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Rachel Amy Luu
AUTEUR DE LA THÈSE / AUTHOR OF THESIS

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TITRE DE LA THÈSE / TITLE OF THESIS

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DIRECTEUR (DIRECTRICE) DE LA THÈSE / THESIS SUPERVISOR

Dr. Lionel Filion
CO-DIRECTEUR (CO-DIRECTRICE) DE LA THÈSE / THESIS CO-SUPERVISOR

EXAMINATEURS (EXAMINATRICES) DE LA THÈSE / THESIS EXAMINERS

Dr. J. Angel

Dr. A. Kumar

Dr. F. Scott

Gary W. Slater
Le Doyen de la Faculté des études supérieures et postdoctorales / Dean of the Faculty of Graduate and Postdoctoral Studies

Characterization of the CD8 T cell Response to *Salmonella typhimurium* Infection in Mice

Rachel Luu

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ABSTRACT

Salmonella typhimurium (ST) causes gasteroenteritis in humans and typhoid-like disease in mice. Since CD8 T cells facilitate acquired immunity, we evaluated the development and function of the CD8 T cell response against ST. Responses were compared to the acute intracellular pathogen, *Listeria monocytogenes* (LM). Because ST replicates within phagosomes and causes chronic infection, it was hypothesized that CD8 T cell priming may be muted and dysfunctional. While LM-induced CD8 T cells differentiated rapidly and displayed a mainly central-memory phenotyope in the long-term, CD8 T cells failed to become activated rapidly during ST infection and differentiated mainly into an effector/effector-memory phenotype. While the CD8 T cells induced against ST were functional, owing to the delay in CD8 T cell activation during ST infection, even conventional memory CD8 T cells failed to respond rapidly. Thus, the phagosomal lifestyle may allow escape from $CD8^+$ T cells, conferring a survival advantage to the pathogen.

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I. List of Abbreviations

Ag	antigen
Anx	annexin V
APC	antigen-presenting cell
BHI	brain heart infusion
BrdU	bromodeoxyuridine
CCR	chemokine receptor
CD	cluster of differentiation
cfu	colony-forming units
Cipro-HCl	ciprofloxacin hydrochloride
CTL	cytotoxic T cell
DC	dendritic cell
ELISPOT	enzyme-linked immunospot assay
ER	endoplasmic reticulum
GFP	green fluorescent protein
HIV	human immunodeficiency virus
IFN	interferon
Ig	immunoglobulin
Ii	invariant chain
IL	interleukin
iNOS	inducible nitric oxide synthase
LCMV	lymphocytic choriomengitidis virus
LLO	listeriolysin
LM	Listeria monocytogenes
LPS	lipopolysaccharide
MHC	Major Histocompatibility Complex
MLN	mesenteric lymph nodes
Mtb	Mycobacterium tuberculosis
NADPH	nicotinamide adenine dinucleotide phosphate hydrogen
Ova	Ovalbumin
SCV	Salmonella-containing vacuole
SPI	Salmonella pathogenicity islands
ST	Salmonella typhimurium
TAP	Transporter associated with Antigen Processing
Tc	cytotoxic T cell
TCR	T-cell receptor
Tet	tetramer
Th	helper T cell
TNF	tumor necrosis factor
TTSS	Type 3 secretion system
VSV	vesicular stomatitis virus

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III. INTRODUCTION

1. T cell response

a. Function of CD4 and CD8 T cells

T cells form an essential component of the adaptive immune response. CD4 and CD8 T cells are derived from the αβ T cell lineage, and are selected to be either CD4-positive or CD8-positive during maturation in the thymus (1). CD4 and CD8 T cells are capable of performing various functions that serve as important parts of the immune response that result in the curtailing of infections.

CD4 T cells are important mediators of immunity, serving as type 1 or type 2 helper T cells (Th1 or Th2). Th1 cells produce interferon gamma (IFN-γ), enabling B cells to undergo class switching to produce IgG2a antibodies in response to infection (2, 3). IgG2a antibodies are highly protective, in infections such as *Leishmania major* (4) and influenza (5). Th1 cells also stimulate macrophages to secrete IFN-γ and tumor necrosis factor alpha (TNF-α), enabling them to produce intracellular nitrite ions and to fight infection more efficiently (6, 7). Th2 cells produce interleukin (IL)-10 and IL-4, providing help to B cells by inducing class switching to IgG1 and IgE, which play an important role in allergy (8, 9). Th2 cells are protective during infection with worms such as *Schistosoma mansoni* (10), where IL-4 and IL-10 serve to inhibit the progression of the infection and to guard against excessive inflammation (11).

CD8 cytotoxic T cells (CTL) form a critical unit in the immunological battle against intracellular infection (12-14). Since the pathogen resides inside the infected cell, antigen (Ag) remains sequestered away from antibodies. Activated effector CTLs can destroy infected cells by several mechanisms. One mechanism is by IFN-γ production which activates infected macrophages to die (15); another is by perforin which forms large pores in

the plasma membrane, and granzymes that induce signaling in the cell to effect its demise (16, 17). A third method of CTL cytotoxicity is by the Fas death pathway, where the engagement of FasL on the CTL to the Fas receptor on the target cell results in the apoptosis of the target cell (18, 19). By elimination of the infected cells, pathogens are forced out of their intracellular niches and exposed to antibodies and other toxic mediators which eventually results in the control of pathogen proliferation and thus control of the infection.

b. Antigen presentation

In order for Ag recognition to take place, pathogen-derived proteins must first undergo processing and presentation by an Ag-presenting cell (APC). Proteins are partially degraded by enzymes of a host cell (33, 34). The resulting peptides are then incorporated with the Major Histocompatibility Complex (MHC) which is then transported to the surface so that the Ag is displayed by the APC for recognition by T cells (35, 36).

There are two main classes of MHC: MHC class I is generally involved in presentation of peptides derived from endogenous or intracellular Ag to CD8 T cells, whereas MHC class II is generally involved in presentation to CD4 T cells with peptides derived from exogenous or extracellular proteins (37). When the TCR complex binds the pMHC, this forms the immunological synapse (38), promoting a cascade of signaling events to ensue which effect the activation of the naïve T cell.

In some systems it has been shown that Ag presentation to CD8 T cells does not occur via one of the conventional pathways described above; that is, exogenous Ag can also be presented in the context of MHC I. This phenomenon is identified as "cross-presentation", which results in "cross-priming" of the CD8 T cell (39). Cross-presentation is regarded as an important pathway for Ag presentation during infection with pathogens that reside in the phagosome (40-42). When a cell undergoes apoptosis, apoptotic blebs are

formed from the cell membrane encasing remnants of cellular components, which are then scavenged by dendritic cells and presented by MHC I to CD8 T cells (43, 44). Specific pathways have been described for exogenous Ag presentation by MHC I (41). Vesicles from the ER fuse with the phagosome, carrying an ER transport protein Sec61 which could facilitate the transport of Ag into the cytosol (45, 46). These vesicles also carry MHC I to the phagosome (45, 46). Also it has been shown that upon endocytosis, the plasma membrane that is incorporated into the phagosomal membrane includes MHC I from the cell surface (47). The exogenous pathway is thought to be inefficient due to the likelihood that MHC I molecules in the plasma membrane or endocytic compartment are already occupied by other peptides (41). These exogenous pathways that facilitate cross-presentation may play an important role in pathogen recognition and response to infection.

c. T cell activation

In order for T cells to perform their specific functions, proper activation of the T cell must take place. There are similarities among T cell subsets as well as several distinguishing factors in their requirements for activation.

Activation of a naïve T cell depends mainly on three signals (20): first, by the recognition by the T cell receptor (TCR) of the peptide displayed in the context of the Major Histocompatibility Complex (MHC); second, by binding of costimulatory molecules expressed on the APC (eg. B7.1 or B7.2) with ligands (eg. CD28) expressed on the T cell surface (21); and third, by cytokines produced by APCs that amplify the T cell response to infection. If these requirements are not met, then a T cell may become unresponsive, or anergic (22).

CD4 T cells may differentiate into either a Th1 or a Th2 phenotype (23), depending on the cytokines to which they are exposed as naïve cells (24, 25). Development into a Th1

phenotype requires IL-12 (26), while the Th2 lineage depends on IL-4 (27). Another factor that can influence the pathway of differentiation towards Th1 or Th2, involves the strength of the signal produced by the binding of the TCR with the peptide presented in the context of MHC II (28). Some studies have shown that a higher dose of Ag tends to lead to a Th1 response, while the response to a lower dose of Ag tends towards Th2 (29, 30).

CD8 cytotoxic T cells also differentiate, into either a Tc1 or Tc2 phenotype (31, 32). Similar to Th1 and Th2 CD4 T cells, these subsets also differ in the cytokines produced; that is, Tc1 express IL-2 and IFN-γ whereas Tc2 cells express IL-4, IL-5, IL-6 and IL-10. (31, 32).

d. T cell memory development

i. Generation and maintenance of T cell memory

As infection is cleared, pathogen-specific CD4 and CD8 T cells decrease in number and differentiate to yield memory cells. Memory T cells respond more efficiently upon pathogen rechallenge, produce cytokines such as IFN-γ and TNF-α, and mediate rapid cytolytic activity (48). The magnitude of the memory population is dependent on the magnitude of the primary response (49). A small proportion of 5-10% of the Ag-specific T cell population generated at the peak of the response survives to form the memory pool; this 5-10% fraction of primed cells that becomes memory is constant, regardless of the infection and dose used (50-52).

It is not clear what distinguishes this 5-10% of surviving T cells from the rest of the population. However, it has been found that expression of the cytokine receptor IL-7Rα decreases at the peak of CD8 T cell activation, and that the subset of T cells that are still IL-7Rα^{hi} at the peak of the response, are those that continue on and become memory cells (53). Additional factors have been shown to distinguish this population, such as increased

expression of anti-apoptotic molecules including Bcl-2 (54, 55). Thus, by expression of anti-apoptotic molecules, some T cells are able to avoid apoptosis and survive long-term.

The phenomenon by which clonally expanded populations of Ag-specific T cells decrease in size following infection is known as contraction. Recent studies have indicated that contraction may actually be programmed early on, even in the first days of infection, rather than as a feedback response to having eradicated the pathogen (56, 57). There may be competition between cells for resources such as cytokines and growth factors that are important to survival, so that those cells that do not gain access to such resources undergo apoptosis due to deprivation, while those that do acquire the necessary factors survive long-term (58).

The memory pool of T cells is maintained by homeostatic proliferation which displays a higher rate of turnover compared to naïve T cells (59). This maintenance has been shown to occur independent of the persistence of Ag (60), and also independent of MHC (61). In order to maintain the size of the population, the rate of turnover must equal the rate of proliferation (48). There is some evidence that maintenance of functional CD8 T cell memory also depends on CD4 T cells (62, 63). The key cytokines involved in maintenance of memory are IL-7 and IL-15, where IL-7 is important for survival and IL-15 plays a role in proliferation (64, 65). Homeostatic mechanisms involve signaling through cytokine receptors such as IL-7R, as well as the correlation with expression of anti-apoptotic molecules (53).

Expression of various molecules on CD8 T cells is used to distinguish between effector and central memory subsets, since central memory cells ($CD44^{hi}CD62L^{hi}CCR7^{hi}$ IL-$7R\alpha^{hi}$) reside in the lymph nodes while effector memory cells ($CD44^{hi}CD62L^{low}CCR7^{low}$IL-

7Rα^{hi}) home to non-lymphoid organs (66, 67, 68). Effector memory T cells have been described as "functionally charged" (69), referring to their rapid response to Ag by producing IFN-γ and by their high CTL activity. Central memory T cells also respond to Ag rapidly, but they display a more potent proliferative capability in contrast to effector memory cells (70).

ii. The role of CD4 T cells in the development of CD8 T cell memory

In a wide variety of infection models, CD8 T cell responses seem to be dependent on the presence of CD4 T cells. How the CD4 T cells help CD8 T cell responses is currently an area of active investigation. One view is that the CD4 T cell and the CD8 T cell recognize the Ag on the same APC simultaneously (71-73). The ligation of CD40L from the CD4 helper T cell with CD40 on the APC is the key in the activation of the APC (3, 74). This ligation induces upregulation of an array of important stimulatory components, including MHC molecules, adhesion molecules such as ICAM-1 (75), costimulatory molecules including CD80 and CD86 (76) as well as an increased ability of the APC to produce stimulatory cytokines such as IL-12 (77), all of which translate to enhanced activation of the CD8 T cell.

Another model of CD4 T cell help has been developed, where it is not necessary for the CD4 helper T cell and the CD8 T cell to recognize Ag on the APC simultaneously. Based on experimental data showing that DCs previously activated by CD4 T cells were just as capable of stimulating CD8 T cells as when CD4 T cells were actually present, Matzinger and colleagues proposed a model where a CD4 helper T cell first interacts with the APC by CD40/CD40L interactions, and then this activated APC goes on to stimulate the CD8 T cell (78). This mechanism was later designated as DC "licensing" by Smith *et al.*, who

confirmed the requirement for initial activation of DC by CD4 helper T cells during HSV-1 infection in vivo (79).

2. Infection

a. Introduction to ST and LM Infection

Typhoid in humans is an infectious disease caused by the bacterial pathogen *Salmonella typhi* which results in more than 16 million cases of infection and 600,000 deaths worldwide each year (80). Current vaccines against typhoid, including the live attenuated strain Ty21a and the subunit vaccine composed of Vi Ag, confer protection only about 50-75% of the time, and it is thought that the generation of functional CD8 memory T cells is insufficient (81, 82). In order to better understand the CD8 T cell response to *S. typhi,* the mouse model of ST infection is used, which closely resembles *S. typhi* infection in humans (83). It is anticipated that by obtaining a sound understanding of the manner in which the CD8 T cell population responds to ST infection in mice, we might use this information to design more effective typhoid vaccines.

Listeria monocytogenes (LM), also an intracellular enteric bacterial pathogen, has been extremely well-studied and is considered a model pathogen for studying intracellular bacterial infection and immunity (84). This study of the CD8 T cell response to ST has been performed along with that of LM so that responses induced against ST can be compared to this well-characterized, potent pathogen.

b. Infection model: LM replicates within the host cell cytosol and causes acute infection.

LM is a gram-positive pathogen which is acquired by the host through ingestion of contaminated food (85). LM crosses the intestinal epithelium through M cells or enterocytes (86); this pathogen has been found to replicate within the phagocytic cells underlying the Peyer's patches (86) and in the lamina propria (87). LM replicates and induces apoptosis in its host cells, causing neutrophils to arrive on scene which then phagocytose the pathogen,

where it can survive intracellularly and hitch-hike to disseminate to the liver, spleen, (88, 89), and mesenteric lymph nodes (MLN) (86).

LM can induce its own uptake by host cells (Figure 1a). Studies suggest that LM enters by a "zipper mechanism" (90), as its surface protein internalin binds to E-cadherin on the host cell (91, 92). Once LM has been taken up by the host cell, it uses a key virulence factor, the pore-forming enzyme listeriolysin O (LLO), to break up the surrounding phagosomal membrane and to thereby escape into the cytosol where it undergoes rapid proliferation (93, 94). This is in sharp contrast to the intraphagosomal replication strategy of ST. In fact, it has been shown that without the production of functional LLO to escape the phagosome, LM cannot replicate within the host cell (95). A second key strategy of LM lies in cell-to-cell spread - using the protein ActA to exploit actin from the host cytoskeleton, LM polymerizes actin to form filaments or "comet tails" to propel itself from one host cell to another (96, 97).

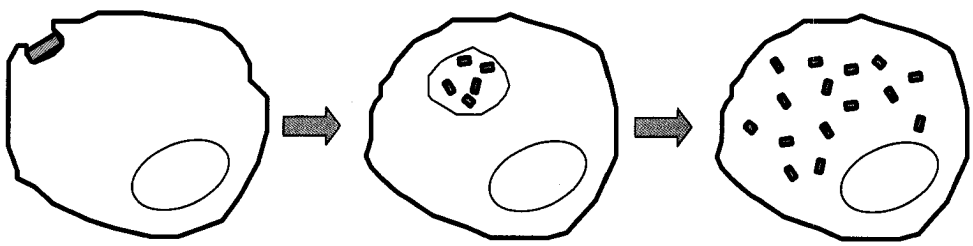
LM also manipulates host cell signaling. In the cytosol, LM expresses Hpt to acquire hexose phosphates from the host cell cytosol for energy (98). LLO and PI-PLC have been implicated in raising the levels of intracellular calcium and activating the PKC signaling pathway; this facilitates the release of LM from the phagosome (99). LM also recruits a host cell protein complex, Arp2/3, which it activates by ActA, to begin the polymerization of the actin filaments (100).

LM causes acute infection, as it proliferates rapidly but is detected early and cleared quickly by the host (101). Previous work in this lab has demonstrated that the splenic burden is rapidly eliminated in mice within the first week of infection (102).

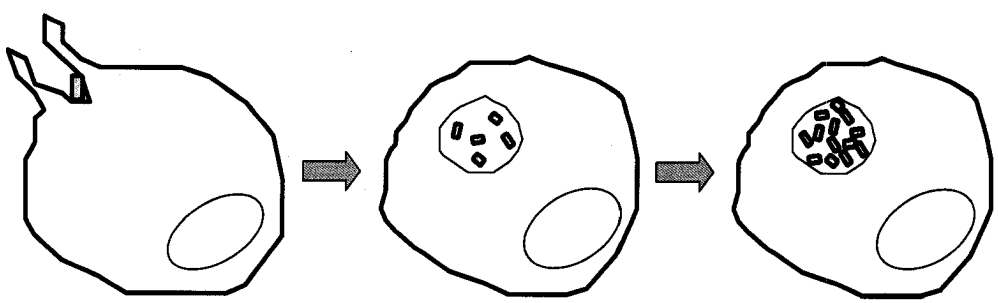
Altogether, LM employs an array of virulence factors to facilitate invasion of host cells, replicate in the cytosol, and translocate itself from cell to cell; however, it is evidently

Figure 1. Differential uptake and intracellular localization of *Listeria monocytogenes* and *Salmonella typhimurium.* (a) *Listeria monocytogenes* invades the host cell via the "zipper" mechanism, then escapes from the phagosome and replicates within the host cell cytosol. (b) *Salmonella typhimurium* invades the host cell via the "trigger" mechanism and replicates and persists within the phagosome of the infected cell.

(a) *Listeria monocytogenes*



(b) *Salmonella typhimurium*



detected by the host immune response and effectively controlled following several days of infection (101).

c. Infection model: *Salmonella typhimurium* replicates with the phagosome of the host cell and causes chronic infection.

Salmonella typhimurium (ST) is a gram-negative pathogen which begins its infection of the host via the oral route, in a manner similar to that of LM as described above.

ST infection of mice is used as a model of human typhoid which is typically an acute infection in humans, however mice that are resistant to ST infection develop a chronic infection that lasts for several months (103, 104). Infection in resistant mice is classified as chronic because the pathogen persists at high levels for the 60-90 days of infection and subsequently persists at sub-clinical levels (105).

Following ingestion of contaminated food or water, the pathogen invades the intestinal epithelium of the host. ST forces its way through the gut epithelium by invading and killing M cells (106, 107). It then infects the underlying macrophages (108), in which it induces caspase-1-dependent cell death, leading to the production of proinflammatory cytokines IL-1β and IL-18 (109) and subsequent recruitment of additional cells - which ST essentially hijacks to disseminate throughout the body including the liver and spleen (110). Although in vitro studies have indicated that ST can be taken up and survive within murine macrophages (111, 112) as well as dendritic cells (111, 113) and B cells (114), in vivo studies of murine ST infection have only shown that ST resides within macrophages of the spleen (115), liver (116, 117), and MLN (105). ST has been shown to establish itself in the host and persist long-term (105).

ST virulence genes are found mainly on two *Salmonella* pathogenicity islands, SPI-1 and SPI-2 (118, 119). PhoP/PhoQ is a two-component regulatory system that controls

transcription of numerous genes essential to virulence (120). These genes include those that encode proteins for a Type III secretion system (TTSS). The TTSS functions as a syringe, forming a needle-like structure from the bacterium that binds to the host cell, and injects soluble proteins into the host cell cytoplasm, inducing the uptake of the bacteria via a "trigger mechanism" (90, 121). Characteristics of this uptake consist of rearrangements of the actin cytoskeleton and membrane ruffling of the host cell (122) (Figure 1b).

ST further uses its virulence factors to interfere with host cell trafficking and signaling. Once ST has been engulfed by the host cell, it survives in the phagosome, or *Salmonella*-containing vacuole (SCV), where it inhibits fusion with the lysosome (123). The precise mechanism responsible for this inhibition is unknown; however, a recent study has shown that the SopE protein is involved in recruiting the early endosomal marker Rab5 to the SCV, and is thus thought to contribute to inhibiting fusion with the lysosome (124). The SPI-2 type III secretion system enables ST to avoid killing by NADPH oxidase in macrophages (125), including the expression of SPI-2 effector proteins (126) such as SifA (127) to promote its survival and replication. Consequently, ST is able to replicate within the phagosome and avoid killing while it evades the immune surveillance of the host.

3. Immune responses to intracellular pathogens

a. Acquired immune responses to intracellular pathogens and the role of CD8 T cells

The immune response to intracellular bacteria is generally dominated by a Th1 response. The important role of CD8 T cells in fighting intracellular pathogens has been established by a vast collection of studies on numerous pathogenic infections (14). Effective protection against rechallenge with LCMV is conferred by the ability of CD8 T cells to rapidly proliferate upon Ag recognition and to lyse infected cells (128). CD8 T cells are recruited to the lung during infection with *Mycobacterium tuberculosis* (Mtb) where they have been shown to produce IFN-γ and TNF-α (129). CD8 T cells also contribute to immunity against infection with HIV (130, 131), *Leishmania major* (132), *Borrelia burgdorferi* (133), Mtb (129), and ST (134).

b. Immune response to LM infection

Innate immunity is the first line of defense against infection, since it is a rapid and non-specific response and also provides a foundation for the development of the adaptive immune response (135). Neutrophils are recruited in large numbers and are essential in the host response to LM infection (136, 137); they contribute to combating infection by killing LM directly (138), or by lysing infected hepatocytes (88). Macrophages and hepatocytes produce the chemokine MCP-1 to recruit additional monocytes to the site of infection (139). Macrophages also produce TNF-α and IL-12 stimulating NK cells to produce IFN-γ, which then in turn activates the killing capacity of macrophages (140). IFN-γ may also be produced by dendritic cells (DC) and macrophages in response to LM infection (141, 142).

There is little evidence for the role of antibodies in combating LM infection. One study showed that some resistance is conferred following immunization of mice with anti-

LLO Ab; however, following natural LM infection, very low titres of Ab were detected (143).

CD4 T cells promote a Th1 response by producing TNF-α, IFN-γ, and IL-2 during infection (144). Interestingly, CD4 T cells have been found to contribute to the maintenance of CD8 T cell memory to LM infection, while they are not required during the earlier stages of infection for a stable CD8 T cell memory population to develop (63). Thus many cells and cytokines come into play during the host response to LM infection, inducing a Th1 response. This response also involves CD8 T cells which are central to the control of LM infection. The CD8 T cell response and the immunity it provides against LM infection has been extensively studied (145-147). CTLs are essential in conferring immunity to infection by perforin-mediated lysis (148); they recognize and lyse hepatocytes and macrophages that are infected by LM (149), as well as protect against rechallenge (150). Ag derived from LM are recognized by CD8 T cells, such as peptides from the key virulence factor LLO (151). The response to LM is rapid, potent, and effective (152). Previous work in this lab has demonstrated that Ag-specific CD8 T cells develop into a large population within days of infection; there are numerous IFN-γ-producing cells present early on, and the contraction of this population occurs quickly after the first week of infection (102).

c. Immune response to ST infection

The host response to ST has been extensively studied using the mouse model of infection. Innate immunity is essential to the control of ST growth early on, involving the recognition of components of ST that initiate the production of proinflammatory cytokines. Lipopolysaccharide (LPS) from ST is recognized by TLR4, and TLR4-deficient mice that are immunized with attenuated ST succumb rapidly to infection (153, 154). Macrophages and dendritic cells respond to LPS from ST by secreting IL-12 and TNF-α (155). Recognition of

ST flagella by TLR5 leads to production of TNF-α (156) by DC (134) and macrophages, which has been shown to contribute to controlling bacterial replication (157). Macrophages produce IL-12 in response to ST infection (158), and IFN-γ is produced mainly by macrophages and neutrophils early on (159). Mice deficient in IL-12, IFN-γ, IL-18, or TNF-α demonstrate a reduced ability to control the bacterial burden (160-162). Neutrophils are critical in early control of ST infection, as high numbers of neutrophils have been shown within the spleen, liver, MLN and peyer's patches within 1 day of infection of mice (163) and depletion of neutrophils causes mice to succumb to infection early on (159). Natural killer (NK) cells as well as NKT cells are activated in the early stages of infection (159, 164) and are also sources of IFN-γ (165). The action of phagocytic NADPH oxidase is important early on, as mice deficient in NADPH oxidase succumbed to infection within 3 days of infection compared to iNOS deficient mice which controlled infection for 2 weeks but still succumbed to infection (166). The *Nramp* gene in mice encodes a divalent cation transporter located in phagosomal membranes which confers immunity against ST, since mice with mutations in this gene are highly susceptible to ST infection (167-169).

Since ST is effective at establishing a protective niche within macrophages, the adaptive immune response becomes an imperative measure of combat to control and eliminate the infection. The humoral arm of the adaptive immune response in mice includes antibodies that are produced against many Ag of ST comprising of outer membrane protein (OMP), LPS, Vi, flagella, and heat shock proteins (HSP) (105). In human typhoid patients, IgG Ab has been observed specific to porins (170), OMPs (171), and Vi antigen (172). The mucosal immunity induced by oral infection of mice with ST includes IgA antibodies which play an important role since mice lacking polymeric Ab are unable to control infection (173). Antibodies also provide protective immunity upon re-infection; IgA-secreting hybridoma

tumors have been shown to protect mice from oral ST infection (174). When mice with defective maturation of B cells ($Ig\mu^{-/-}$) are immunized with attenuated ST and then rechallenged with a virulent strain, they succumb to infection compared to their $Ig\mu^{+/+}$ counterparts (175). Furthermore, B cells provide help to cellular-mediated immunity since the Th1 response is diminished in B cell-deficient mice (176).

T cells play an essential role in the adaptive immune response to ST infection. The T cell response to ST involves costimulation requirements; this includes a requirement for CD28 in order to clear infection. The response in CD28-deficient mice is characterized by a lack of IgG1, IgG2a production and very low production of IFN-γ (177).

CD4 T cells bear a central role in the eradication of ST infection. Without CD4 T cells, mice succumb to infection (178). CD4 T cells mount a predominantly Th1 response to ST, controlling bacterial replication and producing IL-2 and IFN-γ (179), which is dependent on the transcription factor T-bet (180). Th2 cytokines are produced such as IL-4 by murine CD4 T cells in response to ST porins (181) and flagella (182). During the 2-5 weeks following murine infection with virulent ST, 20% of CD4 T cells produce IFN-γ upon restimulation in vitro; and from 2 weeks up to as long as 10 weeks after infection, 70% of CD4 T cells showed activation by reduced CD62L expression (179). There are few epitopes that are characterized from the T cell response to ST infection, however immunodominant epitopes from the antigen FliC were found in the CD4 T cell response (183, 184), and another epitope recognized by CD4 T cells is derived from the SPI-1 effector protein SipC (185).

Research has shown that CD8 T cells are also of great importance in combating ST infection, although there is not a great deal of information regarding the nature of the CD8 T cell response to virulent ST. It is known that CD8 T cells are activated in response to murine

infection with attenuated strains of ST. This is shown by increased expression of the adhesion molecule CD44 and the early activation marker CD69, as well as production of IFN-γ, by CD8 T cells present in the spleen (134). In mice deficient for the MHC I component β_2-microglobulin, there is reduced protection against rechallenge with virulent ST (186), indicating that CD8 T cells are important in protective immunity against this pathogen. In a separate study, when mice were depleted of CD8 T cells, the splenic bacterial burden was significantly increased, but survival was not negatively affected as greatly as when CD4 T cells were depleted (178). In humans immunized orally with the current vaccine, the live attenuated strain Ty21a of *S. typhi*, it was found that CD8 T cells showed an effector memory phenotype ($CCR7^-$ $CD27^-$ $CD45RO^+$ $CD62L^{low}$) and expressed adhesion molecules used for homing to the gut (187).

Compared to the epitopes known for CD4 T cells responding to ST infection, there have been no epitopes defined for CD8 T cells. However, it has been found that some CD8 T cells are specific to ST epitopes presented by MHC class Ib molecule Qa-1 (186). From humans immunized with live attenuated *S. typhi*, CD8 T cells exert cytolytic activity against *S. typhi*-infected target cells (188). In humans immunized with Ty21a, examination of the *S. typhi*-specific CD8 T cell repertoire showed that a large proportion of responding CD8 T cells possess Vβ specificities, with some clones producing a higher amount of IFN-γ while others exert a higher percentage of cytotoxicity (187).

Thus, there is much to be learned in terms of how CD8 T cells respond specifically to virulent ST infection, and it is anticipated that this information might be used in designing a more effective vaccine to combat infection.

4. Hypothesis

Salmonella typhi causes infection world-wide and current vaccines possess immunogenicity and provide immunity that are less than ideal (189, 190). CD8 T cell memory is important in combating intracellular infections (191, 192). Thus, it is anticipated that by studying the CD8 T cell response in a murine model of typhoid, that information may be obtained that will be useful in improving vaccines against typhoid in humans. In order to provide a relative analysis of the response, the Ag-specific CD8 T cell response to ST is compared here to another intracellular bacterium, *Listeria monocytogenes* (LM), which also replicates within macrophages and is considered to be an effective inducer of CD8 T cell memory.

ST and LM differ in their intracellular niche, where LM breaks out of the phagosome to survive and replicate within the cytosol while ST is sequestered within the phagosome itself (123). Previous studies by this group have shown that antigen presentation during infection with phagosomal pathogens such as *Mycobacterium bovis* (BCG) and ST is substantially delayed (102). This is in comparison to other pathogens such as LM where antigen presentation is very high early on and is over within the first week of infection (193).

This leads me to hypothesize that infection of mice with virulent ST would result in the development of a delayed and muted CD8 T cell response. Since ST causes a chronic infection, the CD8 T cells may become dysfunctional in terms of their functional abilitites.

5. Aims and Specific Objectives

In order to comprehensively evaluate the CD8 T cell response to ST-Ova in comparison with LM-Ova, the following aims are addressed:

Aim #1: How do the growth rates of ST and LM compare in vitro?

Specific Objectives: Grow cultures of ST-Ova and LM-Ova in vitro and measure optical density (O.D.) of these cultures at various intervals to construct growth curves.

Aim #2: How does the bacterial burden in the spleen change throughout infection of mice with ST compared to LM?

Specific Objectives: Infect B6.129 F1 mice with ST-Ova or LM-Ova, then sacrifice these mice at various time points of infection and obtain cfu data from the spleen.

Aim #3: What is the proportion of the antigen-specific CD8 T cell population in response to ST infection?

Specific Objectives: Adoptively transfer splenocytes from transgenic OT-1 mice into naïve B6.129 F1 mice; infect these mice with ST-Ova or LM-Ova; stain lymphocytes from spleen or blood at various time points of infection with Ova Tetramer and anti-CD8 antibody; analyze by flow cytometry to determine the percentage of CD8 T cells which are Ag-specific.

Aim #4: What is the profile of proliferation for the antigen-specific CD8 T cells that are generated against ST-Ova?

Specific Objectives: Adoptively transfer splenocytes from transgenic OT-1 mice into naïve B6.129 F1 mice; infect these mice with ST-Ova or LM-Ova; then stain lymphocytes from spleen at various time points of infection; measure proliferation by determining with flow cytometry the proportion of Ag-specific CD8 T cells that are

bromodeoxyuridine (BrdU)-positive, since the cells that have divided will have incorporated the thymidine analogue BrdU into their DNA.

Aim #5: What is the activation profile of the antigen-specific CD8 T cells that are generated against ST-Ova?

Specific Objectives: Adoptively transfer splenocytes from transgenic OT-1 mice into naïve B6.129 F1 mice; infect these mice with ST-Ova or LM-Ova; stain lymphocytes from blood and spleen at various time points of infection; measure activation by determining with flow cytometry the proportion of Ag-specific CD8 T cells that have a central phenotype ($CD44^{+}CD62L^{hi}$) and those that have an effector phenotype ($CD44^{+}CD62L^{lo}$).

Aim #6: What is the memory development profile of the Ag-specific CD8 T cells that are generated against ST-Ova?

Specific Objectives: Adoptively transfer splenocytes from transgenic OT-1 mice into naïve B6.129 F1 mice; infect these mice with ST-Ova or LM-Ova; stain lymphocytes from spleen; measure memory development by determining with flow cytometry the proportion of Ag-specific CD8 T cells that bear IL-7Rα.

Aim #7: What is the profile of commitment to death for the antigen-specific CD8 T cells that are generated against ST-Ova?

Specific Objectives: Adoptively transfer splenocytes from transgenic OT-1 mice into naïve B6.129 F1 mice; infect these mice with ST-Ova or LM-Ova, then measure memory development by determining with flow cytometry the proportion of Ag-specific CD8 T cells that stain positive for Annexin V.

Aim #8: What is the role of CD4 T cell help in the antigen-specific CD8 T cell response?

Specific Objectives: Adoptively transfer splenocytes from transgenic OT-1 mice into naïve B6.129 F1 mice; deplete CD4 T cells from these mice; infect these mice with ST-Ova or LM-Ova; stain lymphocytes from spleen at various time points of infection with Ova Tetramer and anti-CD8 antibody; analyze by flow cytometry to determine the percentage of CD8 T cells which are Ag-specific.

Aim #9: Do the antigen-specific $CD8^+$ T cells that are generated against ST-Ova exhibit CTL abilities when presented with antigen?

Specific Objectives:

a) Evaluate IFN-γ production - Adoptively transfer splenocytes from transgenic OT-1 mice into naïve B6.129 F1 mice; infect these mice with ST-Ova or LM-Ova; stain lymphocytes from blood and spleen at various time points of infection; measure activation by determining with flow cytometry the proportion of Ag-specific CD8 T cells that are IFN-γ^+.

b) Evaluate ability to kill specific targets in vivo – Use in vivo CTL assay to measure % specific lysis at various time points of infection of B6.129 F1 mice with ST-Ova or LM-Ova.

Aim #10: Are the memory CD8+ T cells induced against ST-Ova capable of responding to re-infection?

Specific Objectives: Adoptively transfer splenocytes from transgenic OT-1 mice into naïve B6.129 F1 mice, then infect these mice with ST-Ova or LM-Ova. After infection has been mostly resolved, sacrifice these mice, isolate the ST-Ova-induced or LM-Ova-induced CD8 T cells from the splenocytes; inject these CD8 T cells into naïve mice; then re-infect them with either ST-Ova or LM-Ova. Measure the proportion of Ag-specific CD8 T cells in the blood of these rechallenged mice to

assess the ability of the ST-Ova-induced or LM-Ova-induced memory cells to respond to rechallenge to either ST-Ova or LM-Ova.

6. Experimental Approach

a. The mouse model of typhoid is a valuable tool in the study of ST infection

The mouse model of ST infection closely resembles infection of *S. typhi* in humans (83). Conventional C57BL/6 mice are highly susceptible to infection with low doses (10^2) of ST as the mice die within the first week of infection. On the other hand, 129X1/Sv mice, which also express the same H-2K^b haplotype, are better at controlling ST infection with low doses due to possession of the *Nramp* allele (167); that is, rather than mice dying within the first week of infection, a chronic infection develops. 129X1/Sv mice were crossed with C57BL/6J mice to yield B6.129 F1 mice. These F1 mice are required since they are resistant to ST infection, possess the MHC class I H-2K^b haplotype, and their immune system accepts the establishment of OT-1 transgenic splenocytes upon adoptive transfer. OT-1 transgenic mice possess >90% CD8 T cells bearing TCR specific to the Ovalbumin (Ova) peptide, $OVA_{257-264}$; thus by adoptively transferring splenocytes from OT-1 transgenic mice, to the B6.129 F1 mice, the Ag-specific CD8 T cell response is amplified upon infection and easily detectable by flow cytometry.

b. Recombinant bacteria ST-Ova and LM-Ova are useful in evaluating the Ag-specific response to the same Ag between two pathogens

The pathogens that are used in this project are LM-Ova and ST-Ova; these are wild-type strains of LM and ST that have been designed to constitutively express the protein Ovalbumin (Ova) (194). In this way, the comparison of the immune responses is more stringent so that CD8 T cell responses are evaluated against the exact same protein, while it is expressed by two pathogens in different intracellular environments.

c. Adoptive transfer enables the amplification of the Ag-specific T cell response

Conventional C57BL/6 mice are highly susceptible to ST infection and cannot be used for long-term studies (153). Since C57BL/6 mice die within 7 days of infection, T cell priming and memory development cannot be studied in such mice. On the other hand, 129X1/Sv mice (195) can be used for these studies because they harbor a chronic, non-lethal infection (196). However, the frequency of Ova-specific CD8 T cells is low in such infection models. To overcome this problem, an adoptive transfer model was used. OT-1 transgenic cells are useful in studying the Ag-specific response in that >90% of the CD8 T cells in an OT-1 mouse, bear TCR specificity for the $Ova_{257-264}$ peptide SIINFEKL (197). OT-1 transgenic cells are accepted by conventional C57BL/6 mice but not by 129X1/Sv mice. To overcome this problem and considering the high susceptibility of C57BL/6 mice, B6.129 F1 mice are used instead, since these mice are resistant to ST and the transgenic OT-1 cells are also accepted by this mouse strain. OT-1 splenocytes are transferred into recipient mice, which are challenged with the Ova-expressing recombinant pathogens. This presence of naïve OT-1 transgenic cells amplifies the numbers of Ag-specific cells so that they can be detected easily by flow cytometry.

d. Tetramer technology facilitates the detection of the Ag-specific T cell population

The term "Tetramer" is used to describe a complex of four MHC molecules that are each folded with a peptide (pMHC). These four pMHC complexes are biotinylated and then bound to one streptavidin molecule – this forms one tetramer molecule (198).

The use of the tetramer technology allows the detection of T cells that are specific to a particular Ag (199). Using a fluorescently labeled MHC class I H-$2K^b$ tetramer complexed with the CD8 epitope $Ova_{257-264}$, this allows the detection by flow cytometry of the Ag-specific CD8 T cells that are induced during an infection.

IV. MATERIALS AND METHODS

Mice. B6.129 F1 mice were bred in the animal facility at the Institute for Biological Sciences of the National Research council (NRC-IBS, Ottawa, ON, Canada), by crossing 129X1/SvJ female x C57BL/6J male; both were acquired from The Jackson Laboratory (Bar Harbor, Maine). B6.129 F1 mice were required since they are less susceptible to ST infection, possess the MHC class I H-2K^b haplotype, and their immune system accepts the establishment of OT-1 transgenic splenocytes upon adoptive transfer. OT-1 transgenic mice were acquired from The Jackson Laboratory; these mice were required since they possess >90% CD8 T cells bearing TCR specific to the Ovalbumin (Ova) peptide, $OVA_{257-264}$; thus by adoptively transferring splenocytes from OT-1 transgenic mice to the B6.129 F1 mice, the Ag-specific response is amplified and easily detectable by flow cytometry. At various time points of infection, B6.129 F1 mice were sacrificed by administration of CO_2. Mice were maintained at the NRC-IBS animal facility in accordance with the procedures outlined by the Canadian Council of Animal Care.

Adoptive transfer. Adoptive transfer was performed by transferring splenocytes from OT-1 transgenic mice to naïve B6.129 F1 mice in order to amplify the Ag-specific response; specifically, to detect the response of CD8 T cells to the Ova peptide $Ova_{257-264}$ upon infection with LM-Ova or ST-Ova (described below). Spleens from OT-1 mice were homogenized in RPMI 1640 (Invitrogen Life Technologies, Grand Island, NY) using the frosted ends of two glass slides, then filtered through a 100 μm Falcon strainer (BD Biosciences, Mississauga, ON, Canada), centrifuged 8 min at 450xg, resuspended in HBSS and enumerated. OT-1 splenocytes were injected *i.v.* into naïve B6.129 F1 mice, with $1x10^6$ lymphocytes among total splenocytes, in 200 μl HBSS, 3-5 days prior to bacterial infection.

Bacterial strains. The recombinant LM-Ova strain expresses and secretes a portion of Ovalbumin ($Ova_{134-387}$). LM-Ova was previously generated from LM wild-type strain 10403S; it was then grown to mid-log phase and stored at -80°C as previously described (200). ST-Ova was generated (193) by electroporation of the virulent ST strain SL1344 with the pKKA plasmid, which contains the full-length gene encoding Ova (201). Cultures were grown and at mid-log phase (O.D. 600 = 0.8) ST-Ova was harvested and frozen in 20% glycerol at –80°C. Cfu were determined by performing serial dilutions in 0.9% NaCl, which were spread on BHI-streptomycin (50 μg/ml) agar plates.

Evaluation of bacterial replication in vitro. Cultures of ST-Ova and LM-Ova were grown in Brain Heart Infusion (BHI) (Difco, Detroit, MI) broth at 37°C with shaking. Optical density (O.D.) at 600nm was measured every 20min and used to construct a growth curve for each strain.

Infection of mice. Mice were infected by injection *i.v.* with $1x10^3$ bacteria LM-Ova or ST-Ova, in 200 μl 0.9% saline.

Enumeration of bacterial burden in the spleen and mesenteric lymph nodes. Colony forming units (Cfu) were enumerated by plating dilutions of homogenized spleen or mesenteric lymph nodes (MLN) in 0.9% saline onto Brain Heart Infusion (BHI) (Difco, Detroit, MI) agar followed by overnight incubation at 37°C with 5% CO_2.

Isolation of splenocytes. Spleens were excised from mice and homogenized in RPMI 1640 using the frosted ends of two glass slides, then filtered through a 100 μm Falcon strainer, centrifuged 8 min at 1600 rpm, resuspended in R8 (RPMI 1640 supplemented with 8% FCS [HyClone, Logan, UT] and 50 μg/ml gentamycin [Invitrogen Life Technologies]), and filtered once more. Splenocytes were enumerated with a hemocytometer by trypan blue exclusion.

Isolation of peripheral blood. Mice were bled following approximately 5 min of gentle warming beneath a 250 W heat lamp. A small nick was made in the lateral tail vein with a razor blade, and approximately 200 μl was collected into a Microtainer tube containing lithium heparin (BD Biosciences) to prevent aggregation of RBC.

Staining of cell surface markers. For splenocytes: Samples of 5 x 10^6 cells were washed with 3 ml PBS, resuspended in 80 μl PBS containing 1% BSA (PBS-BSA), incubated on ice with 1 μl anti-CD16/32 (BD Biosciences), and then incubated with antibody and PE-H-2K^{b}OVA$_{257-264}$ iTAg Tetramer (Immunomics, San Diego, CA) in the dark at room temperature for 30 min. Anti-CD16/32 was used to block Fc receptors in order to prevent non-specific binding of antibodies. Ova tetramer was used to bind TCR specific to the Ova peptide in order to detect the Ag-specific cells. For blood: PE-H-2K^{b}OVA$_{257-264}$ iTAg Tetramer and antibody were added directly to a sample of 100 μl blood. Following 30 min of incubation in the dark at room temperature, samples were resuspended in 1 ml Red Blood Cell (RBC) Lysis Buffer (Sigma-Aldrich) and incubated 8 min, washed with PBS, and resuspended in fixative (0.5% formaldehyde from a 37.5% formaldehyde stock, in PBS). The following reagents and their volumes were used: PE-H-2K^{b}OVA$_{257-264}$ iTAg Tetramer –

1 µl for splenocytes, 5 µl of 1:10 for blood; anti-CD8-PerCPCy5.5 (BD PharMingen, San Diego, CA) – 1 µl for splenocytes, 1 µl of 1:10 for blood or anti-CD8-ECD (Caltag Laboratories, Burlingame, CA) – 1 µl for splenocytes; anti-CD44-FITC (BD PharMingen), anti-CD62L-PECy7 (eBioscience) or anti-CD62L-CyChrome (eBioscience), and anti-IL7Rα-FITC (eBioscience) – 1 µl per sample. The combinations of stains were: anti-CD44-FITC, Ova Tetramer-PE, anti-CD8-ECD, and anti-CD62L-CyChrome. Later experiments involved the following combination: anti-CD44-FITC, Ova Tetramer-PE, anti-CD8-PerCPCy5.5, and anti-CD62L-PECy7. Anti-CD44 and anti-CD62L were used to detect activation status of the Ag-specific CD8 T cells.

Flow cytometry. Samples were acquired on an EPICS-XL 4-colour flow cytometer and analysed using the EXPO software (Beckman Coulter, Fullerton, CA); or, later experiments on a BD Biosciences FACS Canto 6-colour flow cytometer and analysed using the BD FACS Diva software.

Quantification of apoptosis by Annexin V staining. For splenocytes and blood: Following the cell-surface Ab staining as described above, samples were washed with 3 ml Annexin Binding Buffer (BD PharMingen) and centrifuged 8 min at 1600 rpm; then washed a second time to avoid non-specific Annexin V binding. Samples were then resuspended in 3 ml Annexin Binding Buffer and 10 µl Annexin-FITC (BD PharMingen), incubated 15 min in the dark at room temperature, then centrifuged 8 min at 1600 rpm, and resuspended in 500 µl Annexin Binding Buffer for acquisition by flow cytometry.

Quantification of cell cycling by BrdU incorporation assay. Mice received 1 mg/ml bromodeoxyuridine (BrdU) (Sigma-Aldrich) in the drinking water, changed fresh daily, for 3 consecutive days prior to harvest. Splenocytes were harvested as described above; $10x10^6$ splenocytes were used per sample. Cell surface staining was performed as described above. Using the Cytofix/Cytoperm kit (BD Pharmingen), splenocytes were permeabilized with Cytofix/Cytoperm for 20 min on ice and then washed with PermWash. DNA was partially fragmented by incubation with DNase (Sigma-Aldrich) in a 37°C water bath for 1 hr. Samples were washed with PBS, then resuspended in PermWash with 1 µl anti-BrdU-FITC (BD Biosciences) and incubated 30 min on ice, then washed with PBS and resuspended in 500 µl fixative for acquisition by flow cytometry.

Quantification of intracellular cytokine expression. $10x10^6$ splenocytes were used per sample. Following the cell-surface antibody staining as described above, cells were incubated for 1 hr at 37°C with GolgiStop (BD Biosciences) to inhibit protein translocation, in the presence of $Ova_{257-264}$ peptide to restimulate the Ag-specific CD8 T cells. $Ova_{257-264}$ peptide was previously obtained as a gift from Dr. Gordon Willick of the Institute for Biological Sciences at the National Research Council. Cells were then permeabilized with the Cytofix/Cytoperm kit from BD Biosciences: cells were incubated with Cytofix/Cytoperm for 20 min on ice, washed with PermWash, then resuspended in PermWash with 1 µl anti-IFN-γ-FITC (BD Biosciences). Samples were then incubated 30 min on ice, washed with PBS, and then resuspended in 700 µl fixative for acquisition by flow cytometry.

Measurement of the killing of specific targets by in vivo CTL assay. This assay was adapted from a previously published protocol (202). Splenocytes from naïve mice were briefly

incubated in RBC Lysis Buffer, washed, and counted. Next, splenocytes were incubated in the presence or absence of 10 μM $Ova_{257-264}$ peptide, stained with the membrane dye PKH26 (4 μM) using the PKH26 Cell Linker Kit (Sigma-Aldrich) and then with CFSE (Sigma-Aldrich): 0.4 μM for the unpulsed population, and 4 μM for the peptide-pulsed population. The pulsed and un-pulsed populations were then mixed 1:1 and $20x10^6$ cells were injected into B6.129 F1 mice that had been previously infected with ST-Ova or LM-Ova, as well as a control group of naïve B6.129 F1 mice, using 3 mice per group for each time point. Percentage of specific lysis was calculated using the following formula:
[100-[(Infected pulsed/Infected Unpulsed)/(Uninfected pulsed/Uninfected Unpulsed)]] x 100.

Purification of CD8 T cells. Using the CELLection Biotin Binder Kit (Dynal, Lake Success, NY), Dynabeads were bound to anti-CD8β-biotin-conjugated rat anti-mouse CD8β.2 mAb (53.5.8; BD Pharmingen) according to the manufacturer's instructions. Spleens from infected or naïve B6.129 F1 mice were processed as described above. Splenocytes were then incubated with Dynabeads conjugated with anti-CD8 antibody for 1 hr on ice; approximately 50×10^6 beads were used for every 100×10^6 splenocytes. Dynabeads were washed gently with R8 and incubated with DNase at 37°C for 10 min, then pipeted up and down vigorously to separate the beads from the CD8 T cells, which were washed in R8, then resuspended in R8 for use in the ELISPOT assay, or in HBSS for injection into naïve mice for rechallenge.

Enumeration of the frequency of Ova-specific CD8 T cells by ELISPOT assay. CD8 T cells were purified from the splenocytes of the infected mice as described above, then seeded $1x10^5$/well and $1x10^4$/well in a 96-well MultiScreen IP Filter plate (Millipore, Cambridge, ON, Canada) that had been coated overnight at room temperature with 5 μg/ml anti-IFN-γ

(clone R46A2) in sodium bicarbonate buffer. Spleen cells from naïve C57BL/6 mice were added as feeder cells to a total of $5x10^5$ cells per well. CD8 T cells were incubated 48 hr at 37°C in R8 containing recombinant murine IL-2 (ID Labs, London, ON, Canada), in the presence or absence of $Ova_{257-264}$ peptide. Next, the plate was washed once with ddH_2O, and five times with PBS containing 0.01% $Tween_{20}$ (PBST). The plate was then incubated with biotinylated anti-IFN-γ (clone XMG) for 2 hr at 37°C, and washed five times with PBST; then incubated with streptavidin-conjugated horseradish peroxidase (SA-HRP) (1 μg/ml) for 1 hr at room temperature, washed three times with PBST, and then three times with PBS. Incubation with 3-amino-9-ethylcarbazole (AEC) substrate (Sigma-Aldrich) was performed according to the manufacturer's instructions, and the reaction was stopped by washing the plate in water. The plate was stored overnight in paper towel at 4°C, and spots were enumerated using an Olympus dissecting microscope after the plate had dried.

Depletion of T cells and cytokines. Antibody was administered *i.p.*, 50 μg in 200 μl PBS, to B6.129 F1 mice twice per week. The following antibodies were used: anti-CD4 clone GK1.5; anti-IFN-γ clone XMG; anti-CD8 clone 2.43. Antibodies were produced by high density cell culture of hybridoma cell lines, all of which were obtained from the American Type Culture Collection (Manassas, VA). Supernatant was harvested twice per week, and then the IgG was purified on a Protein G Sepharose column, concentrated, and dialysed in PBS. Protein was measured by ELISA using IgG standard.

Long-term persistence of ST-Ova. B6.129 F1 mice were infected with 10^3 bacteria ST-Ova intravenously, then received Cipro-HCl (1 mg/ml) in the drinking water for 2 months beginning 60 days post-infection. Following antibiotic treatment, anti-CD4 or anti-IFN-γ

antibody was administered *i.p.,* 50μg twice per week, for 2 weeks; then spleens were isolated and bacteria determined by plating dilutions of homogenized spleen on BHI agar.

Inhibition of ST-Ova growth in vivo. Ciprofloxacin hydrochloride (Sigma-Aldrich) was administered to mice in the drinking water at 1mg/ml and replaced fresh weekly.

Statistical analysis. Mean +/- standard error of the mean is displayed for all data. For time course studies, an unpaired t-test was used to compare LM-Ova vs. ST-Ova for each time point. Differences were considered statistically significant with $P<0.05$. For more than two groups, ie. Fig 13 b,c,d ANOVA was used with Tukey's test to determine P values for each pair combination.

V. RESULTS

1. ST-Ova replicates faster than LM-Ova in vitro

To evaluate the comparative replication of ST-Ova in vitro, cultures of LM-Ova and ST-Ova were grown in parallel in BHI medium. In vitro growth curves (Fig. 2) were constructed by plotting absorbance readings at 600nm with GraphPad Prism. It was determined from these growth curves that the doubling time for LM-Ova and ST-Ova were 46 min. and 23 min. respectively.

2. ST-Ova induces a chronic infection.

To evaluate the bacterial burden in the spleen during infection with LM-Ova or ST-Ova, B6.129 F1 mice were infected *i.v.* with 10^3 bacteria, then sacrificed at various time points of infection. Spleens were homogenized in RPMI, spleen cells were quantified by trypan blue exclusion, and dilutions were plated on BHI agar for enumeration of bacterial burden (cfu).

In uninfected mice, spleen cell numbers are typically around $100x10^6$. As shown in Figure 3, LM-Ova induced a very slight change in the number of spleen cells which peaked at $175x10^6$ by 7 days post-infection, and was restored to approximately $150x10^6$ shortly thereafter. On the other hand, ST infection resulted in massive splenomegaly as the cell numbers reached as high as $1000x10^6$ at 30 days post-infection. Following day 30, the spleen cell numbers declined appreciably; however, the numbers never declined by the amount seen following LM-Ova infection.

The bacterial burden of ST-Ova in the spleen at various time points of infection was compared with that of LM-Ova (Fig. 4). Spleens of LM-Ova-infected mice reached $2.2x10^4$ bacteria just one day post-infection, while the ST-Ova burden was slightly lower initially, but

Figure 2. In vitro growth curve of LM-Ova and ST-Ova. Bacterial cultures were grown in BHI medium and the absorbance measured at various time points.

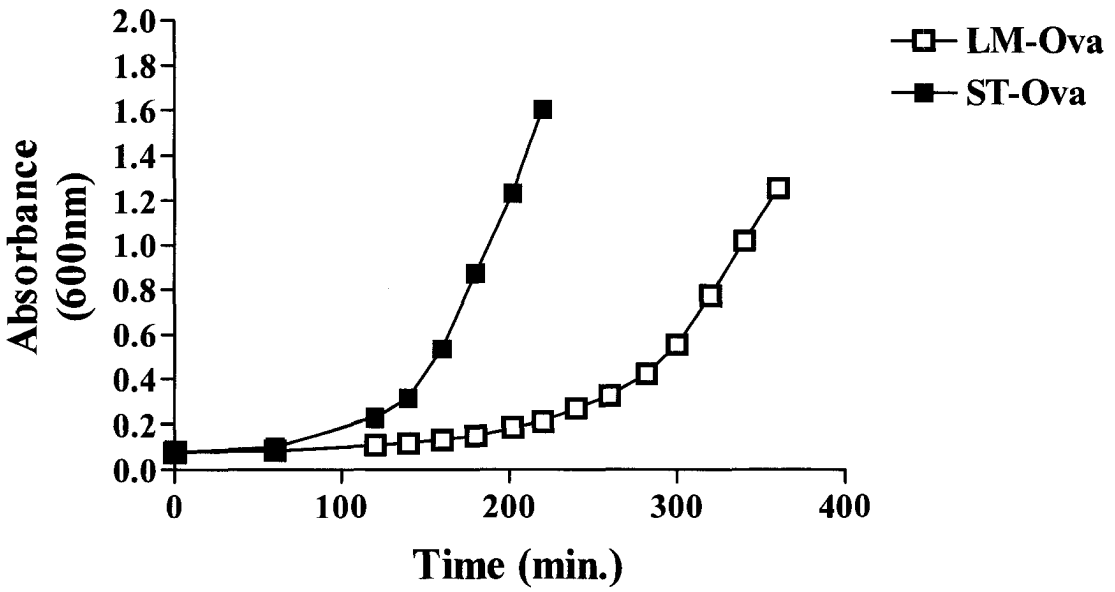


Fig. 3. Profile of splenocyte number during infection. B6.129 mice were infected with $1x10^3$ cfu LM-Ova or ST-Ova; mice were sacrificed and spleens were isolated at various time points post-infection. Spleens were homogenized in RPMI and splenocytes quantified using trypan blue exclusion. n=10 mice per group. Points represent mean +/- standard error of the mean. Asterisks indicate statistical significance ($P<0.05$).

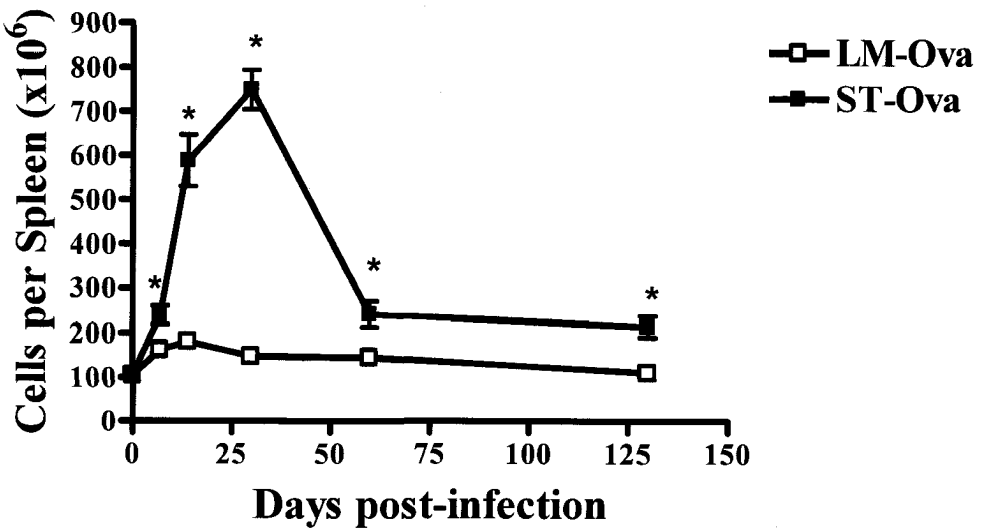
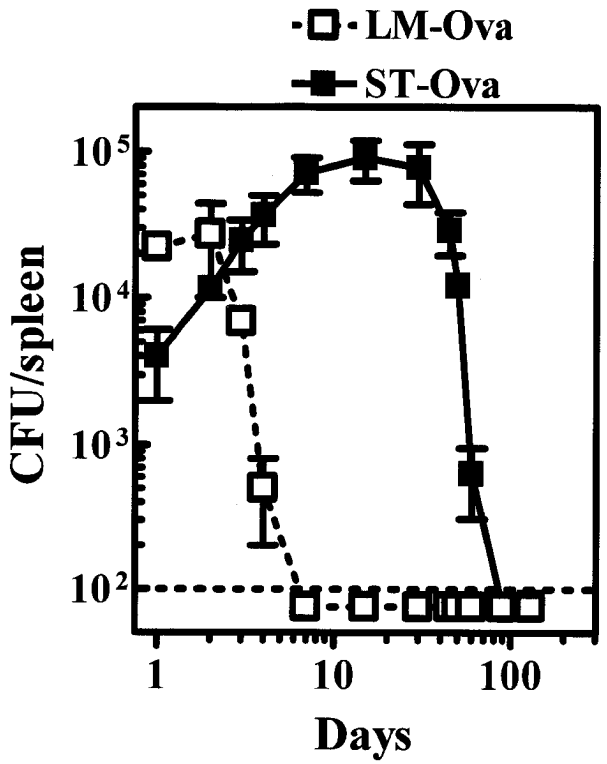


Fig. 4. Bacterial burden in the spleen. B6.129 mice were infected with 10^3 cfu LM-Ova or ST-Ova; mice were sacrificed and spleens were isolated at various time points post-infection. Spleens were homogenized in RPMI, samples diluted in 0.9% saline and plated on BHI agar, and incubated at 37°C overnight. Points represent mean +/- standard error of the mean. n=2-12 mice per group.

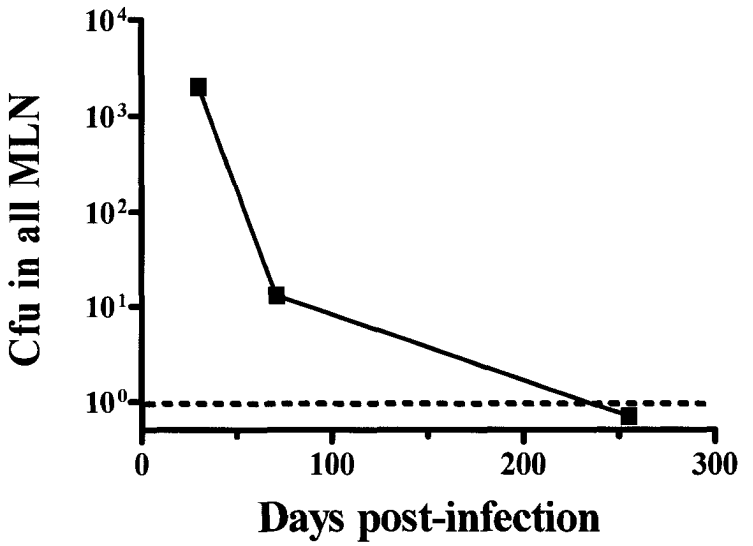


the bacterial burdens were similar by day 3 of infection, and the ST-Ova burden was higher at later time intervals than LM-Ova, by about 5-fold. Although ST-Ova reached levels as high as LM-Ova, LM-Ova bacteria were eliminated rapidly - after day 3 of infection, when the ST-Ova burden was still increasing, LM-Ova bacteria were undetectable by day 5. The ST-Ova burden remained at high levels for an extended period of time, persisting at approximately $5x10^4$ bacteria per spleen from day 7 to day 30. At day 60, ST-Ova burden had markedly decreased to less than 10^3 bacteria per spleen, and in some mice the burden was even undetectable. By day 90 the burden of ST-Ova was below the detection limit of the assay.

ST, when administered to mice orally with a high dose, has been shown to selectively persist within mesenteric lymph nodes (MLN) for prolonged periods (105). Although ST-Ova was not administered orally in this study, it was still investigated whether ST-Ova was persisting within the MLN when injected *i.v.* Mice were infected with 10^3 bacteria *i.v.* and after various time points of infection, the MLN were extracted and homogenized in RPMI. All aliquots were plated on several plates of BHI agar for enumeration of cfu, so that the bacteria in the entire organ could be accounted for. At day 30 there were approximately $2x10^3$ bacteria in all MLN, 13 bacteria at day 76, and no bacteria were detected at 255 days post-infection (Fig. 5).

However, it remains possible that bacteria may persist at low levels. Therefore it was reasoned that if mice are immunosuppressed at a later time interval after ST infection, then perhaps a subclinical ST burden may become reactivated and then replicate to detectable levels. To this end, mice were infected with ST-Ova; then, at day 60, mice were given antibiotics (Cipro-HCl) which was provided in the drinking water and changed twice per week. After two months of antibiotic treatment, CD4 T cells and IFN-γ were depleted; these

Fig. 5. Bacterial burden in the mesenteric lymph nodes. B6.129 mice were infected intravenously with 10^3 cfu ST-Ova; mice were sacrificed and MLN were isolated at various time points post-infection. MLN were homogenized in RPMI, samples diluted in 0.9% saline and plated on BHI agar, and incubated at 37°C overnight. n=1 for days 7 and 31; n=3 for day 255.



are very important in controlling intracellular bacteria (6, 7). Antibodies specific to CD4 and to IFN-γ were administered by *i.p.* injection twice per week. Two weeks after antibody injections, all mice were sacrificed, the entire spleens were homogenized and all aliquots plated, and total bacteria per spleen enumerated (Fig. 6). Although the IFN-γ-depleted mice did not show a significant difference, 50% of the CD4 T cell-depleted mice had a higher ST-Ova burden relative to the control group. Two mice from the CD4-depleted group did not show any bacteria in the entire spleen, one spleen contained 200 bacteria, and another nearly 1000 bacteria. These results indicate that while ST remains undetectable in the long-term, it persists at a very low level and can be reactivated when the immune system is compromised. These results reinforce the notion that ST induces a chronic infection in the murine host.

3. Ag-specific CD8 T cell expansion is delayed and reduced during ST-Ova infection

a. Endogenous Ag-specific response

To evaluate the expansion of the $Ova_{257-264}$-specific CD8 T cells in response to ST-Ova and LM-Ova infection, B6.129 F1 mice were infected with 10^3 LM-Ova or ST-Ova. After various time points of infection, mice were sacrificed, and CD8 T cells were purified from homogenized spleens. These purified CD8 T cells were then used for ELISPOT assay to determine the numbers of IFN-γ-producing CD8 T cells during infection.

As shown in Figure 7a, there was a great difference in the numbers of IFN-γ-producing Ova-specific CD8 T cells, especially early after infection, between LM-Ova and ST-Ova-infected mice. LM-Ova infection resulted in the development of a high proportion of IFN-γ-producing Ova-specific CD8 T cells that reached as high as 10,000 per million CD8 T cells, that is, 1%, of the total CD8 population at day 7 and then decreasing by day 14 but persisting around 2000 per million CD8 T cells even at day 60. By contrast, ST-Ova

Fig. 6. Long-term persistence of ST-Ova. Mice were infected with 10^3 cfu ST-Ova, then received Cipro-HCl in the drinking water for 2 months beginning 60 days post-infection. Following antibiotic treatment, anti-CD4 or anti-IFN-γ antibody was administered *i.p.*, 50μg twice per week, for 2 weeks; then spleens were isolated and cfu determined by plating dilutions of homogenized spleen on BHI agar. n=4 mice per group. Horizontal lines indicate mean. P value (CD4 vs PBS) = 0.25. P value (IFN-γ vs PBS) = 0.35.

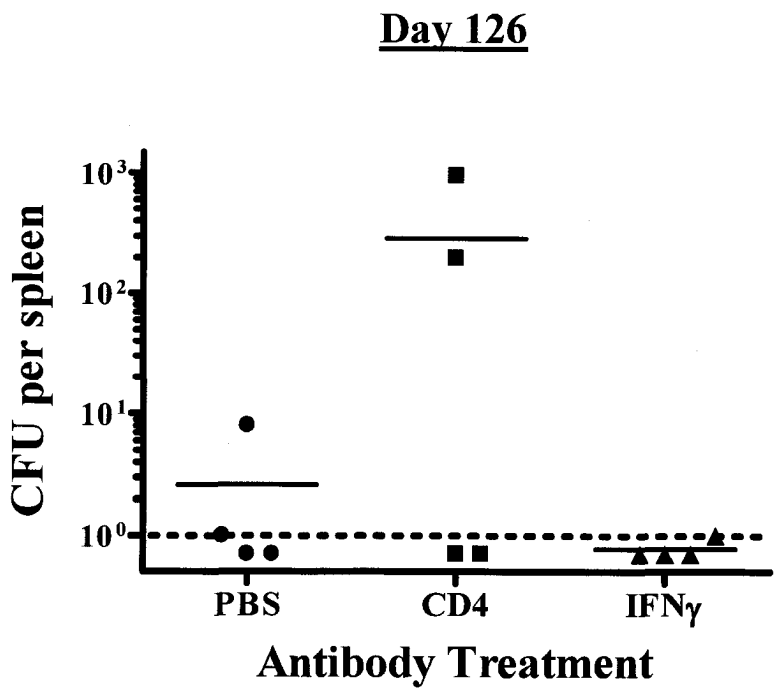
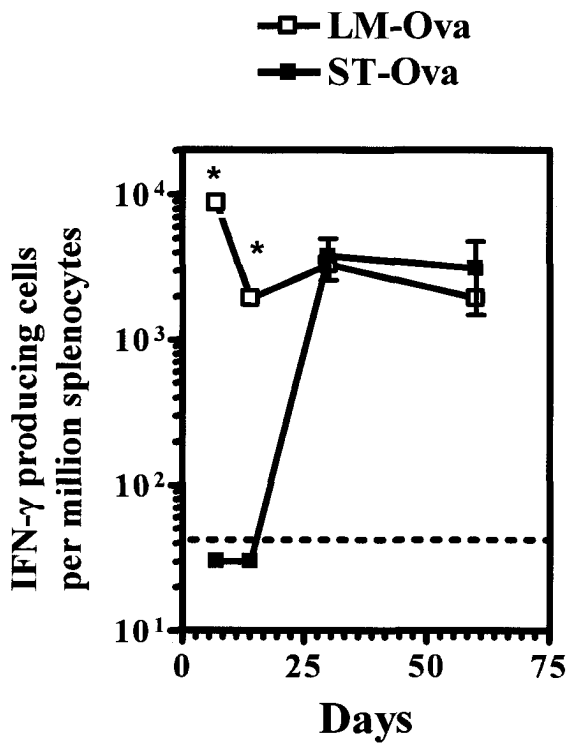
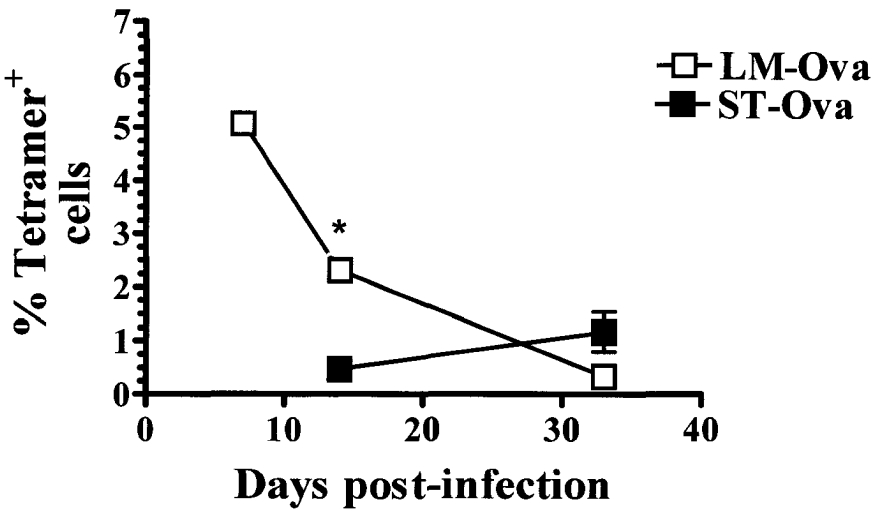


Fig. 7. Ability of Ova-specific CD8 T cells to produce IFN-γ during infection as determined by ELISPOT. CD8 T cells were purified from spleen homogenate, then incubated overnight with IL-2 and in the presence or absence of Ova peptide, using an IP plate that had been coated with anti-IFN-γ Ab (R46A2). After a 48-hr incubation, the plate was washed, then incubated with biotinylated anti-IFN-γ Ab (XMG), followed by SA-HRP and developed using AEC substrate. (a) ELISPOT results (n=4) (b) Endogenous %Tet+ of CD8 T cells from blood of mice infected with 10^3 LM-Ova or ST-Ova (n=3). Points represent mean +/- standard error of the mean. Asterisk indicates statistical significance ($P<0.05$).

(a)



(b)



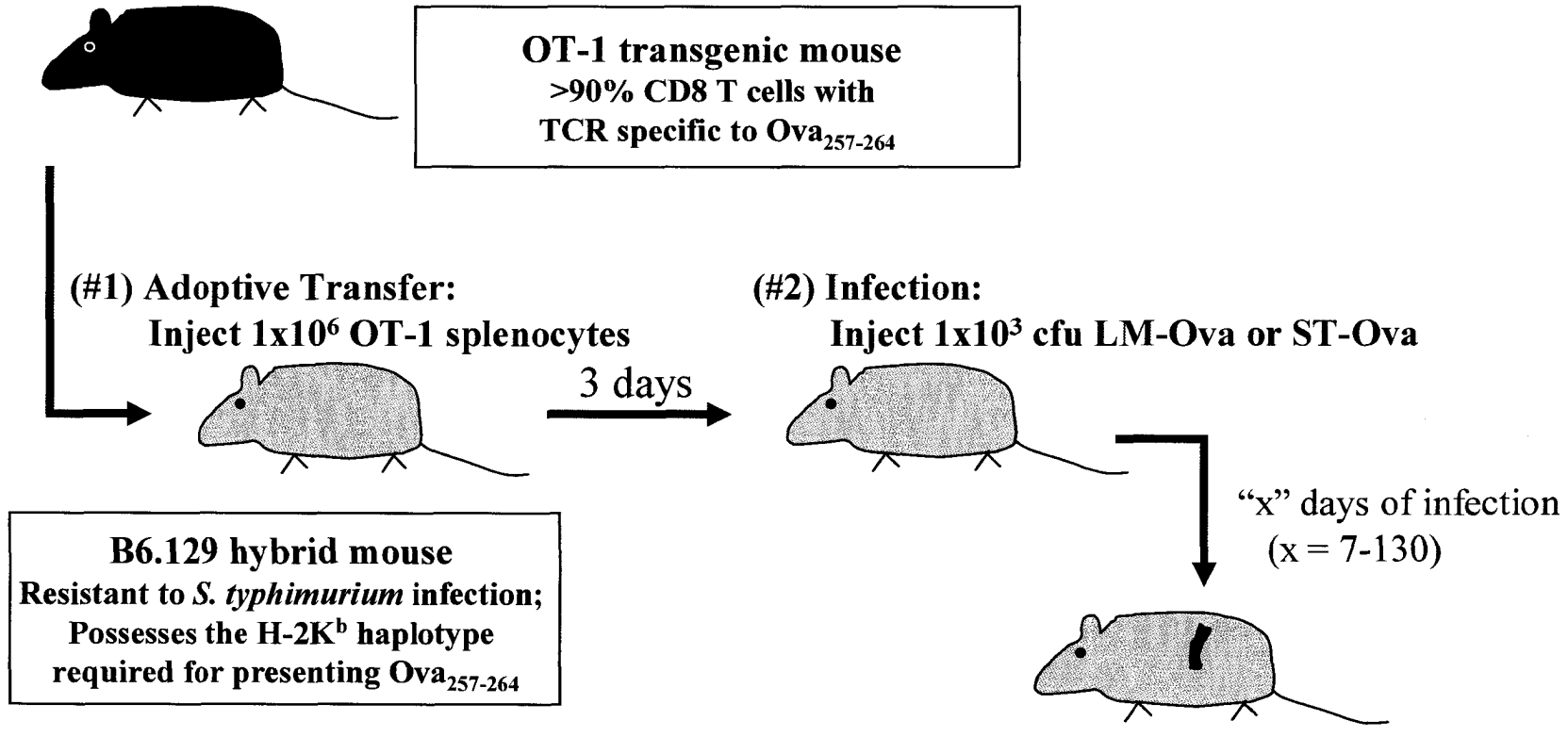
infection did not induce detectable numbers of IFN-γ-producing Ova-specific CD8 T cells at days 7 or 14. However, at days 30 and 60 the numbers were just as high as those induced in the LM-Ova-infected mice. This indicates that the Ova-specific CD8 T cell response is delayed during ST-Ova infection, compared with the response to LM-Ova.

Although there is a large difference in the proportion of IFN-γ-producing Ova-specific CD8 T cells, it is quite possible that there are in fact Ova-specific CD8 T cells that are simply not producing IFN-γ. In order to assess the percentage of Ova-specific CD8 T cells within the total CD8 T cell population, the use of H-2K^b Ova Tetramer was applied. The use of the tetramer technology allows the detection of T cells that are specific to a particular Ag (199). Using a fluorescent labeled MHC class I H-2K^b Tetramer complexed with the CD8 epitope $Ova_{257-264}$, this allows the detection by flow cytometry of the Ag-specific CD8 T cells that respond to infection; that is, the "Tetramer-positive" (Tet$^+$) population. Mice were infected with 10^3 ST-Ova or LM-Ova and at various time points of infection peripheral blood lymphocytes were stained with antibody specific for CD8, in addition to H-2K^b Ova tetramer. Figure 7b shows that at the peak of LM-Ova infection, 5% of CD8 T cells are Ova-specific and this number then declined to very low levels. The percentage of Ova-specific CD8 T cells induced during ST-Ova infection barely reached 1% at day 30. Thus, the endogenous Ova-specific CD8 T cell population during LM-Ova and ST-Ova infection is very low and is thus difficult to detect accurately by flow cytometry.

b. Amplification of the Ag-specific response

As can be seen by the results in Figure 7b, it is difficult to detect the Ova-specific CD8 T cells during LM-Ova and ST-Ova infection directly by Tet$^+$ analysis in the long-term as the relative percentage of Tet$^+$ cells falls near the background levels. Thus we proceeded with the adoptive transfer model as outlined in Figure 8. In this model, OT-1 transgenic

Figure 8. Adoptive transfer and infection model. First, splenocytes from the OT-1 transgenic mouse are transferred by *i.v.* injection to the B6.129 mouse. Next, 3 days later, the B6.129 mouse is infected with LM-Ova or with ST-Ova. Then, after a determined duration of infection, the spleen or blood is isolated and the $Ova_{257-264}$-specific CD8 T cell population is analyzed.



mice are used, since they possess greater than 90% of their CD8 T cells bearing the TCR specific to the $Ova_{257-264}$ peptide (197). OT-1 splenocytes are transferred into naïve B6.129 F1 mice, which are challenged a few days later with either LM-Ova or ST-Ova. This presence of naïve OT-1 transgenic cells in the host results in amplification of the response following infection, so that the responses can be measured in the long-term and the phenotype of Ag-specific CD8 T cells can be discerned.

For staining of the Tet^+ CD8 T cell population within splenocytes or peripheral blood lymphocytes, antibody specific to CD8 was used as well as $H2K^b$ Ova tetramer. Following flow cytometry, the population was determined by gating on those events that were double-positive for tetramer and CD8 as shown in Figure 9a. In this way, the Ova-specific CD8 T cells are tracked directly using $H\text{-}2K^b$ Ova tetramer.

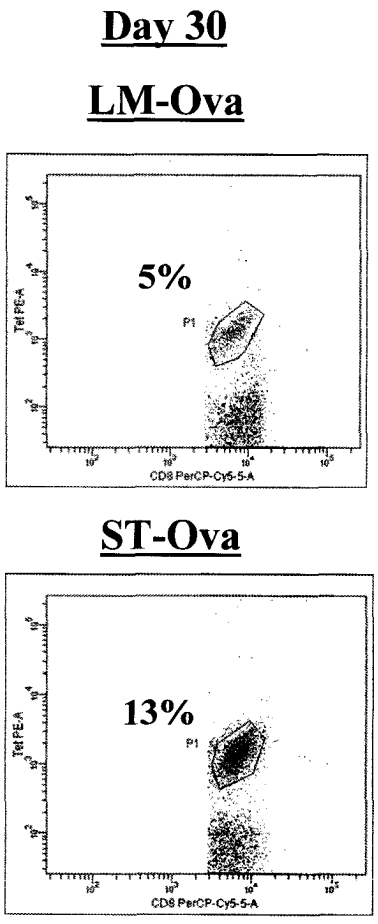
In mice infected with LM-Ova, the number of Tet^+ CD8 T cells in the spleen (Fig. 9b) increased rapidly from $< 1x10^4$ at day 1, to $5x10^5$ at day 5, and reached $4x10^6$ per spleen at day 7. Similarly, in peripheral blood (Fig. 9c), the $\%Tet^+$ of CD8 T cells in the blood of LM-Ova-infected mice peaked around 40% at day 7. The $\%Tet^+$ decreased 10-fold by day 14 of LM-Ova infection.

By comparison, the number of Tet^+ CD8 T cells in the spleen of mice infected with ST-Ova increased slowly, peaking only around day 14-21, and reaching only $5x10^5$ per spleen at this point. Interestingly, this peak of ST-Ova-induced Tet^+ CD8 T cells remained relatively constant until day 45, decreasing slightly at day 60. In the peripheral blood the peak response (10% Tet^+) was delayed until day 21, but was prolonged in a similar manner as that of the spleen, as the $\%Tet^+$ cells remained constant until after day 45.

The proliferation of Tet^+ CD8 T cells was evaluated at specific time points of infection; that is, it was determined how many Tet^+ CD8 T cells are in the process of cycling

Fig. 9. Expansion of Ag-specific CD8 T cells during infection. At various time points of infection with either LM-Ova or ST-Ova, spleens or blood were isolated from B6.129 mice, lymphocytes stained with Ova Tetramer and anti-CD8 Ab, and analysed by flow cytometry. (a) The Tetramer-positive population was determined by gating on Tet+CD8+ T cells. Profile of Tet+ population in (b) spleen and (c) blood. n=2-3 mice per group. Points represent mean +/- standard error of the mean. Asterisk indicates statistical significance ($P<0.05$).

(a)



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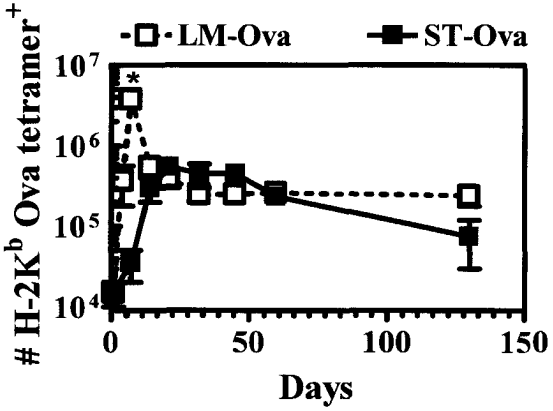

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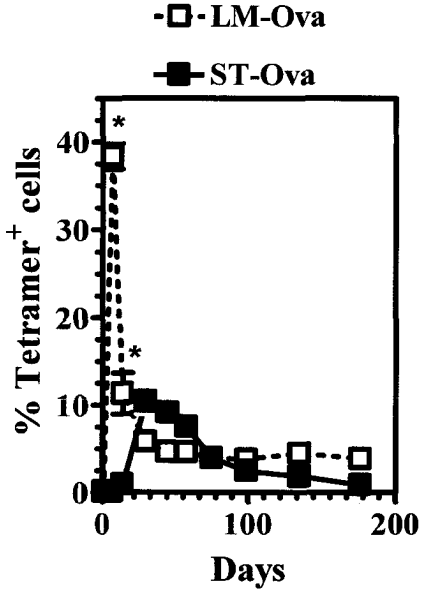


at a particular time point. Mice were given BrdU in their drinking water for 3 days prior to harvest; to determine BrdU incorporation, splenocytes were stained intracellularly with anti-BrdU antibody and analysed by flow cytometry. The $BrdU^+$ population was gated on the $CD8^+Tet^+$ population (Fig. 10a), and the profile of $\%BrdU^+$ within the population of Tet^+CD8 T cells was plotted through the course of infection (Fig. 10b). In LM-Ova-infected mice, the highest percentage of proliferating cells was shown at day 7, with nearly 100% of the Tet^+ CD8 T cells found to have incorporated BrdU; and, by day 14, the BrdU incorporation declined back to a level of homeostatic proliferation (~10%). In ST-Ova-infected mice, proliferation was high early on, with approximately 75% of the Tet^+ CD8 T cells positive for BrdU incorporation, but proliferation was much lower by day 30 with only 35% of the Tet^+ CD8 T cells proliferating, and down to basal levels by day 60. Thus, LM-Ova shows a high proportion of the population proliferating early on and then decreasing very quickly after infection has been cleared. Ova-specific CD8 T cells induced by ST-Ova also proliferated early; however, their proliferation continued to decrease even at day 45, when the bacterial burden was still at high levels.

4. The development of an Ag-specific effector population is gradual and sustained during ST-Ova infection

Besides the proliferation of the Tet^+ CD8 T cells, evaluation of their activation status also provides information relating to their function. To evaluate the phenotype of Ag-specific CD8 T cells, splenocytes and peripheral blood from mice that had received adoptive transfer of OT-1 transgenic cells and LM-Ova or ST-Ova challenge were analyzed at different time points of infection for CD44 and CD62L expression. The population was first gated on $CD8^+Tet^+$, then analyzed by two distinct phenotypes (Fig. 11a): while $CD44^+$ shows all of the Ova-specific CD8 T cells are Ag experienced, $CD62L^{low}$ indicates the "effector"

Fig. 10. Proliferation of Ova-specific CD8 T cells determined by BrdU incorporation. Mice received 1 mg/ml BrdU in the drinking water 3 days prior to sacrifice. Spleens were isolated and splenocytes stained intracellularly with anti-BrdU Ab, as well as with Ova Tetramer and anti-CD8 Ab. (a) $BrdU^+$ cells were determined by gating on Tet^+BrdU^+ population as shown. (b) Profile of $BrdU^+$ incorporation throughout infection. n=2 mice per group. Points represent mean +/- standard error of the mean. Asterisk indicates statistical significance ($P<0.05$).

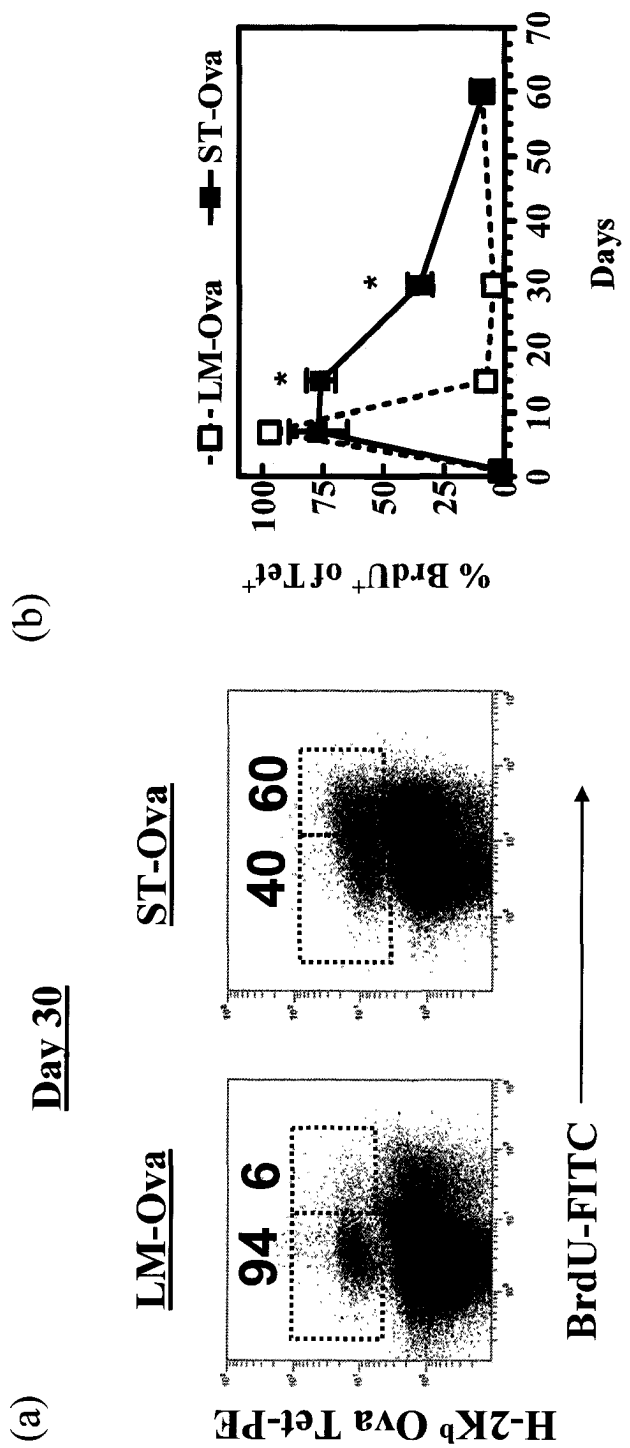
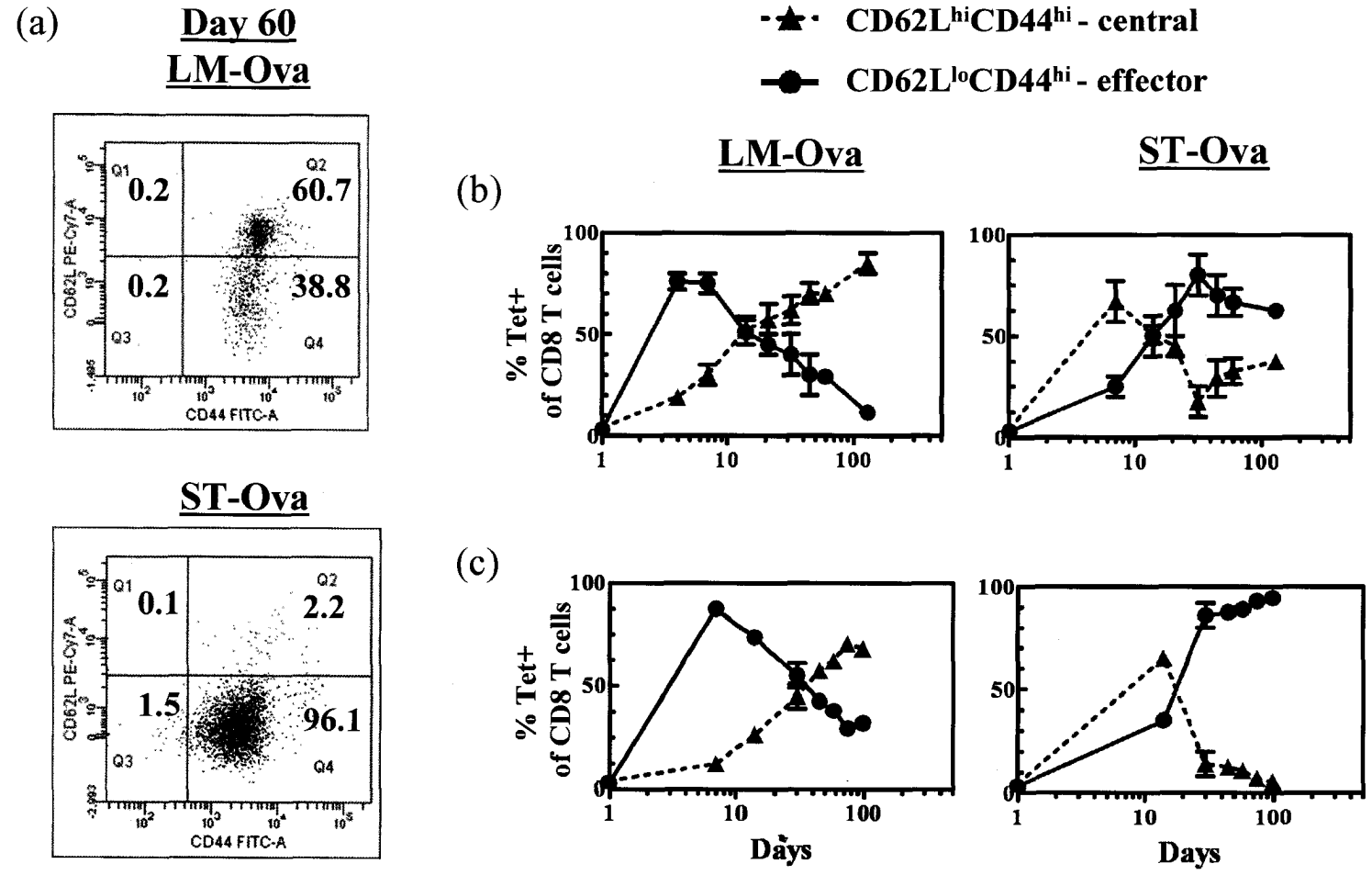


Fig. 11. Differentiation of Ag-specific CD8 T cells to an effector phenotype. Mice were injected with OT-1 cells and challenged with 10^3 cfu LM-Ova or ST-Ova; spleens or blood were isolated at various time points of infection. Lymphocytes were stained with Ova tetramer, anti-CD8 Ab, anti-CD44 Ab, and anti-CD62L Ab, and analysed by flow cytometry. Effector and central populations were gated based on CD44 and CD62L expression as shown (a). $CD62L^{low}$ indicates the effector population while $CD62L^{hi}$ marks the central CD8 T population. (b) Spleen; (c) Blood. n=2 mice per group. Points represent mean +/- standard error of the mean.

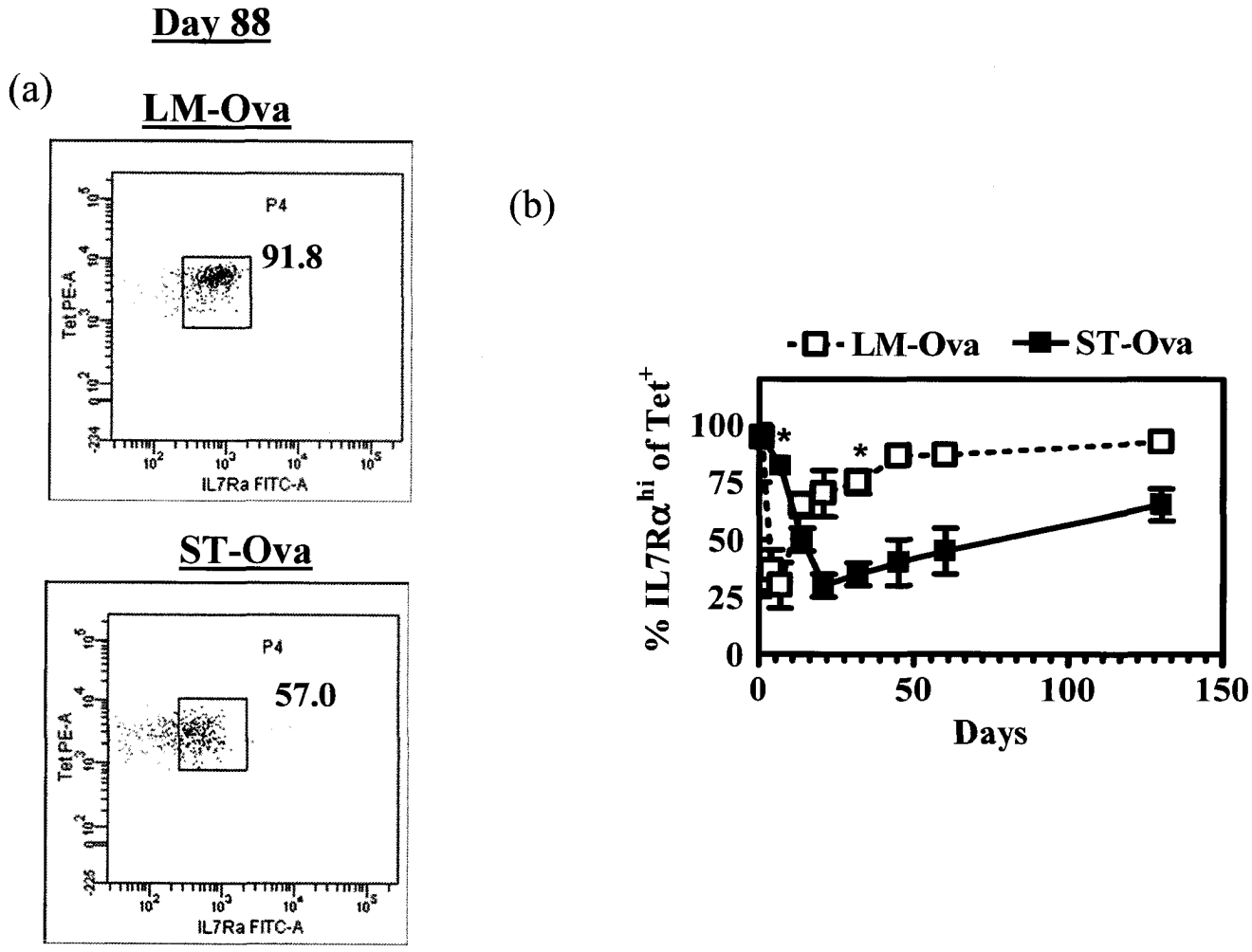


subset and CD62L^{hi} denotes the "central" subset. The profiles of effector and central subsets were plotted separately for spleen (Fig. 11b) and blood (Fig. 11c), comparing ST-Ova with LM-Ova infection. In LM-Ova-infected mice, the Tet^{+} CD8 T cell population consisted of approximately 80% effectors by day 7, decreasing by day 14 and continuing to decline over time. However, in ST-Ova-infected mice, the effector population increased gradually, reaching only 20% effectors at day 7, and gradually increasing to reach 80% by day 30. Interestingly, whereas the LM-Ova-induced effectors had decreased after day 7, the percentage of effectors induced by ST-Ova persisted at a very high level, even as late as 100 days post-infection.

5. The development of Ag-specific memory CD8 T cells is delayed and gradual in ST-Ova infection

The differentiation of CD8 T cells into memory cells has been characterized by expression of the proliferative cytokine receptor IL7Rα. It has been shown that IL7Rα expression is high in naïve CD8 T cells, decreases at the peak of CD8 T cell activation, and then the subset of T cells that are still IL-7Rα^{hi} at this point are those that continue on and become memory cells (53). As it was interesting to note that the Tet^{+}CD8 T cell population remains in an effector state during ST-Ova infection, we examined whether there is a development of a memory phenotype within this population. Mice that had received adoptive transfer of OT-1 transgenic cells were analyzed at various time points of infection for IL7Rα expression by Ag-specific CD8 T cells. The population was first gated on CD8^{+}, and then gated on Tet^{+} cells (Fig. 12a). The profile of the IL7Rα^{hi} subset within the Tet^{+}CD8 population was plotted throughout the course of infection with LM-Ova or ST-Ova (Fig. 12b). It is evident in Figure 12b that the %IL-7Rα^{hi} subset within the Tet^{+}CD8 T cell population decreases rapidly to 25% by day 7 following LM-Ova infection. At day 14, 75%

Fig. 12. Differentiation of Ova-specific CD8 T cells to a memory phenotype. Mice were infected with 103 cfu LM-Ova or ST-Ova, and blood was isolated at various time points of infection. Lymphocytes were stained with Ova Tetramer, anti-CD8 Ab and anti-IL7Rα Ab, and analyzed by flow cytometry. (a) IL7Rαhi population was gated based on Tetramer positive population as shown. (b) Profile of IL7Rαhi population during the course of infection. n=2 mice per group. Points represent mean +/- standard error of the mean. Asterisk indicates statistical significance ($P<0.05$).



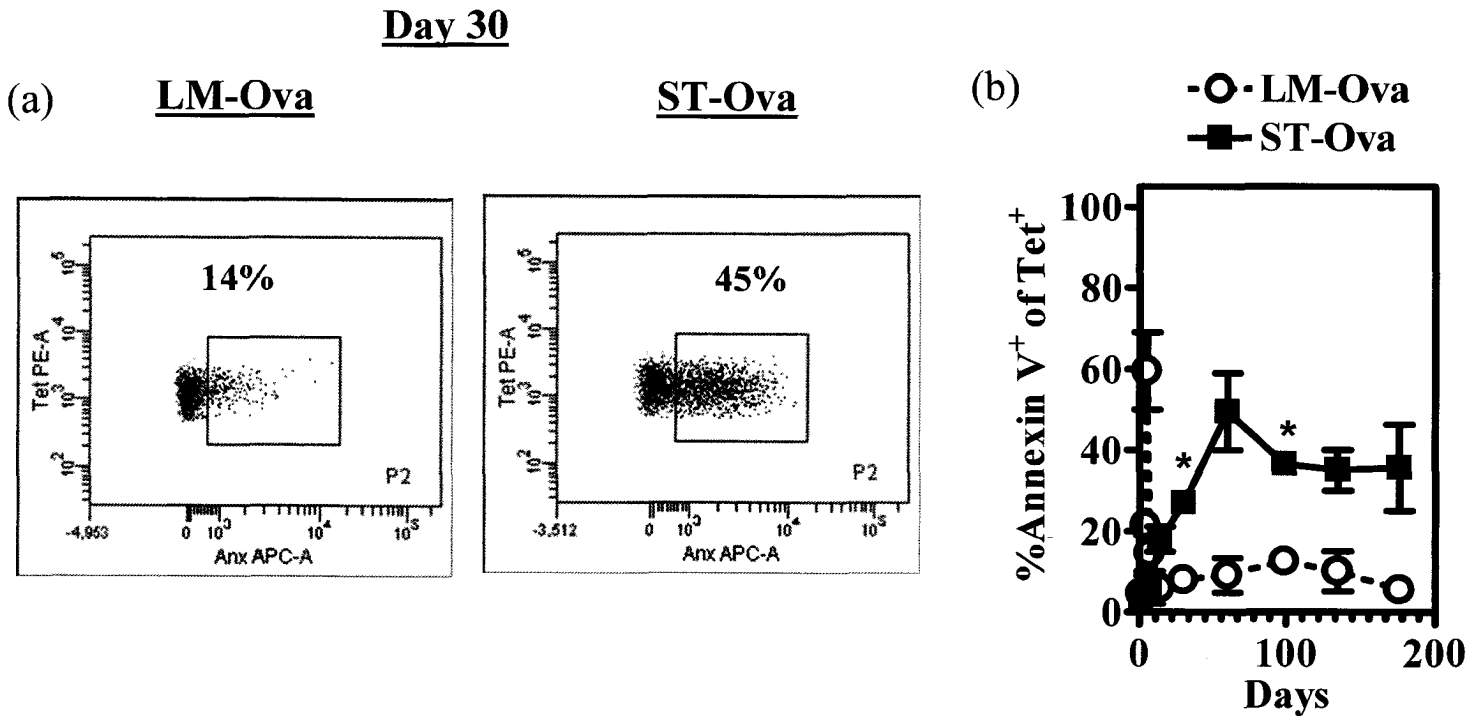
of cells are IL-7Rα^{hi} and the number increases to 90% by day 45. By contrast, following ST-Ova infection, the IL7Rα^{hi} subset takes 3 weeks to decline to 30%, and then begins to gradually increase. It is important to note here that the numbers of IL-7Rα^{hi} cells begin to increase gradually even when the bacterial burden is high, indicating that the programming for memory development occurs even when the pathogen burden is not completely eliminated.

6. Prolonged apoptosis of Ag-specific CD8 T cells during ST-Ova infection

To evaluate the extent of apoptosis of the Tet$^+$CD8 T cell population, the Annexin V binding assay was applied. Annexin V is a protein that specifically binds phosphatidylserine (PS), which is the only phospholipid that, in a healthy cell, is located solely in the inner leaflet of the phospholipid bilayer in the plasma membrane. During apoptosis, PS is flipped to the outer leaflet; this serves as a flag on the dying cell, in order to be scavenged by dendritic cells (203). Hence, by incubating a population of cells with fluorescently labeled Annexin V, those cells that are apoptotic will be visualized as Anx$^+$ by flow cytometry.

Mice that had received adoptive transfer of OT-1 transgenic cells were analyzed at various time points of infection for Annexin V binding. The population was first gated on CD8$^+$, and then gated on Tet$^+$ (Fig. 13a). The profile of the Anx$^+$ subset was plotted throughout the course of infection with LM-Ova or ST-Ova (Fig. 13b). Following LM-Ova infection, the Tet$^+$ population displayed increased commitment to apoptosis at 4 days post-infection with 20% Anx$^+$, and as high as 60% Anx$^+$ only one day later. However, by day 7, the Anx$^+$ subset was reduced to 15% and then remained hovering around 5-10% at later time points. By contrast, the ST-Ova-induced Tet$^+$ population demonstrated a delayed, prolonged and gradual commitment to apoptosis; the Anx$^+$ subset increased to 20% only at day 14, continued to increase to 50% at day 60, and remained relatively high around 30%, even 6

Fig. 13. Contraction of Ova-specific CD8 T cells following infection. Mice were infected with 103 cfu LM-Ova or ST-Ova, and blood was isolated at various time points of infection. Staining was performed with Ova Tetramer and anti-CD8 Ab, followed by Annexin V in Annexin Binding Buffer, then analysed by flow cytometry. (a) Anx+ CD8 T cells were gated on the Tet+ population as shown. (b) Profile of Anx+ Tet+ CD8 T cells throughout infection. n=2-3 mice per group. Points represent mean +/- standard error of the mean. Asterisk indicates statistical significance ($P<0.05$).



months post-infection. Thus, while there is a high proportion of LM-Ova-induced Tet^+CD8 T cells committed to apoptosis over a short period of time, ST-Ova-induced Tet^+CD8 T cells show a moderate proportion undergoing apoptosis over a prolonged period of time.

7. Accelerated removal of ST-Ova does not influence the development of memory

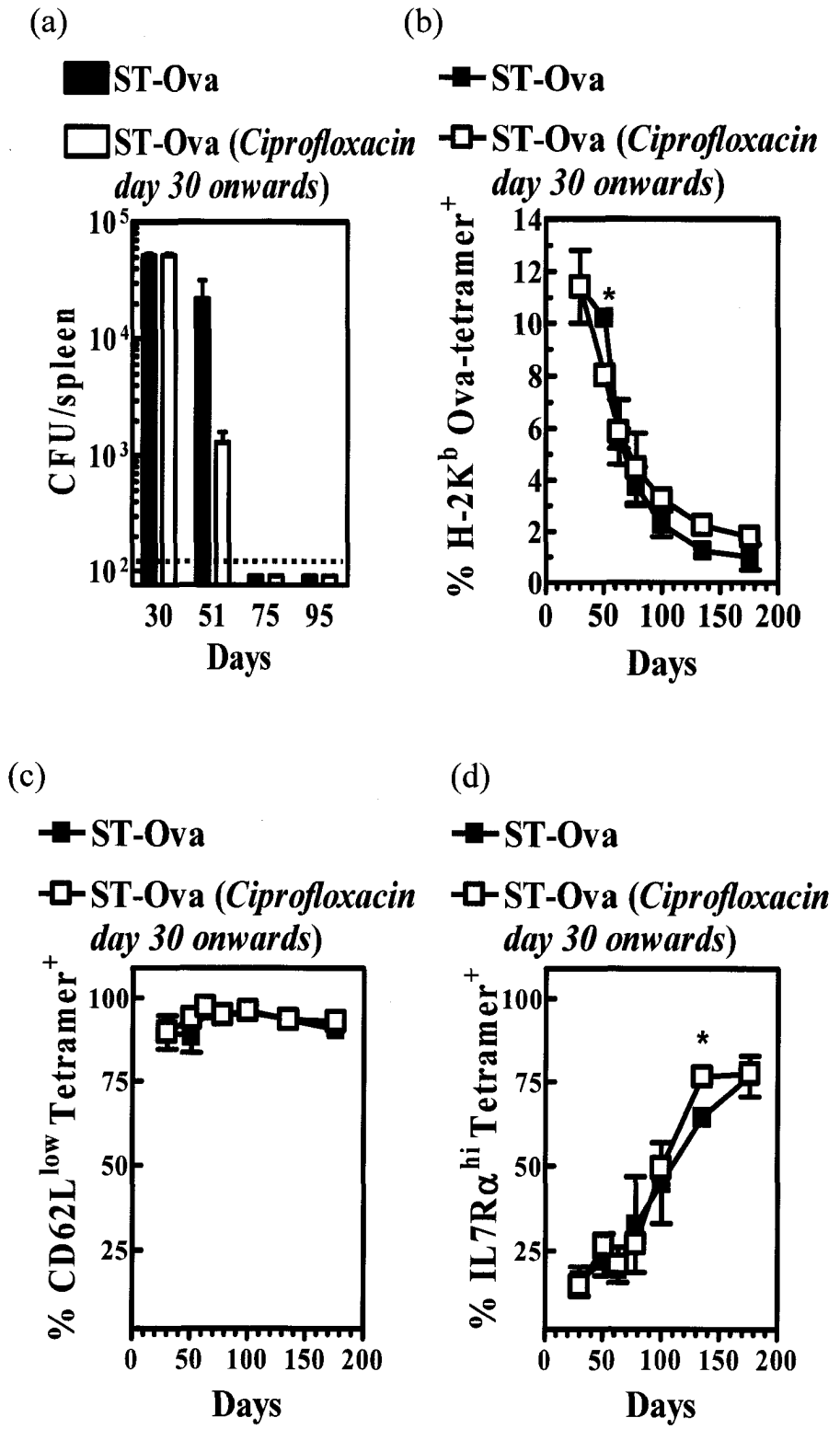
Due to the data showing the persistence of an effector phenotype in the Tet^+CD8 T cell population, and the persistence of a high burden of ST-Ova in the spleen, it was explored whether this persistent burden affects the rate of contraction of the Tet^+CD8 T cell population. B6.129 F1 mice were infected with ST-Ova, and then given Cipro-HCl in their drinking water beginning 30 days post-infection. The bacteria were determined at various time points of infection, and the $\%Tet^+$ of CD8 T cells, $\%CD62L^{lo}$ of Tet^+ cells, and $\%IL7R\alpha^{hi}$ of Tet^+ cells were determined for one group that received antibiotic, and for a control group that did not receive antibiotic.

Figure 14a shows that after 3 weeks of antibiotic treatment, the ST-Ova burden was decreased by 10-fold relative to the group of mice that had not received antibiotic. Bacteria were undetectable at subsequent time points. Figure 14b demonstrates that the $\%Tet^+$ did not change significantly due to the decrease in the ST-Ova burden. Similarly, the $\%CD62L^{lo}$ and $\%IL7R\alpha^{hi}$ Tet^+ populations were not significantly altered (Fig. 14c, d). Taken together, these data show that decreasing the ST-Ova burden from day 30 onwards does not influence the development of the Tet^+CD8 T cell population, and that this population develops to become a persistent effector memory population.

8. CD8 T cell priming during ST-Ova infection depends on the presence of CD4 T cells

CD4 T cell help, in some infection models, is required for the survival, function, or long-term development of the Ag-specific CD8 T cells. For example, infection with Mtb in

Fig. 14. Decreasing the bacterial burden earlier does not increase the rate of contraction of Ova-specific CD8 T cells. Mice were infected with 103 cfu LM-Ova or ST-Ova, and blood (for staining) or spleens (for cfu) were isolated at various time points of infection. Mice were given Cipro-HCl (1mg/ml) in the drinking water beginning 30 days after infection. Staining was performed with Ova Tetramer and anti-CD8, -CD62L, and -IL7Rα antibodies, then analysed by flow cytometry. (a) cfu, (b) %Tet+, (c) %CD62Llow, (d) %IL7Rαhi. n=2-3 mice per group. Points represent mean +/- standard error of the mean. Asterisk indicates statistical significance ($P<0.05$).



the absence of CD4 T cells, results in CD8 T cells that yield decreased CTL activity (204). The primary CD8 T cell response to LM infection is competent in the absence of CD4 T cells, however the function of memory CD8 T cells is impaired (63). In light of this, we explored whether the priming of CD8 T cells during ST-Ova infection depends on the presence of CD4 T cells.

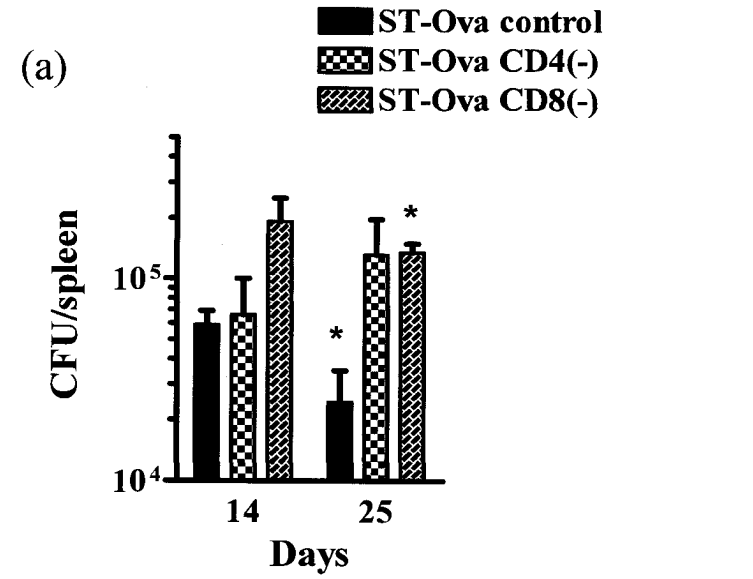
Mice received *i.p.* injection of anti-CD4 Ab, 50 μg twice per week, beginning 2 weeks prior to infection, and continuing throughout the course of infection. The %Tet^+ of CD8 T cells was measured by flow cytometry. In one experiment, cfu per spleen were enumerated and found to significantly increase relative to the control group (Fig. 15a). In a separate experiment, at day 20, the spleen cell numbers were determined and the data indicate a large decrease in the spleen cell number in the CD4- and the CD8-depleted mice, relative to controls (Fig. 15b). In CD4-depleted mice, there was a massive decline in the numbers of Ova-specific CD8 T cells (Figs. 15c, d). On the other hand, CD4 T cells did not appear to play a key role in priming CD8 T cells during LM-Ova infection (Fig. 15c). Overall, this data shows that while CD4 T cells are not important for the priming of the Tet^+ population in LM-Ova infection, they are critical for the priming of the CD8 Tet^+ population in ST-Ova infection.

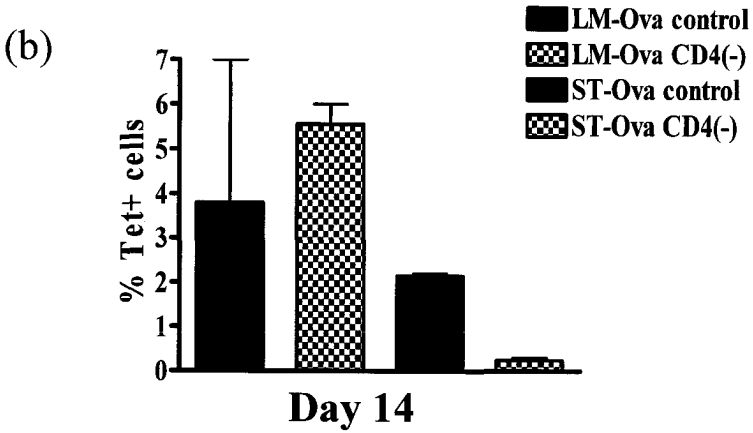
9. Ag-specific CD8 T cells induced by ST-Ova infection are functional

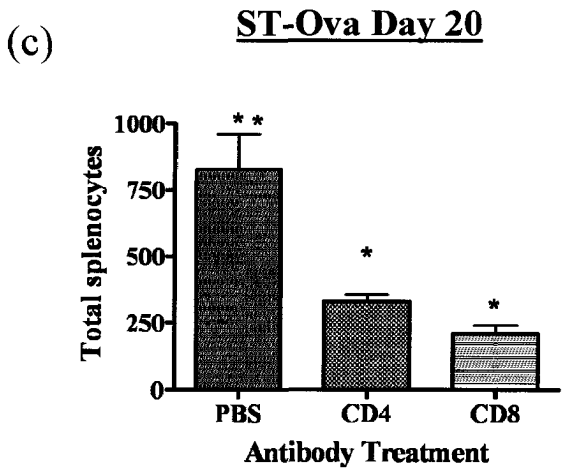
To evaluate the functionality of the Ova-specific CD8 T cells that are induced during ST-Ova vs. LM-Ova infection, two different approaches were employed: intracellular staining for detection of IFN-γ production by flow cytometry, and in vivo CTL assay for measurement of the killing of specific targets by Ova-specific T cells.

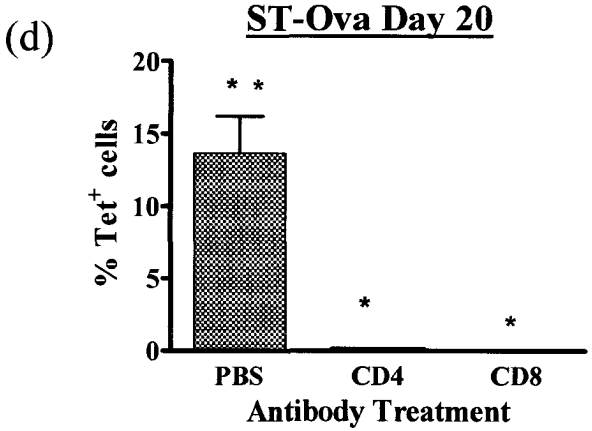
Intracellular staining was performed on splenocytes from mice that had received adoptive transfer of OT-1 cells, then infected with LM-Ova or ST-Ova. The IFN-γ^+

Fig. 15. Dependence of Ova-specific CD8 T cells on CD4 T cells for priming. Mice received injections *i.p.* with 50 μg anti-CD4 Ab or anti-CD8 Ab, twice per week, beginning 2 weeks prior to infection with 103 cfu LM-Ova or ST-Ova. At various time points of infection, spleens or blood were isolated and processed for cfu or for staining with Ova Tetramer and anti-CD8 Ab. (a, b and c) n=2 mice per group; (d) n=5 mice per group. Bars indicate mean +/- standard error of the mean. Asterisk indicates statistical significance ($P<0.05$).









population was first gated on $CD8^+Tet^+$ as shown (Fig. 16a), and the profile of the %IFN-γ^+ population was plotted over the course of infection (Fig. 16b). The flow cytometry data show that the ST-Ova-induced Tet^+ CD8 T cells are fully capable of producing IFN-γ when restimulated with the $Ova_{257-264}$ peptide. Similarly, when the frequency of Ova-specific CD8 T cells was evaluated by ELISPOT assay as previously mentioned (Fig. 7), both ST-Ova as well as LM-Ova induced Ova-specific IFN-γ-secreting CD8 T cells.

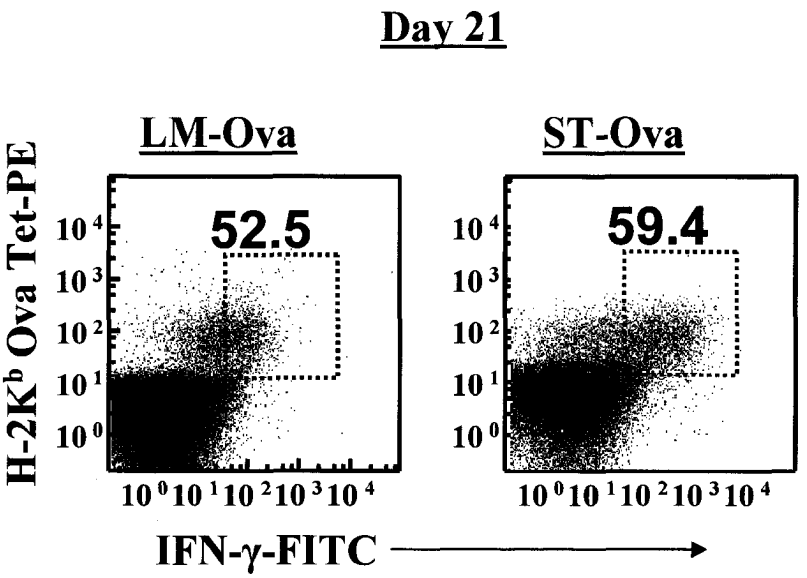
The in vivo CTL assay was used to measure the Ag-specific cytolytic ability of Ova-specific CD8 T cells induced by LM-Ova versus ST-Ova. The peptide-pulsed and -non-pulsed populations of naïve splenocytes were gated as shown in Figure 17a, and the profile of % specific lysis during the course of infection is shown in Figure 17b. In LM-Ova-infected mice, killing of peptide-pulsed target cells was extremely high at day 7 and lower at day 14 onwards. By contrast, in ST-Ova-infected mice, specific killing was undetectable at days 7 and 14, but was quite high at day 30 and day 60, then declining significantly at day 120. Thus, two of the most important functions of CD8 T cells, expression of IFN-γ and cytolytic killing of specific targets in vivo, are maintained appropriately in CD8 T cells induced during virulent ST-Ova infection.

10. Ag-specific memory CD8 T cells induced by ST-Ova infection are capable of responding to rechallenge

In light of the results indicating the development of a persistent effector memory population of CD8 Tet^+ T cells following ST-Ova infection, it was investigated whether these memory CD8 Tet^+ T cells are capable of responding to rechallenge. B6.129 F1 mice were infected with $1x10^3$ LM-Ova or ST-Ova; then, after 4 months, CD8 T cells were purified from these mice and injected into naïve recipient mice. These recipient mice were

Fig. 16. Ability of Ova-specific CD8 T cells to produce IFN-γ during infection as determined by intracellular staining and flow cytometry. Mice were infected with 10^3 cfu LM-Ova or ST-Ova, and spleens were isolated at various time points of infection. Briefly, splenocytes were stimulated with $Ova_{257-264}$ peptide for 1 hr., then permeabilized and stained intracellularly with anti-IFN-γ Ab as well as with Ova Tetramer and anti-CD8 Ab. (a) IFN-γ+ CD8 T cells were gated based on Tet+ population as shown. (b) Profile of IFN-γ+ population during the course of infection. n=2 mice per group. Points represent mean +/- standard error of the mean.

(a)



(b)

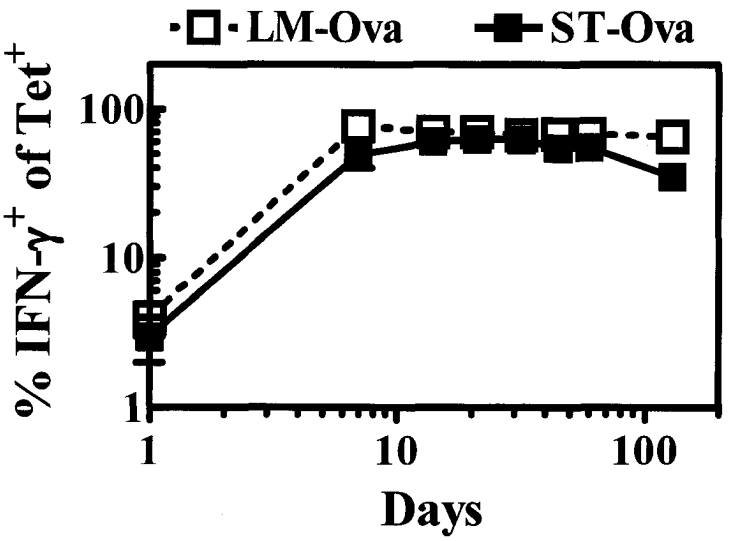
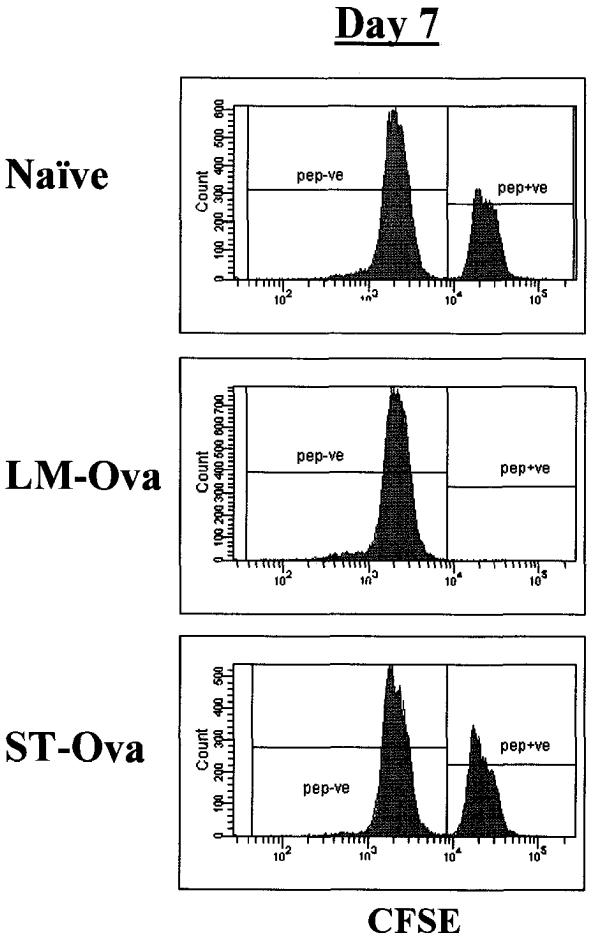
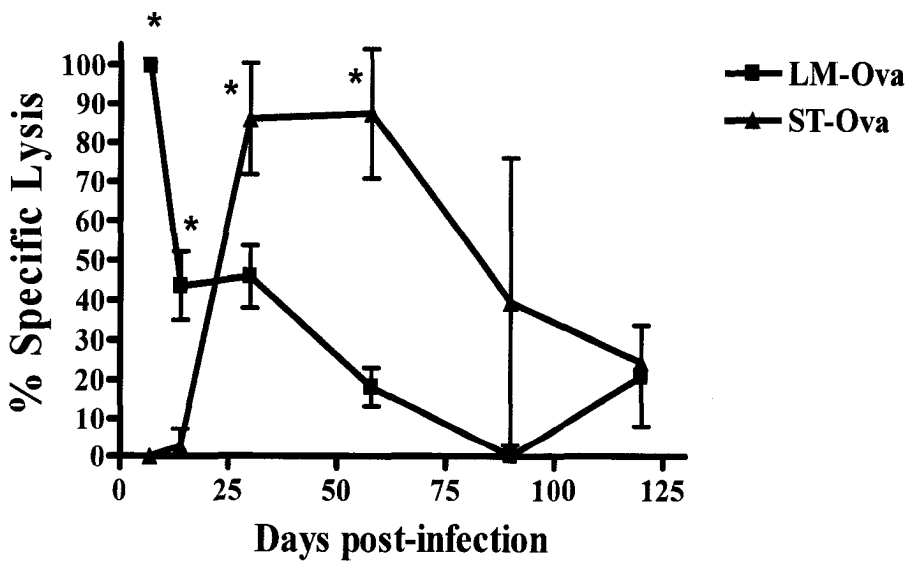


Fig. 17. Specific killing of targets in vivo by Ova-specific CD8 T cells during infection. Mice were infected with 10^3 cfu LM-Ova or ST-Ova. At various time points of infection, splenocytes from naïve mice were stained with PKH26 and CFSE, where the unpulsed population received one-tenth the concentration of CFSE compared to the $Ova_{257-264}$ peptide pulsed population. 10 x 10^6 of each population of these donor cells were injected into LM-Ova- and ST-Ova-infected mice as well as a control group of naïve mice. The next day, the spleens were isolated and specific killing was evaluated by flow cytometry, as shown in (a), using the percentages of peptide-pulsed and – unpulsed populations to calculate the percent of specific lysis. (b) Profile of % specific lysis throughout infection. n=3 mice per group (with the exception of n=1 for ST-Ova on day 120). Points represent mean +/- standard error of the mean. Asterisk indicates statistical significance ($P<0.05$).

(a)



(b)

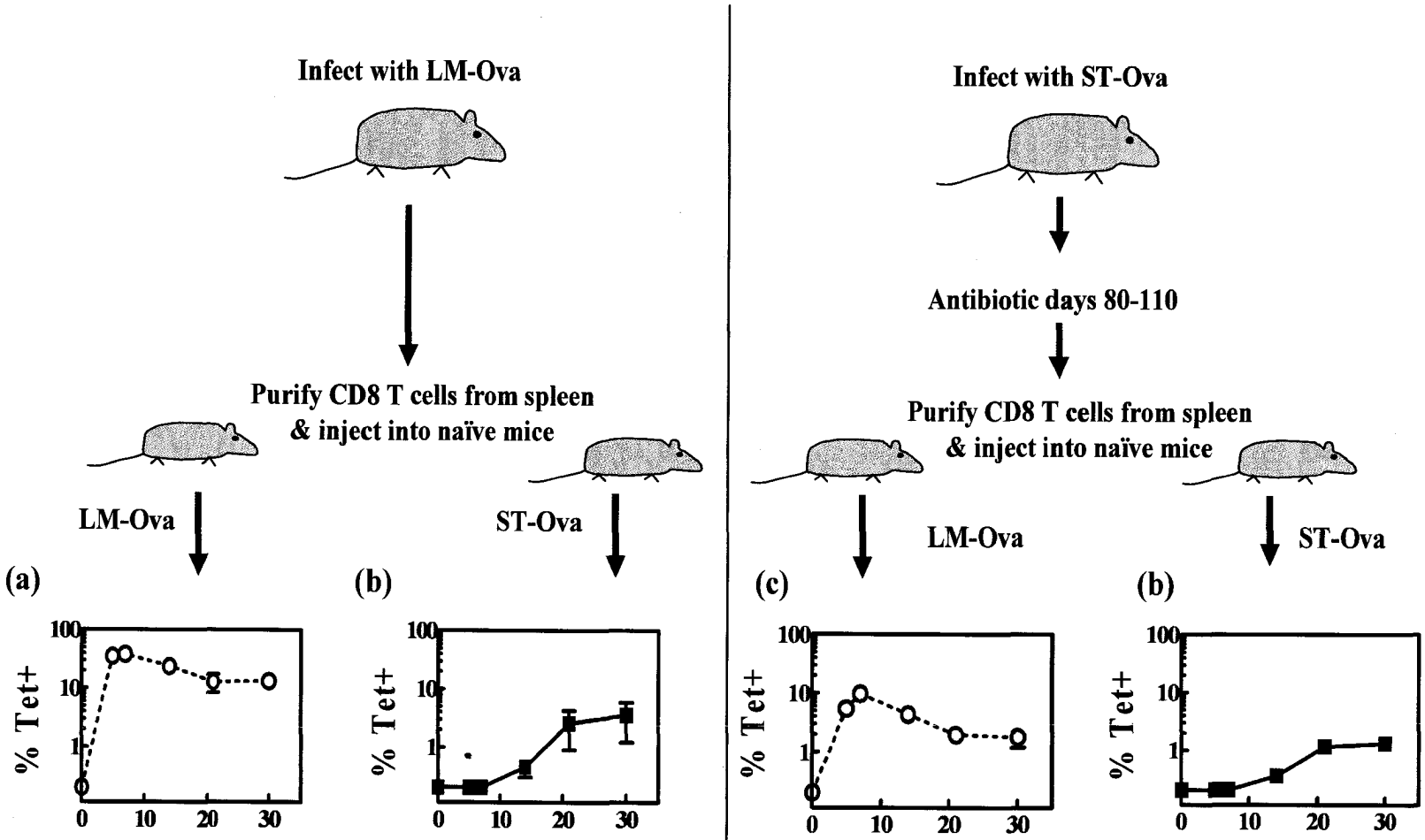


then rechallenged with either LM-Ova or ST-Ova, and the %Tet$^+$ of CD8 T cells was evaluated by staining lymphocytes in the peripheral blood at various time points of infection. The memory CD8 T cell population induced by LM-Ova expanded rapidly in response to LM-Ova rechallenge, with ~40% Ova-specific CD8 T cells at day 5 after rechallenge (Fig. 18a). On the other hand, these same memory cells exhibited a delayed and muted expansion in response to ST-Ova rechallenge (Fig. 18b). Therefore, as with naïve CD8 T cells, conventional memory CD8 T cells (generated against LM-Ova) also mount a delayed response to ST-Ova infection. Thus, memory CD8 T cells are rendered ineffective in responding to ST-Ova infection early on.

The memory CD8 T cell population induced by ST-Ova also expanded early upon LM-Ova rechallenge, with ~5% Ova-specific CD8 T cells at day 5 after rechallenge (Fig. 18c). However, this expansion was much lower in comparison to the expansion of memory CD8 T cells induced by LM-Ova. Furthermore, memory CD8 T cells induced by ST-Ova expanded poorly in response to rechallenge with ST-Ova (Fig. 18d).

These results indicate that the Ova-specific memory CD8 T cells induced by ST-Ova infection are capable of responding rapidly to rechallenge. That LM-Ova-induced memory cells show a reduced expansion following ST-Ova rechallenge, suggests that T cell activation is regulated during ST-Ova infection.

Figure 18. Ability of Ova-specific CD8 T cells to respond to rechallenge. Mice were infected with 10^3 cfu LM-Ova or ST-Ova, then after four months, CD8 T cells were purified and transferred to naïve mice, which were then challenged with either LM-Ova or ST-Ova. Staining of peripheral blood lymphocytes was performed with Ova Tetramer and anti-CD8 Ab, then analysed by flow cytometry. n=2 mice per group. Points represent mean +/- standard error of the mean.



VI. DISCUSSION

This project aims to elucidate the dynamics of the CD8 T cell response to murine infection with virulent *Salmonella typhimurium* (ST), a chronic intraphagosomal bacterium that replicates within macrophages and causes chronic infection in resistant mice. In order to provide a relative analysis of the response, the Ag-specific CD8 T cell response to ST is compared here to another intracellular bacterium, *Listeria monocytogenes* (LM), which also replicates within macrophages and is considered to be a potent inducer of T cell memory. A major limitation in interpretations of the immune response to pathogens stems from the fact that the responses are seldom compared to other pathogens. For example, a 10- to 100-fold increase in T cell numbers during ST infection may be viewed as highly potent; however, this response may appear to be highly muted upon comparison to LM where >5000-fold expansion in the T cell response occurs within the first week of infection. Thus, the dynamics of the response always need to be qualified in relative terms.

1. Why Ova was chosen as a model Ag

Intracellular bacteria express numerous proteins and the identities of key CD8 T cell epitopes of Ag derived from ST are not known. In the absence of this knowledge, it is difficult to evaluate CD8 T cell responses specifically. The gene for ovalbumin (Ova) was cloned into ST and LM so that we can monitor and compare the responses against the same protein expressed by different intracellular bacteria, and thereby provide a comparative analysis of CD8 T cell priming and memory. Recombinant pathogens have been used extensively in evaluating immune responses and the results are considered to be reminiscent of the responses against pathogen derived antigens (205-210).

2. Chronic inflammation induced by ST

Splenomegaly is a prominent feature of ST infection (211). During ST-Ova infection, the spleen cell numbers increased progressively. The overall increase in spleen cell numbers during ST-Ova infection relative to LM-Ova infection can be attributed to massive inflammation that occurs during ST infection (109). It is likely that a similar influx occurs in other lymphoid sites of the body since this is a model of systemic infection.

3. The persistence of ST

The rapid elimination of LM-Ova infection during the first week of infection is due to the innate immune system which controls primary infection of mice with LM (95). On the other hand, ST induces a chronic infection that lasts for several months, indicating that both innate as well as acquired immune system are not able to rapidly eliminate the pathogen. Although ST appears to be cleared from the system altogether, the possibility remained that ST-Ova may still persist in the mouse. Monack and colleagues have shown that even one year after oral infection of mice with a high dose of virulent ST, bacteria were found in the mesenteric lymph nodes (MLN) (105). From our results, ST-Ova when given through the intravenous route is cleared from the MLN in the long term, indicating that the B6.129 F1 mice manage to clear ST-Ova infection altogether. However, depletion of CD4 T cells indicated that some mice do harbour residual ST-Ova at very low levels. These results indicate that intraphagosomal pathogens, such as ST or Mycobacterium tuberculosis may never be completely eliminated by the host, and a state of co-adaptation develops that persists for long-term. Only when the hosts immune system is suppressed, then such chronic subclinical infection gets re-activated and presents a serious problem (246).

4. Low frequency of endogenous Ag-specific CD8 T cells in response to ST-Ova infection

Infection with intracellular bacteria induces 10-100-fold lower CD8 T cell responses in comparison to viral infections. While viruses that induce potent CD8 T cell responses are cytosolic, so that viral antigens are efficiently presented by MHC class I molecules, intracellular bacteria are mainly intraphagosomal. LM is an exception as it has evolved to replicate in the cytosol despite being an intracellular bacterium. Thus, viral infections typically induce stronger CD8 T cell responses. The low frequency of IFN-γ-producing Ova-specific CD8 T cells induced by ST-Ova as determined by ELISPOT and by flow cytometry may therefore be due to the sequestration of Ag as ST-Ova replicates within the phagosome (123). It takes time for this pathogen to become established in its niche within host cells, and since the presentation of exogenous Ag by MHC I is thought to be inefficient (41), there would be a limited availability of Ova peptide presented to CD8 T cells. This would result in the activation of very few Ova-specific CD8 T cells, which would then take a long time for these few cells to proliferate and form a population that can be detected by flow cytometry. Work done in this lab has previously shown that antigen presentation during infection with phagosomal pathogens, such as Mycobacterium bovis and ST is delayed and muted (193, 222). It is unclear what causes the delay and reduction of antigen-presentation. However, the fact that antigen is not present in the conventional MHC class I processing environment (cytosol) presents one possible answer. ST has been shown to inhibit the expression of MHC class II and CD86 on DCs which would limit the activation of CD4, and hence CD8 T cells. Muted CD8 T cell priming does not appear to be due to the reduced engagement of pathogen-associated molecular patterns (PAMPs), since supplementing ST-Ova infected mice with other innate immune signaling molecules (such as Poly I:C, CpG, or even LM) does not result in rapid CD8 T cell activation (unpublished observations from the lab).

To overcome the problem of the low frequency of Ag-specific CD8 T cells that are induced against intracellular bacteria, an adoptive transfer model was employed for further studies in order to amplify the Ova-specific CD8 T cell response, using splenocytes from transgenic OT-1 mice. Adoptive transfer models have been used extensively by various investigators with different infection models and the results obtained in these models indeed correlate very well with the responses of endogenous T cells (212-215). In this way, the population of Ova-specific CD8 T cells is raised to a level that can be detected by flow cytometry.

5. Differentiation of Ag-specific CD8 T cells during ST-Ova infection

The differentiation of the CD8 T cells has been segregated into three phases: the initial expansion phase, the differentiation to effector phase, and the differentiation to memory cells.

a. Expansion of the Ag-specific CD8 T cell response

The gradual increase in $\%Tet^+$ in both blood and spleen during ST-Ova infection may be due to the sequestering of Ag in the phagosome, especially at earlier time points of infection where the majority of the ST-Ova avoid detection by subverting the immune response and hiding away in the phagosome (123).

b. Differentiation to effector CD8 T cells

Various cell surface markers have been used to identify the differentiation of T cells; however, CD44 appears to be the most reliable marker that is expressed at high levels in all Ag-experienced T cells in mice, regardless of their activation status (48, 216). During LCMV (217) and Sendai virus (218) infection of mice, it has been reported that CD8 T cells segregate into two distinct populations: a $CD44^{hi}CD62L^{lo}$ population which is predominately

located in the tissues and exerts a rapid effector function ("effector CD8 T cells"), and a $CD44^{hi}CD62L^{hi}$ population which is found in the spleen and lymph nodes with no immediate effector function ("central CD8 T cells"). Several recent reports have confirmed the presence of distinct effector vs. central T cell subsets (219-221).

Shortly after activation during potent pathogens such as LM or LCMV, T cells down-regulate CD62L (73, 86-88). In chronic ST-Ova infection, the profile of CD62L expression by the Tet^{+} CD8 T cells appears instead to be progressive. The degree of antigenic stimulation may be below a threshold required for the rapid differentiation of the CD8 T cells to an effector phenotype. This is in accordance with data from this lab which previously showed that reduced stimulation of Ova-specific CD8 T cells during infection with attenuated BCG-Ova induces a predominantly central phenotype early on (222).

c. Differentiation to effector memory CD8 T cells

The expression of IL-7Rα by CD8 T cells is down-regulated following antigenic encounter – but, at the peak of the response, a small proportion of CD8 T cells are still IL-$7R\alpha^{hi}$: this is the population that survives and becomes the memory CD8 T cell pool (53). Furthermore, the expression of antiapoptotic proteins such as Bcl-2 is found in this same subset that retains IL-$7R\alpha^{hi}$ expression at the peak of the response (53).

It is important to distinguish the difference between the "*effector* CD8 T cell" and the "*effector memory* CD8 T cell". While both subsets possess the phenotype of $CD62L^{lo}$, the effector cells are IL-$7R\alpha^{lo}$ whereas effector memory cells are IL-$7R\alpha^{hi}$.

Compared with LM-Ova infection, the numbers of Ova-specific IL-$7R\alpha^{hi}$ CD8 T cells in ST-Ova infection demonstrate a gradual differentiation of effector CD8 T cells ($CD44^{+}CD62L^{lo}$IL-$7R\alpha^{lo}$) into the "effector memory" phenotype ($CD44^{+}CD62L^{lo}$IL-$7R\alpha^{hi}$).

However, the numbers of IL-7Rα^{hi} Ova-specific CD8 T cells do not reach the level as is seen with LM-Ova infection, suggesting a low level of Ag presentation occurring during the later stages of ST-Ova infection.

The differentiation of CD8 T cells into the central memory versus effector memory phenotype has been an area of intense investigation. Previously it was believed that the cells possessing an effector memory or central memory phenotype represent cells that are derived from a common lineage (223). However, this view has been challenged by various studies which have indicated that the effector memory and central memory populations of CD8 T cells may be distinct lineages (70, 224, 225). This study indicates that CD8 T cells against the same antigen can display completely different phenotypic profiles: central phenotype in LM-Ova infection and effector profile in ST-Ova infection. Thus, unique host-pathogen interactions can have a profound influence on the differentiation of CD8 T cells.

6. Programming of the development of effector memory CD8 T cells occurs despite pathogen persistence

During ST-Ova infection, it is interesting to note that the primed CD8 T cells differentiated to become effector memory cells despite the persistence of high levels of ST-Ova. Considering that there was never a stable population of Ova-specific CD8 T cells during ST-Ova infection, nor did the cells upregulate CD62L expression, it was postulated that by reducing the bacterial burden of ST-Ova early on, a more stable population of memory CD8 T cells would develop that would perhaps display a central memory (CD62L^{hi}) phenotype more readily.

Because the reduction of bacteria by antibiotic did not affect the size of the Tet^{+} population or the effector memory phenotype, the programming of these effector memory CD8 T cells evidently occurs despite the persistence of the pathogen. It is important to note,

however, that from the immunosuppression experiment, ST-Ova may still not be completely eliminated from each host. The persistence of low levels of bacteria would possibly generate an insufficient level of Ag to influence the differentiation of cells any further.

An important question that arises is how would CD8 T cell expansion decrease, allowing differentiation to effector memory cells, despite the persistence of the pathogen? It is possible that the *ova* gene is lost during the chronic stage of infection. This gene is still present in all of the bacteria that are harvested 60 days post-infection (unpublished results from this lab); yet, it is possible that the Ova Ag display falls below a certain critical threshold of Ag presentation that may be required for T cell activation (226). This would result in a failure to stimulate T cells in the long term, causing effector T cells to differentiate further into effector memory cells.

7. Gradual and prolonged contraction of Ag-specific CD8 T cells during ST-Ova infection.

Contraction involves the decrease in size of a population of clonally expanded, Ag-specific T cells following infection (50). This is a beneficial process in that it reduces excessive use of resources, since such a large proportion of Ag-specific T cells is no longer needed once the pathogen is eliminated. A population of T cells contracts as the cells undergo apoptosis. During apoptosis, phosphatidylserine (PS) is flipped to the outer leaflet of the plasma membrane, which is detected by Annexin V binding (203). Thus, by quantifying the proportion of Ova-specific CD8 T cells that was Anx^+, the profile of contraction of the Ova-specific CD8 T cell population was evaluated.

Compared with LM-Ova infection, the contraction by Ova-specific CD8 T cells responding to ST-Ova infection was delayed, gradual, and also sustained. The gradual contraction may be due to the reduced stimulation of T cells during ST-Ova infection (193).

In Mtb-infected mice, contraction was enhanced by eliminating the bacterial burden by antibiotic (227). However, studies of LM infection in mice have shown that a higher dose of LM results in the same kinetics of contraction as with a lower dose (228).

The continued commitment of primed T cells to apoptosis may alternatively be explained by the chronic induction of inflammatory mediators by ST-Ova. Several studies have demonstrated that inflammation, and IFN-γ in particular, is important in influencing contraction of CD8 T cells (228). Evidence that IFN-γ promotes contraction is found in a study of LCMV infection where the contraction phase of responding CD8 T cells was dampened in IFN-γ-deficient GKO mice compared to that of wild-type mice (229).

Considering the high proportion of Anx^+ CD8 T cells that persists throughout the later part of ST-Ova infection, it is unclear how the overall response contracts gradually and less. It is possible that new Ag-specific CD8 T cells may be continually generated in the presence of Ag, so the net outcome may be that the T cell numbers may be maintained, yet this is actually due to a combination of cell death and the generation of new cells. One aspect of homeostasis that is not revealed in these studies is the rate at which the CD8 T cells are proliferating or dying. Since the $\%Anx^+$ increases from 20% at day 14 to 40% at day 60, yet the overall $\%Tet^+$ remains high near 10% from day 14-30, this indicates there are cells that are proliferating, thus compensating for the death of the Anx^+ cells in the net $\%Tet^+$. However, since the BrdU data indicates peak proliferation at day 14, decreasing at day 30 until day 60 where proliferation is down to minimal levels, it seems that most Tet^+ cells are not proliferating. Thus, it may be suggested that Ag presentation is still occurring at later time points, priming new Ova-specific CD8 T cells; these cells may be somehow prevented from proliferation, perhaps by the contraction-promoting effects of IFN-γ and inflammation mentioned above (228).

8. Ag-specific CD8 T cells require CD4 T cells for survival during ST-Ova infection

CD4 T cell help is essential for an effective CD8 T cell response against numerous pathogens. CD8 T cell priming against Mycobacteria is dependent on CD4 T cells (200). CD4 T cell help is also critical in the CD8 T cell response to parasitic infections such as *Plasmodium* and *Toxoplasma*, where initial responses appear intact; however, as infection progresses, the potency of the response becomes greatly reduced (230, 231). On the other hand, the role of CD4 T cell help is deemed unnecessary for the development of the CD8 T cell response in some infection models, such as those of acute LCMV (63) and LM (232).

In ST infection it has been shown that CD4 T cells play an important role in combating infection (179, 180, 183); although, whether their presence is necessary for CD8 T cell priming is unknown. Accordingly, it was examined whether the development of an Ova-specific CD8 T cell response would be hindered by depleting CD4 T cells in ST-Ova-infected mice. The results show that the Tet^+ population is largely non-existent in the absence of CD4 T cells; therefore, the CD8 T cell response to ST-Ova infection relies on the presence of CD4 T cells. Several factors may be involved in determining the role of CD4 T cell help. Firstly, the level of costimulation provided to the CD8 T cells by DC may influence the need for CD4 T cell help. In situations where costimulation is weaker, CD4 T cell help may be more crucial. A second factor in determining the level of requirement for CD4 T cell help is the affinity of a given epitope for MHC I. Mice immunized with peptides of highest affinity to MHC I show the strongest CTL response with the least dependence on CD4 T cell help, whereas the peptides with lower affinity induce a response that is largely dependent on CD4 T cell interactions (233).

9. Ag-specific CD8 T cells are functional in response to ST-Ova infection

Effector CD8 T cells are characterized by production of IFN-γ upon antigenic stimulation, and the ability to kill upon recognition of Ag during infection (15-17).

In other chronic infection models it has been shown that T cells may lose responsiveness with time, via anergy or exhaustion (234). When a T cell is effectively activated, the ligation of its CD28 co-receptor with B7 on an activated APC promotes proliferation of the T cell and enhances its production of IL-2 (21). "Clonal anergy" occurs when a T cell binds its TCR with MHC but there is an absence of costimulation provided to the T cell through this CD28/B7 interaction (235), rendering the T cell tolerant to its cognate Ag. Exhaustion occurs when T cells receive a large amount of antigenic stimulation over a long period of time, as with chronic LCMV infection, where the LCMV-specific CD8 T cells gradually lose their ability to produce IL-2 and IFN-γ in response to infection (236, 237). A form of exhaustion is exhibited during HIV infection where CD8 T cells develop replicative senescence (238). It is thought that the high Ag load and constant antigenic stimulation of the CD8 T cells causes them to divide so many times that they eventually become incapable of dividing further (239).

Functional inactivation evidently does not occur during chronic ST-Ova infection as evidenced by IFN-γ production and ability to kill specific targets in vivo. This is indeed a long-term infection where CD8 T cells could potentially be inundated by continuous stimulation with Ova peptide. However it is possible that the Ag derived from ST-Ova infection is not available in large amounts, due to the intraphagosomal localization of this pathogen and the inefficiency of the cross-presentation of exogenous Ag by MHC I (41). Thus, the limited Ag availability and the reduced T cell stimulation during ST-Ova infection may prevent the CD8 T cell response from going into exhaustion despite the persistence of ST-Ova.

10. Ag-specific memory CD8 T cells induced by ST-Ova are capable of responding to rechallenge

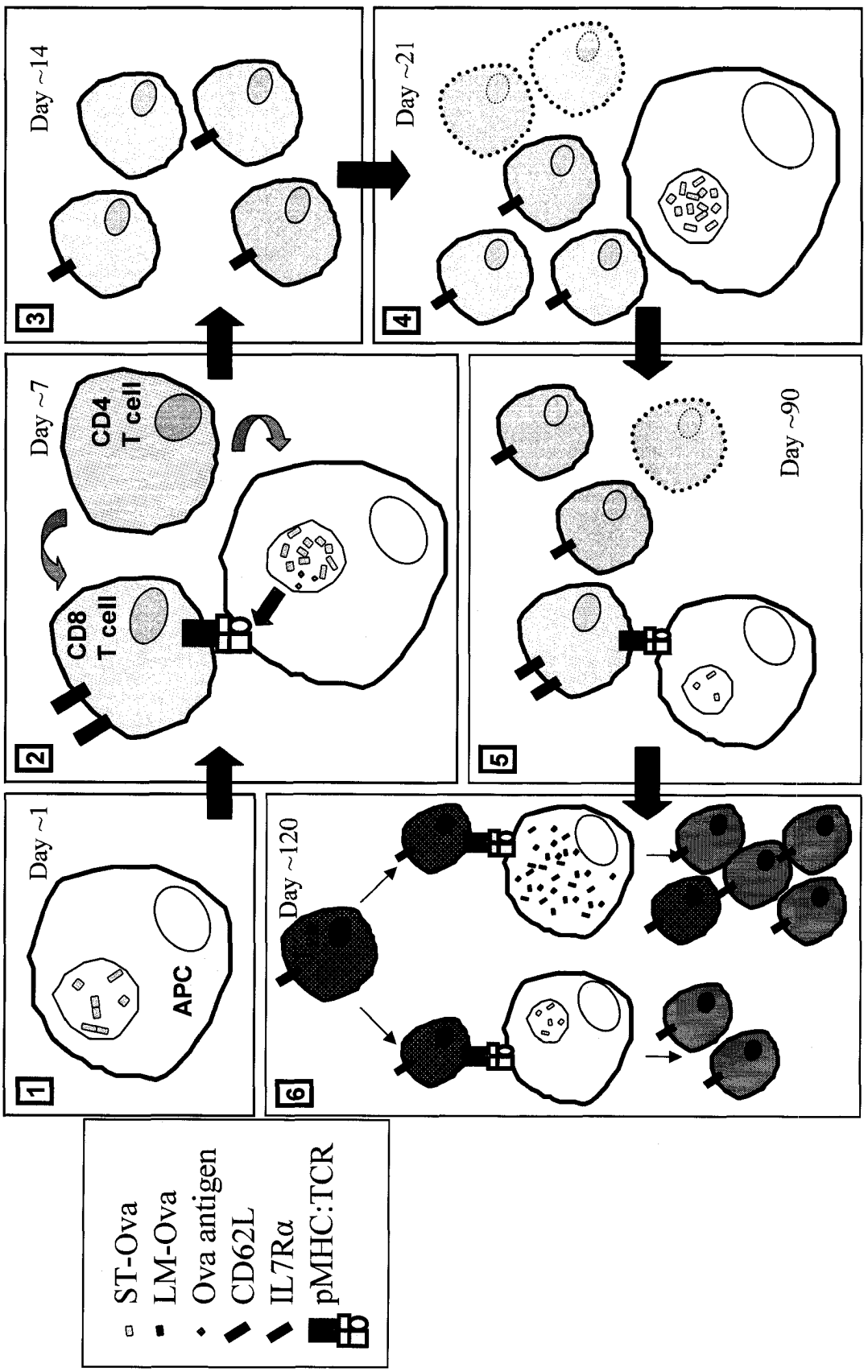
It has been shown in these studies that an effector memory population results from ST-Ova infection. However, effector memory cells may not proliferate as readily as central memory cells (223). Furthermore, the delayed and gradual profiles of expansion and contraction suggest that the resulting memory pool could be relatively ineffective at responding to rechallenge. Therefore, it was tested whether these ST-Ova-induced predominantly effector memory cells are capable of responding to rechallenge, by comparison with LM-Ova-induced memory cells which have a predominantly central memory phenotype.

That memory CD8 T cells from ST-Ova-infected mice expanded rapidly when rechallenged with LM-Ova shows that these effector memory cells are in fact capable of proliferating early on during infection. However, their proliferation was lower in comparison to the mainly central memory phenotype cells from LM-Ova-infected mice. Conventional memory CD8 T cells (induced by LM-Ova) failed to proliferate rapidly in response to ST-Ova infection, indicating that either the Ag levels are below the threshold needed for T cell activation, or T cell activation is inhibited during ST-Ova infection. This poses problems for vaccine development, since enhancement of memory T cells may not facilitate protection against ST. Why T cell activation does not happen early on during ST infection is an important question that needs to be addressed.

11. Model of the CD8 T cell response throughout ST-Ova infection

In light of the findings presented herein, I propose a model of the CD8 T cell response during ST-Ova infection (Fig. 19). One possibility for the delayed CD8 T cell response is reduced Ag presentation. Previous studies in this lab indicate that there is a very

Figure 19. Model of Ag presentation during ST-Ova infection. (1) ST-Ova requires time to establish its niche within the host phagosome. (2) Ag is sequestered in the phagosome and must be cross-presented by MHC I for the priming of the CD8 T cell to take place, which is aided by CD4 cells. (3) Due to Ag sequestration, there is a delayed expansion and differentiation of the CD8 T cells. (4) Contraction begins early on, when cfu are still high. (5) Contraction is prolonged, and some CD8 T cells are still being activated later on during infection. (6) The development of the CD8 T cell population yields effector memory CD8 T cells, which proliferate gradually when rechallenged with ST-Ova; yet, they are capable of rapid proliferation when rechallenged with LM-Ova. This demonstrates that there is a limited Ag availability during ST-



low level of antigen presentation occurring throughout ST-Ova infection (193). This may be mediated by a feedback response causing the elimination of APC following a transient period of priming, so that less Ag is presented despite the persistence of viable intracellular pathogen (240). Also, ST has been shown to regulate antigen presentation by inhibiting expression of MHC II and CD86 on DCs, limiting recognition of Ag by CD4 T cells (241). Alternative explanations for a delayed CD8 T cell response could be due to the great deal of inflammation present early on after infection (200, 211, 229), as well as other potential mechanisms of ST immunosuppression such as inhibition of T cell proliferation (242).

As shown by the gradual increase in splenic bacterial burden, ST-Ova requires 2-3 weeks to establish its niche within host cell phagosomes (123). Because ST-Ova replicates within the phagosome, Ag is sequestered, especially at earlier time points of infection, as shown by the incredibly gradual increase in Ag-specific CD8 T cells.

Cross-presentation is an important mechanism of Ag presentation during intracellular infections such as ST (40-42). Because the pathogen inhabits the host cell, it evades immune surveillance. Also, because ST survives within the phagosome, it is concealed from detection even further. In order for detection of the pathogen to occur, it is necessary for the exogenous Ag to be presented by MHC I to CTLs that may then destroy the infected cell and eliminate the pathogen (35, 36).

During early infection, the processing of Ag within the *Salmonella*-containing vacuole may be inefficient due to the low initial quantity of bacteria in the host cells; secondly, due to the fact that ST infects primarily macrophages (105, 116, 117), which are less efficient APCs than dendritic cells (243, 244); and thirdly, due to the inefficiency of noncytosolic pathways of exogenous processing (41). Altogether, this poses a possible

scenario of low Ag availability combined with inefficient presentation, which may trigger very low T cell priming early on during ST-Ova infection.

Thus, it takes weeks for these few primed CD8 T cells to proliferate and expand to form a significant population of clonally expanded effectors. The degree of antigenic stimulation may be below the threshold required for rapid differentiation of CD8 T cells to an effector phenotype. However, the few cells that are being primed are expanding rapidly. At the peak of the response (3 weeks) the Ova-specific CD8 T cell population has reached its full capacity so that a lesser degree of proliferation is required to maintain the population.

The contraction of the Ag-specific CD8 T cells during ST-Ova infection begins early on and occurs gradually. This is likely due to the continued presence of high levels of inflammatory cytokines (211), since numerous studies have shown that IFN-γ promotes contraction of the CD8 T cell population (200, 228, 229).

The IL-7Rα^{hi} subset that develops during ST-Ova infection is not restored to a level similar to that seen after LM-Ova infection; it is possible that a low level of Ag presentation may be occurring particularly during the later time intervals. This correlates with the data showing that ST-Ova persists at very low levels even 120 days post-infection.

The Ag-specific CD8 T cells require CD4 T cell help for survival, possibly due to a low level of costimulation by DCs. Also, the fact that less Ova-specific CD8 T cells are able to respond due to the low availability of Ag means that CD4 T cell help is more valuable in activating the low number of responding CD8 T cells.

The Ova-specific CD8 T cells maintain functionality in their ability to produce IFN-γ as well as the high % specific cytotoxicity. Lack of exhaustion or anergy in CD8 T cells induced against ST could be because of attenuated T cell activation that happens during ST

infection. Even if T cells are activated in a chronic manner, they may be receiving attenuated stimulation, precluding them from differentiating into an anergic phenotype.

The Ag-specific effector memory CD8 T cells induced by ST-Ova infection proliferated in response to LM-OVA infection, but their response was lesser in magnitude than the response of memory CD8 T cells induced by LM-OVA, indicating the central memory phenotype cells mediate better recall responses than effector memory cells. Conventional memory CD8 T cells induced against LM-Ova failed to proliferate rapidly in response to ST-Ova infection, underscoring the limited T cell activation that proceeds during ST infection. In such a scenario, no matter how many memory T cells are induced by a vaccine, they will be rendered ineffective due to curtailment in T cell activation during ST infection.

VII. FUTURE DIRECTIONS

The data presented herein suggest delayed and muted CD8 T cell priming during ST-Ova infection. However it is not clear whether delayed proliferation and prolonged contraction of CD8 T cells are a direct result of a low level of Ag, or whether additional factors such as inflammation may play a role. To further study the mechanisms that are responsible for the delayed activation of CD8 T cells during ST-Ova infection, measurement of the relative levels of Ova during LM-Ova versus ST-Ova infection would be important. This could be done by performing quantitative RT-PCR analysis of infected spleens at various time intervals after infection. In addition, the direct ex vivo antigen display (DEAD) assay could be used, which evaluates the proliferation of CFSE-labeled, naïve, Ag-specific CD8 T cells when incubated with splenocytes from infected mice (228). Immunohistochemistry would be useful in visually determining the level of Ag presentation by staining splenocytes or peripheral blood lymphocytes at various time points of murine infection with ST-Ova, with a fluorescently-labeled Ova-specific Ab, in combination with antibodies to phagosome markers Rab5 and Rab18 which are recruited by ST-containing phagosomes to prevent lysosomal fusion (245). This would also enhance the understanding of the role of Ag presentation - that is, whether Ag from ST-Ova is predominantly derived from cross-presentation by infected cells, or via DC that have phagocytosed apoptotic cells.

VIII. CONCLUSIONS

The aim of this study was to evaluate the Ag-specific CD8 T cell response to *Salmonella typhimurium*, using recombinant ovalbumin (Ova)-expressing ST in a chronic murine typhoid model, and comparing this response with the well-characterized acute intracellular pathogen *Listeria monocytogenes* (LM-Ova).

Chronic infection with ST-Ova was characterized by massive and sustained inflammation as seen by splenomegaly and high splenic bacterial burden which persisted up to 60 days post-infection. At later time points, ST-Ova was controlled to subclinical levels but could be reactivated by depletion of CD4 T cells. To evaluate the endogenous Ova-specific CD8 T cell response, IFN-γ ELISPOT as well as flow cytometry with MHC I H-2K^b Ova Tetramer were used, however the frequency of endogenous Ova-specific CD8 T cells was too low to detect. To solve this problem, an adoptive transfer model was used by injecting splenocytes from OT-1 transgenic mice (with >90% CD8+ T cells specific to the $Ova_{257-264}$ peptide) into the resistant B6.129 F1 mice prior to infection with ST-Ova or LM-Ova. In this way, the Ag-specific response was amplified so that the Ova-specific CD8 T cell response could be readily evaluated by methods such as ELISPOT and flow cytometry.

The response of the Ova-specific CD8 T cells to ST-Ova infection overall was characterized as delayed and reduced by comparison with the response to LM-Ova infection. The expansion of the Ova-specific CD8 T cell population only reached its peak 21 days post-infection, compared to the expansion of the response against LM-Ova which peaked 7 days post-infection. The differentiation of the Ova-specific CD8 T cells was also delayed, peaking 3 weeks post-infection. T cell priming may occur at a low level during ST infection, which perhaps may increase as infection progresses, due to further ST-Ova replication as more Ag becomes available.

The Ova-specific CD8 T cell population undergoes gradual and prolonged contraction during ST-Ova infection, as shown by the gradual increase in the Anx^+ subset which remained higher than that of LM-Ova even 6 months after infection. Contraction appears to be programmed early on during ST-Ova infection, since the accelerated removal of the pathogen at day 30 did not change the rate of contraction in terms of proportion of Ag-specific CD8 T cells, proportion of effectors, or the proportion of IL-7Rα^{hi} cells within this population.

The development of memory despite the presence of high ST-Ova burden suggests that perhaps the low level of Ag is below a threshold of activation that may be required for the maintenance of the effectors, so that the Ag-specific effector CD8 T cells differentiate to become effector memory. Accelerated removal of ST-Ova did not change the dominant effector memory phenotype, showing that the effector memory population is persistent. However, the effector memory population is unstable in the long-term as the $\%Tet^+$ decreases gradually over time in the later stages of infection. This may be due to an insufficiency of antigenic stimulation, as effector memory cells have been reported to depend on the persistence of Ag during a chronic infection.

The Ova-specific CD8 T cell response was also evaluated for its dependency on CD4 T cells for priming. By depleting CD4 T cells in mice infected with ST-Ova, the Ova-specific CD8 T cell population was abolished, proving the fundamental dependence of this population on the CD4 T cells, while the Ova-specific CD8 T cell population in LM-Ova infection did not change. It has been shown that CD4 T cell help is required in situations of weaker stimulation to Ag-specific CD8 T cells, thus this adds further support to the idea that there is low Ag presentation during ST-Ova infection.

Functionality of the Ova-specific CD8 T cells in ST-Ova infection was demonstrated by the capacity to produce IFN-γ as well as CTL activity in vivo. The Ova-specific CD8 T cells induced by ST-Ova infection are indeed fully functional, as the proportion of cells producing IFN-γ was comparable to that of LM-Ova infection, and the % specific lysis during ST-Ova infection was high during the peak of infection. Thus in this model of chronic infection the Ag-specific CD8 T cell population does not undergo anergy or exhaustion which has been shown to occur in other models of chronic infection. This could be because of reduced T cell activation that occurs during ST infection, possibly preventing T cells from becoming anergic.

Finally, the ability of ST-Ova-induced effector memory CD8 T cells was tested by rechallenge of mice with either LM-Ova or ST-Ova. Upon LM-Ova rechallenge, the ST-Ova memory cells proliferated rapidly although not as profoundly as the LM-Ova-induced memory cells. More importantly, conventional memory CD8 T cells failed to proliferate rapidly in response to ST-Ova infection, indicating that T cell activation is inhibited during ST infection.

It was anticipated that by elucidating the dynamics of the CD8 T cell response to ST-Ova infection, that this information may be useful in developing improved vaccines that may be more effective at providing protection against typhoid infection. Our results have suggested that the problem may lie not with the memory T cells but with the mechanisms that regulate T cell activation during the virulent challenge with ST. A thorough understanding of the key virulence factors of ST that cause inhibition of phagosome-lysosome fusion and facilitate phagosomal persistence is needed in order to develop strategic ways of facilitating antigen-presentation during ST infection.

IX. References

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X. Curriculum Vitae

EDUCATION

Sep 2004 – June 2007	**University of Ottawa** Master's of Science, Microbiology & Immunology	**Ottawa, ON**
Sep 1999 – Apr 2004	**University of British Columbia** Bachelor's of Science, Microbiology & Immunology	**Vancouver, BC**
Sep 1998 – Apr 1999	**University College of the Fraser Valley** Bachelor's of Science	**Abbotsford, BC**

Specialized Courses:

Canadian Council on Animal Care - Animal User Training Program
National Research Council Canada - Radiation Safety
Workplace Hazardous Materials Information System

PUBLICATIONS

Luu, R.A., Gurnani, K., Dudani, R., van Faassen, H., Sirard, J.C., Krishnan, L., Sad, S. 2006. Lack of antigen presentation despite rapid pathogen proliferation: Delayed expansion and contraction of the CD8+ T cell response during chronic infection with *Salmonella typhimurium*. J. Immunol. 177(3):1516-25.

Marr, N., **Luu, R.A.**, Fernandez, R.C. 2007. *Bordetella pertussis* binds the human C1 esterase inhibitor during the Bvg+ phase in order to evade complement-mediated killing. J. Infect. Dis. 195(4):585-8.

Carrillo, C.D., Taboada, E., Nash, J.H., Lanthier, P., Kelly, J., Lau, P.C., **Verhulp, R.**, Mykytczuk, O., Sy, J., Findlay, W.A., Amoako, K., Gomis, S., Willson, P., Austin, J.W., Potter, A., Babiuk, L., Allan, B., Szymanski, C.M. 2004. Genome-wide expression analyses of *Campylobacter jejuni* NCTC11168 reveals coordinate regulation of motility and virulence by *flhA*. J. Biol. Chem. 279(19): 20327-38.

STRENGTHS

- Work diligently independently as well as a team player
- Possess strong organizational and communication skills
- Show motivation and perseverance
- Demonstrate initiative and trouble-shooting capability
- Learn new skills and methodologies quickly and competently

EXTRA-CURRICULAR ACTIVITIES

- Participated in the Ottawa Dragon Boat Festival
- Held an executive position in the UBC Microbiology & Immunology Student Association
- Interests include jogging, kayaking, and playing the flute

WORK EXPERIENCE

Coley Pharmaceutical Group, Inc. **Jun 2006 – Present**
Research Associate **Ottawa, ON**

- Perform and develop assays for the evaluation of novel therapeutics in various models of disease

Techniques: Processing of tumor and various organ samples; Preparation of DNA and RNA; Isolation of various cell types using MACS beads; Cell-surface and Intracellular staining with Flow Cytometry of Murine Lymphocytes; Cytokine measurement by Luminex, ELISA, and ELISPOT assays; CTL assay; Tissue culture.

University of Ottawa, Department of Microbiology & Immunology Sep 2004 – Jun 2007
with the Institute for Biological Sciences, **Ottawa, ON**
National Research Council of Canada
Master's Research Project: "Characterization of the CD8 T cell response to Salmonella typhimurium infection in mice"

- Planned and conducted studies to analyse the Antigen-specific CD8 T cell response to bacterial infection in mice using the Tetramer technology
- Examined Apoptosis in CD8 T cells by Annexin V binding and Caspase activity assays
- Assessed Memory CD8 T cell function following adoptive transfer into naïve mice

Techniques: Cell-surface and Intracellular staining and Flow Cytometry of murine spleen and peripheral blood lymphocytes (BD Bioscience FACS-CANTO: 6-colour and Beckman-Coulter EPICS-XL: 4-colour); Handling of mice for intravenous injections, tail-bleeding and splenectomy; Annexin V binding and Caspase activity assays; Confocal Microscopy; CD8-T-cell Purification using Magnetic Biotin CeLLection Beads; CTL assays; ELISA- and ELISPOT-based Cytokine Measurement assays; Production of mAb using "High Density Culture Systems"; Purification of mAb using Protein G Columns; Maintenance of various cell cultures (splenocytes, hybridomas, EL-4, EG-7, JAWS II)

Abgenix Biopharma, Inc. **May – Dec 2003**
Co-op Education Work Term: Antibody Discovery Team **Burnaby, BC**

- Spearheaded a new project to isolate antibodies from tumour-infiltrating lymphocytes
- Executed native binding assays to screen monoclonal antibodies on cancer cell lines

Techniques: Processing of tumour samples, FACS staining and analysis, Maintenance of various cell cultures (HeLa, CHO, HUVEC).

University of British Columbia, **Sep 2003 – Apr 2004**
Department of Microbiology & Immunology **Vancouver, BC**
Directed Studies Project: "Characterization of the role of BrkA in mediating serum resistance by Bordetella pertussis"

- Examined binding of the *B. pertussis* virulence factor BrkA to human complement

Techniques: Affinity chromatography, Protein purification, Bacterial culture

Institute for Biological Sciences, National Research Council Canada **Jan – Aug 2002**
Co-op Education Work Term: Pathogenomics Group **Ottawa, ON**

- Evaluated the virulence of *Campylobacter jejuni* by constructing knock-out mutants in a flagellar regulatory gene and sialic acid biosynthesis genes

Techniques: Invasion assays, Gel electrophoresis of lipo-oligosaccharide, Western blotting, Tissue culture

Cellulase Laboratory, UBC Chemistry Department **Oct 2000 – Aug 2001**
Research Assistant: Agrobacterium Glycosidase **Vancouver, BC**

- Contributed to studies in Enzyme kinetics, Protein purification, and Site-directed mutagenesis