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Exploring the Mechanisms that Lead to Delayed $CD8^+$ T Cell Response Against *Salmonella* Typhimurium

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Abstract

Salmonella Typhimurium (ST) infection of murine hosts results in a critical delay in $CD8^+$ T cell expansion, and survival is therefore solely dependent on the alternate, innate immune system. A compromised innate immune system is therefore catastrophic for the host. The mechanism of delayed $CD8^+$ T cell response was therefore explored. Results indicate that the delay in $CD8^+$ T cell activation is related to the inefficient interaction of antigen-presenting cells with ST: ST is not readily taken up by antigen-presenting cells, a significant fraction of ST escapes into the extracellular niches to avoid detection, and within the infected cell ST resides within phagosomes which does not lead to antigen-processing through the direct antigen-processing pathway. All these mechanisms lead to activation of $CD8^+$ T cells by the alternate, cross-presentation pathway, which does not operate efficiently in all the strains of mice.

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List of Abbreviations

APC	Antigen presenting cell
BMDC	Bone marrow-derived dendritic cell
BMM	Bone marrow-derived macrophage
CFSE	Carboxyfluorescein diacetate, succinimidyl ester
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
ER	Endoplasmic reticulum
IFNα	Interferon alpha
IFNγ	Interferon gamma
IFN-I	Type I interferons
IFN-I R	Type I interferon receptor
IL	Interleukin
IRF	Interferon regulatory factor
i.p.	Intraperitoneal(ly)
i.v.	Intravenous(ly)
LCMV	Lymphocytic choriomeningitis virus
LLO	Listeriolysin O
LM	*Listeria monocytogenes*
LPS	Lipopolysaccharide
MHC I, II	Major histocompatibility complex I, II
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer cell
NRAMP	Natural resistance associated macrophage protein
OVA	Ovalbumin
SPI-1, 2	*Salmonella* pathogenicity island 1, 2
ST	*Salmonella* Typhimurium
T cell	T lymphocyte
TAP	Transporter associated with antigen processing
TLR	Toll-like receptors
TNF-α	Tumour necrosis factor alpha
TTSS	Type three secretion system

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1. Introduction

1.1 *Salmonella* Typhimurium

Salmonella enterica subspecies *enterica* serovar Typhimurium (ST) is a Gram-negative, facultative intracellular enteric pathogen. ST is a serovar of the species *Salmonella enterica,* which encompasses a number of other serovars that infect and cause a range of disease in numerous different hosts. Over 2500 serovars of *Salmonella* are known to exist (2, 42). Several of these serovars can cause gastroenteritis in humans, while others are known to cause lethal typhoid fever. *Salmonella enterica* subsp. *enterica* Serovars Typhi and Paratyphi are the serovars responsible for typhoid fever in humans (1). Typhoid fever is primarily a serious public health issue in developing countries and has largely been eradicated as a health concern in industrialized nations (1). An estimated 22 million people are thought to suffer from typhoid fever each year causing over 200 000 deaths annually, however, due to poor reporting techniques and the prevalence of other enteric bacteria in these developing regions, these figures are difficult to estimate.(1) The large number of cases in the developing world highlights salmonellosis as a critical global health problem. Importantly, typhoid disease caused by *S.* Typhi infections is not reproducible in animal models (21), however, ST is a related serovar and results in a typhoid-like disease in mice, yet only causes gastroenteritis in human hosts (21, 42). 20-30% of the *Salmonella* genome is specific to the *Salmonella* genus (26), yet each subspecies of *S. enterica* differs from the others by an estimated 2.8-4.4% nucleotide difference (56). Despite the relatively minimal genetic differences between *S.* Typhi and ST, mice and humans exhibit crucial specificity in the susceptibility to these infections (21, 42, 88, 119). *S.* Typhi causes typhoid in humans and gastroenteritis in mice, and the reverse is true for ST. Due to these similarities, the murine model of ST is used to delineate the host-pathogen interactions during

typhoid fever (reviewed in 72). Several vaccines currently exist against human typhoid disease, yet efficacies remain low (2). There are currently 2 main groups of vaccine that are currently administered throughout the world, and efficacies are generally in the 60-70% range for all age groups (2). Other groups have synthesized more effective vaccines (1); however, they tend to be significantly less cost effective, which prevents their widespread distribution. In order to develop better vaccines against ST, a clear understanding of its mechanism of virulence is therefore needed.

1.2 ST as a facultative intracellular pathogen

ST has been described as a facultative intracellular pathogen (47). Thus, ST can exploit a wide variety of host niches and organ systems upon infection. Many pathogens are intracellular at some point in time in their life cycle, as inhabiting the host cells can be an effective way of avoiding the host's own defences (20). In the case of viruses, intracellular existence is a requirement for their propagation. Viruses require the host's own biological cellular systems to assemble new viral particles, thus promoting viral propagation and further infection of neighbouring cells (4, 121). Some bacteria have stricter metabolic requirements as well, which outfits them for a nearly exclusively intracellular lifestyle. *Salmonella* species, however, exhibit the facultative intracellular lifestyle (47, 87, 119), which allows a bacterium to exist both within host cells and in the extracellular matrix of the infected tissue or the gut lumen. This is beneficial for enteric pathogens, as the most common route of uptake of enteric bacteria is through the fecal-oral route (1, 42). The fecal-oral route is usually related to food and water sources that have been contaminated with human waste. It is this contamination of water that spreads the bacterium, and what makes these infections so prevalent in developing nations with

poor sanitation (1). Facultative intracellular bacteria are at an evolutionary advantage in comparison to other pathogens that are obligate intracellular pathogens.

Several bacterial pathogens, including *Salmonella*, exploit the intracellular niche as a way to evade key immune defence mechanisms, such as the effects of antibodies and complement proteins. In the case of ST, subsequent to uptake/invasion into immune cells, ST remains within the vesicles (phagosomes) of infected cell for prolonged periods, which may cause a delay in T cell activation (5, 14, 105). Phagosomes typically fuse with lysosomes which facilitates antigenic breakdown and antigen-processing. However, ST prevents the fusion of the phagosome with the lysosomes, and can therefore impair antigen-processing (20, 77, 118). ST is able to replicate within the cells without the danger of being degraded by powerful lysosomal enzymes (20). ST has been specifically visualized as predominantly intracellular by Salcedo et al. (87), with at least 70% of ST being characterized as intracellular post-infection. However, the location of ST *in vivo* may be variable, as the pathogen's facultative intracellular lifestyle may be influenced by small changes in the environment.

Intracellular lifestyle has costs to the pathogens as well. Viruses and bacteria capable of solely inhabiting the intracellular niche are generally exposed to the numerous intracellular enzymes that exist in many cells. Macrophages in particular are equipped to degrade particulate matter, as these cells contain numerous lysosomes and reactive oxygen species (ROS) that enable the cell to efficiently breakdown most proteins, lipids and carbohydrates (83). However, ST do not survive within these cells passively, but they seem to actively modulate the biochemistry of the phagosomes to suit their lifestyle. It is estimated that approximately 4% of the genome of ST is required for causing fatal disease in mice (19), and a significant part of that is related to the

ability of ST to thrive within the phagosomes of infected cells. Virulence of ST has been shown to depend upon the ability of ST to thrive within the intracellular environment (60, 95, 119).

ST penetrate the intestinal mucosa, usually at Peyer's patches, proliferate in epithelial cells, and are eventually phagocytosed by macrophages (27, 42, 107). The infected macrophages then migrate to the intestinal lymph nodes where ST replicate before disseminating throughout the body. Polymorphonuclear cells and activated macrophages control bacterial growth (41, 47, 68, 78, 119). The bacterial genes that are involved in *Salmonella* sp. invasion are mainly clustered at one location on the bacterial chromosome (Salmonella pathogenicity island-1, or SPI-1) (64, 101). They encode several factors, including an operon encoding a type III secretory apparatus that exports specific proteins out of the bacteria and into the host cell. These secreted proteins, whose regulation is highly complex, are involved in triggering host signal transduction pathways, culminating in actin polymerization leading to the production of pro-inflammatory cytokines (14, 64). Mutations in genes that are involved in bacterial invasion decrease *Salmonella* virulence when delivered orally (77, 101). Two major virulence loci allow *Salmonella* to replicate intracellularly. The two-component regulatory system *phoP/phoQ* controls more than 40-different genes, termed *phoP*-activated and *phoP*-repressed genes, necessary for intracellular proliferation and avoidance of lysosomes (77). Deletion of *phoP* or *phoQ* significantly decreases virulence (71). Another pathogenicity island (SPI-2) encodes a second type III secretion system that mediates resistance to intracellular killing, and is key to bacterial virulence (64, 101). SPI-2 genes are induced inside host cells, and mediate resistance to NADPH oxidase-dependent killing by macrophages (77).

1.3 Immune responses to ST

Salmonella serovars can effectively infect a wide variety of species, including birds and mammals. However, the pathogenesis of a given serovar can be significantly different based on the species that it infects. In our models, ST is used. ST can infect both humans and mice, yet causes typhoid-like symptoms in mice, while only causing gastroenteritis in immunocompetent adult humans (2, 88, 119). As described earlier, this discrepancy in symptoms is reversed in *S.* Typhi infections (88, 119), making ST an ideal model organism for understanding the pathogenesis of typhoid disease. In addition, within the mouse species, different strains of mice will react with an array of symptoms. C57BL/6J mice, for example, succumb to infection with initial doses as low as 100 ST within 7 days and are hence referred to as susceptible mice. On the other hand, 129SvJ mice develop a chronic infection with ST which is controlled but never fully eliminated, and these mice are referred to as resistant mice (5). This survival difference has been largely attributed to a mutation in *Nramp* gene in C57BL/6J mice. *Nramp* has a role in increasing the production of cation channels in macrophage phagosomes (5, 41, 68), thereby depriving phagosomes of metal ions that are key for pathogen survival. Macrophages are a primary immune cell, and are crucial in the elimination of the early ST infection (61, 78, 97). Mice with a mutation in *Nramp*1, such as C57BL/6J, are therefore susceptible to infection (5, 41). In fact, it has been observed *in vivo* that *Nramp1*-/- mice (which lack the gene entirely) are susceptible to a range of other pathogens that reside in the phagosomes such as *Mycobacterium* and *Shigella* implying that *Nramp* plays a key role in controlling phagosomal pathogens (41). While *Nramp* has no effect on bacterial uptake, it contributes to the efficacy of the subsequent degradation of the bacteria within the macrophage phagosome (119). Efficient degredation of phagosomal bacteria will confer greater resistance to the host.

The most common mode of uptake of ST by hosts is through the gastrointestinal system. Within the gut, tolerance is usually favoured, due to the abundance of commensal bacteria that inhabit it (27, 30). However, in sufficient doses, ST can induce the gut mucosa to adopt an inflammatory state. Dendritic cells (DCs) in lamina propria are specialized to penetrate the tight junctions that exist between gut epithelial cells in order to sample the gut lumen (27, 107). By doing this, these DCs can activate the immune response if a sufficient bacterial load is determined within the lumen. Specialized M cells within the Peyer's patches also act to sample the lumen in order to efficiently present to DCs (27, 81, 107). However, ST is also capable of invading the gut epithelial cells in order to access the rest of the host's body. ST can achieve this using its type three secretion system (TTSS), which will inject effector proteins into the host cells (71, 77, 101). If the route of infection is through the gut, the cells that will be first infected are the epithelial cells (64). While immune cells are phagocytic and will readily take up ST, epithelial cells are induced to undergo extensive membrane ruffling by proteins injected by the TTSS (64). The TTSS is encoded by SPI-1 and SPI-2 genes, both of which have roles in membrane rearrangement and intracellular survival, respectively (64). In addition, the gut epithelium contains specialized lymphoid structures within the tissue itself that primarily exists to target enteric pathogens. A variety of specialized DCs exist in the mesenteric lymph nodes, Peyer's patches and lamina propria, which secrete tolerenogenic IL-10 in order to promote tolerance to commensal microbes, but can be rapidly activated upon infection (27).

Macrophages containing ST migrate to the draining lymph nodes and eventually to the bloodstream. Initially, innate immune response is engaged to control the pathogen burden. A number of innate responses also assist the development of acquired immune responses. Antibodies and defensins are part of the first line of defence against most pathogens. ST has

been shown to render defensins ineffective using serine proteases (118). Antibodies can target foreign antigen for engulfment by macrophages for destruction. Macrophages have a key role in the interaction of ST with the immune system as a whole, as macrophages operate on both the innate and adaptive arms of the immune system. On the innate side, macrophages can engulf particulate matter and degrade it within phagosome-lysosomes. Degraded proteins can then be presented on the macrophage cell surface, which will result in T cell activation and development of acquired immune response. The inflammatory response to ST is characterized by the secretion of a number of pro-inflammatory cytokines (such as IFNγ, IL-12, TNF-α, etc.), and is largely triggered by the presence of lipopolysaccharide (LPS) on Gram-negative bacteria (31). LPS is a hallmark of Gram-negative bacteria, and stimulates toll-like receptor 4 (TLR4) on immune and epithelial cells (4, 33, 90). It is the lipid A portion of LPS that binds to TLR4 which results in stimulation of the inflammatory immune response (33, 70). LPS must first bind to CD14 via LPS-binding protein (LBP) prior to binding to TLR4 (4, 33). The resultant signalling cascade activates IFN regulatory factors (IRFs), which are essential in both type I and type II IFN signalling (4, 74). LPS also has roles in inducing the maturation of dendritic cells, making them more efficient in processing and presenting antigen and thus enhancing the acquired immune response. Engagement of TLR4 results in expression of cytokines such as IFN-γ or TNF-α, which promotes the induction of bactericidal mechanisms in macrophages (24, 90). During the initial stages, cell envelope components of *Salmonella*, such as LPS, induce a massive inflammatory response resulting in the expression of inflammatory cytokines (eg, TNF-α, IL-1, IL-6, IL-12 and IL-18), and a variety of chemokines that recruit cells of the immune system to these sites (50, 88). IFN-γ is also produced during early infection, with natural killer (NK) cells being an important source during this stage (8, 75). However, this inflammatory

response fails to completely eradicate the bacteria, and it has been suggested that *Salmonella* may actually limit the development of immune responses by preventing the expression of the high affinity IL-12 receptor IL-12Rβ2 and IFN-γ (36, 88).

The acquired immune response to ST is not fully elucidated. It is known that ST is taken up by macrophages and DCs in mouse models, albeit not as readily as other facultative intracellular pathogens, such as *Listeria monocytogenes* (5). It is also thought that ST may evade antigen presentation by hiding within the phagosome of the APC, preventing it from fusing with lysosomes and surviving in the acidic environment to the point that ST can successfully replicate (14, 20, 101). In C57BL/6J mice, ST infection results in lethal sepsis, while this does not occur in the resistant strain (129SvJ). Both strains of mice fail to induce $CD8^+$ T cell response within the first 7 days of infection. Thus, susceptible hosts die without engaging acquired immune response (5, 63), and resistant mice appear to control the pathogen through functional *Nramp* and possibly better innate immune response (42). A number of factors have been proposed to contribute to this lethal delay in acquired immune response. The presence of LPS has been suggested to stimulate the release of IL-10 (118), which dampens the inflammatory response and directs it to a tolerenogenic response instead. ST can also directly inhibit T cells. Co-culture of T cells with ST *in vitro* led to a surprising inhibition of T cell response (102). It was determined that this response was not due to a lack of DC signalling or DC death, but instead was posited to be due to a release of unknown inhibitory molecules into the culture supernatant (102). This suggests that ST likely contains a number of genes that are solely responsible for the inhibition of the immune system, allowing the pathogen to extensively proliferate without the interference of the immune system.

1.4 Antigen presentation

Antigen presentation is the process by which antigen is taken up into APCs and processed into immunogenic epitopes for display on the cell surface to be detected by specific T cells. The presentation of antigen to T cells results in a cascade of acquired immune response, and depending on the cell types stimulated can range from pro-inflammatory cytokine release to cytotoxicity against infected cells. Antigen presentation of protein antigen occurs on the surface of cells where the short peptide fragments are found in complex with MHCI or MHCII. Engagement of the MHC-peptide complex on APC by its cognate receptor on a T cell causes a cascading reaction in both cells initiating an immune response. (reviewed in 39, 82, 108). MHC II processing presents to $CD4^+$ T cells, which results in the release of a cytokine cascade that will target extracellular antigen.

1.5 Direct (classical) MHC I processing pathway

MHC I-mediated direct presentation is generally the mechanism by which cells present intracellular antigen that is localized in the cytosol of APC to $CD8^+$ T cells. Nearly all cells in the body possess the machinery necessary to present intracellular antigen (reviewed in 43, 108). Functional MHC I complexes allow host cells to alert the immune system to intracellular anomalies such as misfolded proteins, which can be a sign of stress, age or oncogenesis (43). However, pathogen-derived proteins which reside in the cytosol can also become available to the acquired immune system by way of MHC I processing. For example, viral particles generally must infect cells in order to take over the replicative machinery in order to propagate (121). By doing this, viral proteins will also be processed through the direct MHC class I processing pathway (121). Assembly of the functional MHC I protein plus peptide is a multi-step process involving numerous enzymes and cofactors (43, 82, 108). Briefly, antigen is available within the

cytoplasm and is thus tagged for degradation by ubiquitin. Tagging by ubiquitin directs the protein to the proteasome, where it is degraded into shorter, usable peptide fragments (reviewed in 82, 108). Each peptide fragment is then transported to the endoplasmic reticulum (ER) by the transporter associated with antigen processing (TAP) complex. The TAP complex is crucial, and it has been shown in several models that TAP-deficient mice are impaired in presenting antigen, although this largely depends on the pathogen (discussed below) (43, 82, 104, 121). Peptide is loaded onto empty MHC I in the ER and subsequently transported to the cell surface by the Golgi complex. MHC I loading requires numerous chaperones, such as calnexin (39, 43). Calnexin as a chaperone is crucial and it has been shown that after uptake of a highly immunogenic bacterium, *Listeria monocytogenes* (LM), calnexin will surround the LM-containing phagosome, priming the antigen for MHC I presentation (40). A deficiency in any of the proteins involved can either mildly or severely impair antigen processing and presentation. In addition, the inflammatory response is crucial in enhancing the efficacy of this antigen-processing pathway. For example, IFNγ has been implicated in the enhancing of antigen presentation in some bacteria models (66). In the case of a bacterial infection, the bacteria may have mechanisms in place to circumvent the MHC I pathway in order to evade immune response and encourage their own survival and replication instead. In the case of *Listeria monocytogenes*, Listeriolysin O (LLO) is secreted by the bacteria in order to perforate the cells and spread to adjacent cells (13, 89, 114, 118). However, this comes at the cost of becoming available in the cytoplasm. LM first has to break free of the phagosome before it can spread. By using LLO, LM leaves the phagosome by the dissolution of the membrane (89). As a result, antigen becomes available in the cytoplasm, which can undergo direct MHC I-processing resulting in potent activation of $CD8^+$ T cells (89). In the case of *Salmonella* or *Mycobacterium tuberculosis*, these

bacteria may evade MHC I machinery by remaining within the phagosome (47, 77, 101, 111). This prevents antigen from becoming available, and various factors are secreted by the pathogens to prevent phagosomal fusion with lysosomes and subsequent degradation (101, 111).

1.6 Alternate, cross presentation, pathway

As noted above, sometimes antigen is not available in the cytosol for classical MHC I direct presentation. In such cases, $CD8^+$ T cells are still induced by an alternative pathway, which is referred to as cross-presentation pathway. This is considered to be an alternate pathway, as usually exogenous antigen is presented via MHC II, and does not prime $CD8^+$ T cell responses. In the cross-presentation pathway, $CD8^+$ T cell responses can be elicited against antigen that is not present in the cytosol (6, 23, 113, 116). This could be antigen that is present in the extracellular compartment or within phagosomes of infected cells (16, 52, 61, 78, 116). Generally, cross presentation is the MHC I presentation of exogenous antigen by APCs (usually DCs) to specific $CD8^+$ T cells, resulting in cytotoxic T cell responses. Early on, this was suggested as an alternate MHC I pathway in response to a study that showed that cell-associated ovalbumin (OVA) could stimulate a specific CTL response (93). Further study showed that LCMV nucleoproteins and synthetic virus-like particles alone were also capable of stimulating CTL responses (11). Since then, rapid growth in this field has shown how exogenous antigens from a variety of sources that are not present in the cytosol, are capable of stimulating MHC I-dependent CTL responses (reviewed in 79).

Cross presentation can result in either cross priming or cross tolerance of $CD8^+$ T cells. Cross tolerance occurs when the APCs (most often DCs) do not express the activating/licensing signal to activate $CD8^+$ T cells, which then results in the targeted deletion of specific $CD8^+$ T cells instead of the promotion of CTL responses (52, 57, 58, 86). The absence of the DC

licensing signal is often a characteristic of an immature DC (57). The presence of the licensing signal allows $CD8^+$ T cells to respond to MHC-I-peptide complex presented by DC and to amplify CTL response to the specific antigen (57, 58).

Several models of cross presentation have been proposed, in response to the wide variety of antigens that show some degree of cross presentation both *in vitro* and *in vivo*. The wide range of potential cross presentation mechanisms highlights the differences between antigens and the variability of the immune system in its response to invading pathogens. There is no one unifying cross presentation mechanism, as all the mechanisms are related in some way to the ability of the immune system to adapt to the many methods of evasion that pathogens utilize. Several reviews (16, 82, 108, 114) have outlined the dominant cross presentation hypotheses, but for the scope of this thesis, only a few are highlighted.

In general, protein antigens are by far the majority of cross-presented antigens. Cross presentation has been shown in response to both viral and bacterial protein antigens. While B cells have a minor role as capable APCs, it is primarily macrophages and DCs that cross present antigen (10, 15, 51, 82). More specifically, since macrophages have a primary role of antigen degradation, DCs largely represent the majority of cross-presenting APCs. Macrophages have been noted to require higher loads of protein antigen in order to prime T cells as effectively as DCs (40, 61, 78). Cross presentation pathways can be divided into two groups, based on which portion of the MHC I loading pathway is altered: 1) where the loading of MHC I is altered; and 2) where the processing of antigen is altered.

The loading of the empty MHC I complex is the crucial final step of direct MHC I antigen presentation. One proposed method of presentation of extracellular antigen is that of

endocytic exchange (79, 114). In this pathway, exogenous antigen is taken directly into an early endosome, where it is subsequently loaded onto a free MHC I. The exogenous peptide may also replace the original peptide which results in a direct MHC I recycling to the cell surface. This method has been implicated in the cross presentation of hepatitis B surface antigen (114), and is considered to be the dominant form of TAP-independent cross-presentation (16, 52, 59, 61).

The most common methods of cross-presentation involve altered antigen processing pathways. This is due to the fact that regardless of where an antigenic protein came from, it is likely not processed to a point where it will adequately bind to a free MHC-I and be effectively presented. The majority of natural antigens must be processed prior to presentation. In the case of *Listeria monocytogenes*, the bacteria are capable of being released from the phagosome using LLO. This allows the LM to inhabit the cytoplasm freely. This is known as phagosome egression, and leads to LM being targeted for proteasome degradation despite being taken up by the cell in a manner that would traditionally lead to MHC II presentation (i.e., uptake into a phagosome) (80, 114). Phagosomes themselves have also been implicated as being "leaky" and lose their integrity when infected with certain pathogens (108). Both *Cryptococcus neoformans* (108) and *Mycobacterium tuberculosis* (111, 116) have been implicated in the dissociation of phagolysosomes shortly after uptake, which allows the pathogens to propagate further after escape. This, however, also allows the antigen to be available within the cytoplasm.

A second type of processing-pathway modification involves the interaction of the ER and the phagosome prior to the uptake of exogenous antigen. ER-phagosome interaction has many deviations, however, the common factor is that the "phagosome" contains membrane proteins that are normally only found in the ER. These phagosomes may be referred to as autonomous

phagosomes (3, 116), or "ERgosomes,"(61) or the phagosome may temporarily fuse with the ER in order to directly feed exogenous particles to the ER. The TAP complex (3, 16, 52, 61, 82, 104) as well as Sec61 (46, 52, 61) are implicated in these ER-phagosome pathways. Both of these proteins are typical of the ER and yet have been found to co-localize with exogenous antigen within the phagosome (3). Furthermore, treatment of macrophages with Brefeldin A suggests that these MHC I complexes are newly synthesized, and thus do not correlate with the MHC I-recycling pathway (55). This suggests that in the case of cross-presentation, the intracellular membranes are extremely plastic and can alter their protein components in order to best present exogenous antigen.

A final potential pathway for cross presentation is that of the detour pathway. This pathway was proposed in response to the observation that a number of pathogens cause extensive apoptosis of infected cells. However, the presence of apoptosis does not seem to inhibit the ability of these infections to prime $CD8^+$ T cells. In the detour pathway, apoptotic blebs are engulfed by bystander DCs and subsequently presented as exogenous antigen via cross presentation (31, 111, 115, 116). However, DCs seem to be significantly more able to take up apoptotic blebs as bystander cells than macrophages (122). Apoptosis has been demonstrated in both viral (influenza) (6) and widely in bacterial (*Salmonella* (35, 45, 88, 115), *Mycobacterium* (111, 115), *Shigella* (116)) infections. The near-ubiquity of apoptosis during pathogen invasion suggests it a likely candidate for cross-presentation pathway in a wide variety of infections.

1.7 Type I interferon

Type I interferons (IFN-I) are a family of cytokines that have a wide range of action *in vivo* upon viral, and more recently elucidated, bacterial infections. While the type II interferon group is exclusively composed of IFNγ, the type I IFN group encompasses a broader range of

cytokines, including IFN-α, IFN-β, IFN-δ, IFN-ω and IFN-τ (18, 99). Thus far, only IFN-α and IFN-β are well-characterized in the immunological sense, and are prominent in both murine and human immune responses (99). Thus, Type-I interferons generally refers to IFN-α/β. Type I IFNs are known to be produced and secreted by a wide variety of cell types when infected, however, the major producers of IFN-I are macrophages and plasmacytoid DCs (pDCs) (18, 54, 62, 94, 99). Macrophages in particular have been shown to secrete low levels of IFN-I even in a resting state. Secreted IFNαβ binds to IFNαβ receptor on T cells, macrophages, DCs and fibroblasts (31, 57, 76, 99). The IFNαβ receptor consists of 2 subunits: IFNAR-1 and IFNAR-2 (18, 99). Binding to the IFNAR leads to activation of the Jak-Stat (99) or the MAPK (31, 76) pathway, and the resultant signalling cascade can influence either pro- or anti-inflammatory responses, based on the infection, cell type stimulated, and cytokine milieu. Generally, type I IFN inhibits cell growth, kills tumor cells, promotes homeostasis, and has regulatory effects on cellular and humoral responses (8). Many pathogens will attempt to evade immune responses by inducing the production of anti-inflammatory cytokines, such as IL-4 (18, 48, 94) or IL-10 (18, 98, 110). These anti-inflammatory mediators have been observed to suppress type I IFN function as well.

1.8 Type I IFN and viral infection

While type II interferons are associated with infection in general, type I interferons are largely associated with viral infections where much research has been done to elucidate their role. Generally, type I IFN is secreted in large quantities from macrophages and pDCs upon viral infection. Viruses signal to cells through a number of cell-surface and cytoplasmic receptors, and many of these receptors will initiate a signalling cascade that will culminate in type I IFN responses. Many viruses contain double-stranded RNA as their genetic material.

Detection of dsRNA is crucial in order to respond effectively to a viral infection. It has been established that dsRNA signals through intracellular TLR3 (4, 7), and subsequently has an immune cascade similar to LPS-binding of TLR4 (74). TLR3 promotes a signalling cascade which uses both NF-κB and IRFs (4, 74). Activation of these intracellular mediators will lead to IFNβ signalling in all immune cells, and IFNα secretion in immune cells (74).

During infection with Lymphocytic Choriomeningitis Virus (LCMV), IFNαβ production is upregulated and has direct pro-survival effects on both $CD4^+$ and $CD8^+$ T cells (54, 58, 94, 100). Further studies with LCMV have shown that IFNAR-/- mice are highly susceptible to viral infections (54, 58). The elimination of an effective type I IFN signalling cascade leads to aberrant innate immune responses as well as deficiencies in T cell responses only against viral infections. IFNAR-/- $CD8^+$ T cells will respond at modest levels (54, 99), however, the survival signal is lost and these cells will undergo rapid exhaustion or deletion upon continuous exposure to antigen (54). This may be due to IFNαβ-deficient mice being inadequately activated. Type I IFN has been suggested to act as a signal for $CD8^+$ T cell activation (8, 28, 76), allowing the T cells to be better equipped to respond to APCs and to subsequently promote effector functions. There are additional effects on the cytotoxicity of the activated $CD8^+$ T cells and the size of the memory pool after infection, both of which are reduced when IFNαβ signalling is abrogated (54, 96). IL-12 has been shown to interact with type I interferon to promote many of the pro-inflammatory effects in response to viral infections (54, 75, 100, 110). IL-12 and type I IFN tend to work both synergistically and antagonistically, depending on the situation. Both cytokines seem to exhibit similar effects on the adaptive immune response, and one can compensate for the loss of the other (28, 54). Generally, the presence of IL-12 can overcome an

IFNαβ deficiency, and vice versa. IFNAR-/- mice do not have complete type I IFN pathways, and thus lack the positive feedback that occurs upon infection (8, 54). However, there is evidence that in these mice, IL-12 levels are upregulated in order to compensate somewhat for this loss. This allows the modest amount of clonal expansion that is observed in virally-infected T cells. Deficient mice treated with exogenous IL-12 also helped to rescue T cell proliferation (54). Conversely, IL-12-deficient mice have normal CTL responses, suggesting that type I IFN signalling is compensating for the loss (28).

It is of some importance to note that not only do type I IFNs promote the survival and activity of T cells, but they also promote antigen-presentation to T cells. IFNαβ is produced in large quantities by APCs, and is upregulated further in these cells upon infection. The effects on these cells are largely related to antigen presentation. Firstly, virally-infected APCs exposed to IFNαβ upregulate MHC I, enhancing APC-T cell interactions which leads to effective activation of specific T cells (ineffective interactions can lead to tolerance) (31, 34, 57, 58, 94, 96, 121). This act of "licensing" DCs leads to the secretion of IL-15 from DC, which further stimulates T cells (57, 96, 120). Type 1 IFN can also "license" DCs to become effective in cross presentation (59). This type of licensing requires DCs to interact with $CD4^+$ T cells that have previously been stimulated by IFNαβ induced during infection, through CD40-CD40L interactions (59). Importantly IFNαβ has also been shown in LCMV infections to be crucial in enhancing apoptosis of infected cells (100). The enhancement of apoptosis is crucial in not only the clearing of virally-infected cells in order to overcome the infection, but also plays a role in cross presentation. As noted above, one method of cross presentation is that of the detour pathway, which involves the uptake of apoptotic blebs. Encouraging apoptosis of infected cells increases

the likelihood of these cells being subsequently picked up by bystander DCs and cross presented to specific T cells. LCMV models have supported this theory, showing that type I IFN-deficient mice have abrogated cross presentation potential. Finally, IFN-I has shown to have direct effects on the actual process of cross presentation itself, independent of apoptosis or receptor upregulation.

1.9 Type I IFN and bacterial infection

Type I IFN has been shown to be crucial in many viral infections, and is likely related to the obligate intracellular lifestyle that viruses exhibit. However, many bacteria are intracellular, or at least facultatively intracellular. Several viral studies have included *Listeria monocytogenes* as a potential bacterial comparison to viral infections. One study comparing LM to a number of viral pathogens showed that, compared to LCMV, LM infections resulted in less type I IFN production (100). However, the amounts produced were similar to vaccinia virus (VV) and vesicular stomatitis virus (VSV) infections (100). Thus, at the very least, LM infections have a similar type I IFN profile to some viruses, in that it is produced, and does aid in T cell function. Other bacteria have been implicated in production of type I IFN due to the downstream effects of LPS and TLR4 signalling (31, 91, 96). This is analogous to IFNαβ production due to TLR3 signalling in response to double-stranded DNA (10). A number of Gram-negative intracellular (facultative or obligate) bacteria have been observed to stimulate type I IFN production based on binding or invasion of the host cells. In addition, IFN-I has been shown to contribute to anti-inflammatory effects coupled with IL-10 production upon stimulation with LPS (24). However, this is in response to only a single inflammatory mediator, as opposed to the entire infectious bacteria, which could easily secrete other factors that play a role in cross presentation. Interestingly, IFNαR-/- mice show an increased susceptibility to some extracellular Gram-

positive infections, including group B streptococci (65). Susceptibility to these infections suggests a common role for type I IFN against the majority of bacterial infections, which is that of increased proinflammatory responses (65). However, the specific role that type I IFN plays in bacterial infection is still largely unknown, and is crucial to the understanding of antigen presentation in the context of an intracellular bacterial antigen.

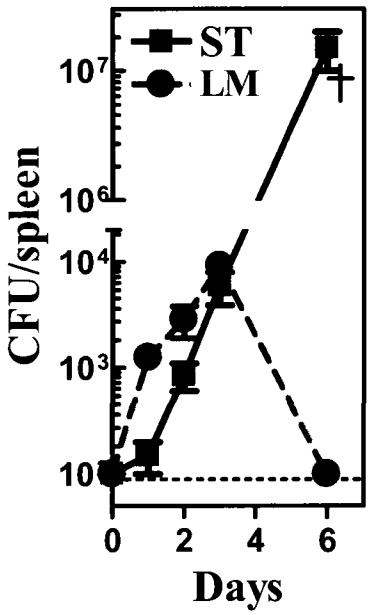
2. Rationale, Hypothesis and Objectives

2.1 Rationale

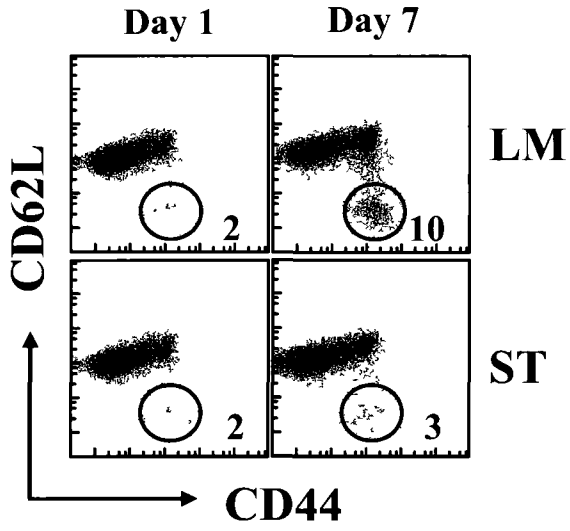
Antigen presentation is a crucial host-defence strategy of the immune system. The innate immune system is occasionally not able to control infection comprehensively and antigen presentation allows engagement of the acquired immune response, which can lead to complete resolution of infection. Antigen presentation occurs against both bacteria and viruses, and against both intracellular and extracellular pathogens. The mechanism by which the immune cells target specific pathogens is highly dependent on the pathogen itself, including its lifestyle, metabolism, and secreted factors that can alter immunity. Our lab has shown previously that *Listeria monocytogenes* induces an acute infection in C57BL/6J mice, which is immediately followed by the development of a massive and early LM-specific T cell response, which facilitates complete elimination of LM from the system by day 7 post-infection (Fig. 1). However, when mice are infected with an equivalent dose of *Salmonella* Typhimurium, T cells are not engaged early on. C57BL/6J mice infected with ST instead succumb to bacterial infection. Bacterial burdens in the spleen increase to lethal levels with no simultaneous T cell activation by day 7. Conversely, resistant mice (129x1SvJ) or F1 hybrids between resistant and susceptible mice (B6.129F1) exhibit a chronic infection profile with detectable $CD8^+$ T cell

response occurring only after day 15. Pathogen burden is controlled to undetectable levels after >90 days, although the pathogen is never eliminated (Fig. 2).

T cell activation is not a single process; it requires the complex interactions of a number of cell types and cytokines which results in T cell activation and proliferation only when optimal conditions are met. The lack of early T cell activation during ST infection suggests that some aspect of the cell-cell interaction is likely altered by specific characteristics of the infection. T cell activation and proliferation are intimately tied to antigen-presentation; without effective presentation of the antigen, T cell proliferation will not occur or will be driven towards tolerance. Modulating the parameters of the ST infection may give some insight into what causes this delay. Additionally, the activation status of the antigen-presenting cell is crucial for effective antigen-presentation. Inflammatory mediators such as LPS and IFN-I are known to enhance maturation of these cells and can shed light on the ability of APCs to present ST early on. IFN-I has been reported to be extremely important in viral infections, and promotes maturation of APCs and T cells and presentation of antigen. Ultimately, antigen-presentation can occur in a number of different ways, and depends largely on the pathogen being presented. Cross-presentation has been suggested as an alternate pathway for a number of viral and bacterial antigens, and determining the degree to which this pathway plays a role in anti-ST immune response may illuminate the nature of immune responses to ST and other facultative intracellular pathogens.


ST
LM
CFU/spleen
10^7
10^6
10^4
10^3
10^2
0
2
4
6
Days

(a)


Day 1
Day 7
CD62L
LM
ST
2
10
2
3
CD44

(b)

Figure 1: C57BL/6J mice succumb to ST infection without T cell response. C57BL/6J mice were infected intravenously with 10^3 ST or LM. At day 1, 2, 3, and 6 spleens were removed. Spleens were homogenized in RPMI to a single-cell suspension and splenic bacterial burden was determined via serial dilutions in saline on BHI agar. BHI agar plates were incubated for 24 hours at 37°C, and cell colonies were enumerated (CFU: colony forming units) (a). Concurrently, spleen cells were counted to ascertain spleen cell numbers, and subsequently incubated with fluorescent antibodies to surface molecules of interest (CD62L and CD44). Cells were washed, fixed and acquired on BD FACS Canto Flow cytometer. Activated T cells are considered to be $CD62L^{low}$ and $CD44^{high}$. Activated T cell populations are outlined on the dot plots at both day 1 and day 7 as percent of total cells acquired (b). S. Sad et al., unpublished data.

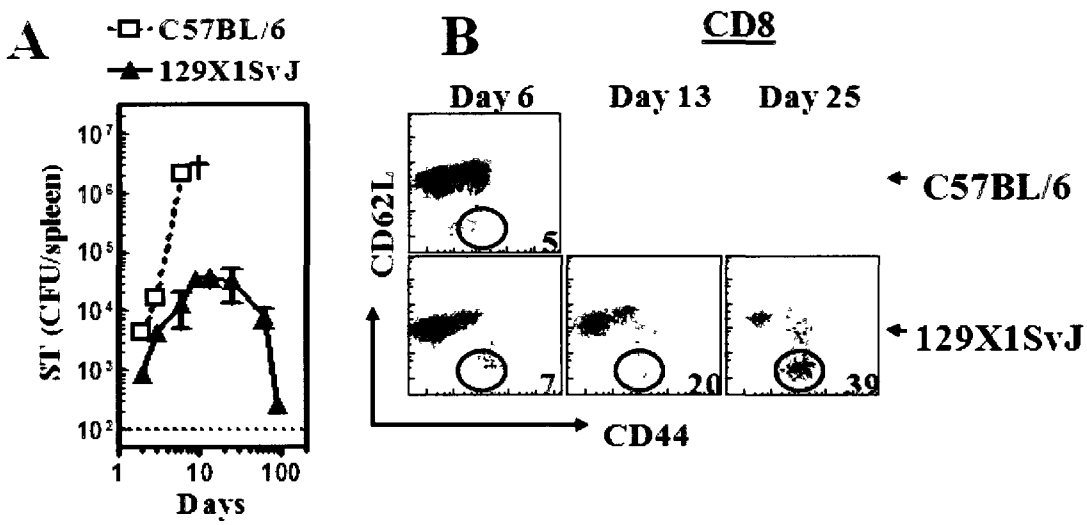

A
C57BL/6
129X1SvJ
ST (CFU/spleen)
Days
B
CD8
Day 6
Day 13
Day 25
CD62L
CD44
C57BL/6
129X1SvJ
5
7
20
39

Figure 2: T cell activation is delayed in response to ST infection. C57BL6 and 129X1SvJ mice were infected with 10^3 ST i.v. At day 1, 3, and 6, spleens were removed from C57BL6 mice in order to maintain humane endpoints. At day 1, 3, 6, 13, 25, 30, 60 and 90, spleens were removed from 129X1SvJ mice. All spleens were homogenized into single cell suspensions and subsequently plated on BHI agar (a) and stained with fluorescent antibodies against CD62L and CD44. Activated T cells are characterized by a $CD62L^{low}$ $CD44^{high}$ phenotype, and are outlined and enumerated on the dotplots (b). S. Sad. et al unpublished data.

2.2 Hypothesis

Cross-presentation is largely regarded as an alternate pathway for MHC I presentation whereby exogenously derived, processed antigens that are not in the cytosol can be presented. It is utilized by the immune system against a number of viral and bacterial pathogens, and can promote effective specific T cell responses. Cross-presentation can occur through a number of pathways, and is largely dependant on the specific pathogen, and has been suggested as a way to control bacteria that otherwise may not be detected by classical MHC I-dependent processes.

Salmonella Typhimurium infection in C57BL/6J mice results in early death and a lack of T cell activation, while B6.129SvJ mice show a delay in T cell activation and proliferation which peaks 21 days after initial infection. **I propose that T cell activation against *Salmonella* Typhimurium is delayed due to a lack of direct (classical) antigen presentation and is primarily mediated by APCs via cross-presentation.**

2.3 Objectives

1) To determine if the level of splenic pathogen burden is related to the lack of early T cell activation.

2) To determine when T cell activation occurs.

    a) To determine if antigen presentation against ST infection is instantaneous at a late time point, or accumulates in magnitude gradually.

    b) To evaluate the relative timing of antigen-presentation using antibiotic treatment.

3) To determine the influence of the location of ST within the spleen and blood on T cell activation.

   a) To evaluate the proportion of *Salmonella* that are extracellular in the spleen during the course of the infection.

   b) To use a non-penetrative antibiotic to evaluate the influence of extracellular bacteria on the T cell response.

4) To determine how antigen-presenting cells (APCs) interact with ST.

   a) To evaluate whether the APCs from infected mice can stimulate OVA-specific T cells *ex vivo*.

   b) To evaluate whether reduced pathogen uptake is the reason for poor antigen-presentation against ST.

   c) To assess whether increasing the pathogen uptake by APC maturation influences T cell activation against ST.

5) To determine how antigen-presentation could be occurring during ST infection.

   a) To use IFNαR-/- mice to evaluate the mechanism of antigen-presentation against ST.

   b) To test if antigen translocation directly to the cytosol of APCs influences the kinetics of antigen-presentation and T cell activation.

3. Materials and Methods

3.1 Mice

6-8 week old female C57BL/6J mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). IFN-I $R^{-/-}$ mice on the B6 background were bred in-house at the animal care facility at NRC (Ottawa, ON, CA). B6.129F1 mice were generated by mating female 129xSvJ mice with male C57BL/6J mice at the animal care facility at NRC. $CD45.1^{+}45.2^{-}$OT-1 TCR transgenic mice were also bred in-house by mating $CD45.1^{+}$OT-1 mice (generated in house) with B6.SJL mice. Similarly, $CD45.1^{-}CD45.2^{+}$ OT-1 TCR transgenic mice were also bred in-house. All mice were housed under pathogen-free conditions in the animal care facility at NRC in accordance with the regulations of the Canadian Council on Animal Care.

3.2 *Ex vivo* Cell Culture

i) **Bone marrow derived dendritic cells**: Bone marrow was harvested from the femur and tibia of B6.129F1 mice. Bones were extracted from euthanized mice and kept in cold, sterile phosphate-buffered saline (PBS) from which all muscle and connective tissue was removed. The marrow cavity of the bones was exposed by removal of the ends, and subsequently flushed with Roswell Park Memorial Institute medium 1640 (RPMI) in order to remove the cells from the cavity. After clearing of the bones, the cell suspension was centrifuged and cells were resuspended in RPMI + 8% fetal calf serum (FCS) (R8) and counted. Cells were cultured in T-25 vented tissue culture flasks at a concentration of $2x10^{6}$/ml. Cells were cultured in media containing 50μg/ml gentamicin and 5ng/ml granulocyte macrophage colony-stimulating factor (GM-CSF). Culture flasks were incubated at 37 °C for 7 days. Every 2 days, culture flasks were cleared of all non-

adherent cells by slight agitation and aspiration. Fresh R8 and GM-CSF were added back into the adherent cells in the culture flask. From day 5 onwards, dendritic cells became non-adherent, while the macrophage contaminates were highly adherent. Non-adherent cells were isolated by gentle washing, and the phenotype of these cells was confirmed by flow cytometry (>90% $CD11c^{+}$).

ii) **Bone marrow derived macrophages**: Bone marrow harvesting and cell culture enumeration and culture proceeded as above. Instead of culturing cells with GM-CSF, they were cultured with 5ng/ml macrophage colony-stimulating factor (M-CSF). Cell flasks were incubated at 37 °C for 7 days. Every 2 days culture flasks were cleared of all non-adherent cells by slight agitation and aspiration. Fresh R8 and M-CSF was added back into adherent cells in the culture flask. After day 5, all the adherent cells were found to be macrophages (BMMs) ($F4/80^{+}$).

3.3 Bacteria and Infection Model

Previously, a recombinant strain of *Listeria monocytogenes* (10403S) (LM) expressing the gene for ovalbumin was generated (32). This recombinant LM (LM-OVA) was generated via the electroporation of plasmid pJJD-OVA into LM strain 10403S, as previously described (32). This rLM-OVA expresses a truncated OVA (amino acids 134-387), which has been fully integrated into the genome. LM-OVA was grown at optical density (OD) 600 = 0.4 in BHI medium, and aliquots were stored in 20% glycerol at -80 °C. A recombinant strain of *Salmonella* Typhimurium (SL1344) (ST) expressing the gene for ovalbumin (ST-OVA) was also generated. Full-length OVA-containing plasmid (pKK-OVA) was electroporated into ST (63). Bacteria were grown in BHI medium, and at mid-log phase (OD600 = 0.8), bacteria were

harvested and frozen at -80 °C in 20% glycerol. The naturally occurring strain SL3261 (*aroA*) is an attenuated strain that lacks the enzyme aromase that is necessary for synthesis of aromatic compounds and was provided by Dr. Brett Finlay (University of British Colombia, Canada). Thus, greater numbers of SL3261 can be injected into all strains of mice in comparison to wild-type ST. AroA-ST was also generated as an OVA-expressing strain using the same pKK-OVA plasmid. Both AroA and AroA-OVA were grown to mid-log phase (OD600 = 0.8). AroA bacteria were subsequently harvested and frozen at -80 °C in 20% glycerol. All bacterial burdens were determined by performing serial dilutions of bacteria in 0.9% saline and plating 100µl aliquots on BHI agar plates.

i) ***in vivo* infection:** Frozen bacterial stocks were thawed immediately prior to use and were diluted to the desired infectious dose in 0.9% saline. Infectious doses ranged from 10^1 bacteria/200µl to 10^7 bacteria/200µl, depending on the experiment. All bacteria were prepared in the same way, and post-injection bacterial numbers were confirmed by enumeration of colony-forming units (CFU) on BHI agar plates. These post-injection CFUs were kept at 37 °C for 24 hours in order to assess growth of bacterial colonies. All mice were infected either intravenously (i.v.) via the lateral tail vein or subcutaneously (s.c.) on the lower back of the animal where indicated.

ii) ***in vitro* infection of bone marrow-derived antigen-presenting cells:** Bone marrow was harvested from B6.129F1 mice. BMMs were generated as described above, and at day 7 were washed and then incubated at 37 °C with PBS to isolate the adherent BMMs. Cells were subsequently seeded onto a 96-well U-bottom plate at a concentration of 10^5 cells/well. ST or LM stocks were thawed immediately prior to use, washed and resuspended in R8 at 10^6 ST/100µl R8. BMMs were infected with either 100µl of the ST

or LM suspension (10 multiplicity of infection (MOI)) or R8 alone (control). Plates were centrifuged for 10min at 400g and incubated at 37 °C for 20min. Cells were then resuspended in 50μg/ml gentamicin in R8 for 2h to eliminate extracellular bacteria. Subsequently cells were centrifuged, washed and resuspended in 10μg/ml gentamicin in R8 and incubated at 37 °C. At various time points following infection, cells were assessed for intracellular bacterial burden.

iii) ***in vitro* infection of bone marrow-derived dendritic cells:** Bone marrow was harvested from B6.129F1 mice. Bone marrow-derived dendritic cells (BMDCs) were generated as described previously. On day 8, BMDCs were washed and subsequently seeded onto a 96-well U-bottom plate at a concentration of 10^5 cells/well. *In vitro* infection proceeded as described above for BMM infection.

iv) ***in vitro* maturation of bone marrow derived cells:** Bone marrow of B6.129F1 mice was harvested and cultured in GM-CSF or M-CSF-enriched media to derive BMDC or BMM, respectively. At day 8, cells were harvested and plated at a concentration of 10^5 cells/100μl in a 96-well U-bottom plate. At this point, BMM and BMDC were stimulated with 10 ng/ml *E. coli*-derived LPS to induce maturation of APCs. BMM and BMDC were also kept unstimulated as a control. Cells were incubated at 37 °C for 18h prior to *in vitro* infection with ST. At this point, all cells were washed and subsequently infected with 10^6 ST per well (10 MOI). Plates were centrifuged for 10min at 400g and incubated at 37 °C for 20min. Cells were resuspended in 50μg/ml gentamicin in R8 for 2h to eliminate extracellular bacteria. Subsequently cells were centrifuged, washed and resuspended in 10μg/ml gentamicin in R8 and incubated at 37 °C. At various time points

following infection, cells were spread on BHI agar plates to assess intracellular bacterial burden.

3.4 Assessment of Bacterial Burden:

i) **Whole Organ:** Mice were euthanized by CO_2 asphyxiation and cervical dislocation and spleens were removed aseptically. Spleens were homogenized between the frosted ends of two glass slides in 10ml RPMI. Ten-fold serial dilutions of the tissue homogenate were diluted in 0.9% saline and 100µl samples from each were plated on BHI agar. Bacterial colonies were counted after 24h incubation at 37 °C.

ii) **Supernatant fraction:** Whole spleen homogenates in R8 were centrifuged in order to distinguish between cell-associated and non-cell associated fractions. Tissue homogenates were centrifuged at 200g for 10 min. The supernatant fraction was plated on BHI agar and serial dilutions were performed in 0.9% saline. Bacterial colonies were counted after 24h incubation at 37 °C.

iii) **%Supernatant calculation:** Burden in the splenic supernatants was calculated as a function of the full spleen burden. Thus,

% Supernatant = 100 x CFU in the supernatant/ CFU in the full spleen

iv) ***In vitro*** **intracellular Burden:** Intracellular burden was assessed in BMM and BMDC cultures. Supernatants were removed from BMM and BMDC, and cells were lysed using lysis buffer (0.1% TritonX100 v/v, 0.01% SDS w/v in PBS, pH 7.2). Cell lysates were serially diluted and plated on BHI agar. Colonies were counted after 24h incubation at 37

°C. Additionally, enumerations of CFUs were assessed by a similar protocol in purified cell samples ($CD11c^+$ cell fractions).

3.5 Flow Cytometry: T cell populations in the spleen were monitored using the BD FACSCanto™ flow cytometer (BD Biosciences, Mississauga, ON, Canada). Depending on the experiment, cell populations were obtained either from the spleen or the blood.

i) To monitor splenic populations, spleens were removed aseptically at various time points following infection and homogenized between the frosted ends of two glass slides. Cell suspensions were strained and resuspended in PBS + 1% v/v bovine serum albumin (BSA) after counting. Cells were incubated with FcR block followed by combinations of antibodies of interest. All antibodies (CD45.1, CD8, CD62L) were purchased from BD Biosciences. $OVA_{257-264}$-Tetramer was purchased from Beckman Coulter (Missisauga, ON, Canada). Cells were washed and resuspended in 0.5% paraformaldehyde/PBS fixative and acquired immediately.

ii) Blood was obtained from the lateral tail vein of the animals in heparin-lined tubes to prevent clotting. Blood was immediately incubated with the antibodies of choice, without FcR block. Red blood cells were then lysed used RBC lysis buffer (Sigma-Aldrich, Oakville, ON, Canada) until the cell suspension appeared translucent. Cells were then washed with PBS and resuspended in 0.5% paraformaldehyde/PBS fixative and acquired immediately. All analysis was performed using BD FACSDiVa™ software.

3.6 Assessment of Antigen Presentation *in vivo*

i) **CFSE-labelled cells:** Spleens from CD45.1^{+}OT-1 mice were removed aseptically and homogenized in RPMI to a single-cell suspension. These cells were subsequently labeled with carboxyfluorescein diacetate, succinimidyl ester (CFSE) dye in order to track T cell divisions over time. Briefly, spleen homogenates were counted to determine cell numbers and then labeled at a concentration of $20x10^{6}$cells/ml. CFSE dye was diluted to 2.5μM in PBS and incubated with an equal volume of cells for 8 min. at 37°C in the dark. The CFSE-cell suspension was then quenched with an equal volume of equine serum on ice for 5 min., followed by centrifugation and counting of the resuspended cells. After counting to determine relative amount of cell loss due to staining, cells were resuspended in Hank's Buffered Salt Solution (HBSS) for adoptive transfer. Cells were injected intravenously at a concentration of 10^{7} labeled cells/200μl per mouse. 2-3 days after OT-1 injection, mice were intravenously infected with LM-OVA, ST-OVA, or AroA-OVA.

ii) **Unlabelled OT-1 Cells:** Spleens from CD45.1^{+}OT-1 mice were removed aseptically and homogenized in RPMI to a single-cell suspension. Cells were passed through a 45μm strainer, then centrifuged. Cells were resuspended in HBSS, then counted. Cells were injected i.v. at a concentration of 10^{6}/200μl into recipient mice.

i) **Assessment of early antigen presentation:** Five days following infection, spleens from OT-1 recipient B6, or B6.129F1 mice were harvested, processed and stained with PerCP-Cy5.5 conjugated anti- CD8 antibody and Allophycocyanin (APC) conjugated CD45.1 antibody and analyzed by FACS. A splenic aliquot was also collected and bacterial burden was assessed using serial dilutions on BHI agar (as outlined above). $OVA_{257-264}$-specific T cell proliferation

was evaluated by gating on $CD45.1^+$ $CD8^+$ cells and determining the degree of CFSE reduction, which gets halved with each cell division.

ii) ***In vivo* stimulation of APCs by LPS:** LPS is known to induce maturation of antigen-presenting cells. Either 10 μg of *E.coli*-derived LPS or PBS control was administered intraperitoneally to B6.129F1 mice with 10^6 OT-1 cells adoptively transferred 1 day prior to infection. These mice were subsequently infected intravenously via the lateral tail vein with 10^3 ST-OVA. Bacterial burdens and T cell proliferation were assessed 5 days post-infection, as described previously.

iii) **Assessment of T cell accumulation:** In order to assess the accumulation of $OVA_{257-264}$-specific T cells *in vivo* in response to ST infection, B6.129F1 mice were parked with 10^7 CFSE-labeled OT-1 cells prior to infection. Mice were infected with 10^3 ST or ST-OVA at day 0, and groups of mice were euthanized at day 5, 10 and 15 post-infection. T cell proliferation was determined by the relative amount of CFSE dilution by the same parked OT-1 cells that had occurred by each finite timepoint. Spleens were aseptically removed from mice and homogenized into single-cell suspensions as above. Spleen homogenates were labeled with anti-CD8 antibody and $OVA_{257-264}$-Tetramer and analyzed by FACS for specific T cell proliferation. T cell divisions were measured by a marked reduction in CFSE fluorescence and were used as a sign of the magnitude of divisions by specific T cells.

3.7 Purification of DCs:

B6.129F1 mice were infected with 10^3 bacteria at day 0. Bacteria used for cell purification were ST or LM, either lacking or expressing OVA. At various time points following infection, mice were euthanized using CO_2 asphyxiation and cervical dislocation and spleens were aseptically removed. $CD11c^+$ cell purification was performed using the Stem Cell $CD11c^+$ Selection Kit™ as described by the manufacturer's instruction (STEMCELL Technologies, Vancouver, British Colombia, Canada). Briefly, spleens were homogenized in dissociation medium and rocked at room temperature for 30min. Spleens were further dissociated by passing the suspension through a 16 gauge needle. Suspensions were incubated with 10mM EDTA and subsequently labeled with anti-CD11c-PE antibody followed by incubation with PE selection cocktail and magnetic nanoparticles. Cell suspensions were placed in StemCell magnets to achieve separation of the CD11c-labelled cells from the remaining homogenate. By pouring off the supernatant, only magnetically labeled cells remained, thus purifying only $CD11c^+$ cells (dendritic cells). Purified cells were resuspended in R8. Cell counts and bacterial burden of the purified cells were enumerated and purity was assessed by flow cytometry.

3.8 Assessment of antigen presentation *in vitro*

Spleens from $CD45.1^+$ OT-1 mice were removed aseptically and homogenized in RPMI to a single-cell suspension. After washing and enumeration of the OT-1 cells, splenic homogenates at a concentration of $20x10^6$cells/ml were labeled with 0.125μM CFSE in PBS. CFSE-labeled cells were incubated in the dark for 8 min., followed by quenching with an equal volume of equine serum and recovery on ice for 5 min. Cells were subsequently washed and assessed for cell loss due to CFSE labeling. In order to culture these cells with dendritic cells (DCs), they were resuspended in R8 at a concentration of 10^6/100μl. 100μl of the labeled OT-1 cell

suspension was seeded into 96-well U-bottom plates. OT-1 cells were co-incubated with either $1x10^5$, $3x10^5$, or $9x10^5$ purified DCs. Some wells received either 1μg of OVA-peptide (positive control) or media (negative control). T cell proliferation *in vitro* was analyzed using flow cytometry, as above. Antibodies used were anti-CD45.1 and anti-CD8 as well as PE-labelled $OVA_{257-264}$-Tetramer, and cells were acquired by FACS.

3.9 Antibiotics

In order to control bacterial burden during infection, antibiotics were used in some experiments. Ciprofloxacin is a front-line antibiotic for many bacterial infections and has been used as a treatment for non-resistant *S.* Typhi infections (117), and is traditionally administered in the clinical setting as an oral antibiotic. Gentamicin is another clinically relevant antibiotic and is generally regarded as non-penetrative at the concentration administered *in vivo* in this work (53). Gentamicin is nearly always administered either intravenously (i.v.) or intramuscularly (i.m.) in the clinical setting. Here, we administer gentamicin i.m. to minimize distress on the animal upon repeated, daily injections.

i) **Ciprofloxacin treatment:** Prior to ciprofloxacin treatment, B6.129F1 mice were parked with 10^6 unlabelled OT-1 cells. OT-1 cells were obtained by aseptically removing the spleens from OT-1 mice of the same gender as the recipients. These spleens were homogenized, counted and resuspended in HBSS for subsequent injection. The recipient mice were subsequently infected intravenously 2-3 days later with 10^3 ST-OVA. Ciprofloxacin powder was dissolved in fresh water at a concentration of 1mg/ml and administered *ad libitum* in the drinking water. Water

was replaced once per week with fresh ciprofloxacin-water preparations. In order to assess $CD8^+$ T cell response as a function of bacterial presence *in vivo*, groups of mice were administered ciprofloxacin preparations beginning at day 3, 6, 15, or 30 post-infection, and treatment continued for the duration of the experiment. In order to assess the extent of T cell proliferation in the presence or absence of antibiotic, mice were bled through the lateral tail vein every second week. Bleeding procedures conformed to CCAC-approved protocols. Blood was collected into heparin-coated tubes to prevent clotting of the blood prior to staining. 200µl of collected blood per mouse was stained with anti-CD8 antibody and $OVA_{257-264}$-Tetramer immediately after collection and incubated for 30 min. Following incubation, blood was lysed and fixed using Red Blood Cell Lysis Buffer (Sigma-Aldrich) as described above. Fixation was followed by immediate acquisition using FACSCanto.

ii) **Gentamicin treatment:** Prior to infection, B6.129F1 mice were parked with 10^6 unlabelled OT-1 cells. 2-3 days after parking, mice were infected with 10^3 ST-OVA intravenously. Gentamicin was administered to infected B6.129F1 mice *in vivo* via intramuscular injection. Gentamicin was prepared to a concentration of 1mg/ml in sterile PBS and each mouse was administered 100µl of the preparation in the hind leg each day following initiation of the treatment. Treatment was initiated at day 3 post-infection in one-half of the infected mice, while the control group received sham inoculations. At day 10, 15, 21 and 30 post-infection, both gentamicin-treated and sham mice were euthanized and spleens were removed for analysis. Spleen homogenates were assessed for bacterial burden via serial dilutions on BHI agar as

above, and T cell proliferation was assessed using FACS analysis of anti-CD8 antibody and $OVA_{257-264}$-Tetramer-labelled spleen cells.

3.10 MHC class I cross-presentation model

The cross-presentation assay was utilized to evaluate the ability of an *in vivo* ST infection to stimulate the uptake of soluble OVA protein. C57BL/6, $IFN\alpha R^{-/-}$ and B6.129F1 mice were used for this assay. Each mouse strain was injected subcutaneously with either 1mg soluble OVA protein alone (dissolved in 100µl sterile PBS) or OVA protein ad-mixed with 10^4 ST subcutaneously. At day 7 post-infection, mice were sacrificed and spleens were processed as outlined above. Homogenized spleens were assessed for bacterial burden as described previously, and assessed for endogenous T cell proliferation by staining with anti-CD8 antibody and $OVA_{257-264}$-Tetramer and subsequent analysis by flow cytometry.

3.11 Evaluation of direct MHC class I antigen-presentation

ST-OVA-T is a construct of ST-OVA which secretes and translocates OVA antigen into the host cell cytosol. This contruct differs from the standard ST-OVA, where OVA remains in the phagosome with the ST bacterium. Evaluation of T cell proliferation in response to ST-OVA-T infection was assessed as above. C57BL/6J and $IFN\alpha R^{-/-}$ mice received 10^7 CFSE-labelled OT-1 splenocytes prior to infection with 10^3 ST-OVA-T or ST-OVA . Mice were also kept as naïve controls. Mice were evaluated for bacterial burden and T cell proliferation at day 5 post-infection. T cell proliferation was assessed by determining the total numbers of $CD8^+$ $OVA_{257-264}$-$Tetramer^+$ cells per spleen, as well as by evaluating the percentage of $CFSE^{low}$ cells in this population by flow cytometry.

4. Results

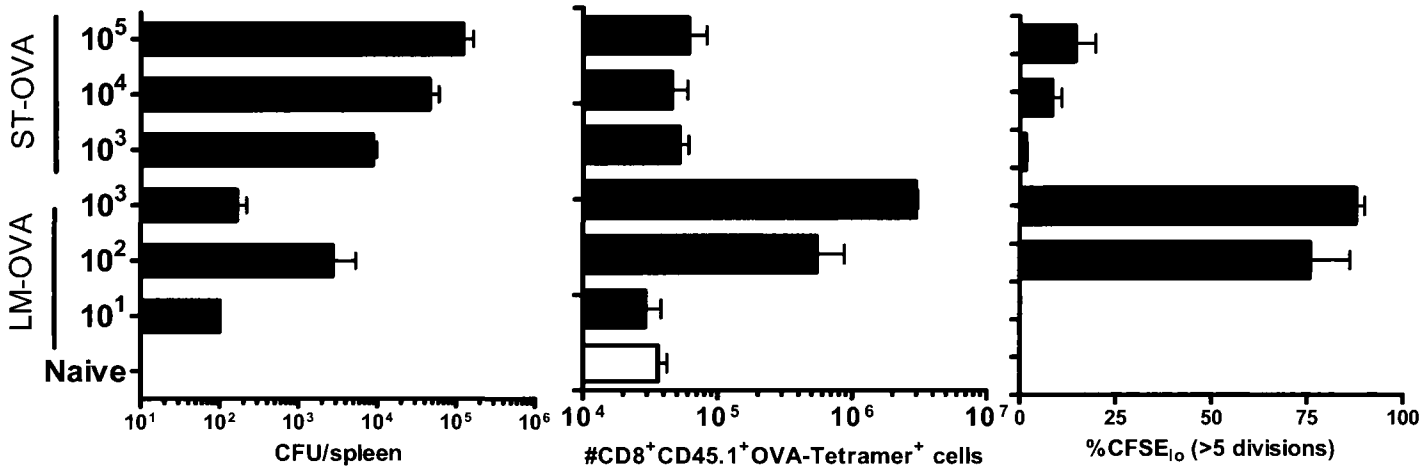
4.1 Pathogen burden does not explain the lack of early T cell response to *Salmonella*

Previous results from our lab have shown that mice infected with 10^3 *Listeria monocytogenes* (LM) intravenously exhibit an acute infection and rapidly recover from bacterial infection (Fig. 1). By day 7 post-infection, C57BL/6J mice mount a potent T cell response to the infection and consequently eliminate the bacterial burden. However, when C57BL/6J mice are infected with a similar dose of *Salmonella* Typhimurium (ST), no such response occurs. Instead, C57BL/6J mice succumb to high bacterial burdens by day 7 (at levels of approximately 10^8 ST/spleen), and show no significant T cell response to infection (Fig. 1). Similar results were noted in the resistant strain (129X1SvJ) of mice (Fig. 2). B6.129F1 mice, which are the offspring of C57BL/6 and 129SvJ mice, do not show any signs of early T cell activation either. In fact, during an ST infection of B6.129F1 mice, there is a slow accumulation of sub-lethal levels of ST, followed by a similar slow decline in bacterial burden after day 21 post-infection (5). Additionally, these mice do not show any large T cell expansion early on, but exhibit a massive T cell response by day 21.

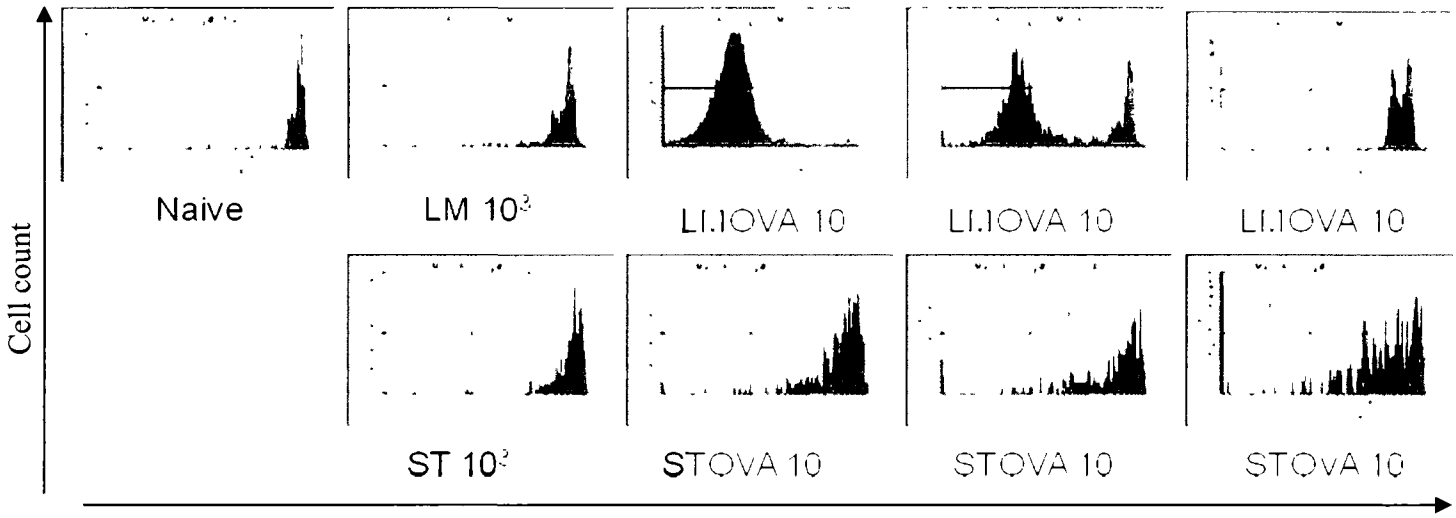
Thus, seeing as there are stark differences between the progressions of an ST infection versus an LM infection, we sought to examine early immune activating parameters in an effort to induce an early T cell response to ST infection. T cell activation and response will not occur in a system if there is insufficient antigen available for presentation, or insufficient co-stimulation provided by the antigen presenting cells (12, 92). This is a parameter set by the immune system in order to avoid activating an unnecessary and damaging acquired immune response to commensal or non-harmful bacteria. ST manages to exploit this immune dampening parameter

by minimizing the availability of its proteins to MHC class I processing machinery; as a result an inadequate immune response occurs, allowing ST to survive.

We reasoned that LM-OVA infection may allow more antigen to be available to the MHC I-processing machinery than ST-OVA infections. To address this, we increased the dose of ST-OVA and evaluated the effects on antigen-presentation. Adoptive transfer method of tracking T cell response was used wherein OT-1 cells were parked in B6.129F1 recipient mice that were subsequently challenged with varying doses of LM-OVA, ST-OVA or AroA-OVA. Five days post-infection, a timepoint where antigen-presentation against LM-OVA is complete $CD8^+$ T cell response was evaluated. An LM-OVA infectious dose of 10^3 resulted in higher numbers of responding $CD8^+$ T cells (Fig. 3b), and visible control of bacterial burden by day 5 (Fig. 3a, red bars). By this point in the infection, there are approximately 10^2 LM-OVA in the spleen, while OVA-specific $CD8^+$ T cell numbers have increased to $5x10^6$, well above the baseline of $5x10^4$ specific T cells (Fig 3b, white bars). In addition, T cell proliferation can be observed based on the dilution of the intensity of CFSE expression in the adoptively transferred cells (Fig 3c, red bars). As these cells undergo division, the intensity of the intracellular dye will dilute, which is exhibited by reduction in CFSE fluorescence as the cells divide (Fig 3d). Interestingly, LM-OVA infections seem to be highly immunogenic, as even doses as low as 10^2 induced an appreciable albeit reduced $CD8^+$ T cell response compared to an infective dose of 10^3 as confirmed by FACS analysis (Fig. 3b&c); interestingly, the control of splenic bacterial burden was comparable (Fig. 3b). While LM infections do tend to require less bacteria to stimulate an effective $CD8^+$ T cell response, our results indicate that the lower threshold for this effect is greater than 10^1 LM, as this infectious dose displayed no T cell responses, despite exhibiting a modest splenic CFU.


ST-OVA
LM-OVA
10^5
10^4
10^3
10^3
10^2
10^1
Naive
10^1 10^2 10^3 10^4 10^5 10^6
CFU/spleen
10^4 10^5 10^6 10^7
#CD8+CD45.1+OVA-Tetramer+ cells
0 25 50 75 100
%CFSE$_{lo}$ (>5 divisions)

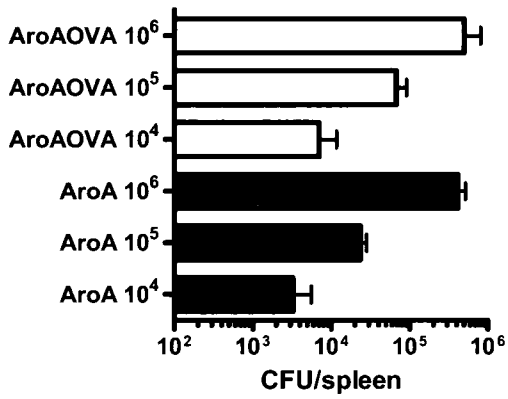
(a) (b) (c)


Naive
LM 10^3
LI.IOVA 10
LI.IOVA 10
LI.IOVA 10
ST 10^3
STOVA 10
STOVA 10
STOVA 10
Cell count
CFSE expression

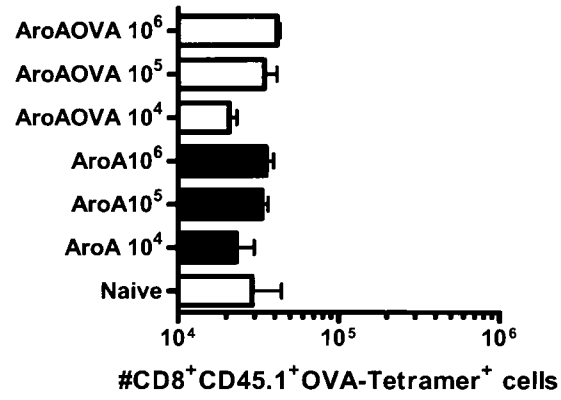
(d)

Figure 3: Differential pathogen burden does not explain the lack of early T cell activation against ST-OVA compared to LM-OVA. All mice received an i.v. injection of 10^7 CFSE-labelled OT-1 splenocytes. Two days post-adoptive transfer, mice received either (i) 10^3 LM, (ii) 10^1-10^3 LM-OVA, (iii) 10^3 ST, (iv) 10^3-10^5 ST-OVA or (v) no bacteria (naive). At day 5 post infection, all mice were euthanized and spleens were removed for processing to determine bacterial burden. Data represents mean CFU values ±SEM (a). T cell responses were characterized by flow cytometry. Cell suspensions were incubated with anti-CD8 antibody, anti-CD45.1 antibody, and PE-labelled OVA-Tetramer fluorescent stains. $CD45.1^+$ and $Tetramer^+$ cells were elucidated using flow cytometry to determine the number of adoptively transferred cells responding. Data represents mean cell counts, as determined by flow cytometry ±standard error of the mean (SEM) (b). CFSE labelling of OT-1 cells allowed visualization of the relative number of divisions that $CD8^+$T cells had undergone, as displayed by a $CFSE^{low}$ phenotype. Data represents mean % $CFSE^{low}$ values ±SEM (c, d). All data are representative of 3 independent experiments with n=4 replicates per independent experiment.

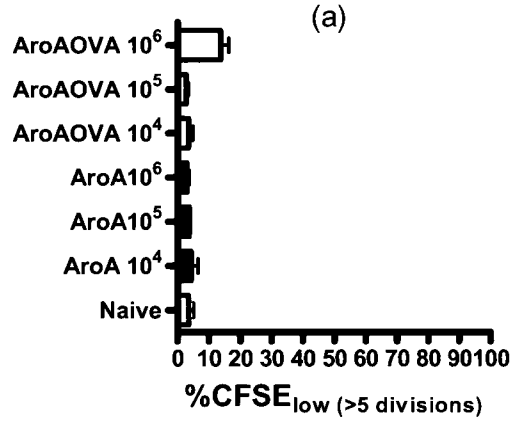
As previously reported, low-level ST-OVA infection in B6.129F1 mice does not result in a rapid, massive $CD8^+$ T cell response (5). This was reaffirmed in these experiments, which show that an infectious dose of 10^3 ST-OVA does not result in $CD8^+$ T cell proliferation well above that which is observed in naive mice (Fig 3 b, blue bars). This lack of $CD8^+$ T cell activation is reiterated by a lack of CFSE dilution, which is in spite of an increasing bacterial burden in the spleen (Fig 3c). Increasing the infectious dose of ST-OVA by 10- and 100-fold did not result in induction of $CD8^+$ T cell proliferation (Fig 3b). $CD8^+$ T cell numbers remained similar to naive, and very little CFSE dilution was observed in response to higher dose (Fig 3c). Again, these doses do increase the level of bacterial burden observed in the splenic compartment (Fig 3a), but do not seem to be presented efficiently to the adoptively transferred $CD8^+$ T cells. *AroA* is a mutant strain of *Salmonella*, which has a defect in its ability to synthesize aromatic hydrocarbons. Due to this defect, *aroA* has been proposed as a potential *Salmonella* vaccine agent, as it has decreased virulence related to its decreased metabolic abilities. Thus, *aroA* can be infected both *in vivo* and *in vitro* at higher bacterial burden levels than wild-type ST. In these experiments, *aroA* was infected at doses ranging from 10^4 to 10^6 *aroA*/mouse. Bacterial burdens at day 5 were high, and corresponded to the dosage level (Fig 4a). However, even at these increased bacterial dosages, *aroA* did not stimulate an efficient early $CD8^+$ T cell response (Fig 4b, c). Our lab has previously reported that both LM-OVA and ST-OVA express similar levels of OVA protein (5). Increasing the ST-OVA dose by 100-fold did not induce early antigen-presentation, implying that lack of early antigen-presentation is not be due to poor generation of antigen levels in case of ST-OVA infection.


AroAOVA 10^6
AroAOVA 10^5
AroAOVA 10^4
AroA 10^6
AroA 10^5
AroA 10^4
10^2 10^3 10^4 10^5 10^6
CFU/spleen

(a)


AroAOVA 10^6
AroAOVA 10^5
AroAOVA 10^4
AroA10^6
AroA10^5
AroA 10^4
Naive
10^4 10^5 10^6
#CD8+CD45.1+OVA-Tetramer+ cells

(b)


AroAOVA 10^6
AroAOVA 10^5
AroAOVA 10^4
AroA10^6
AroA10^5
AroA 10^4
Naive
0 10 20 30 40 50 60 70 80 90100
%CFSE low (>5 divisions)

(c)

Figure 4: High-dose attenuated ST-OVA fails to induce early T cell proliferation. AroA is an attenuated ST pathogen that can be infected at a higher dose without comparable damage to the host. Prior to AroA infection, B6.129F1 mice were adoptively transferred with 10^7 CFSE-labelled OT-1 cells. Mice were infected i.v. at concentrations from 10^4 to 10^6, with both AroA and AroA-OVA. Mice were sacrificed at 5 days post infection. Spleens were homogenized into single-cell suspensions which was subsequently plated on BHI agar and incubated for 24 h at 37°C to determine bacterial count (a); and incubated with anti-CD8 antibody, anti-CD45.1 antibody, and PE-labelled OVA-Tetramer fluorescent stains. Incubation with fluorescent stains allowed the visualization of $CD8^+$ T cell response to infection. $CD45.1^+$ and $Tetramer^+$ cells were elucidated using flow cytometry to determine the number of responding adoptively transferred cells (b). CFSE labelling of OT-1 cells allowed visualization of the relative number of divisions that $CD8^+$ T cells had undergone, as displayed by a $CFSE_{lo}$ phenotype (c). All data are represented by mean values ±SEM and are representative of 2 independent experiments, with n=4 replicates per independent experiment.

4.2 $CD8^+$ T cell response to ST-OVA occurs as an accumulation over time

As shown previously, ST-OVA infections in B6.129F1 mice do not result in $CD8^+$ T cell proliferation early on, yet a large pool of specific $CD8^+$ T cells is detectable by approximately day 21 post-infection. Two alternate ways in which this $CD8^+$ T cell response can manifest itself are: either $CD8^+$ T cells rapidly proliferate in a short amount of time around 3 weeks post-infection, resulting in a perceived "burst" of $CD8^+$ T cell response, or $CD8^+$ T cells gradually accumulate in response to subtle levels of antigen-presentation, with increasing numbers of specific $CD8^+$ T cells being observed at all timepoints past day 5. In order to determine the $CD8^+$ T cell response trajectory, the adoptive transfer model was once again used in B6.129F1 mice, with 10^3 ST-OVA infectious dose. At day 5 post-infection, the results for both ST and ST-OVA infections were equivalent and corresponded to $CFSE_{lo}$ levels shown in Fig 3, indicative of poor antigen-presentation at this time interval (Fig 5). By day 10 post-infection, the population of $CFSE_{lo}$ had not increased appreciably above that of day 5 for ST or ST-OVA-infected mice. However, by day 15 post-infection there was a visible divergence between the ST and ST-OVA-infected groups. While ST, as expected, did not result in an increase in the $CFSE_{lo}$ population, ST-OVA-infected mice displayed a significant increase in the numbers of proliferating $CFSE_{lo}$ $CD8^+$ T cells, , with ~50% of the OVA-specific cells exhibiting a $CFSE_{lo}$ phenotype. This suggests that, at least past day 10 $CD8^+$ T cell proliferation occurs as a accumulation, with the $CD8^+$ T cell response becoming progressively stronger as exposure to antigen continues.

4.3 Continued presence of bacteria is not necessary for $CD8^+$ T cell response

If $CD8^+$ T cell response to ST infections occurs as an accumulation over time, it is useful to consider the role that antigen has in the host during the infection. Persistent antigen in the

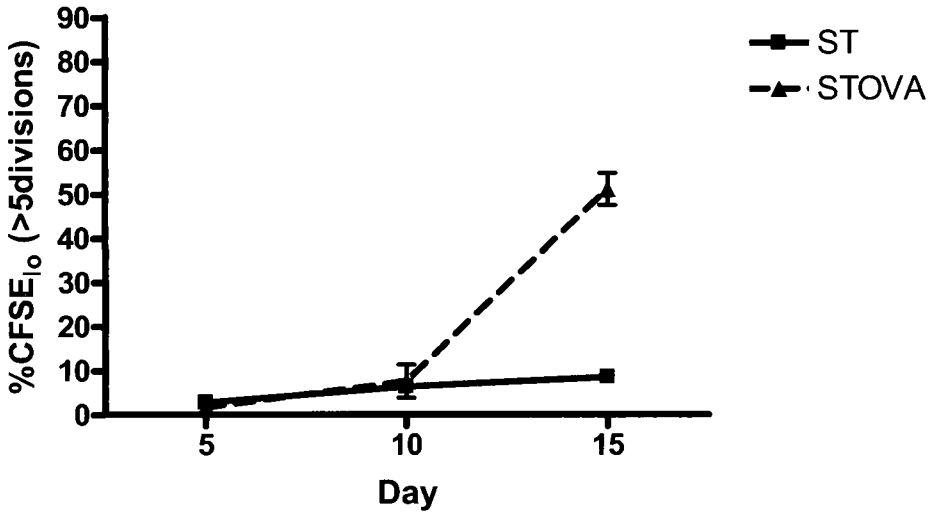
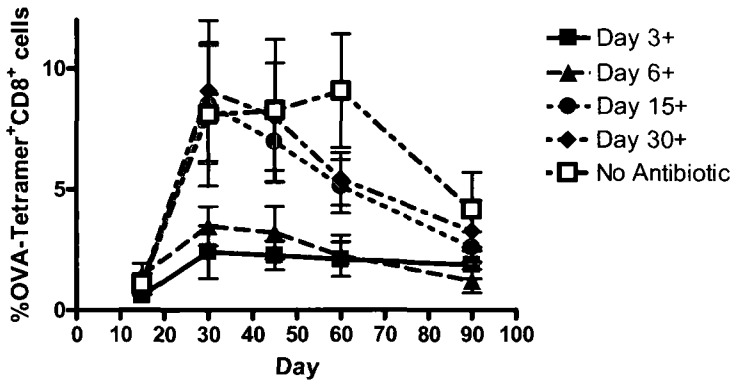

90
80
70
60
50
40
30
20
10
0
%CFSE$_{lo}$ (>5divisions)
5
10
15
Day
ST
STOVA

Figure 5 $CD8^+$ T cell response to ST-OVA occurs as an accumulation over time. B6.129F1 mice were initially adoptively transferred with 10^7 CFSE-labelled OT-1 cells prior to infection. At day 0, mice received an i.v. injection of either 10^3 ST or 10^3 ST-OVA. At each timepoint, 3 mice from each group were euthanized and spleens were collected. Spleens were homogenized to a single-cell suspension, which was counted and incubated with fluorescent antibodies to markers of interest. Flourescent detection of CD8, Tetramer and CFSE was performed by flow cytometry. $\%CFSE_{lo}$ is used as a measure of gauging the number of proliferations that adoptively transferred $CD8^+$ T cells have undergone within the host. Here, it is representative of >5 specific $CD8^+$ T cell divisions. Data are represents mean $\%CFSE_{lo} \pm$ SEM and are representative of 2 independent experiments, with n=4 replicates per independent experiment.

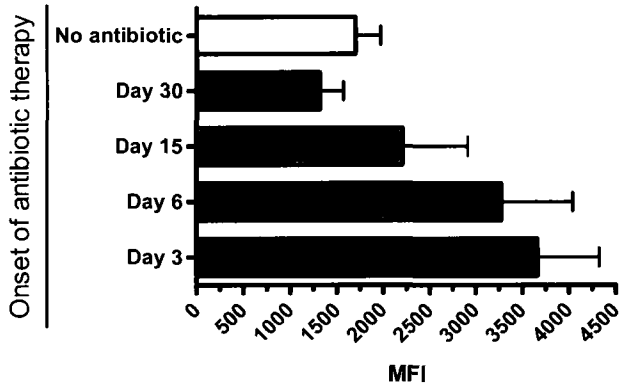
host can be the cause of $CD8^+$ T cell response, but can also contribute to $CD8^+$ T cell exhaustion and/or lethal sepsis or irreversible damage to crucial systems. In order to evaluate whether persistent antigen is necessary (particularly within the first 21 days) to promote continual $CD8^+$ T cell response, adoptively transferred ST-OVA infected B6.129F1 mice were treated for varying intervals of time with the antibiotic ciprofloxacin. Treatment with ciprofloxacin yielded 2 distinct patterns of $CD8^+$ T cell response: 1) visibly abrogated in response to treatment or 2) no change from untreated mice (Fig 6a). Only the groups that received ciprofloxacin from day 3 or 6 onwards exhibited an attenuation of $CD8^+$ T cell response, where tetramer percentages did not increase above 5% at any point in time during the infection. Surprisingly, the mice that were treated with ciprofloxacin at day 15 post-infection did not display any altered $CD8^+$ T cell response. The difference between the treatment groups is further highlighted when the CD62L profiles are considered (Fig 6b). CD62L is a marker for naive and memory $CD8^+$ T cells, and is downregulated upon activation of $CD8^+$ T cells to an effector phenotype. OVA-specific CD8+ T cells in mice that received antibiotics from day 3 or day 6 onwards had the highest levels of CD62L expression, implying faster transition towards memory cells when persisting antigen is removed. Coupled together, these data show that abrogation of $CD8^+$ T cell response by elimination of bacteria will only have an effect prior to day 15. Removal of bacteria after this timepoint has little effect on the $CD8^+$ T cell response or the phenotype, which is likely already fixed to proceed. This majority of antigen-presentation must occur between day 6-15.

4.4 Extracellular ST plays a key role in antigen presentation

Since ST is known to be a facultative intracellular pathogen, this definition implies that there may be some fraction of ST that is extracellular at some point in time during the infection. In order to investigate whether this extracellular fraction plays a role in the subsequent immune


%OVA-Tetramer+CD8+ cells
10
5
0
0 10 20 30 40 50 60 70 80 90 100
Day
Day 3+
Day 6+
Day 15+
Day 30+
No Antibiotic

(a)

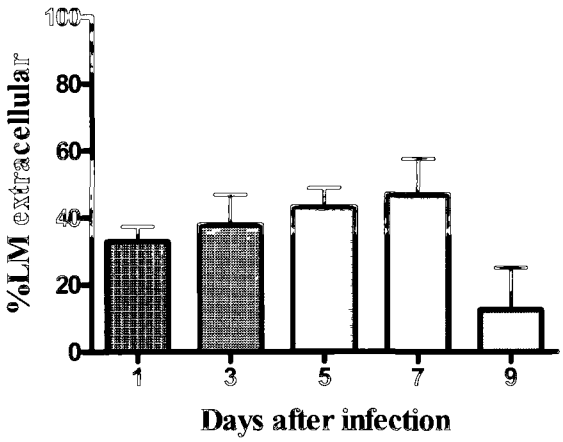

Onset of antibiotic therapy
No antibiotic
Day 30
Day 15
Day 6
Day 3
0 500 1000 1500 2000 2500 3000 3500 4000 4500
MFI

(b)

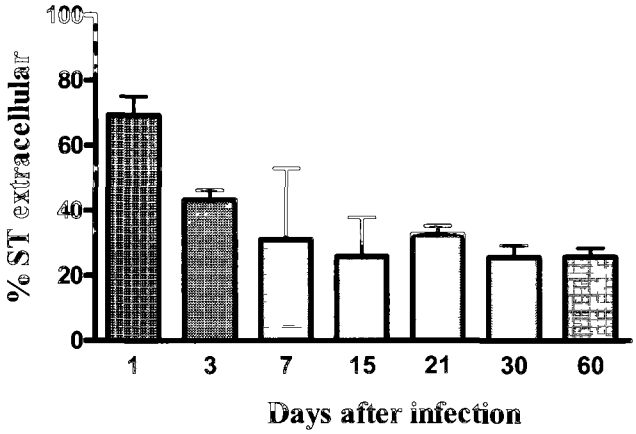
Figure 6 Continued presence of bacteria does not influence $CD8^+$ T cell response against ST-OVA B6.129F1 mice were adoptively transferred with 10^6 unlabelled OT-1 cells prior to infection. At day 0, all mice received 10^3 ST-OVA i.v. Four of the five groups of mice (5 mice per group) received ciprofloxacin treatment. At day 3, one group began receiving ciprofloxacin through the water at a concentration of 1 mg/ml for the duration of the experiment. Groups 2, 3 and 4 received antibiotic water from day 6, 15, and 30 onwards, respectively. One group remained untreated. Blood was monitored in every mouse in order to track the numbers of responding specific T cells over time. Every 2 weeks, 100µl of blood was collected from mice via the lateral tail vein into heparin-coated tubes. The blood was subsequently incubated with fluorescent markers (anti-CD8, anti-CD62L and PE-labelled OVA-Tetramer) in order to detect specific $CD8^+$ T cell response. Each line is representative of one antibiotic treatment group, over time (a). Mice were also assessed for the expression of CD62L in OVA-specific $CD8^+$ T cells as measured by mean fluorescence intensity (MFI) (b). Data represents mean values ± SEM; n=5.

response to the infection, first it must be determined how many bacteria are extracellular at any given point during the infection. This is crucial to determine, as if there is relatively little bacteria extracellularly, it can be posited that there would be relatively little effect on the overall immune response. In order to determine the relative numbers of bacteria that are extracellular in an infected B6.129F1 mouse spleen, centrifugation was utilized to separate the cell-associated bacteria from the non cell-associated. Both LM- and ST-infected B6.129F1 mice have representative early timepoints, while only ST-infected mice exhibited visible bacterial burden past day 7. It was immediately observed that both LM and ST-infected mice displayed a substantial extracellular bacterial burden at all points during the infection. In both of the groups, 15-20% of bacteria were extracellular at all points in time during the infection. However, LM-infected individuals maintain a relatively constant percentage of extracellular bacteria during the course of the infection (Fig 7a), while ST-infected mice exhibit an early peak in extracellular fraction followed by a lower plateau for the remainder of the infection (Fig 7b). LM-infected hosts do not exceed an average of 50% extracellular bacteria during the infection. In ST-infected mice, ~70% of all splenic bacteria were extracellular at day 1 post-infection and this percentage does not fall below 20% at subsequent time intervals. Thus, *in vivo,* both ST and LM-infected mice harbour a sizeable portion of their bacterial burden extracellularly at all points in time. This indicates that this bacterial population may be crucial to both bacterial and immune responses.

If extracellular bacteria are so prevalent during the entirety of an *in vivo* ST infection, it can be inferred that extracellular bacteria likely have an effect on the ability of the immune system to eradicate the infection. In order to determine if this is true, antibiotic was used to eliminate extracellular ST for increasing lengths of time during the infection. The antibiotic of



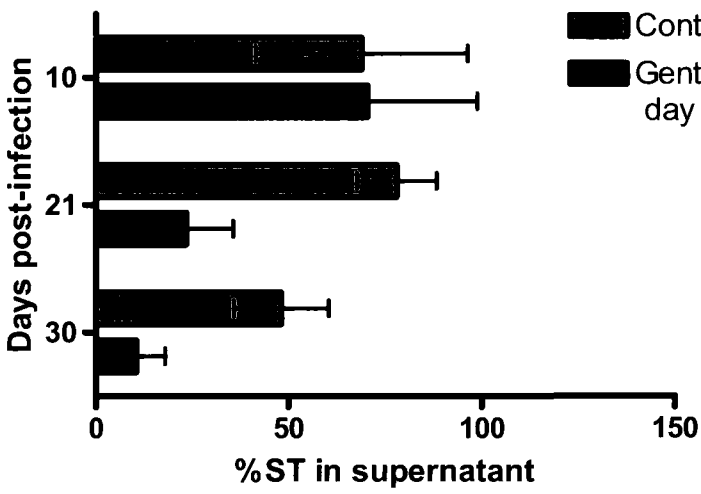
Listeria monocytogenes



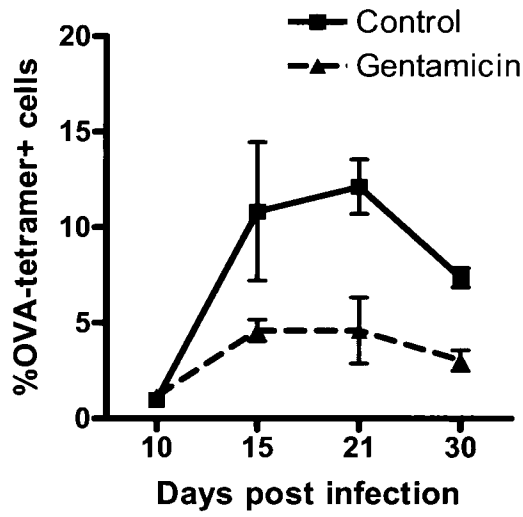
Salmonella Typhimurium

Figure 7 LM and ST-infected B6.129F1 mice exhibit non-cell-associated burdens at all timepoints. B6.129F1 mice were infected i.v. with either 10^3 ST or 10^3 LM at day 0. For LM-infected mice, spleens were removed for analysis at day 1, 3, 5, 7 and 9 post-infection (a). ST-infected mice were sacrificed at day 1, 3, 7, 15, 30 and 60 post-infection (b). Spleens were homogenized into a single-cell suspension and subsequently plated on BHI agar using serial dilutions in 0.9% saline. The remaining cell suspension was centrifuged at 1000rpm for 10 min and the resultant supernatant was plated onto BHI agar to enumerate non cell-associated colonies. BHI plates were incubated at 37°C for 24 h prior to counting. % Extracellular bacteria = 100 X ($CFU_{supernatant}$/ $CFU_{full\ spleen}$). Data represents mean CFU values ±SEM, representative of 3 independent experiments; n=5.

choice was gentamicin, which would not penetrate host cells *in vivo* at the concentrations used here, leading to extracellular bacteria being favourably eliminated. B6.129F1 mice were adoptively transferred with 10^6 unlabeled OT-1 cells prior to 10^3 ST-OVA infection i.v. Beginning day 3 post-infection, one-half of the mice received 100µl of 1mg/ml gentamicin preparation given intramuscularly every day for the duration of the treatment. The remaining mice received sham treatment. Time points were day 10, 15, 21 and 30 post-infection. To confirm gentamicin as a primarily extracellular antibiotic, separation of the cell-associated and non-cell-associated fraction was performed and non cell-associated fractions were serially diluted and plated on BHI agar. These CFUs were compared to that of the whole spleen. Fig 8a shows that early gentamicin treatment causes a substantial decrease in the "% bacteria in supernatant" parameter compared to control by day 21 post-infection. Day 21 represents the peak in overall splenic bacterial burden, thus, the elimination of such large numbers of bacteria at this timepoint was expected to have profound effects on $CD8^+$ T cell responses. It was previously shown (Fig 6) that the administration of ciprofloxacin (a penetrative antibiotic) abrogated $CD8^+$ T cell responses when treatment begins early in the infection. With gentamicin, similar results are observed. The application of gentamicin *in vivo* to ST-infected mice resulted in an overall abrogated $CD8^+$ T cell response, based on the %OVA-tetramer$^+$ cells detected in the splenic fraction (Fig 8b). Similar to the ciprofloxacin-treated mice, those treated with gentamicin did not mount more than 5% OVA-specific $CD8^+$ T cells at any point in time during the infection in contrast to controls where the OVA-specific $CD8^+$ T cell response reached >10%. Thus, the extracellular compartment of the bacterial infection is likely crucial as a reservoir for antigen, and the elimination of this key reservoir allows the immune


Control
Gentamicin, day 3 onwards
Days post-infection
10
21
30
0
50
100
150
%ST in supernatant

(a)


Control
Gentamicin
%OVA-tetramer+ cells
0
5
10
15
20
10
15
21
30
Days post infection

(b)

Figure 8 Gentamicin decreases extracellular bacterial burden to control $CD8^+$ T cell response. 10^6 unlabelled OT-1 cells were adoptively transferred into B6.129F1 mice. All mice were subsequently infected with 10^3 ST-OVA 2 days later. At day 3 post-infection, half of the mice began gentamicin treatment, while the remaining mice underwent sham control. Gentamicin treatment schedule was one 100µl injection of 1mg/ml gentamicin diluted in sterile PBS in the hind leg musculature once a day for the duration of the experiment. At day 10, 15, 21 and 30, 3 mice per group were sacrificed. Spleens were homogenized into single cell suspension and centrifuged to separate the supernatant fraction. A portion of both the cell homogenate and the supernatant was serially diluted in 0.9% saline and plated on BHI agar. Bacterial colonies were enumerated on agar after 24 hours at 37°C (a). The remaining homogenate was counted to assess cell numbers and incubated with labelled anti-CD8 antibody and PE-labelled OVA-tetramer. After incubation and washing, cells were acquired using flow cytometry to determine the relative numbers of OVA-specific $CD8^+$ T cells (b). Date represents mean values ±SEM, representative of 2 independent experiments; n=3.

system to control and eliminate pathogen without requiring the activation of the acquired immune response.

4.5 Dendritic cells isolated from ST infected mice fail to present antigen to $CD8^+$ T cells.

Since we failed to detect rapid, antigen-presentation to OT-1 $CD8^+$ T cells upon transfer to ST-OVA infected mice early on, we evaluated whether this was due to some suppressive mechanisms that inhibited the ability of DC's to present antigen to OVA-specific $CD8^+$ T cells *in vivo*. We therefore purified dendritic cells from the infected mice and evaluated their ability to stimulate OVA-specific $CD8^+$ T cells *ex vivo*. Dendritic cells were purified since they are considered to be the primary professional antigen presenting cell (pAPC). Thus, the efficacy of DCs purified from LM-OVA and ST-OVA infected mice to induce antigen-presentation *ex vivo* was evaluated.

B6.129F1 mice were infected with 10^3 ST-OVA or LM-OVA i.v., and spleens were obtained at day 1, 3, 7, 15, 30 and 60 after infection (post-day 7 are only ST). DCs were purified using StemCell EasySep^{TM} and co-cultured with 10^6 CFSE-labeled OT-1 cells for 5 days to evaluate the capacity of purified DCs to stimulate $CD8^+$ T cells to divide *ex vivo*. The effect of culturing the infected DCs with $CD8^+$ T cells yielded different results, depending on whether the mice were infected with ST-OVA or LM-OVA. We have already established that LM bacterial burden peaks early and is followed by a rapid, large clonal expansion of specific $CD8^+$ T cells (Fig 3). This is in contrast to the ST infection, where the $CD8^+$ T cell proliferation does not begin until day 15 post-infection and does not peak for one week after that (Figs 2, 8). *Ex vivo*, $CD8^+$ T cell proliferation was at its peak when OT-1 cells were co-cultured with DC's that were obtained at day 1 of LM-OVA-infection (Fig 9a). At this time point, 40% of OT-1 cells had

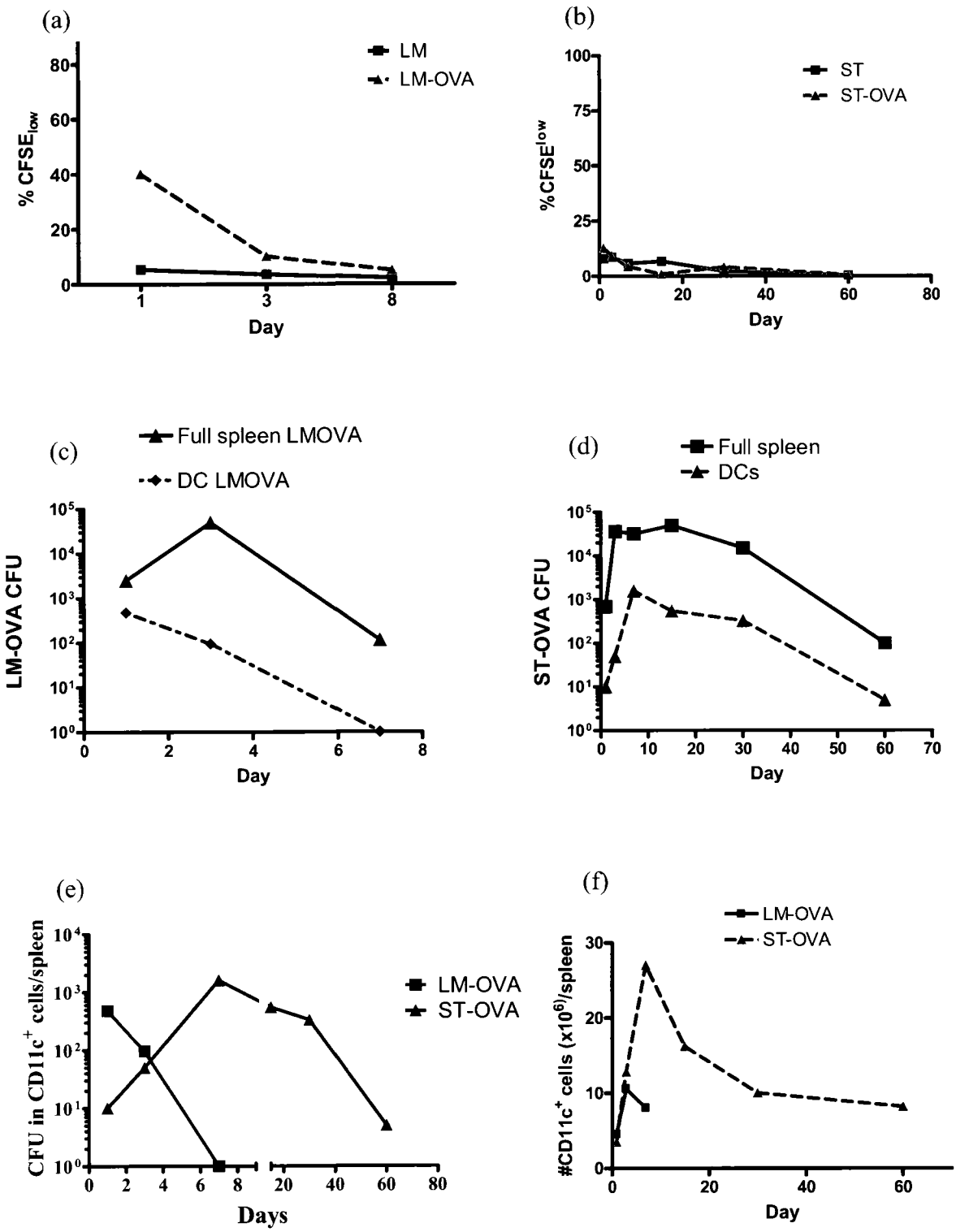

(a)
LM
LM-OVA
% CFSE$_{low}$
Day
(b)
ST
ST-OVA
%CFSE^{low}
Day
(c)
Full spleen LMOVA
DC LMOVA
LM-OVA CFU
Day
(d)
Full spleen
DCs
ST-OVA CFU
Day
(e)
LM-OVA
ST-OVA
CFU in CD11c$^+$ cells/spleen
Days
(f)
LM-OVA
ST-OVA
#CD11c$^+$ cells (x10^6)/spleen
Day

Figure 9 Antigen presentation is related to timing of peak burden *ex vivo*. B6.129F1 mice were infected at day 0 with either 10^3 LM-OVA or ST-OVA. At specific time points, spleens were removed from these mice and homogenized to a single cell suspension. Subsequently, DCs were purified according to the StemCell EasySep protocol . The purified DCs were then cultured for 5 days with 10^6 CFSE-labelled OT-1 cells in R8 in 96-well plates to determine T cell proliferation in response to the *ex vivo* DCs. At day 5, all wells were washed, stained with anti-CD8, anti-CD45 antibodies and PE-labelled OVA-tetramer, followed by acquisition and analysis by Flow cytometry (a,b). Bacterial burden was determined by plating the single-cell suspension on BHI agar (c,d). Comparison between LM-OVA and ST-OVA burdens in the DCs is shown in panel (e) and expansion of DC numbers in response to infection in panel (f). Data is representative of 2 independent experiments; each point represents DCs of 2 independantly infected, pooled spleens.

undergone cell division ($CFSE_{lo}$ phenotype). All purified DCs from subsequent time points during LM-OVA infection failed to induce proliferation of OVA-specific $CD8^+$ T cells, indicating that DCs from these points during infection are less efficient at presenting antigen. Interestingly, DCs isolated from ST-OVA infected mice at any given time point do not induce proliferation of OT-1 cells during *ex vivo* culture (Fig 9b).

Upon removal of the spleen, a full-spleen CFU on BHI agar was performed. Upon isolation of the DCs from the infected spleens, an aliquot of these isolated cells was also plated on BHI agar to determine the bacterial burden that exists solely associated with the DCs *in vivo*. Comparison of the full spleen to the DC burdens yields interesting results between the LM and ST-infected mice. LM-infected mice displayed peak in DC burden early on; 1 day after i.v. infection. After DC burden peaked, there was a steady decline in bacterial burden which continued through to day 7. Thus, at day 1 post-infection, the DC burden represents a larger fraction of the overall bacterial burden (represented by the full spleen CFU), than at all other points. This suggests that at least in LM-OVA infection, early antigen-presentation is due to higher numbers of bacteria that are taken up selectively by DCs. The same cannot be said for ST-OVA-infected DCs. In the ST-OVA-infected hosts (Fig 9d), full spleen burden peaks by day 21, as was expected, based on previous *in vivo* observations. However, the peak in DC burden occurs much earlier; day 7 post-infection. This is interesting as we do not see an equivalent *ex vivo* $CD8^+$ T cell response when cells from day 7 are co-cultured with OT-1 cells. Thus, the antigen presentation abilities of ST-OVA-infected DCs may be altered in some way that prevents antigen from being efficiently loaded and/or presented to the specific $CD8^+$ T cells. Aside from the obvious differences in the bacterial burden (Fig 9e) and antigen presentation capacity of dendritic cells, there was also a sizeable difference shown between the numbers of splenic

dendritic cells that respond to *in vivo* infection (Fig 9f). Regardless of spleen size, DCs were isolated nearly entirely at high purity from the infected spleens. Thus, the number of isolated DCs should accurately reflect the level of DC accumulation that is occurring in response to infection. The numbers of DCs in LM-OVA-infected mice declined corresponding to the relative decline in bacterial burden: a peak of $10x10^6$ DCs/spleen at day 3 post-infection, followed by a decline (Fig 9e, f). The number of DC's in ST-OVA infected mice also followed a trajectory similar to the ST-OVA burden, suggesting that the increase in the number of DCs is directly related to the massive increase in ST-OVA burden on day 7. The number of responding ST-OVA-infected DCs far surpasses that of LM-OVA-infected DCs, with a peak of nearly $3x10^7$ DCs/spleen on day 7; however, antigen-presentation is still poor. Similar to the DC-associated ST-OVA burden, the number of DCs also gradually declines after this peak.

4.6 Increase in early uptake of ST does not induce antigen-presentation

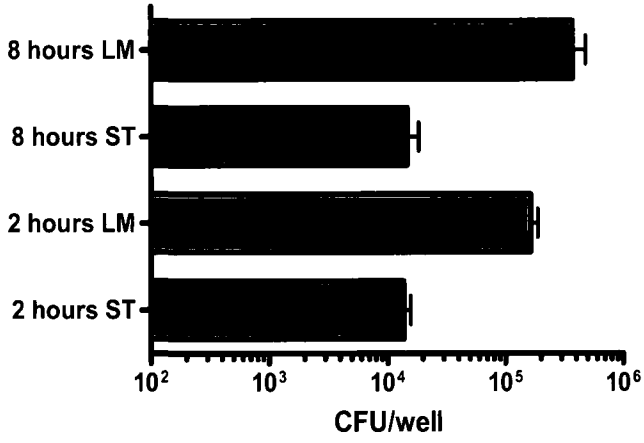
Ex vivo co-culture of purified DCs with OT-1 cells allowed us to determine the ability of these APCs to present antigen at various points during infection. It was noticeable when comparing the CFU data between LM-OVA and ST-OVA infected B6.129F1 mice, that DCs tended to peak in LM-OVA burden earlier than ST-OVA burden. These data appeared to indicate that DC's differed in their ability to phagocytose LM versus ST and the phagocytosis of ST appeared to be influenced by the maturation state of DCs. To address these questions, we generated bone marrow derived DCs from B6.129F1 mice. Isolation of B6.129F1 bone marrow from mice can lead to differentiation of cells into either macrophages or DCs, depending on the cytokine milieu. Macrophages were derived from bone marrow when cultured in MCSF, while DCs were derived when cultured in GMCSF. Thus, these were considered the model populations of the two main types of antigen presenting cells. Infection of the bone marrow-

derived APCs involved the incubation of the cells in a 96-well plate for 2 hours with 10 MOI (10X more bacteria than cells) of either LM or ST (not expressing OVA, as this is an uptake-only experiment). After 2 hours, the cells were washed with non-penetrating levels of gentamicin in order to eliminate all extracellular bacteria, for accurate CFUs of only intracellular bacteria. It was determined that at both 2 and 8 hours post-infection LM was taken up by both types of APCs more readily than ST (Fig 10). In fact, at both time points for each type of APC, at least 10-fold more LM was taken up than an equivalent dose of ST. Since each bacterium had equal time for uptake/invasion into the plated APCs, this suggests that the capacity of APCs to take up ST versus LM can have an influence on antigen-presentation.

To overcome this handicap, we addressed whether mature DC's would take more ST in comparison to immature DCs, which may subsequently influence their ability to stimulate $CD8^+$ T cells. LPS is a component of Gram-negative bacteria outer membranes, and is a key effector in many signalling pathways that result in the general upregulation of the immune system. One such effect of LPS is the stimulation of APC (particularly DC) maturation. Once mature, DCs become much more efficient at antigen presentation, and thus, the addition of LPS to the BM cell cultures, was expected to promote positive responses against ST infection. After culture at 37°C in GMCSF (DCs) or MCSF (Macrophages) for one week, macrophages and DCs were isolated and plated in 96-well plates. Cells were incubated with *E.coli*-derived LPS for 18 hours. At the 18 hour timepoint, cells were washed and infected with ST. Pre-treatment with LPS did indeed enhance the uptake of ST in both the macrophages and the DCs (Fig 11).

We determined whether this increase in ST uptake by LPS-induced maturation of macrophages and DCs would translate to induction of antigen-presentation early on *in vivo*. 10^7

(a)


Dendritic Cells
8 hours LM
8 hours ST
2 hours LM
2 hours ST
10^2
10^3
10^4
10^5
10^6
CFU/well

(b)

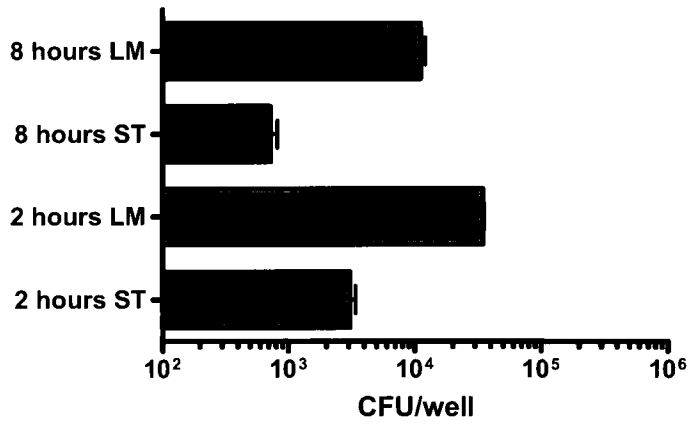
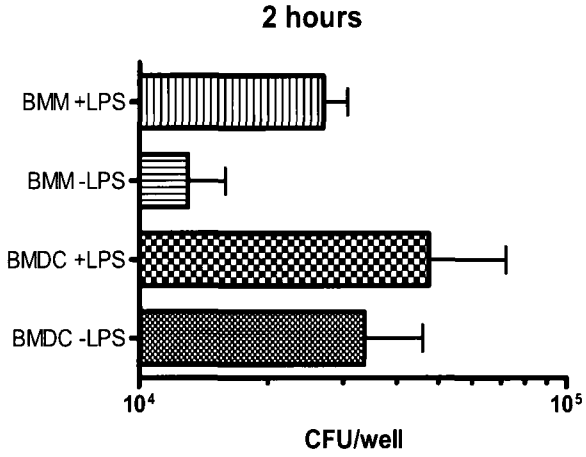
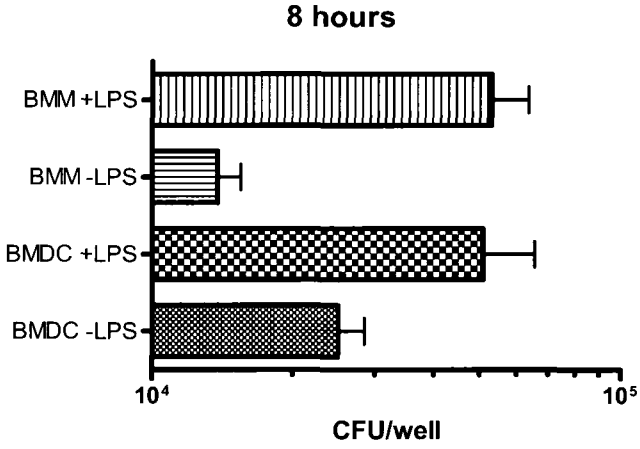

Macrophages
8 hours LM
8 hours ST
2 hours LM
2 hours ST
10^2
10^3
10^4
10^5
10^6
CFU/well

Figure 10 APCs accumulate LM more readily than ST *in vitro*. Bone marrow of B6.129F1 mice was isolated and cultured for 8 days at 37°C in R8. Bone marrow dendritic cells (BMDC) (a) were derived after cell culture in GM-CSF, while bone marrow macrophages (BMM) (b) were derived after cell culture in M-CSF. Media was replaced with fresh R8 + cytokines every 2 days. Upon maturation, cells were removed from flasks and plated in 96-well plates at 10^5 cells/well. Cells were infected with 10 MOI bacteria (10^6) in 100µl, and after subsequent cycles of washing in high (50µg/ml) and low (10µg/ml) gentamicin, cells were returned to 37°C. At 2 hours and 8 hours post-infection, supernatants are suctioned off and cells were briefly lysed with Triton lysis buffer. Lysates are subsequently serially diluted in 0.9% saline and plated on BHI agar. BHI agar plates were incubated at 37°C for 24 hours prior to enumeration of bacterial colonies. Data represents mean CFU values ± SEM, representative of 3 independent experiments; n=3.


2 hours
BMM +LPS
BMM -LPS
BMDC +LPS
BMDC -LPS
10⁴
10⁵
CFU/well

(a)


8 hours
BMM +LPS
BMM -LPS
BMDC +LPS
BMDC -LPS
10⁴
10⁵
CFU/well

(b)

Figure 11 LPS stimulation prior to *in vitro* infection increases ST uptake. B6.129F1 bone marrow was isolated and differentiated into BMM and BMDCs, as described previously. After plating 10^5 bone marrow cells per well in 96-well plates, *E.coli*-derived LPS was added to the culture and cells were kept at 37°C for 18 hours. At 18 hours, cells were subsequently infected with 10 MOI (10^6) ST per well followed by consecutive washings in 50 μg/ml and 10 μg/ml gentamicin were performed to eliminate extracellular bacteria. Cells were kept at 37°C until 2 hours (a) and 8 hours (b) post-infection. Cells were lysed with Triton lysis buffer and suspension was serially diluted in 0.9% saline and plated on BHI agar. Cell colonies were enumerated 24 hours later. Data represents mean CFU ±SEM, representative of 2 independent experiments; n=3.

CFSE-labelled OT-1 cells were injected into recipient mice two days prior to infection. One day prior to infection, mice were injected with 10 micrograms of LPS given intraperitoneally. Mice were infected with ST-OVA, and the influence on bacterial burden and antigen-presentation evaluated. The CFU data was reflective of data observed *in vitro:* mice pre-treated with LPS showed 10-fold higher splenic CFU than their counterparts that did not receive the pre-treatment with LPS (Fig 12a). Surprisingly, despite the known maturation effects of LPS, there was no induction of early antigen-presentation as OT-1 $CD8^+$ T cells failed to undergo cell division in LPS treated mice (Fig 12b, c). The numbers of input OT-1 cells remained unchanged in PBS versus LPS injected mice (Fig. 12b). Thus, these results indicate that reduced early ST-OVA burden in APCs may not be the main reason for the absence of early antigen-presentation in ST-OVA infected mice

4.7 The effect of type I interferons on $CD8^+$ T cell response against ST

C57BL/6J mice succumb to ST infection rapidly without any evidence of T cell activation (Fig. 1). We were interested in determining whether these susceptible mice would induce $CD8^+$ T cell response if their death was delayed by immune modulation, which could subsequently influence their survival. Indeed, previous work in our lab indicated that mice that are deficient in IFN-I receptor (IFN-IR-/-), and also on B6 background can maintain the ST burden for extended duration and succumb only after 20-30 days. We were thus interested in testing whether $CD8^+$ T cell response would be induced in these mice since they did display extended survival in comparison to WT C57BL/6J mice that die by day 7. Since C57BL/6J mice die early, antigen-presentation and $CD8^+$ T cell response cannot be evaluated at later time intervals. We thus used B6.129F1 mice

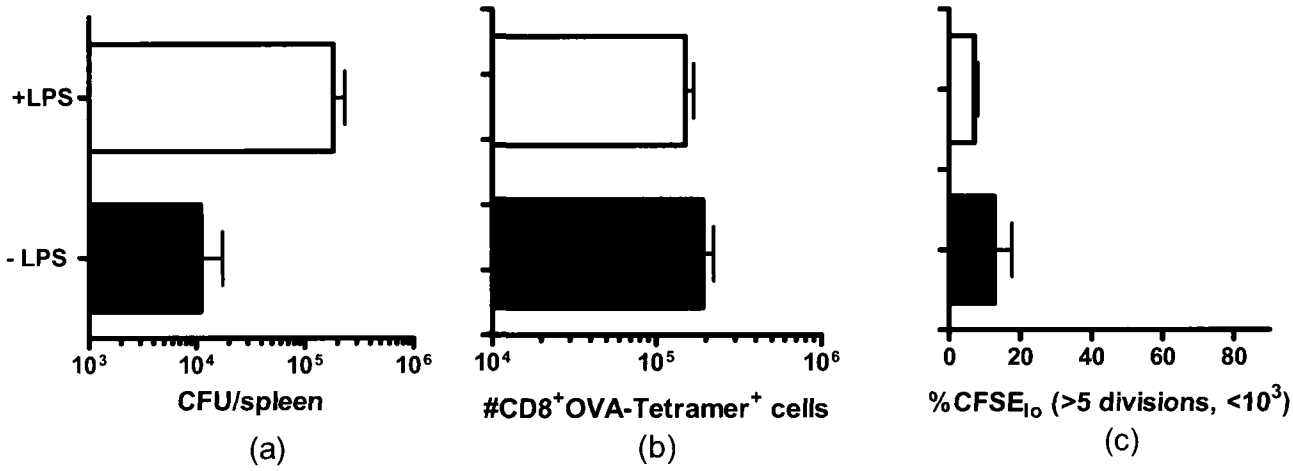

+LPS
- LPS
10³
10⁴
10⁵
10⁶
CFU/spleen
(a)
10⁴
10⁵
10⁶
#CD8⁺OVA-Tetramer⁺ cells
(b)
0
20
40
60
80
%CFSE$_{lo}$ (>5 divisions, <10³)
(c)

Figure 12 LPS pre-treatment increases bacterial burden but does not induce antigen-presentation early on. 10^7 CFSE-labelled OT-1 cells were adoptively transferred to B6.129F1 mice prior to i.v. challenge with 10^3 ST-OVA. One group received LPS pre-treatment. 10μg of *E.coli*-derived LPS was injected intraperitoneally (i.p.) into the experimental group 1 day prior to ST-OVA infection. The control group received no LPS. All mice were sacrificed at day 5 post-infection and spleens were removed and homogenized into a single cell suspension. Homogenates were assessed for bacterial burden by performing serial dilutions of the cells in 0.9% saline on BHI agar. These plates were incubated at 37°C for 24 hours prior to enumeration of colonies (a). Remaining cell suspension was labelled with labelled anti-CD8 antibody and PE-labelled OVA-tetramer. Cells were fixed and acquired using flow cytometry. $CD8^+$ T cell proliferation was assessed by measuring the absolute number of OVA-specific $CD8^+$ T cells in the spleen (b), as well as the percentage of CFSE-labelled OT-1 cells that were expressing the $CFSE_{lo}$ phenotype; an indicator of cell division (c). Data represents mean values ±SEM, representative of 2 independent experiments; n=3.

for comparison since these mice mount a chronic, non-fatal infection. B6.129F1 and IFN-IR-/- (on B6 background) were infected with 10^3 i.v. ST-OVA. At day 17 post-infection endogenous OVA-specific $CD8^+$ T cell response was measured. Day 17 was chosen as the desired timepoint because later points would see $IFN\alpha R^{-/-}$ mouse death when infected with 10^3 ST-OVA. B6.129F1 was the control breed, due to its ability to survive past day 7 and to exhibit the initial stages of $CD8^+$ T cell response by day 17. Bacterial burdens were much higher in the IFN-IR-/- mice in comparison to B6.129F1 mice (Fig 13a). B6.129F1 mice exhibited a full splenic bacterial burden of ~$5x10^4$ ST-OVA/spleen, in line with what we had observed previously (5). However, IFN-IR-/- mice showed a much higher bacterial burden; nearly 10^7 ST-OVA/spleen, indicating that these mice were indeed nearing the point at which they would succumb to lethal sepsis. As expected, the B6.129F1 mice were beginning to exhibit a strong OVA-specific $CD8^+$ T cell response. Approximately 2% of all $CD8^+$ T cells in the spleens of B6.129F1 mice were specific to OVA (Fig 13b). However, $IFN\alpha R^{-/-}$ mice, showed very little specific $CD8^+$ T cell response, with numbers falling below the limit of detection (Fig. 13b, c). These data suggest that despite the elevation of bacterial burden to extremely high levels and extension of mice survival, due to the absence of type I IFN signalling, detectable $CD8^+$ T cell response is not induced even at day 17.

4.8 Cross presentation as the primary mode of antigen presentation

The ability to effectively present antigen to T cells is crucial to the host's ability to control pathogen invasion. Nearly all cells possess the ability to directly present antigen in an MHC I dependent manner (intracellular antigen), and only activated phagocytic cells can induce MHC II presentation (extracellular antigen). However, many infections have been shown to trigger an alternate MHC I pathway of presentation that will present exogenous antigen (not in

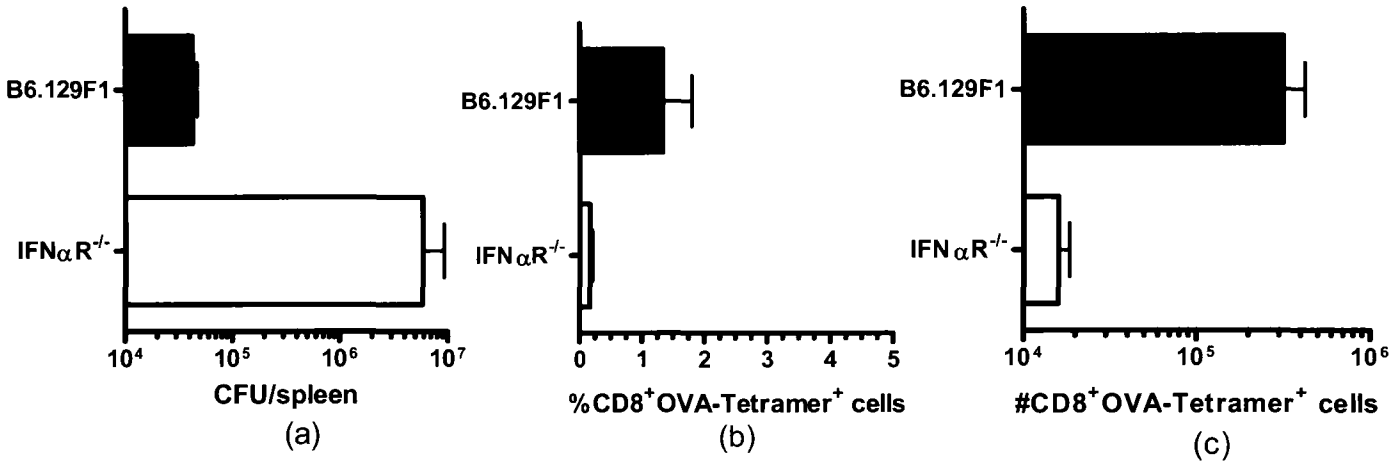

B6.129F1
IFNαR-/-
10⁴
10⁵
10⁶
10⁷
CFU/spleen
(a)
B6.129F1
IFNαR-/-
0
1
2
3
4
5
%CD8+OVA-Tetramer+ cells
(b)
B6.129F1
IFNαR-/-
10⁴
10⁵
10⁶
#CD8+OVA-Tetramer+ cells
(c)

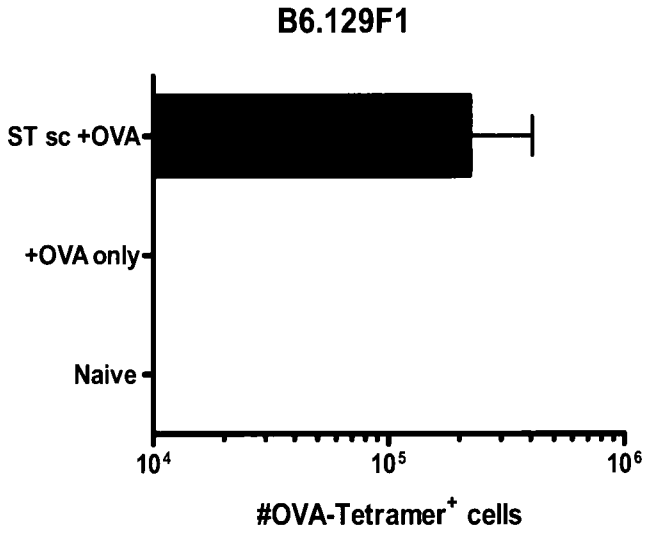
Figure 13 Type I IFN-receptor deficient mice fail to generate $CD8^+$ T cell response to ST-OVA. $IFN\alpha R^{-/-}$ mice lack the IFNα receptor and are thus deficient in signalling in response to type I IFN. To investigate the effect that the lack of IFNαR has on $CD8^+$ T cell proliferation in response to ST-OVA infection, both B6.129F1 and $IFN\alpha R^{-/-}$ mice were infected i.v. with 10^3 ST-OVA. No adoptive transfer was used in these experiments. 17 days post challenge, spleens were removed from the mice and homogenized to single cell suspensions. Cell suspensions were serially diluted in 0.9% saline and plated on BHI agar to determine bacterial burdens (a). The remaining portion of the cell suspension was incubated with anti-CD8 antibody and PE-labelled OVA-Tetramer. These cells were then fixed and acquired using flow cytometry. $CD8^+$ T cell proliferation was measured as both an absolute number of OVA-specific $CD8^+$ T cells (c) and as a percentage of OVA-$tetramer^+$ cells among all splenic $CD8^+$ T cells (b). Data represents mean values ±SEM, representative of 3 independent experiments; n=4.

cytosol) to $CD8^+$ T cells. This pathway is broadly known as cross-presentation. In order to determine if ST antigen can be presented via cross-presentation early on, a cross presentation model was set up.

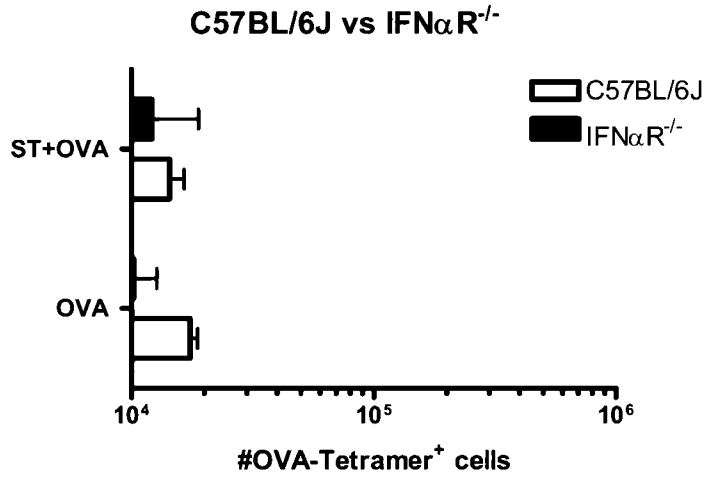
In order to simulate the availability of exogenous antigen for cross presentation, 1mg of soluble OVA protein was injected subcutaneously into mice. Injection of whole soluble OVA would not gain entry into the MHC class I processing pathway. These mice were compared to those that received not only the soluble OVA, but 10^4 ST (not expressing OVA) concurrently in the injection. The added ST was proposed to induce the characteristic ST inflammatory response, whereas OVA alone would only be available in the extracellular matrix, without additional inflammation. Seven days post- injection, mice were sacrificed and spleens were evaluated for endogenous $CD8^+$ T cell response. In B6.129F1 mice, $CD8^+$ T cell proliferation was only evident in the mice injected with both OVA protein and ST together (Fig 14a). Those mice that received no treatment or OVA alone did not show any appreciable increase in OVA-specific $CD8^+$ T cells in the spleen. However, when C57BL/6 and IFN$\alpha R^{-/-}$ mice underwent the same protocol, neither mouse showed any substantial increase in specific $CD8^+$ T cells in response to any treatment (Fig 14b). Thus, at least in B6.129F1 mice, by inducing cross presentation, antigen presentation occurs readily at early timepoints; much sooner than would be observed normally with ST-OVA i.v.

4.9 Direct presentation of ST is possible if antigen is available in the cytosol

Cross presentation requires extracellular antigens to become available in order for the MHC I processing machinery to access it. Fig 14 illustrates that B6.129F1 hosts are capable of acquiring extracellular antigen for efficient presentation, but C57BL/6J and IFN$\alpha R^{-/-}$ mice seem to be less capable. Is it possible then that the death of these two mice (C57BL/6J and IFNaR-/-)


B6.129F1
ST sc +OVA
+OVA only
Naive
10^4
10^5
10^6
#OVA-Tetramer+ cells

(a)

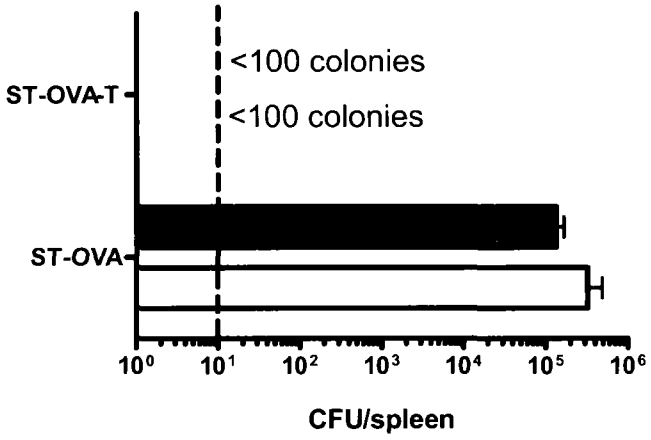

C57BL/6J vs IFNαR-/-
C57BL/6J
IFNαR-/-
ST+OVA
OVA
10^4
10^5
10^6
#OVA-Tetramer+ cells

(b)

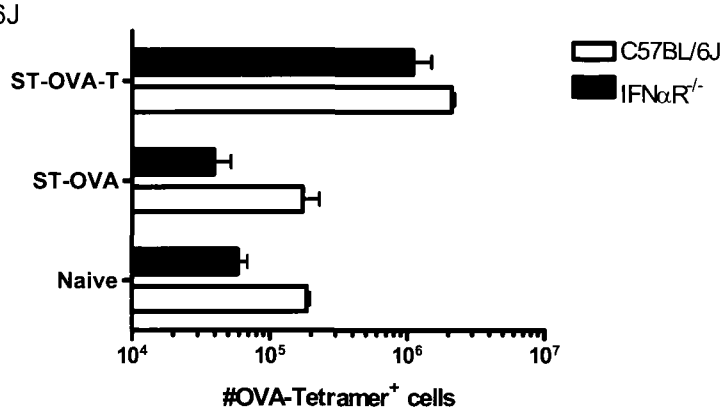
Figure 14 Cross presentation model results in early $CD8^+$ T cell activation in B6.129F1 mice. No mouse strain observed here activates $CD8^+$ T cells early on upon i.v. infection with ST-OVA. The cross presentation model simulates an extracellular antigen that can be picked up and processed via MHC I machinery and presented to $CD8^+$ T cells. To simulate a cross presentation-favourable environment, soluble OVA protein was used as the extracellular antigen. B6.129F1 (a), C57BL/6J and IFN$\alpha R^{-/-}$ mice (b) were infected in the same way. Mice were treated with OVA alone or OVA + ST (not-ST-OVA). OVA alone treatments received 100µl of 10mg/ml OVA protein in PBS subcutaneously (s.c.). Mice that were treated with ST + OVA received the same injection of OVA, but also received a 10^4 s.c. ST dose concurrently. Mice were sacrificed at day 5 post-injection. Spleens were removed and homogenized into single cell suspensions. Bacterial burden was assessed (not shown) and cells were incubated with fluorescent anti-CD8 antibody and PE-labelled OVA-Tetramer to detect OVA-specific $CD8^+$ T cell response. Cells were acquired using flow cytometry. B6.129F1 data represents mean cell counts as determined by flow cytometry ±SEM, representative of 4 independent experiments; n=3. C57BL/6J/IFN$\alpha R^{-/-}$ data represents mean cell counts as determined by flow cytomerty ±SEM, representative of 3 separate experiments; n=3.

occurs because they fail to induce antigen-presentation? We therefore addressed the consequences of inducing antigen-presentation by the direct MHC class I processing pathway. ST-OVA-T construct was generated wherein OVA expressed by ST is rapidly translocated to the cytosol of infected cells. In contrast, OVA expressed by normal ST-OVA would remain confined to the phagosomes of infected cells.

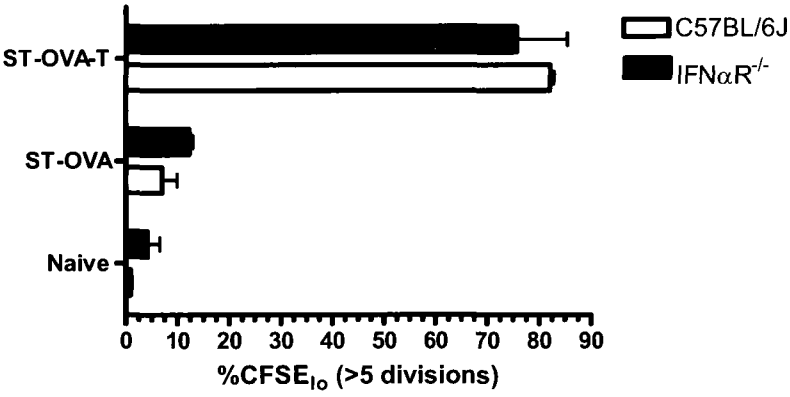
Translocation of OVA into the cytosol of infected cells was made possible through the fusion of OVA gene to *YopE*, a member of the type III secretion system of Yersinia. *YopE* has been shown to translocate fused proteins to the cytosol of infected cells (118). Fused OVA is injected through the type III injectosome of ST directly into the cytosol of infected cells. The adoptive transfer protocol involving CFSE labelled OT-1 cells was used for evaluating the extent of antigen presentation. 10^7 CFSE-labelled OT-1 cells were adoptively transferred into C57BL/6J and IFNαR$^{-/-}$ mice prior to i.v. infection with either 10^3 ST-OVA, ST-OVA-T or sham. At day 5 post-infection, mice were evaluated for splenic burden and CD8$^+$ T cell proliferation. Mice that received ST-OVA-T controlled bacterial burden comprehensively (Fig. 15 a), and showed dramatic, early induction of antigen-presentation as detected by overall increase in the numbers of OVA-specific CD8$^+$ T cells (Fig. 15 b), and the dilution of CFSE expression of OT-1 cells (Fig. 15 c). These results indicate that translocation of OVA into the cytosol of infected cells results in a shift in the type of antigen-presentation similar to the situation in LM-OVA infection. In contrast, those mice that received conventional ST-OVA, where antigen is present mainly in the phagosomes, failed to induce any CD8$^+$ T cell response.


C57BL/6J
IFNαR-/-
<100 colonies
<100 colonies
ST-OVA-T
ST-OVA
10⁰ 10¹ 10² 10³ 10⁴ 10⁵ 10⁶
CFU/spleen

(a)


C57BL/6J
IFNαR-/-
ST-OVA-T
ST-OVA
Naive
10⁴ 10⁵ 10⁶ 10⁷
#OVA-Tetramer+ cells

(b)


C57BL/6J
IFNαR-/-
ST-OVA-T
ST-OVA
Naive
0 10 20 30 40 50 60 70 80 90
%CFSE$_{lo}$ (>5 divisions)

(c)

Figure 15 Direct presentation of OVA results in rapid control of ST infection. ST-OVA-T is a construct of ST-OVA that allows OVA to be secreted and translocated into the cytosol, as opposed to normal ST-OVA which is not. 10^7 CFSE-labelled OT-1 cells were adoptively transferred into C57BL/6J and IFNαR$^{-/-}$ mice. Two days later one group of each strain received 10^3 ST-OVA, one received 10^3 ST-OVA-T, and the third remained naïve. All mice were euthanized 5 days post-infection. Spleens were homogenized and serial dilutions of the single cell suspensions were done in 0.9% saline and plated on BHI agar. After incubation at 37°C for 24 hours, cell colonies were counted as an assessment of splenic bacterial burden (a). The remaining cell suspension was incubated with anti-CD8 antibody and PE-labelled OVA-Tetramer, and the cells were acquired by flow cytometry. $CD8^+$ T cell proliferation was measured by an increase in the absolute number of OVA-specific T cells (b), and in the percentage of OT-1 cells that were $CFSE_{lo}$ at day 5 (c). Data represents mean values ±SEM, representative of 2 independent experiments; n=3.

5. Discussion

5.1 Prelude

Salmonella infections are known to be a worldwide health care concern, and are of particular note due to their prevalence within impoverished and vulnerable populations. *Salmonella* infections in humans can generally take one of two forms. The first, less lethal pathology is due to infections with *Salmonella enterica* subsp. *enterica* serovars that tend to be more highly pathogenic in other species, such as *Salmonella* Enteritidis or *Salmonella* Typhimurium. These pathogens cause gastroenteritis in immunocompetent adults, and are generally spread via contaminated food products, and occasionally receive significant media coverage in first-world countries due to massive food recalls (2, 1). While antibiotic-resistant forms of these pathogens are becoming more prevalent, these infections are generally confined to a self-limiting gastroenteritis, providing the host is non-pregnant and otherwise healthy (2). The second form of *Salmonella* infection seen in humans tends to be limited to developing areas of the world and is largely caused by *Salmonella* ser. Typhi and *Salmonella* ser. Paratyphi infections. Infections with these serovars can cause typhoid disease in humans, resulting in enteric fever and numerous physiological symptoms which range from fever and diarrhoea to intestinal bleeding and perforations. Treatment with antibiotics has achieved some level of control over these infections, bringing mortality rates down to 1-4% annually, but Typhoid fever can last from several weeks to several months (1). This particular *Salmonella* infection is endemic where sanitation is low, and is generally spread throughout the population through feces-contaminated food and water.

In order to study these diseases, *Salmonella* Typhimurium is used as a model pathogen in mice, as mice will exhibit a range of symptoms which resemble Typhoid fever. *Salmonella*

Typhi and Paratyphi do not cause typhoid in mice, and in fact mice have been observed to mount an extremely efficient clearance when infected with Typhi, showing little bacteremia and few symptoms (119). Thus, *Salmonella* Typhimurium is utilized as the choice model pathogen due to its similarities in mice to human infections with *S.* Typhi. As previously discussed, a number of both host and pathogen factors are instrumental in the effectiveness and severity of a murine *Salmonella* infection. In terms of host response, the *Nramp*1 gene is implicated as crucial in determining severity of ST infection. NRAMP protein functions as a divalent cation efflux pump in the phagosomes of phagocytes (namely macrophages) (37). It is the presence or absence of NRAMP that is attributed to the differences in *Salmonella* infection progression between 129SvJ and C57BL/6J mice, respectively (Fig. 2). As shown in Fig. 1, equivalent doses of LM or ST given intravenously in C57BL/6J mice result in very different host outcomes and immune responses. C57BL/6J mice infected with LM i.v. display an acute infection and rapidly mount an effective T cell response against the pathogen, culminating in the elimination of bacteria by day 7 post-infection. Conversely, an equivalent dose of ST will proliferate to the point of lethal bacteremia and sepsis by day 7 with no significant $CD8^+$ T cell response. However when the same ST infection is administered to B6.129F1 mice, mice display a chronic infection against ST, and mount a delayed $CD8^+$ T cell response which is detectable only during the second week of infection and peaks between days 21-30 post-infection (Fig. 2). It is at this point that the bacterial burden in the spleen begins to be controlled and decrease to sub-lethal levels; however it is important to note that the spleen is not cleared of pathogen until after day 90 post-infection. This significant delay in $CD8^+$ T cell activation indicated a delay in antigen presentation of ST at the host level, and suggests that this may be a mechanism exploited by ST to promote immune evasion and hence survival.

5.2 Characterizing the $CD8^+$ T cell response to ST

In our mouse models, ST infections are shown to proceed in one of two ways; either a) infection results in rapid septicaemia and death (Fig. 1), or b) infection increases bacterial burden gradually over time, and will just as gradually recede with the initiation of T cell response (Fig. 2). However, what takes the $CD8^+$ T cell response so long to develop, and why the response, albeit delayed, fails to clear the pathogen rapidly is not clear. One particularly notable characteristic of the *Salmonella* bacteria is their facultatively intracellular lifestyle. Thus, even when infected in a model resembling a systemic infection, ST can inhabit either the intra- or extra-cellular niche, depending on which is most beneficial to its survival. In addition, ST is well-characterized to utilize its type three secretion system (TTSS) to escape initial lyzosomal degradation (64, 77, 101). The TTSS is encoded by SPI-1 and SPI-2 genes, each of which has a function related to the survival of the pathogen within the cells of the infected hosts. SPI-1 is largely involved in the initial infection of the cell. The SPI-2 mechanism is essential for the injection of effector proteins into the host cell in order for ST to survive within the phagosome (64, 77, 101). It is this viable phagosomal residence that is implicated in the delay of $CD8^+$ T cell response to ST infections. It is suggested that this phagosomal residence effectively "hides" the ST from the MHC I presentation machinery, thus delaying the activation of ST-specific $CD8^+$ T cells (14, 20). Simultaneously, ST can exist extracellularly in some capacity, increasing the level of inflammation and progressing to lethal sepsis if not controlled (47, 105). In B6.129F1 mice, this control is observed. However, in most viral and bacterial infections, the increase in dose of the pathogen leads to earlier onset of symptoms and responses. For example, increasing the dose of *Mycobacterium bovis* by 100-fold results in a significant shift in $CD8^+$ T cell response to earlier on during the infection (84). Thus, in order to evaluate if the infectious

dose of ST was not simply insufficient for induction of a viable $CD8^+$ T cell response early on, the dosage was increased.

An LM-OVA dose of 10^3 bacteria i.v. was sufficient to mount an effective specific $CD8^+$ T cell response. Interestingly, when this dose was decreased 10-fold to 10^2 LM-OVA/200µl, a potent $CD8^+$ T cell activation response was still observed early on (Fig. 3). However, this response was diminished to undetectable levels when the mice were infected with 10 bacteria. These results suggest that LM-OVA is extremely effective at stimulating the adaptive immune system, even at low infectious doses. The results observed with 10 bacteria were likely due to the infection being so minute that the level of variation may be large between mice (i.e., it is likely that some mice received <10 LM-OVA). Clinically, pathogen doses below a certain threshold are considered "safe" and are not expected to result in the clinical manifestation of disease (17). However, in the case of slow-growing pathogens, such as BCG and HIV (29, 84), low doses may be sufficient to prime $CD8^+$ T cell responses. Indeed, we have previously noted that in case of ST, infection with a minimum of 50 organisms is needed to induce a clinically relevant infection consistently (our unpublished data). In B6.129F1 mice, when ST-OVA dose was increased (up to 10^5 ST-OVA/200µl), no appreciable increase in early antigen presentation and $CD8^+$ T cell response was observed (Fig. 3). Thus, we can suggest that even when the ST burden is escalated to near lethal (B6.129F1 mice) or lethal (C57BL/6J mice) levels, immune recognition at least from the perspective of acquired immunity does not set in early.

While our lab has previously shown that antigen-presentation is delayed against ST, it was not clear how $CD8^+$ T cell response, which peaks between days 21-30, is eventually induced. Did antigen presentation occur gradually over the first three weeks, or was it

dramatically initiated after a later time point? During LM infection, the T cell response is dramatic, and extensive $CD8^+$ T cell proliferation occurs as a result of clonal burst by day 5 post-infection (Fig. 3). Similarly, Butz and Bevan (22) showed that acute LCMV infections result in massive clonal burst of specific $CD8^+$ T cells within the first week of infection. This is generally a characteristic of the acute infections where antigen is presented through the classical antigen processing pathway; acute infections have massive bursts of clonal T cell expansion early on. Conversely, $CD8^+$ T cell response to another intracellular bacterium that resides in the phagosomes of infected cells, BCG, shows a delayed $CD8^+$ T cell response, with T cell response peaking between days 21-30 at low doses, yet peaking at day 7 post-infection at higher doses (84). While in the BCG infection model it does appear that the delay in $CD8^+$ T cell response is indeed due to reduced generation of antigenic levels, which can be overcome by infection with very high doses of the bacterium, it is worth noting here that BCG replicates very slowly, with a doubling time of >24 h (103). This would indeed generate very low antigenic levels *in vivo*, and this may be an important factor in the BCG infection model (63, 103). This mechanism may not operate in case of ST infection model since ST (doubling time 26 min) proliferates even faster than LM (doubling time 46 min) (63, 103). Additionally, BCG is a highly attenuated bacterium, which engages different TLRs (38, 44), and may exert inhibitory effects on antigen-presentation that may not be as profound as those induced by ST. Interestingly, $CD8^+$ T cell response to 10^3 ST-OVA *in vivo* appears to be due to a gradual proliferation of $CD8^+$ T cells (Fig. 5). While the numbers of OVA-specific $CD8^+$ T cells at day 5 and day 10 remain low, by day 15 there is an increase in OVA-specific $CD8^+$ T cells to the point at which >50% of OVA-specific $CD8^+$ T cells have undergone more than 5 divisions, implying a rapidly developing $CD8^+$ T cell response at this time point. Since this response takes an additional 7-15 days to peak (Fig 6, 8), these

results indicate that $CD8^+$ T cell response to ST does not occur through a rapid clonal burst, but occurs through a gradual accumulation of primed $CD8^+$ T cells. This gradual accumulation suggests that there may be a number of intracellular processes that are occurring prior to, or in conjunction with antigen presentation. There may be a rate-limiting step in the antigen processing pathway that takes the first 2 weeks to occur, which is subsequently followed by an increasing amount of primed DCs presenting the antigen. Additionally, immature DCs have been known to sequester antigen until sufficient maturation signals have primed the DC (52). This has been implicated as a major mechanistic difference between macrophages and DCs, and may play a role in the ability of DCs to sufficiently present antigen (particularly extracellular antigen) whereas macrophages are much better equipped for rapid degradation of extracellular antigen in order to prevent prolonged inflammation and sepsis

In vivo antigen presentation against ST seems to be related to the time at which antigen is successfully processed and presented to $CD8^+$ T cells, however, it is unknown if antigen is continuously presented and if that may have an impact on the $CD8^+$ T cell response. Generally, when antigen is continuously presented, $CD8^+$ T cell exhaustion can occur if the infection is not being controlled (54, 85). Since ST is detectable within the spleen for a significant amount of time after $CD8^+$ T cell peak, there was a question of whether $CD8^+$ T cells may be continuously stimulated with the antigen, and whether this may have had an effect on the $CD8^+$ T cell response. Continuous treatment with ciprofloxacin beginning at various time points during infection allowed us to see how the $CD8^+$ T cell response varies when antigen is removed from the system. A striking difference was observed between those groups that received early treatment and those that received ciprofloxacin later on in the infection (Fig. 6). Those mice that received continuous antibiotic treatment beginning at day 3 or 6 post-infection displayed a

markedly reduced $CD8^+$ T cell response compared to those that had a treatment onset of day 15 or later (Fig. 6). While the addition of antibiotics to the system is a well-known way to expedite the clearance of pathogens from a host, what is interesting about these results is that any antibiotic added after day 15 of the infection had no significant impact on the T cell response, suggesting that it is at this point during the infection that the $CD8^+$ T cell response is committed to responding to the infection, regardless of the bacterial burden. Here, we can posit that perhaps that elimination of bacteria has little to no impact on the presence of the antigenic epitope. It is this epitope that is presented to the $CD8^+$ T cells for targeting, and is only one small part of the bacteria as a whole. Ciprofloxacin is a fluoroquinolone, a class of antibiotic that targets the DNA gyrase of the bacterium, rendering it unable to proceed with DNA and protein synthesis, which is crucial to replication (117). Thus, by day 15 the elimination of bacteria is only effective in controlling damage done by bacterial-induced inflammation, since the epitope has already been sufficiently processed and presented to the $CD8^+$ T cells. It is also important to note that the entire pathogen burden is not eliminated immediately following antibiotic treatment. Furthermore, while antibiotic treatment will remove any bacteria present in the system, this does not necessarily mean that specific processed antigen will be removed simultaneously. These data match up with the increase in the fraction of responding specific $CD8^+$ T cells (Fig. 6), suggesting that there is a 2-week delay in antigen processing which is the rate-limiting step prior to antigen presentation, which is then committed to proceed, regardless of actual pathogen burden. In other infection models where antigen-presentation occurs mainly by the classical pathway, antigen-presentation is completed within the first 48 h of infection, and proceeds uninterrupted even if the antigen is removed subsequently (49, 106), indicating that $CD8^+$ T cells differentiation proceeds in an autopilot mode when antigen-presentation is

completed. In case of ST that antigen-presentation program takes several weeks, and removal of pathogen before but not after the first two weeks, impairs the development of $CD8^+$ T cell response. On the other hand in chronic viral infection models (67, 112), $CD8^+$ T cell response was reported to be totally dependent on the persistence of antigen. It is possible that this is related to the potency of $CD8^+$ T cell stimulation. During viral infection, $CD8^+$ T cells are stimulated through the direct antigen-processing pathway, which can drive $CD8^+$ T cells into exhaustion if stimulated on a continuous basis during chronic infection (54, 112). Thus, antigenic persistence may be needed during such infection models to allow for maintenance of $CD8^+$ T cell response.

5.3 *Salmonella* lifestyle as an escape mechanism

As previously described, *Salmonella* species are facultative intracellular pathogens, and due to this lifestyle flexibility, they are able to inhabit the most optimal environment in order to escape the immune system. Intracellular viability is crucial to the escape from the immune system, and *Salmonella*'s TTSS are essential for secreting factors that result in the successful phagosomal survival of ST due to the inhibition of lysosomal-phagosomal fusion, thereby preventing exposure to degrading lysosomal enzymes (20). Furthermore, the activation of genes such as the *phoP* induced genes while inhabiting the phagosome increase ST tolerance to reactive oxygen species (ROS) and antimicrobial peptides while inhabit immune cells (77). However, Salcedo *et.al* (87) have shown that there is at least 30% of the splenic ST present extracellularly 3 days post-infection, suggesting that the ST-infected hosts may also harbour a portion of the infection extracellularly. These studies are in agreement with our results.

When our B6.129F1 mice were infected with 10^3 ST or LM i.v., both infections displayed sizeable intra- and extracellular bacterial fractions at all time points (Fig. 7). The extracellular

fraction of the ST-infected mice was noticeably higher on day 1 than at all other time points and higher than all extracellular burdens in LM-infected mice. Mice infected with either ST or LM both showed a minimum of 15% extracellular bacteria at some point during the infection. This supports the current classification of LM and ST as facultative intracellular pathogens. Interestingly, in terms of the immune response to either of these infections, they tend to be evaluated as intracellular pathogens, however, these data indicate that there is a portion of the bacteria present in the system which is extracellular at all points in time. The presence of extracellular bacteria during these infections, particularly in the case of ST, which exhibits a chronic infection phenotype in the B6.129F1 mice, indicates that this extracellular fraction may be important in the ability of the infection to persist. The extracellular fraction would not be targeted by the CTL response, and thus may act as a form of reservoir for the bacteria while the intracellular bacteria are removed from the system due to CTL lysis.

Thus, the significance of the extracellular fraction during *in vivo* infection is essential to evaluate in order to understand the nature of the immune response to ST. If this >15% extracellular fraction is truly important as a reservoir for either further propagation of pathogens or as a reservoir for the release of pathogenic factors, the elimination of this fraction should have drastic effects on the $CD8^+$ T cell response to infection. For example, when macrophages take up bacteria, most bacteria are generally routed to the lysosomes and result in degradation instead of antigen presentation (78). However, if there are more bacteria outside of these macrophages, the immune system could easily become overwhelmed and not be able to keep up with the rate of bacterial replication. Furthermore, $CD8^+$ T cells are known to take much longer to divide (12 hours) in comparison to pathogens they may be acting against (2-3 hours) (29). Administration of gentamicin antibiotic to ST-OVA-infected mice resulted in a noticeable change in the $CD8^+$ T

cell response over the first 30 days of infection. Gentamicin is an aminoglycoside antibiotic, and is non-penetrative at the concentrations used in these experiments (53). Thus, treatment with gentamicin suggests that only extracellular bacteria will be eliminated. This was supported by our data, which showed that those mice treated with the gentamicin exhibited a measurable reduction in extracellular bacteria (Fig. 8). Interestingly, at day 10 post-infection there is no observable difference between those mice that received gentamicin and the untreated group (Fig. 8). Firstly, any therapy used to treat an infection does not have immediate, noticeable activity. Treatment began at day 3 post-infection, so a delay in noticeable effects is to be expected, as the bacteria were given a chance to replicate prior to antibiotic treatment. Secondly, effectiveness of gentamicin would be related to extracellular availability. Early on in infection, ST will stimulate their own uptake in order to hide in the phagosome (14, 77, 101). As the infection progresses, it is more beneficial to reside extracellular in order to be disseminated via the gastrointestinal system to the environment. By day 21, when the adaptive immune response is generated, ST may be exiting the intracellular niche in greater numbers and thus are more available to be targeted by the gentamicin.

5.4 Impaired uptake by DCs as a means to avoid antigen-presentation

Intracellular pathogens are able to survive within a host by one of three ways: the lysis of the phagosomal membrane, the resistance to lysosomal factors, or the prevention of phagosomal-lysosomal fusion (20). These survival mechanisms are extremely important, as once these microorganisms enter into the host cell, either by triggering its own uptake or by phagocytosis, they will be exposed to the harsh intracellular environment while simultaneously requiring the host for essential growth factors. Previous work has shown that *Salmonella* can evade degradation intracellularly by preventing lysosomal-phagosomal fusion (14, 20, 101). Our work

tends to focus on the effect of ST infection once it has become systemic, but there are several important processes that occur at the intestinal epithelial level as well. In both the systemic and enteric infections, a crucial step in *Salmonella* infection is the entry of ST into the host cells. Generally, the epithelial cells at the intestinal interface are not classically phagocytic, and thus, would not be expected to readily take up ST (42, 64, 107). This is where the TTSS of ST comes into play and is a crucial first step in the development of systemic infection. At the splenic level, a large population of immune cells are present, which can indeed be invaded by ST in a manner similar to that of the epithelial cells, but many of these cells are instead specially equipped to engulf foreign antigen on their own. Thus, the uptake potential of ST by either its own mechanisms or by the function of the phagocytic immune cells is crucial to the eventual antigen-presentation and immune response to ST infection.

As shown in Fig. 7, both LM and ST-infected mice will harbour pathogen in both the intracellular and extracellular splenic compartments. Since the spleen itself is largely composed of specialized immune cells, any passing ST would be expected to both trigger its own invasion using the TTSS, and to also be actively taken up by macrophages and DCs that inhabit the spleen. In addition, APCs from other organs that have been infected by the pathogen would be expected to have migrated to the spleen in order to stimulate naive T cells. When DCs were purified from ST-OVA or LM-OVA-infected spleens, these splenic DCs were expected to be capable of presenting OVA antigen to OVA-specific $CD8^+$ T cells *in vitro* and to give a picture of antigen presentation at given time points along the *in vivo* infection. Purification of LM-OVA infected spleens yielded a similar antigen presentation profile to what was previously observed *in vivo* (Fig. 9). This suggests that during systemic infection with LM-OVA, splenic DCs are sufficiently mature and take up a sufficient amount of antigen that allows them to present to

specific $CD8^+$ T cells. This presentation and T cell activation is highest at day 1, and declines during the following time points (Fig. 9). This suggests that LM-OVA-infected DCs are most potent at stimulating $CD8^+$ T cells by day 1, and the 4 day *in vitro* incubation time infers that the following days are a time of massive $CD8^+$ T cell proliferation, supporting the peak of $CD8^+$ T cell numbers seen *in vivo* at day 7. The peak in antigen-presenting ability of DCs coincides with the relative LM-OVA burden in DCs. This is in contrast to ST-OVA infected spleens, where the splenic DC number and DC-specific burden is minimal early on, which peaks later, but fails to induce $CD8^+$ T cell response *in vitro* (Fig. 9). These results suggest that while splenic DCs are clearly taking up ST and accumulating in response to the infection, there is possibly some lack of signalling that prevents the proper *in vitro* T cell proliferation from occurring.

In both *in vivo* and *in vitro* systems, APCs require a specific set of requirements in order to function properly and induce T cell activation. DCs are particularly well-equipped as APCs, while macrophages tend to have functions more in line with the innate immune system; scavenging and eliminating extracellular antigen. Macrophages can be induced artificially to be good antigen presenting cells (78), but DCs are regarded as the primary APC. The slow accumulation of ST burden (compared to LM) observed in the *ex vivo* data suggested that DCs may be impaired somewhat in the initial ST uptake, either by interference by ST itself or as some discrepancy between the ability of DCs to pick up ST over LM. When APCs were cultured *ex vivo* from bone marrow and infected with ST or LM there was at least a 10 to 15-fold difference between uptake of LM over ST (Fig. 10). Within the first 24 hours of infection ST, when compared to LM, seems to be impaired in uptake by APCs. This likely explains the initial difference in the burden of ST-OVA in DCs early on; ST takes a longer length of time to successfully and completely invade the host cells. The deficiency in early uptake is likely

another facet of the observed delay; if ST takes longer to be taken up into host cells, the time of antigen presentation and $CD8^+$ T cell response would also be pushed back accordingly.

In order to signal and interact successfully with other cells, a cell must be properly activated, and this principle is particularly important when it concerns immune cells. Both $CD8^+$ T cells and dendritic cells require crucial maturation signals in order to effectively respond to antigen. Without these maturation signals on both cell types, the immune response can be skewed towards T cell tolerance rather than activation. DC licensing is the process of maturation into a functional professional APC that can induce T cell activation (51, 57, 59). Type I IFN has been implicated as crucial in both T cell and DC licensing (59), and DC maturation tends to rapidly occur when antigenic inflammatory mediators are detected; in this case, LPS (109). The addition of LPS to the bone marrow-derived cell culture results in an upregulation of a number of cytokines leading to maturation of the immune cells. Mature DCs are known to be more efficient at presenting antigen, and thus, we posited that the artificial induction of maturation would lead to increased antigen uptake and presentation. Although ST itself has LPS on its surface which would induce maturation of DCs and macrophages, however, in this case LPS on ST is not available globally, but only inside the phagosome where ST resides (70). Even though addition of LPS to both the *in vivo* and *in vitro* systems resulted in visible increases in ST uptake both early on (Fig. 11) and within 1 week of infection (Fig. 12), there was no appreciable increase in $CD8^+$ T cell response. Again, this supports the theory that regardless of the state of the DCs, antigens of ST will still not be presented early on. It is not the DCs or the $CD8^+$ T cells that are deficient in the antigen presentation process; there is likely some interference of the ST itself, such as the escape into the phagosome, and the non-classical pathway that leads to attenuated antigen presentation.

5.5 Cross presentation as the main antigen-presentation pathway

Presentation of antigen to $CD8^+$ T cells is crucial for the development of CTL responses, which will then eliminate intracellular pathogens. The mechanism of the classical MHC I-$CD8^+$ direct presentation pathway has been extensively unravelled (reviewed in 43, 108). The classical pathway works extremely well when the system is exposed to a misfolded protein within a cell or when that cell is infected with an intracellular virus (reviewed in 43, 82, 108). In these cases, the antigen is within the cytoplasm, which allows it access to the potent MHC I machinery, and subsequent processing and presentation render that cell marked for CTL-directed degradation. Additionally, some intracellular bacteria, such as *Listeria*, become available in the host cytoplasm and also will be detected through the direct presentation pathway (89). However, there are numerous antigens that do not fit into this general pathway, and yet efficiently stimulate $CD8^+$ T cell responses. Extracellular OVA antigen was first detected as able to be presented in an MHC I-type presentation pathway, which is contrary to its classical presentation through MHC II to $CD4^+$ T cells (93). Since then, numerous pathogens have been shown to present somewhat via this non-traditional pathway, known as "cross-presentation."

As previously noted, the removal of the extracellular fraction of an ST infection had dramatic effects on the resultant $CD8^+$ T cell response (Fig. 8). This suggests that the extracellular ST play a significant role in the development of $CD8^+$ T cell response. An assay for cross presentation can be modelled *in vivo* via the subcutaneous injection of the antigen of choice. When B6.129F1 mice were subcutaneously infected with ST (not expressing OVA) and soluble OVA protein together, a measurable endogenous $CD8^+$ T cell response occurred by 7 days post-infection (Fig. 14a). As detected by the number of OVA-specific $CD8^+$ T cells isolated from an infected spleen, only mice who received the ST-induced inflammation along

with OVA gave measurable increases over those mice that were either uninfected or infected with OVA protein alone. These results suggest that it is the ST-type inflammation that causes the immune response, and since the antigen of choice (OVA) is exclusively extracellular in this model (as opposed to being expressed by the ST), OVA is being picked up as a result of the inflammation. Early cross-presentation studies suggested that OVA can only be picked up if in association with injected cells. These data show that B6.129F1 mice are fully capable of presenting OVA to $CD8^+$ T cells, which is a hallmark of cross-presentation.

Interestingly, C57BL/6J and $IFN\alpha R^{-/-}$ mice did not display similar cross-presentation capabilities (Fig. 14b). In the case of the $IFN\alpha R^{-/-}$ mice, this may very well be due to their lack of type I-IFN receptor (discussed below), which has been shown to be crucial for the mechanism of cross-presentation (31, 58). The fact that that the typical cross-presentation model failed to induce a $CD8^+$ T cell response in C57BL/6J mice indicates that the reduced uptake by APCs (discussed earlier), or the mechanism of death of macrophages in this strain of mouse may be key determinants in preventing the successful operation of the detour pathway of cross-presentation in C57BL/6J mice. When $IFN\alpha R^{-/-}$ mice were infected with 10^3 ST-OVA simultaneously alongside B6.129F1 mice, by day 18 post-infection, the $IFN\alpha R^{-/-}$ mice were not observed to have stimulated any $CD8^+$ T cell proliferation *in vivo* (Fig. 15). In contrast, the B6.129F1 mice were, as previously observed, very capable of mounting a successful $CD8^+$ T cell response by this timepoint to the ST-OVA. Between this data and the observed inability of C57BL/6 and $IFN\alpha R^{-/-}$ mice to respond to the cross-presentation assay, the mouse strains were considered as potentially deficient in antigen-presentation pathways. If this were so, neither mouse would be able to successfully present OVA during an ST infection if the OVA were engineered to be expressed in the cytoplasm. In the case of ST-OVA-T, OVA antigen does

exactly that: ST-OVA-T bacteria secrete and translocate OVA into the cytosol of the infected host cell upon infection. This results in OVA being readily available in the cytoplasm, where the classical direct presentation pathway could access it and present to $CD8^+$ T cells. Infection of C57BL/6 and $IFN\alpha R^{-/-}$ mice with 10^3 ST-OVA-T yielded confirmation that these mice are indeed capable of directly presenting OVA antigen during an ST infection, if the antigen is made readily available within the cytoplasm (Fig. 15). In both strains, $CD8^+$ T cell responses at 5 days post-infection were similar to those of LM-OVA infections in B6.129F1 mice by day 5. Furthermore, the CFU numbers detected by this timepoint were below detection level, suggesting that these mice had effectively responded to this infection, rendering it an acute phenotype, and eliminating the bacteria before lethal sepsis could be achieved. These mice survived well beyond day 7 when infected with ST-OVA-T as well (our unpublished observations).

Several viral and bacterial pathogens have been implicated in the ability to induce cross presentation, through a number of alternate antigen presentation pathways. Type I IFN has been considered as a key cytokine that promotes cross-presentation (58). The fact that IFNαR-deficient mice failed to generate $CD8^+$ T cell response implies that cross-presentation is the only pathway through which $CD8^+$ T cell response is induced against ST. The inability of $IFN\alpha R^{-/-}$ mice to present antigen (Fig. 13), despite their continued survival past day 7 suggests that while the lack of IFN-I leads to survival during the innate inflammatory stage, the adaptive immune system requires IFN-I signalling in order to effectively present antigen and initiate $CD8^+$ T cell responses. Previously, our lab has illustrated how the lack of IFN-I in macrophages reduces macrophages death upon infection with ST. This would circumvent the lack of NRAMP1 in macrophages that would otherwise lead to their death. However, many viral models of cross presentation illustrate a key role for IFN-I in cross presentation (7, 58, 96, 100). Mice deficient

in IFN-I are more prone to T cell tolerance due to insufficient T cells and DC licensing, and $CD8^+$ T cells have reduced cytolytic activity (28, 58). These mice have been observed to be more susceptible to LCMV infections, as well as bacterial *E. coli* and *Streptococcus pneumonia* infections (65).

Furthermore, the presence of IFN-I renders infected host cells more susceptible to apoptosis (31, 96). This is crucial for both innate clearance of the bacteria, but also for CTL responses. Both *Salmonella* and *Mycobacterium* species have been shown to increase macrophage apoptosis *in vivo* (35, 88, 115). Taking this with our delayed $CD8^+$ T cell activation and cross presentation data, a strong case is presented in favour of the detour pathway. The detour pathway involves apoptotic blebs of infected cells being engulfed by nearby bystander APCs (31, 116). Once inside the APC, the contents of the bleb will become available to the cross-presentation pathway, and lead to $CD8^+$ T cell CTL activity. The lack of IFN-I signalling in $IFN\alpha R^{-/-}$ mice suggests that the apoptotic portion of the pathway has been abrogated and thus, no blebs are available for bystander DCs to pick up. This will subsequently prevent CTL action, and without $CD8^+$ T cell activity, the infection will proliferate to the point of lethal sepsis.

6. Conclusion

Cross presentation against extracellular antigen is becoming a growing area of investigation, as research shows that numerous viral and bacterial antigens are being presented to $CD8^+$ T cells in this manner. The range of pathways that make up the larger umbrella term of "cross-presentation" illustrate the diversity of the immune system and its ability to target a wide variety of antigen. Research shows that nearly any antigen that can exist in the extracellular environment, whether as a functional pathogen, protein or within an apoptotic bleb, can be

presented via this pathway. Cross-presentation is leading to the clarification of how antigen that is not classically presented can stimulate effective CTL responses. As the field expands, clinical applications of cross-presentation are coming to the forefront. Interventions that can encourage the cross-presentation of extracellular antigen which may have otherwise not responded to traditional therapies are being increasingly explored, with some success (9, 73). Furthermore, cross-presentation can be uniquely stimulated by pro-inflammatory cytokines, such as IFN-I, as addressed here. The host-pathogen interactions observed in both bacterial and viral infections are complex and varied, and cross-presentation is an effective alternate pathway used by the immune system to target antigen, such as *Salmonella* or *Mycobacterium* which would otherwise evade traditional responses. Cross-presentation can occur via number of pathways, and after the initial uptake of the extracellular antigen, generally feeds into the same machinery as the direct MHC I pathway, albeit in a different location within the cell. Our work has demonstrated support for the detour pathway, due to our observed delay in antigen presentation coupled with the known apoptotic properties of *Salmonella* infections (115, 122). This is also a proposed pathway for other pathogens, including *Mycobacterium tuberculosis* (111, 115), *Shigella* (116) and potentially, in the case of phagocytic neutrophils, *Listeria monocytogenes* (89). The prominence of apoptosis in these bacterial infections would suggest that this process would play an important role beyond that of further bacterial dissemination.

In this study, we further elucidated the host response to a *Salmonella* Typhimurium infection, at the level of the adaptive immune response. What had been previously shown was a lack of early antigen presentation and $CD8^+$ T cell response against ST, which resulted in either death or chronic infection, depending on the mouse strain used. This was noted to be in direct contrast with the infection course of another facultative intracellular pathogen, *Listeria*

monocytogenes. During *Listeria* infection, it was noted that regardless of the mouse strain, an acute infection profile was observed and the infection was non-lethal, yielding a functional memory T cell population. Increasing the burden of ST early on in the animals had no effect on the infection course; indeed, the only difference was an elevated splenic burden, suggesting that an increase in antigen early on, should the infection have continued, only correlated with an earlier onset of death. Indeed, the additional ST-derived LPS likely would have led to increased inflammation, causing death. Other early infection interventions, such as the increased maturation of the dendritic cells showed a similar lack of early response, suggesting that response to ST infection was due to a rate-limiting step after infection that preceded antigen presentation.

Interestingly, work with antibiotics illustrated the different phases of antigen presentation and $CD8^+$ T cell response, as elucidated by elimination of either extracellular or all bacteria at given points in time. The onset of antigen presentation 2 weeks after infection, followed by a committed $CD8^+$ T cell response, regardless of antigen suggested that this 2-week incubation period was crucial to the development of effective $CD8^+$ T cell responses. Although apoptosis due to *Salmonella* invasion has been shown to occur relatively rapidly in cultured macrophages (25, 69), there are likely additional factors that influence the speed of antigen presentation. Dendritic cells must be sufficiently primed and matured in order to effectively present to $CD8^+$ T cells and induce $CD8^+$ T cell activation (rather than tolerance). IFN-I is a cytokine mediator that has been implicated in several viral infections as being extremely important in the promotion of $CD8^+$ T cell response to antigen, including during cross presentation. Our data illustrates the importance of type I Interferons, in that those mice lacking IFN-I signalling are deficient in the normal anti-ST $CD8^+$ T cell response. Thus, we propose that apoptosis caused by ST infection

leads to subsequent uptake by bystander DCs. Bystander DCs have been previously observed to pickup apoptotic blebs of ST and other pathogen-infected cells *in vivo*, but we suggest that in conjunction with IFN-I signalling, this detour pathway of cross presentation is the primary pathway by which ST antigen can be presented (Fig. 16).

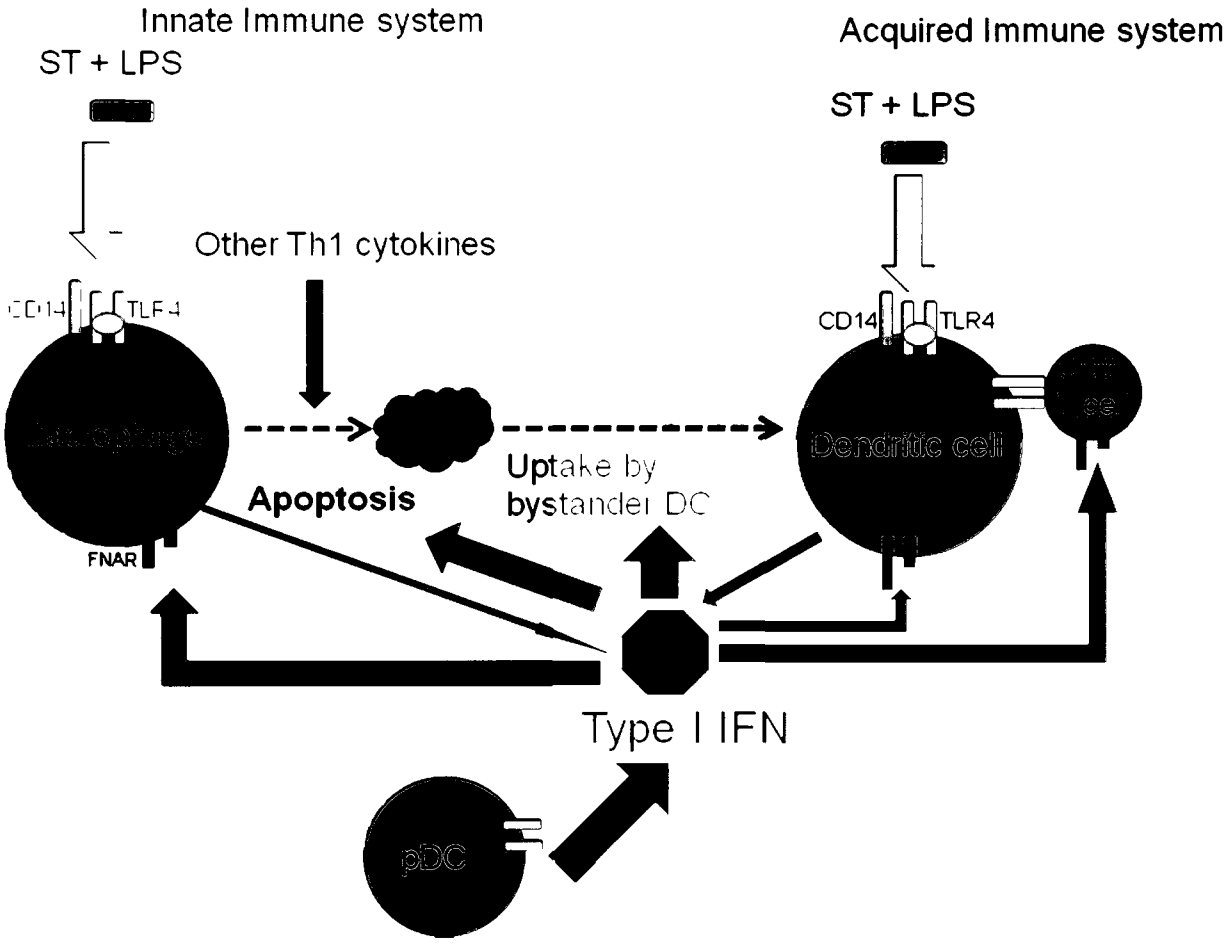

Innate Immune system
ST + LPS
Other Th1 cytokines
CD14
TLR4
Macrophage
FNAR
Apoptosis
Uptake by
bystander DC
Acquired Immune system
ST + LPS
CD14
TLR4
Dendritic cell
Type I IFN
pDC

Figure 16: Model for the cross-presentation pathway induced by *Salmonella* Typhimurium infection *in vivo.* *Salmonella* can infect many different cell types by either cell-mediated uptake or *Salmonella*-induced uptake. The entry of ST into host cells, particularly macrophages and neutrophils, triggers the apoptosis of these cell types. The apoptotic blebs are subsequently engulfed by bystander DCs. Bystander DCs are only functional as antigen presenting cells when fully licensed and matured, a process triggered by LPS from the pathogen, and type I IFN released from infected cells. IFN-I acts to license DCs for presentation, encourages the apoptosis of infected macrophages, and acts as a third activation signal for $CD8^+$ T cells to mature.

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