alternatively, it is possible that some amount of antigen leaks to the cytosol even during infection with ST-OVA, and this process may be inefficient and protracted. Further studies are therefore required to determine the contribution of these pathways during infection with ST-OVA. On the other hand, antigens localized in the cytosol of host cells are processed through the cytosolic pathway, which is dependent on proteasomal degradation (Goldberg et al., 2002). Our results indicate that this seems to be the main pathway that operates when antigen expressed by ST is translocated to the cytosol.

$CD8^+$ T cells can efficiently control a pathogen only when the target cells that harbor the pathogen also display pathogen-derived peptide on the cell surface. It is likely that this does not happen in the case of phagosomal pathogens. Although cross-presentation of antigen after death of infected targets can activate $CD8^+$ T cells by the alternative pathway (Albert et al., 1998; Yrlid and Wick, 2000), this mechanism may not promote recognition of "infected" target cells by primed $CD8^+$ T cells. Thus, target cells that contain the pathogen but fail to display pathogen-derived peptides on the cell surface will be spared

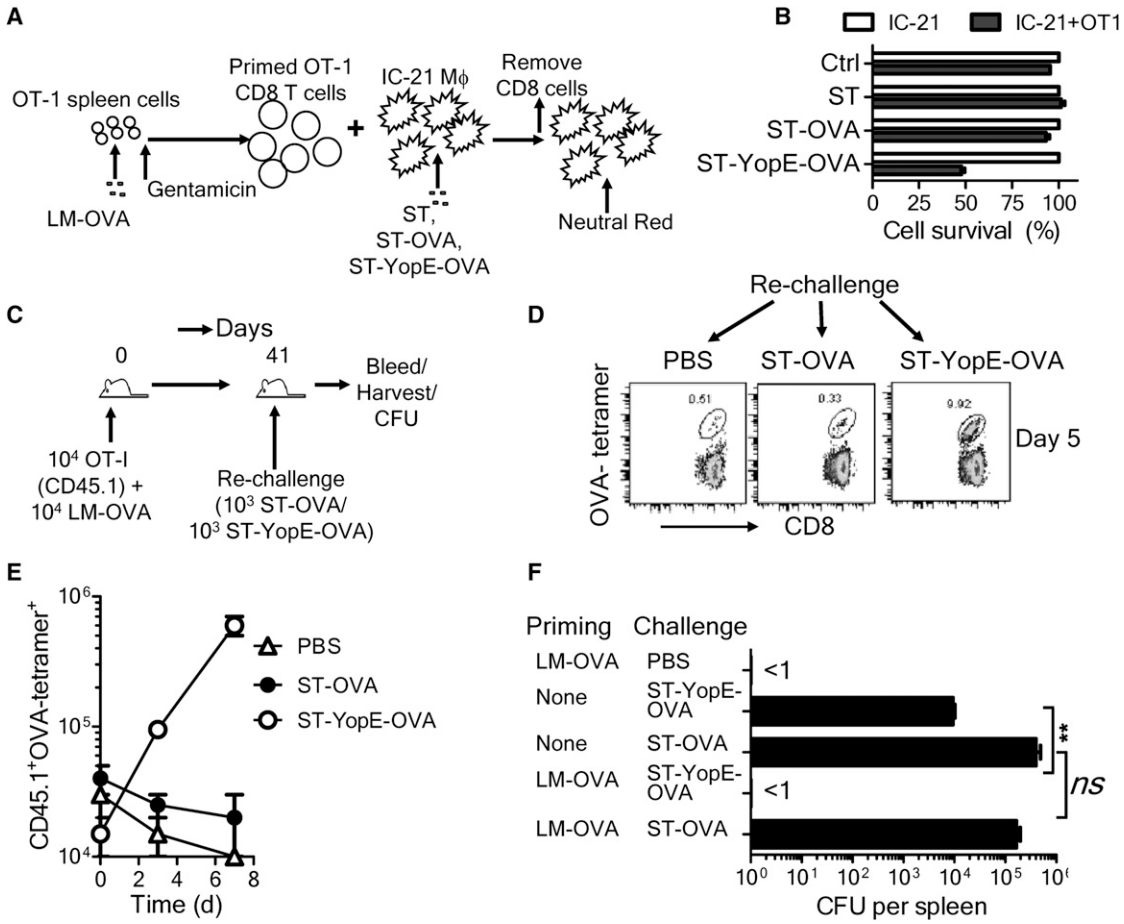

Figure 6. Target Cell Recognition by CD8⁺ T Cells Leads to Control of ST-YopE-OVA

(A and B) OT-1 spleen cells (10^8) were infected with LM-OVA (10^3) followed by washing and culture in media containing gentamicin at 18 hr. Activated OT-1 CD8⁺ T cells were subsequently cultured in media containing interleukin 7 (1 ng/ml) for 7 days. These cells (>99% CD8⁺) were added (1:1) on top of adherent IC-21 macrophages that were previously infected (for 2 hr) with various bacteria as indicated (A). After overnight culture, OT-1 cells were removed by washing and the survival of adherent IC-21 macrophages evaluated by staining with neutral red followed by colorimetric measurement. Results represent the mean ± SD per group.

(C–F) C57BL/6J mice were injected with 10^4 OT-1 (45.1⁺) cells and infected with 10^4 LM-OVA (C). At 41 days postinfection, groups of mice were rechallenged with PBS or 10^3 ST-YopE-OVA or ST-OVA. Control groups of mice that were not primed with LM-OVA were challenged with the same dose of ST-OVA or ST-YopE-OVA. At days 0, 3, and 5 postrechallenge, spleens were isolated and spleen cells stained with OVA-tetramers and antibodies against CD8 and CD45.1. The relative numbers of CD45.1⁺OT-1 cells in the spleen were enumerated (D and E), and bacterial burden in the spleen evaluated (F). Results represent the mean of three mice ± SD per group (E, F) (**$p < 0.01$; ns, nonsignificant).

See also Figure S5.

from a CD8⁺ T cell attack, which will lead to immune evasion and chronicity. ST induces CD8⁺ T cell activation, albeit delayed, suggesting that native ST antigens contain CD8⁺ T cell epitopes, although their identity is unknown. It is unclear whether such CD8⁺ T cell epitopes are present in the virulence proteins of ST that are secreted through the type III secretion system.

Phagosomal pathogens have been shown to engage immunosuppressive mechanisms (Kaufmann, 1993; VanHeyningen et al., 1997; van der Velden et al., 2005; Geijtenbeek et al., 2003; Cheminay et al., 2005; Qimron et al., 2004; Johanns et al., 2010), which have been considered to favor immune evasion and pathogen chronicity. ST inhibits antigen presentation (Tobar et al., 2006; Halici et al., 2008; van der Velden et al., 2005; Cheminay et al., 2005; Qimron et al., 2004). ST has also been shown to inhibit CD8⁺ T cell activation through a direct, contact-dependent mechanism (van der Velden et al., 2005). However, other reports have failed to reveal any inhibition of antigen presentation by ST (Niedergang et al., 2000; Petrovska et al., 2004; Wijburg et al., 2002; Albaghdadi et al., 2009) as potent T cell responses are induced by these pathogens in the long-term (Dudani et al., 2002; Luu et al., 2006; Serbina et al., 2000; Hone et al., 1992). Indeed, the CD8⁺ T cell response induced against salmonella and mycobacteria is highly potent in magnitude (Stover et al., 1991; Pasetti et al., 2011). Our results suggest that the suppression of host immunity during ST infection is a consequence of the persistence of the pathogen and not the reason of chronicity.

When LM was relocated from cytosol to phagosomes, due to disruption of the lisA gene coding for Listeriolysin, this resulted in

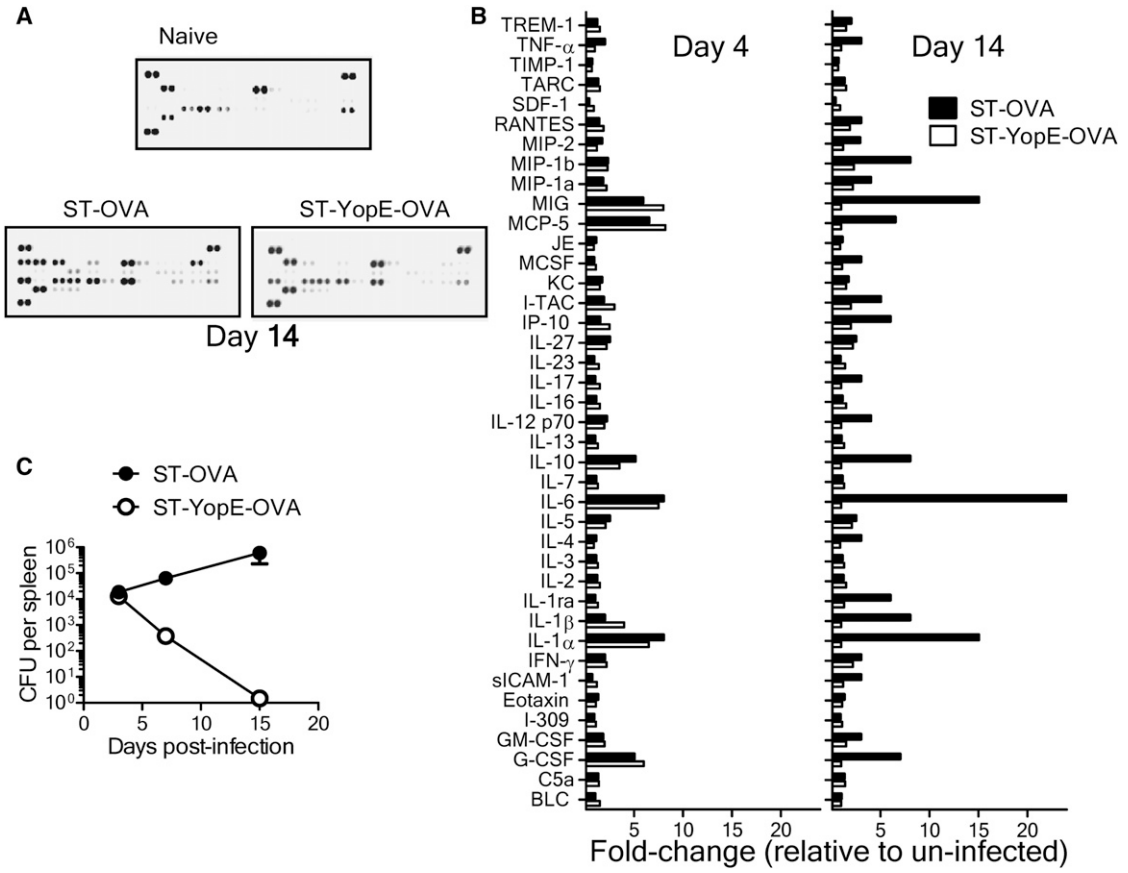

Figure 7. Pathogen Chronicity Exacerbates Cytokine Expression

(A–C) B6.129F1 mice were infected (10^3, i.v.) with bacteria. At days 4 and 14 postinfection, serum was isolated from infected mice and the expression of cytokines/chemokines evaluated by mouse cytokine array as described in Experimental Procedures (A). Fold change in cytokine/chemokine levels in comparison to naive mice was evaluated (B). Bacterial burden in the infected mice was evaluated in the spleens (C). Results represent the mean of three mice ± SD per group and were performed twice.

See also Figure S6.

a poor $CD8^+$ T cell response due to the induction of interleukin 10 expression and poor maturation of DCs (Bahjat et al., 2009). This has led to the notion that phagosomal residence is immunosuppressive. In the case of LM, the entire organism was relocated from cytosol to the phagosomes, where the pathogen would be forced to interact with different sets of signaling molecules (Bahjat et al., 2009). Because LM has not evolved to reside in phagosomes, this may explain why relocation of LM caused attenuation of virulence, and induction of suppression (Bahjat et al., 2009). In our model, only the antigen expressed by ST was relocated to the cytosol. Thus, the innate immune sensors engaged by ST in the phagosomal compartment would be the same whether the antigen was in the cytosol or the phagosome. Furthermore, our results clearly indicate that the expression of immunosuppressive mechanisms, such as the induction of T-regs, gets exacerbated with pathogen-persistence, suggesting that chronicity leads to immunosuppression.

Considering the numerous genes that pathogens such as ST employ for virulence and chronicity (Jones and Falkow, 1996), our results provide insights into the power of the acquired immune system, wherein engagement of the cytosolic antigen presentation can be sufficient to control an otherwise uncontrollable bacterium. Although our model involves translocating OVA and NP, not ST-derived antigens, into the cytosol of ST-infected cells, it provides an important proof of concept demonstrating the power of $CD8^+$ T cells to abridge pathogen chronicity with a single modification of antigenic translocation. Efforts in vaccine development against phagosomal pathogens are often geared toward inducing $CD8^+$ T cell response. However, our results suggest that strategies for controlling phagosomal pathogens have to include ways of modulating antigen presentation such that infected target cells can be readily recognized by $CD8^+$ T cells.

EXPERIMENTAL PROCEDURES

Mice and Infections

C57BL/6J, B6.OT-2, 129x1/SvJ, B6.129S2-*H2*$^{dlAb1\text{-}Ea}$/J, B6.129S7-Rag1^{tm1Mom}/J, B6.129P2-*B2m*^{tm1Unc}/J, and OT-I TCR-transgenic mice were obtained from The Jackson Laboratory. $CD45.1^+$ OT-1 TCR transgenic mice

were generated in-house. B6129F1 mice were generated by mating 129x1/SvJ females with C57BL/6J males. Mice were maintained at the Institute for Biological Sciences, Ottawa, Canada, in accordance with Canadian Council on Animal Care guidelines. For infection, mice were injected intravenously (i.v.) with 10^3 organisms suspended in 200 μl NaCl (0.9%) via the lateral tail vein.

Recombinant Bacteria

Recombinant ST (strain SL1344) that does not translocate antigen (OVA) to the cytosol has been published previously as ST-OVA (Luu et al., 2006). Recombinant ST that translocates OVA to the cytosol (ST-YopE-OVA) was generated by transformation of ST (strain SL1344) with the plasmid pHR-OVA by electroporation. This plasmid was generated through the modification of plasmid pHR-241 (Rüssmann et al., 2001), which contains the sequence for the YopE-specific chaperone SycE and the fusion protein YopE-p60/M45. Briefly, the sequences for p60/M45 were replaced by the OVA sequence coding for amino acids 134 to 386 with BamHI and KpnI restriction sites added to use for inserting the plasmid. The following primers were used for this: 5′-CG GGATCCAACTTTCAAACAGCTG-3′; GGGGTACCTTAAGGGGAAACACATC-3′. We also cloned the LCMV NP encoded by amino acids 396–404 (FQPQNGQFI) into ST. cDNA encoding amino acids 288–508 of the NP protein (GenBank accession number NP_694852.1) was cloned into the plasmid, pHR, to generate ST-YopE-NP. The cDNA insert for this was amplified from a pre-existing full-length clone for NP using oligonucleotides with BamHI and KpnI restriction sites added to use for inserting into the plasmid. The oligonucleotide sequences used for the cDNA amplification were: 5′ CTGGATCCTTTGTTTCA GACCAAGT 3′ and 5′ TAGGTACCCTAGTCCCTTACTATTCC 3′. cDNA encoding amino acids 288–463 of the NP protein was similarly cloned into the plasmid, pKK, to generate ST-NP. In this case, NcoI and HindIII restriction sites were added to the oligonucleotides used for amplification of the insert sequence. The oligonucleotide sequences used for the cDNA amplification were: 5′ TACCATGGCATTTGTTTCAGACCAAGT 3′ and 5′ TAAAGCTTCTAG TCCCTTACTATTCCAG 3′. After confirmation of the sequences, these plasmids were electroporated into the SL1344 ST strain.

Western Blotting

Bacteria grown in liquid culture were tested for OVA and flagellin expression. Spleens were obtained from C57BL/6J mice infected with 10^7 bacteria after 1 day of infection. Cytosolic and noncytosolic proteins were isolated following cell lysing in isotonic buffer and differential centrifugation (Rüssmann et al., 2001; Timmons et al., 2011). Samples were normalized for cell number and were loaded on SDS-10% polyacrylamide gels and OVA and actin expression measured by enhanced chemiluminescence.

Flow Cytometry

Aliquots of spleen cells (5×10^6) were incubated in 80 μl PBS plus 1% BSA (PBS-BSA) with anti-CD16/32 at 4°C. After 10 min, cells were stained with H-2K^{b}OVA$_{257–264}$ tetramer-PE (Beckman Coulter) and various antibodies (anti-CD8 PercP-Cy5, anti-CD62L APC-Cy7, anti-CD127 PE-Cy7, and anti-CD45.1 APC) for 30 min. Cells were washed with PBS, fixed with 1% formaldehyde, and acquired on BD Biosciences Flow Cytometer. All antibodies were obtained from BD Biosciences. T-regs were enumerated using Mouse T-regulatory T cell Staining kit (e-Bioscience Cat#88-8115). Intracellular staining was done according to the manufacturer's protocol.

Infection of Cells and Antigen Presentation

Antigen presentation was done as described previously (van Faassen et al., 2004; Albaghdadi et al., 2009). For in vitro antigen presentation, IC-21 macrophages (H-2^b) were pulsed with the bacteria, followed by removal of extracellular bacteria with gentamicin and incubation with CFSE-labeled OT-1 cells for 3 days. For in vivo antigen presentation, mice were infected with bacteria, followed by adoptive transfer of CFSE-labeled OT-1 cells at various time intervals. Four days after the OT-1 transfer, spleens were removed from recipient mice and the reduction in CFSE expression on donor OT-1 cells was evaluated.

ELISPOT and In Vivo Cytolytic Activity

Enumeration of IFN-γ-secreting cells was done by ELISPOT assay as previously described (Dudani et al., 2002). In vivo cytolytic activity of antigen-specific CD8$^+$ T cells was enumerated as previously described (Tzelepis et al., 2008).

Cytokine Array

Serum was collected for proteomic analysis using the Mouse Proteome Array kit (R&D Systems). Expression of cytokines/chemokines was quantitated by chemiluminescence detected using a Fluorochem 8900 imager (Alpha Innotech). Densitometric expression values were enumerated using AlphaEase software and were corrected to the internal positive controls and expressed as mean fold change over uninfected samples.

Statistical Analysis

The values of samples were compared by one-way ANOVA followed by Tukey HSD tests available at the site http://faculty.vassar.edu/lowry/VassarStats.html. The differences were considered significant when the p value was <0.05.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2012.10.024.

LICENSING INFORMATION

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