HOST RESPONSES TO *SALMONELLA TYPHIMURIUM* INFECTION *IN VITRO* AND *IN VIVO*

by

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Abstract

Host responses to *Salmonella typhimurium* infection *in vitro* and *in vivo*

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Salmonella spp. are facultative intracellular pathogens capable of causing localized and systemic disease of significant morbidity and mortality; disease can be prevented by oral immunization with viable attenuated bacteria. During the *Salmonella*-host interaction, multiple processes occur that determine the outcome of infection. In this dissertation, I examine several events - bacterial induction of programmed host cell death, innate recognition of bacterial motifs, and adaptive immune responses to microbial antigens.

Prior studies observed *Salmonella* invasion of macrophages induced apoptosis, or non-inflammatory programmed cell death. Results presented here suggest that *Salmonella* kills macrophages by a unique mechanism distinct from apoptosis and necrosis. Unlike apoptotic cells, *Salmonella*-infected macrophages displayed diffuse rather than condensed patterns of DNA fragmentation and lacked caspase-3 enzymatic activity, but like necrotic cells, infected macrophages exhibited membrane damage and retained the enzyme PARP in its active uncleaved state. Unique to *Salmonella*-induced macrophage death was the requirement for caspase-1 activity. These results demonstrate that *Salmonella* infection of macrophages triggers caspase-1-dependent pro-inflammatory necrosis.

During *Salmonella* infection, CD4+ T cells respond to FliC, the major subunit protein of the flagellar apparatus. Described here is further examination of the CD4+ T cell response to FliC and identification of four discrete FliC epitopes with varying immunodominance *in vitro* and *in vivo*. Analysis of CD4+ T cell responses to non-FliC antigens identified unique surface organelles as a rich source of natural antigens. *Salmonella* antigens directly stimulated Toll-like receptors (TLRs) or were intimately associated with TLR ligands, suggesting that TLR recognition biases T cell responses to specific antigens. *Salmonella* evaded innate and adaptive immune recognition by modifying or repressing expression of natural antigens during growth *in vivo*; bacterial regulation of antigen expression was shown to occur at transcriptional and post-transcriptional levels. Experimental dysregulation of FliC expression during *in vivo* infection profoundly influenced the ensuing mucosal CD4+ T cell response to FliC, indicating that *Salmonella* preferentially expresses FliC during colonization of the mucosa. These results demonstrate that regulated antigen expression can influence antigen-specific immune responses, and may enable *Salmonella* to evade immune recognition and continue replication in host tissue.

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DEDICATION

I dedicate this dissertation to the taxpayers of the United States of America, whose contributions funded my experiments and training.

CHAPTER 1: INTRODUCTION

Numerous responses occur during microbial infection of hosts. The consequence of individual responses can profoundly influence the outcome of infection, swaying the balance between life and death for pathogen or host. Several responses of note occur at the cellular, multicellular/tissue, and systemic levels during microbial infection. Discussed here are host responses to microbial infection that occur at the cellular, multicellular/tissue, and systemic levels: bacterial-induced eukaryotic cell death, innate recognition of microbial molecules, and generation of adaptive immunity to pathogens, respectively.

BACTERIAL INDUCTION OF EUKARYROTIC CELL DEATH

Apoptosis versus Necrosis

The study of bacterial-induced programmed cell death began recently, starting ten years ago when Zychlinsky and colleagues reported that *Shigella* induced apoptosis in infected macrophages (262). The broader field of eukaryotic cell death has been studied for over a century, receiving more attention following the landmark papers of James Kerr that described the morphological features of eukaryotic cell death and defined programmed cell, or the quiescent deletion of individual cells, as "apoptosis" (Greek, a falling off of leaves). Apoptosis normally functions to remove unneeded cells from an organism, for example, during limb patterning or immune system development (119, 143). Apoptosis initiates via a cell-surface or mitochondrial signals, often utilizes specialized enzymes called caspases, demonstrates morphological features such as nuclear and cellular condensation, and progresses slowly to culminate with phagocytosis by neighboring cells

(232) (Figure 1.1). Necrosis, conversely, is conventionally defined as accidental cell death and results from frank external membrane damage that rapidly leads to extensive cellular swelling and leakage of cytoplasmic contents to extracellular environs (232) (Figure 1.1). However, recent studies of cell death in multiple experimental systems demonstrate that the biochemical and morphological distinctions between apoptosis and necrosis can be blurred (185). For example, some forms of apoptosis occur independently of caspases (124), as during granzyme A-mediated, cytotoxic T lymphocyte-induced cell death (11). DNA fragmentation, often categorically restricted to apoptosis (135), can be detected during some forms of necrosis (54). Additionally, the outcome of cell death *in vivo* can have significant consequences. *In vivo*, apoptotic cells die quietly and are phagocytosed before inflammation begins, and are thus immunologically invisible (131). Conversely, necrotic cells release inflammatory cytoplasmic molecules like high mobility group 1 protein (203), and initiate inappropriate adaptive immune responses to cellular proteins (202). Thus, careful description and definition of a given cell death process is important for precise prediction of events downstream of cell death and for understanding underlying mechanisms of disease pathogenesis.

Prokaryotic induction of programmed eukaryotic cell death is a common phenomenon, as bacteria from over ten different genera are capable of inducing cytotoxicity (Table 1.1). Generally, bacteria adapted to either facultative intracellular or extracellular infection can induce cytotoxicity, while obligate intracellular bacteria and viruses inhibit eukaryotic cell death (10), the latter observation suggesting the teleological hypothesis that some microbes prevent cytotoxicity to maximize intracellular replication. Distinct from previously described systems where microbial cytotoxins alone are sufficient to kill

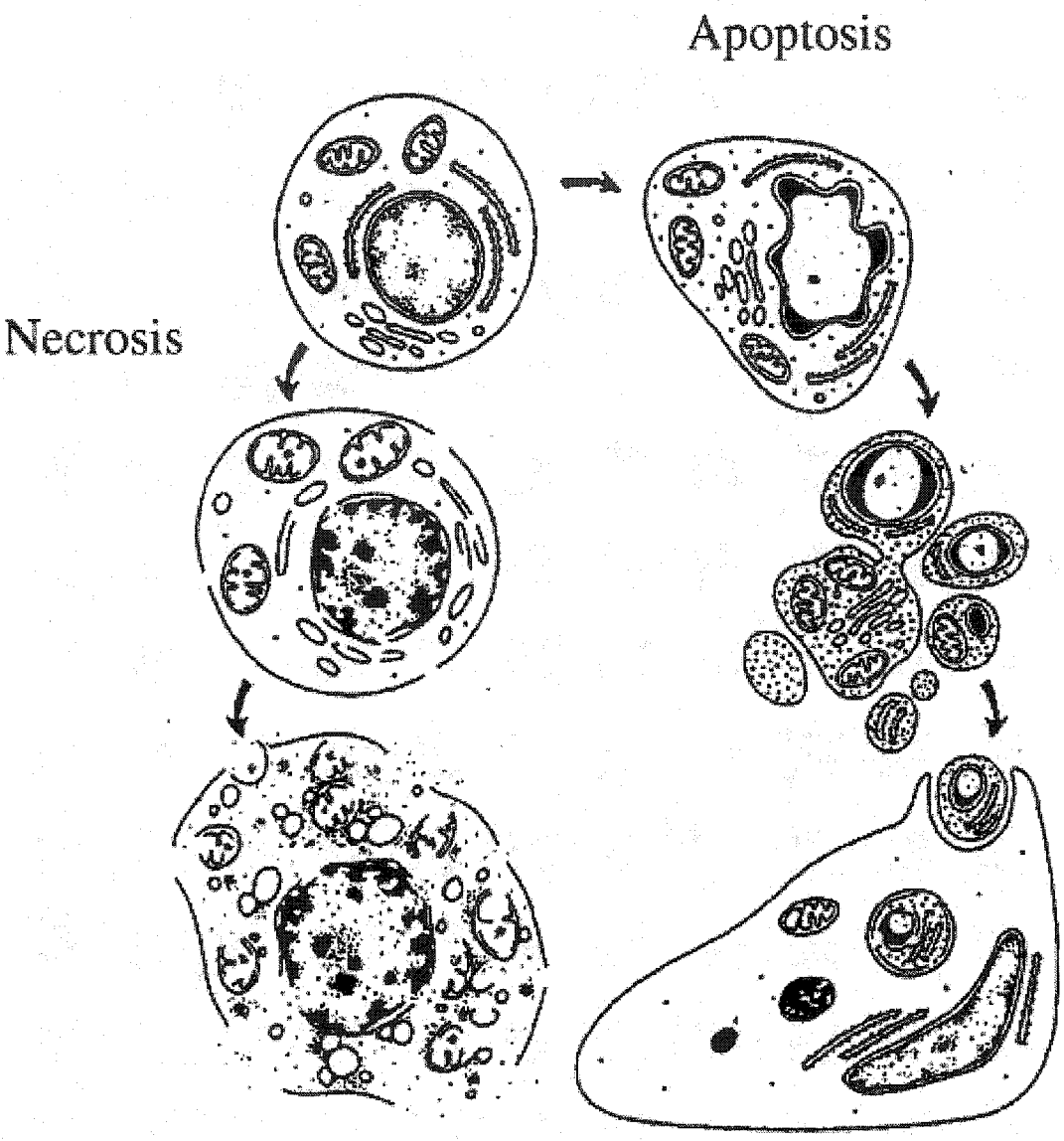


Figure 1.1 Morphology of cells undergoing necrosis or apoptosis. This figure was reproduced from van Cruchten *et. al.* (232). Cells dying by apoptosis present condensed nuclei and fragment into smaller parts (apoptotic bodies) for phagocytosis; necrotic cells swell and burst plasma membrane to leak contents into extracellular space.

Table 1.1 Bacterial Pathogens that Induce Programmed Cell Death Summarized from published literature, beginning with first description of bacterial-induced programmed cell death (1992).

Pathogen	Eukaryotic Cell Type	Features of Death	Concluded Mechanism of Death	Reference
Shigella flexneri	macrophages	fragmented DNA caspase-1 dependent	apoptosis	(261, 262)
Salmonella spp.	macrophages	fragmented DNA caspase-1 dependent	apoptosis and necrosis	(25, 130, 158, 241)
Yersinia spp.	macrophages	fragmented DNA, caspase dependent	apoptosis	(149, 157, 197)
Pathogenic *Escherichia coli*	epithelial cells	fragmented DNA, caspase activation	apoptosis and necrosis	(38, 39, 62, 162)
Pseudomonas aeruginosa	epithelial cells, macrophages	fragmented DNA	apoptosis, acute necrosis	(28, 84)
Bordetella bronchiseptica	epithelial cells	fragmented DNA, caspase-independent	necrosis	(115, 257)
Legionella pneumophila	macrophages, epithelial cells	fragmented DNA, caspase-3 dependent	apoptosis	(70, 71, 161, 168)
Mycobacteria spp.	macrophages	fragmented DNA	apoptosis	(114, 179)
Listeria monocytogenes	macrophages, dendritic cells	fragmented DNA	apoptosis or necrosis (different references)	(7, 81, 193)
Staphylococcus aureus	keratinocytes	fragmented DNA, necrotic morphology	apoptosis and necrosis	(144)
Streptococcus spp.	macrophages, epithelial cells	fragmented DNA, caspase-dependent	apoptosis	(63, 164)

eukaryotic cells (66), bacterial-induced programmed cell death requires active microbe infection or invasion and uses host factors to execute the death process. Despite the common mechanism of infect-and-kill in each system (166), there exist significant differences between systems to merit separate examinations of select pathogens.

Shigella

Shigella flexneri were the first bacteria reported to induce apoptosis in eukaryotic cells (262). *Shigella* infects macrophages, escapes from the vacuole to the cytoplasm and secretes the type III secretion system (TTSS) protein IpaB, which binds and activates caspase-1 to induce rapid cell death (26). *Shigella ipaB* mutant bacteria fail to induce death (261), and caspase-1 inhibition or deficiency prevents death (89). Initial observations of DNA fragmentation and caspase-1 dependency suggested apoptosis, but later studies revealed that macrophages either lacking pro-apoptotic factors (caspase-3, caspase-11, or p53) or overexpressing anti-apoptotic proteins (Bcl-2 or Bcl-X_L) were still infected and killed by *Shigella* (89). Caspase-1, the host enzyme mediating *Shigella*-induced macrophage death, is not required for canonical apoptosis despite homology to pro-apoptotic caspases (121, 127), but is required to process proinflammatory cytokines IL-1β and IL-18 (52) and to induce intestinal inflammation during inflammatory bowel disease (212) and shigellosis (200). The requirement of caspase-1 for biological activities associated with acute inflammation, coupled with the dogma that apoptosis is non-inflammatory (131), strongly suggests that caspase-1 mediates macrophage death by a novel proinflammatory and non-apoptotic mechanism.

Salmonella

Salmonella causes macrophage cytotoxicity by two distinct mechanisms: rapid cell death

mediated by the TTSS encoded on *Salmonella* Pathogenecity Island 1 (SPI1) (158), and delayed cell death induced by the SPI2 TTSS (130, 134, 233). SPI1 TTSS-induced cell death resembles that caused by *Shigella*: the IpaB homologue SipB binds to caspase-1 (86), DNA fragmentation occurs (25), and infected cells die quickly (25). SPI2 TTSS-expressing bacteria induce delayed cell death in a SipB-independent, caspase-1 (154) and caspase-2 (103)-dependent manner. Early reports concluded that SipB induces apoptosis in macrophages (25, 158), although others suggested the death mechanism more closely resembles necrosis (241). The relevance of caspase-1 for *Salmonella* infection *in vivo* has been tested: *Salmonella enterica* serovar *typhimurium* (hereafter called *Salmonella typhimurium*) fails to colonize the mucosal intestinal tissue of caspase-1-deficient mice (155), suggesting that cytotoxicity *in vivo* facilitates bacterial replication; additionally, infected, dying macrophages can be detected *in vivo* (191). However, *sipB* mutant bacteria are not attenuated for induction of inflammation in the bovine intestine (201), suggesting that SipB-independent, caspase-1 dependent mechanisms function to cause macrophage death *in vivo*. It is unknown if *Salmonella*-induced macrophage death demonstrates conventional biochemical and morphological features of apoptosis or necrosis, or if *Salmonella*-specific immune recognition (discussed below) can prevent macrophage death.

Yersinia

Yersinia spp. also infect and kill macrophages by a TTSS-dependent mechanism (149, 157, 197); macrophage death is caspase-1-independent and appears to occur by apoptosis (49, 155, 197). While *Shigella* and *Salmonella* initiate cell death during invasion/phagocytosis, *Yersinia* kills macrophages while simultaneously blocking phagocytosis and perturbing host cell signaling, all via TTSS proteins (36). One protein,

YopJ of *Y. pseudotuberculosis* (called YopP in *Y. enterocolitica*) modulates signaling pathways that control host defense and apoptosis (172), by blocking the superfamily of MAPK kinases to indirectly inhibit TNFα cytokine production and cell survival signals (173, 195). YopJ activates pro-apoptotic factors (caspases and Bid) during *Yersinia* infection of macrophages (49), suggesting the pathogen can directly engage cellular death machinery. YopJ is necessary to induce macrophage death, as *yopJ* mutant bacteria fail to kill cells (149, 157), but lipopolysaccharide potentiates *Yersinia*-induced macrophage death (196, 259), suggesting that YopJ alone may not be sufficient to induce macrophage apoptosis. A *yopJ Yersinia* mutant was severely attenuated for virulence and induction of macrophage apoptosis in mice, demonstrating the relevance of this cellular process for pathogenesis (156).

Other Bacteria

Other studies of bacteria-induced cytotoxicity are less developed, with only microbe or host factors known for any given system. Typically, microbes deliver death effectors via specialized secretion systems, target cells present DNA fragmentation, and researchers conclude that apoptosis occurs. Enteropathogenic *E. coli* initiates epithelial cell death via the TTSS protein EspF; dying cells display features of both apoptosis and necrosis (38). *Pseudomonas aeruginosa* kills epithelial cells and macrophages by a rapidly executed mechanism requiring the TTSS protein ExoU (28, 84). *Bordetella*-induced epithelial cell cytotoxicity uses an unknown TTSS protein (257) and occurs by caspase-1-independent necrosis (221). *Legionella pneumophila* utilizes Type IV secretion to kill alveolar macrophages and epithelial cells (260) by mechanism resembling classic apoptosis, indicated by the requirement for mitochondrial signaling and caspase-3 activity (70, 71, 161, 168). Induction of cytotoxocity is not limited to Gram-negative pathogens, as

Mycobacteria, *Listeria monocytogenes, Staphylococcus aureus* and *Streptococcus* can each infect and kill cells (63, 81, 114, 144, 164, 179, 193). Despite the apparent dominance of microbes that induce cytotoxicity, pathogens like *Rickettsia* (27), *Chlamydia* (61), *Ehrlichia* (254) and *Brucella* (76) can infect eukaryotic cells and inhibit apoptosis, although the molecular mechanisms remain undefined.

The function of pathogen-induced programmed cell death during *in vivo* pathogenesis is unclear. Induction of macrophage death *in vivo* could simply remove cells capable of killing bacteria, thus facilitating extracellular bacterial replication. Ensuing inflammation could recruit new cells permissive for intracellular bacterial growth to the site of infection, or facilitate bacterial dissemination. Alternatively, microbe-induced cell death could be considered a host response strategy to eliminate a niche for infection, analogous to the programmed tissue destruction utilized by plants to limit bacterial dissemination (181), or the anti-viral interferon response of mammalian cells to reduce viral cell-to-cell spread (110). Host recognition of microbial infection via Toll-like receptors can also lead to cell activation and apoptosis (4), supporting the hypothesis that microbial-induced programmed cell death functions as a host response to infection rather than a bacterial virulence strategy.

TOLL-LIKE RECEPTORS AND MICROBES

Ligands and Mechanism of Action

Toll-like receptors (TLR) recognize conserved pathogen-associated pattern molecules (PAMPs) (100). TLRs, expressed by professional phagocytes like dendritic cells and macrophages, interact with a plethora of microbial molecules to stimulate innate immune

responses (Figure 1.2). TLR-initiated signaling events at local infection sites have direct and indirect effects: activation of TLR-expressing cells, and recruitment of adaptive immune cells to infected tissue (100). TLR recognition is critical for innate antimicrobial responses, as TLR stimulation upregulates inducible nitric oxide synthase activity and cytokine production (15), and mice and humans deficient in TLR signaling are highly susceptible to bacterial infection (178, 224, 228). TLRs also enhance adaptive immune responses; TLR ligands like lipopetides and flagellin act as adjuvants to prime T cell responses to model antigens (141, 213), and the absence of TLRs severely impairs T cell responses (209).

Alternate Function of TLRs

The consequences of TLR recognition upon development of specific adaptive immune responses, as compared to the non-specific adjuvant effect, remain enigmatic. One intriguing hypothesis is that TLRs could serve as antigen-capture receptors and deliver TLR ligand-attached antigens to processing and presentation compartments, while simultaneously signaling to activate the infected cell. Supporting this hypothesis, TLR2 localizes to pathogen-containing compartments during phagocytosis (230); TLR2 exploits recognition of the microbial protein OmpA to deliver OmpA-model antigen fusion proteins to MHC Class I processing/presentation machinery and consequently increase T cell responses to model antigen *in vitro* and *in vivo* (102). Correlative evidence also exists for TLR-directed T cell responses to *Mycobacterium tuberculosis*, as mycobacterial lipoproteins are ligands for TLR2 (15) and are also natural antigens for *Mycobacteria*-specific CD4+ and CD8+ T cells (153, 163). The influence of TLR recognition upon T cell responses during natural *Salmonella* infection is unknown.

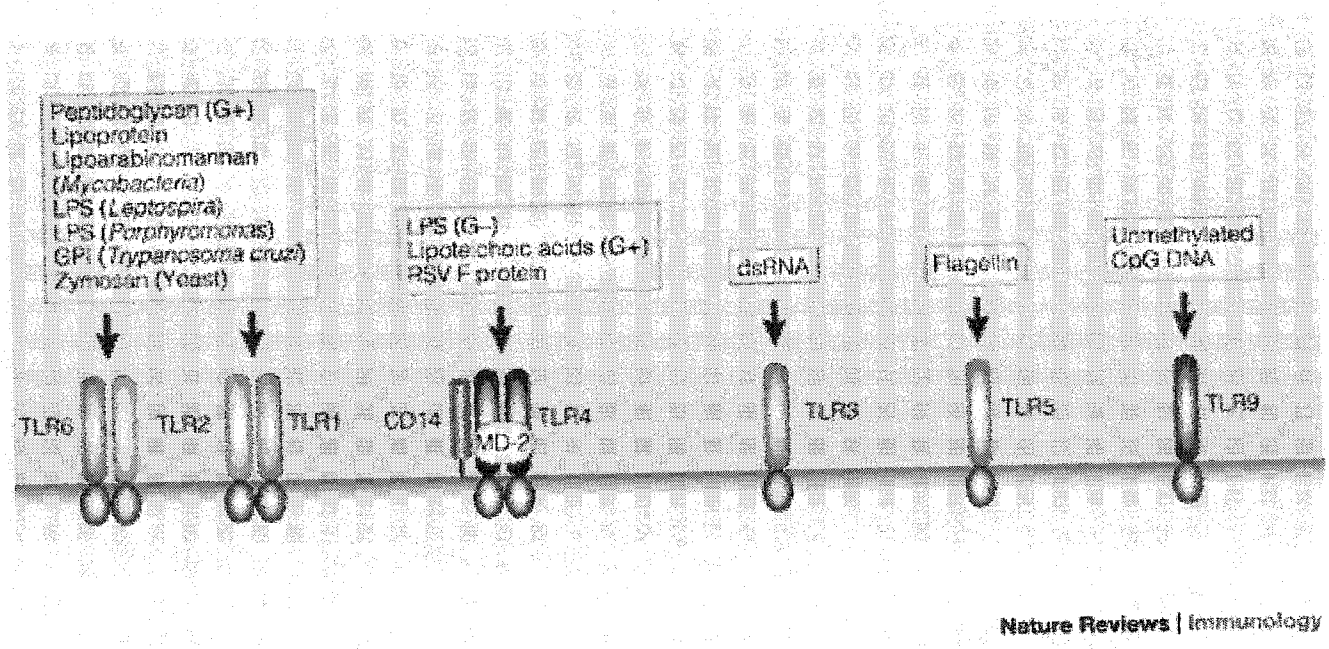


Figure 1.3 Toll-like Receptors and Ligands. This figure was reproduced from Medzhitov (142). Toll-like receptors (TLRs) recognize a variety of pathogen-associated molecular patterns (PAMPs). TLR4, in conjunction with accessory molecules, recognizes glycolipids like lipopolysaccharide. TLR2 combines with TLR1 or TLR6 to recognize a broad range of structurally unrelated ligands from Gram-positive (G+) and G-negative (G-) bacteria. TLR3 responds to double-stranded RNA (dsRNA), TLR5 is specific for bacterial flagellin; TLR9 recognizes molecules abundant in bacterial DNA, unmethylated CpG DNA.

ADAPTIVE IMMUNITY TO INTRACELLULAR PATHOGENS

General Rules

Following innate immune recognition, the host response to intracellular pathogens rests largely on T cells (188), as the intracellular niche prevents effective extracellular surveillance by B cells and neutrophils. T cells recognize pathogen-derived antigens via T cell receptor (TCR) engagement of peptide-MHC complex on the surface of infected cells (99). The compartment housing the pathogen routes antigens into a particular processing and presentation pathway, which then influences recognition of peptide-MHC by different T cell subsets (99). Hence, exogenous antigens from phagosomal pathogens like *Mycobacterium tuberculosis* are presented by MHC Class II to drive CD4+ T cell responses (112), while cytosolic pathogens such as *Listeria monocytogenes* express antigens that are processed by the cytoplasmic proteosome and presented by MHC Class I to stimulate CD8+ T cells (20). Exceptions to the one-compartment/one-subset rule do exist, as *Mycobacteria*-specific CD8+ and *Listeria*-specific CD4+ T cells are generated during natural infection (44, 111), via a mechanism called cross-priming, when specialized dendritic cells acquire microbial antigens and cross-present antigens via Class I or II (48). After naïve CD4+ or CD8+ T cells recognize the appropriate antigen-receptor complex, they become activated and express effector functions to facilitate microbe removal, and may eventually transition into memory T cells. One major effector function of activated CD4+ T cells is secretion of IFNγ cytokine, which activates macrophages and other infected cells to increase microbial killing (production of reactive oxygen/nitrogen species and microbicidal enzymes) (99). CD8+ effector T cells enable pathogen removal in several ways: induction of infected cell death by perforin delivery or Fas-FasL signals, secretion of cytokines IFNγ and TNFα to activate infected cells, and

secretion of chemokines that recruit activated cells to infection sites (99). These are general assignments of effector T cell functions; the contribution of each facet of adaptive immunity to individual pathogens will vary depending on the microbe.

Listeria *as a Model*

Our understanding of adaptive immunity to intracellular pathogens continues to grow. Of all the intracellular pathogens, *Listeria monocytogenes* has excited immunologists and microbiologists equally; hence much is known about how *Listeria* pathogenesis intersects with the host adaptive immune response (183, 251). This Gram-positive facultative intracellular pathogen infects both humans and mice and colonizes deeper tissues of liver and spleen. The intracellular life cycle begins when *Listeria* invades eukaryotic cells, escapes from the vacuole by lysis, replicates and moves through the host cell cytosol by exploiting actin-based motility, pushes through the plasma membrane into neighboring cells, and lyses the double-membrane vacuole to repeat the cycle (183). Infected host cells counter by selecting *Listeria* proteins from the eukaryotic cell cytoplasm for MHC Class I presentation to activate CD8+ T cell responses.

One listerial protein functions in both pathogen and host responses: secreted listeriolysin O (LLO), which serves as both membranolytic agent (184) and target antigen for CD8 + T cells (175). p60 is another secreted protein that stimulates a T cell response (174); both LLO and p60 contain epitopes for CD8+ T cells in mice of two different haplotypes, C57BL/6 ($H2^b$) and BALB/c ($H2^d$) (73). Antigen selection depends on extrinsic and intrinsic features of the proteins: both p60 and LLO are abundant, secreted by the bacteria, and contain destabilizing amino acids that allow rapid turnover in the host cell cytoplasm (46, 53, 184, 216); protein instability coupled with degradation by host

proteosomes generates epitope-containing peptides bound by MHC Class I (215). Conversely, few to no CD8+ T cells are primed to the listerial actin-binding protein ActA (40), likely because ActA remains attached to the bacterial surface and lacks destabilizing amino acid motifs (159). The significance of antigen localization upon generation of anti-listerial immunity was recently tested, using *Listeria* expressing a recombinant model antigen either secreted or retained within bacterial cells (210, 258). Antigen localization minimally influenced priming of antigen-specific T cells during immunization, but significantly altered recognition and protection by antigen-specific $CD8^+$ T cells during challenge, demonstrating that antigen localization does impact immune recognition of antigen. These studies also revealed that antigen processing pathways select from a larger pool of antigens during T cell priming than during situations requiring T cell recognition for protective immunity (251). Host factors can also influence which *Listeria* epitopes are selected for presentation to CD8+ T cells. *In vitro* studies of *Listeria*-infected cells determined that certain epitopes were more efficiently processed and presented than others (217, 218, 238, 239), suggesting a particular hierarchy of epitope immunodominance. Surprisingly, *in vivo* antigen presentation studies demonstrated a different epitope hierarchy (73, 218, 237), demonstrating that *Listeria*-infected cells process and present epitopes differentially *in vitro* and *in vivo*. Epitope-specific CD8+ T cells contribute to clearance of *Listeria* and protective immunity (113) by a poorly defined mechanism: *Listeria*-specific CD8+ T cells are generated in IFNγ-/-, TNFα-/-, and perforin-/- mice and protect against infection comparable to wild-type T cells (83, 245-248), demonstrating that typical effector functions like cytokine production and cytolysis of target cells are unessential. CD8+ T cell production of the chemokine MIP-1α, which induces migration of macrophages, neutrophils and natural killer cells (145), is required for protective immunity to *Listeria* (31), suggesting that the major role of CD8+

T cell recognition of *Listeria*-infected cells may be to recruit additional cells to fight the infection.

SALMONELLA INFECTION AND IMMUNITY

Salmonella: *Model Vacuolar Pathogen*

While murine infection with *Listeria monocytogenes* provides a good model system for adaptive immune responses to cytoplasmic-dwelling pathogens, our understanding of immunity to vacuolar pathogens is more limited. Enter *Salmonella*, the Gram-negative facultative intracellular pathogen that can grow in host phagosomes (104). *Salmonella typhi* infects humans to cause systemic typhoid fever; oral immunization with viable attenuated bacteria induces T and B cell responses that provide protective immunity (126). The murine model system of *Salmonella typhimurium* allows dissection of pathogenic mechanisms and analysis of protective immunity conferred by oral immunization (104). Additionally, knowledge of the host-*Salmonella* interaction can apply to studies of *Mycobacterium tuberculosis* and *Histoplasma capsulatum*, medically significant but experimentally challenging pathogens that also live in phagocyte vacuoles and induce similar immune responses as *Salmonella.*

Pathogenesis and Immunity

Hosts naturally acquire *Salmonella* via ingestion of contaminated food and water. *Salmonella* transit to and penetrate the murine gastrointestinal tract via M cells in Peyer's Patches to colonize underlying mucosal tissue (105), and from there disseminate via the bloodstream and lymphatics to the spleen and liver (22). Continued bacterial replication in infected organs results in death. Critical to successful infection is *Salmonella's* ability

to parasitize cells of the reticuloendothelial system, in particular, macrophages or other CD8+ T cells (21, 133). Accordingly, *Salmonella* mutants incapable of surviving in macrophages are severely attenuated for virulence (65). The two component regulatory system PhoP/PhoQ controls the *Salmonella*-macrophage interaction (146). Composed of sensor kinase PhoQ and response regulator PhoP, the system both activates and represses *Salmonella* gene expression in response to environmental signals (75). PhoP/PhoQ is activated within the phagosome and promotes bacterial surface modifications that engender bacterial resistance to cationic peptides and reduce cytokine production, thereby enabling microbial survival within the phagosome by inhibiting innate immune recognition (59). Our understanding of *Salmonella* biology during intramacrophage growth is facilitated by *in vitro* studies of mutant *Salmonella* strains that express the active, phosphorylated form of PhoP, which effectively locks the bacteria into the intracellular growth state (23, 78, 116, 147).

Infection of naïve susceptible mice with attenuated *Salmonella* or immune mice with virulent *Salmonella* has permitted dissection of the host response to the microbe. The innate immune response to *Salmonella* infection is mounted by macrophages (117, 225) and natural killer cells (186, 204). Highlighting the importance of innate recognition, mice deficient for lipopolysaccharide recognition (Toll-like receptor 4 deficient mice) exhibit increased susceptibility to *Salmonella* infection. Innate recognition of *Salmonella* leads to the development of adaptive immune responses; sterilizing immunity to *Salmonella* infection requires both humoral and cellular immune responses to specific *Salmonella* antigens (42, 43, 58, 90, 231). $CD4^+$ T cells are particularly important, as demonstrated by T cell depletion studies (43, 136, 165), adoptive transfer studies (137, 165), and knock-out mice (88, 244). Both B and T cell responses to *Salmonella* can be

studied using the murine model system of oral immunization with viable, attenuated *S. typhimurium*.

Salmonella-*Specific CD4+ T Cell Responses*

The microbial factors influencing CD4+ T cell responses are undefined. Does *Salmonella*-induced macrophage death affect ensuing T cell responses, or do *Salmonella*-specific T cells contribute to immunity by preventing *Salmonella*-induced cytotoxicity? Additionally, the antigenic specificity of CD4+ T cells responding to natural *Salmonella* infection is largely unknown. *Salmonella typhimurium* infection of mice stimulates antigen-specific CD4+ T cells that recognize multiple antigens (Cookson, unpublished), but the identity of only one is known: FliC, the major subunit of the bacterial flagellar apparatus (32, 140). Murine infection with *Salmonella enteritidis* generates CD4+ T cell responses specific to the fimbrial protein SefA (170), but *sefA* is absent from serovar *typhimurium* genome (229)). Identities of other *Salmonella* antigens recognized by $CD4^+$ T cells remain elusive, as are the characteristics influencing antigen selection and the relative immunodominance of different antigens. It is also unknown if *Salmonella* regulates expression of natural antigens to modify antigen-specific immune recognition.

The major goal of this dissertation was to explore the intersection between *Salmonella* pathogenesis and host response. To this end, I examined host-microbe interactions that occur at the cellular and multicellular levels. I demonstrated that *Salmonella* infection of macrophages triggers caspase-1-dependent pro-inflammatory necrosis. Study of CD4+ T cell responses to *Salmonella* showed that natural antigens localize in bacterial surface organelles with TLR-stimulatory activity. Paradoxically, *Salmonella* represses antigen expression *in vivo* via PhoP/PhoQ at both the transcriptional and posttranscriptional

levels. Finally, I demonstrated that the natural *Salmonella* antigen FliC contains multiple T cell epitopes and is preferentially expressed *in vivo* to stimulate mucosal T cell responses, such that dysregulation of FliC expression profoundly influences the T cell response to FliC. Collectively, these experiments suggest that the *Salmonella*-host relationship has evolved from a series of actions and reactions: *Salmonella* infects macrophages, host cells react with alarm-ringing pro-inflammatory death; host immune responses to *Salmonella* target specific antigens, *Salmonella* responds by repressing or modifying antigen production. The outcome of these interactions thus determines the development of disease or development of protective immunity.

CHAPTER 2: *SALMONELLA* INDUCES MACROPHAGE DEATH BY CASPASE-1-DEPENDENT NECROSIS

ABSTRACT

We provide evidence that *Salmonella typhimurium* kills phagocytes by an unusual proinflammatory mechanism of necrosis that is distinguishable from apoptosis. Infection stimulated a distinctly diffuse pattern of DNA fragmentation in macrophages, which contrasted with the marked nuclear condensation displayed by control cells undergoing chemically induced apoptosis. In apoptotic cells, DNA fragmentation and nuclear condensation result from caspase-3-mediated proteolysis; caspases also subvert necrotic cell death by cleaving and inactivating poly-ADP ribose polymerase (PARP). Caspase-3 was not activated during *Salmonella* infection, and PARP remained in its active, uncleaved state. Another hallmark of apoptosis is sustained membrane integrity during cell death, yet infected macrophages rapidly lost membrane integrity as indicated by simultaneous exposure of phosphatidylserine with the uptake of vital dye, and the release of the cytoplasmic enzyme lactate dehydrogenase. During experimentally induced necrosis, lethal ion fluxes through the plasma membrane can be prevented by exogenous glycine; similarly, glycine completely blocked *Salmonella*-induced cytotoxicity. Finally, inhibition of the IL-1-converting enzyme caspase-1 blocked the death of infected macrophages but not control cells induced to undergo apoptosis or necrosis. Thus, *Salmonella* kills macrophages by an unusual caspase-1-dependent mechanism of necrosis.

BACKGROUND

Salmonella typhimurium infection of mice and *S. typhi* infection of humans is characterized by inflammation at the site of bacterial entry and in deeper infected tissues (104). *S. typhimurium* penetrates the murine gastrointestinal tract via Peyer's Patches (105) to colonize underlying mucosal tissue, where the bacteria preferentially infect phagocytes and consequently disseminate to the spleen and liver (236). Following infection of phagocytes, *Salmonella* spp. can survive and eventually destroy these cells (65, 130). Cytotoxicity occurs both *in vitro* and *in vivo* (25, 130, 191) by a mechanism previously described as apoptosis (86, 158).

Apoptosis and necrosis are two forms of eukaryotic cell death that can be distinguished biochemically and morphologically (135). Apoptosis requires ATP and activation of specific proteases. Dying cells display membrane blebbing, nuclear condensation and eventually cell shrinkage, yet maintain membrane integrity until late in this process. Necrosis results from membrane damage either directly, or indirectly because of energy depletion, and yields swollen cells that leak their cytoplasmic contents. As a result, necrosis is strongly proinflammatory *in vivo*, while apoptotic cells are rapidly phagocytosed and thus generate minimal inflammation (135).

Because the mechanism of host cell death can influence the inflammatory response, discriminating apoptosis from necrosis may provide insight into the pathophysiology of infectious diseases caused by intracellular pathogens. The nuclear changes considered characteristic of apoptotic cells include condensation of nuclei and DNA fragmentation (138). However, the latter event was also recently observed to occur during necrosis (45,

54, 171), such that detecting DNA cleavage in cells does not necessarily identify apoptosis.

Caspases are a family of cysteine proteases important in apoptosis (29). Caspase-3 activates "executioner" caspases and cleaves other substrate proteins during the apoptotic death program (182). Although caspase-3-independent pathways of apoptosis exist, this enzyme is essential for nuclear condensation and DNA cleavage (101, 252). *Salmonella* infection provokes macrophage DNA fragmentation (25, 130, 158, 191), but it is unknown if caspase-3 is required for this event. Caspase-1, an interleukin 1-beta-converting enzyme required for *Salmonella*-induced macrophage cytotoxicity, interacts with the *Salmonella* secreted protein SipB (86). The caspase-1 protease was originally classified as a pro-apoptotic enzyme based on homology to CED-3, a protein important in *Caenorhabditis elegans* cell death (150). Although caspase-1 is required for inflammation, it has been shown to be dispensable for apoptosis (30): caspase-1-deficient mice fail to produce IL-1β and resist endotoxic shock, but demonstrate no significant defects in apoptosis (120, 127, 128).

Macrophage apoptosis can be prevented by interactions with activated $CD4^+$ T cells expressing the CD40L surface receptor and secreting macrophage-activating cytokines IFNγ and IL-12 (60, 223); therefore, $CD4^+$ T cells responding to *Salmonella* (32, 165) may potentially help infected macrophages avoid apoptosis. To begin investigating this possibility, we studied *Salmonella*-infected macrophages using methods that discriminate between apoptotic and necrotic cell death. In contrast to chemically induced apoptosis, infected macrophages fragment their DNA but do not activate caspase-3, and rapidly sustain membrane damage that releases their cytoplasmic contents. Exogenous glycine

prevents pathological ion fluxes through the plasma membranes of necrotic cells, and correspondingly blocks the death of *Salmonella*-infected macrophages. In addition, a key enzymatic substrate, poly-ADP ribose polymerase, is normally cleaved during apoptosis to avoid the stimulation of necrotic cell death, but remains in its active state in infected cells. However, unlike apoptosis or necrosis, death of infected macrophages requires caspase-1. Thus, *Salmonella*-infected macrophages are killed by an unusual caspase-1-dependent mechanism of necrosis.

MATERIALS AND METHODS

Bacterial Strains, Macrophages and Growth Conditions

Salmonella typhimurium strain SL1344 and its *prgH1::TnphoA* derivative (9) were used for all experiments. Bacteria were grown as previously described (25). Briefly, overnight cultures back-diluted 1:15 into L broth containing 0.3M sodium chloride, were grown at 37°C with shaking for 3h, washed and resuspended in cold sterile PBS, and kept on ice prior to macrophage infections. The macrophage-like cell line J774A.1 was obtained from the American Type Culture Collection. Peritoneal exudate macrophages were selected as the plastic-adherent population from C3H/HeJ mice 3 days after 1mL of Brewer's thioglycollate i.p. (32). Macrophages were cultured at 37°C in 7% $C0_2$ in Dulbecco's minimal essential medium (DMEM, Gibco BRL) supplemented with 10% FCS, 5mM HEPES, 0.2mg/mL L-glutamine, and 0.05mM β-mercaptoethanol.

Reagents

Gliotoxin (Sigma) was used at 5μM to induce macrophage apoptosis (158, 240). To deplete cells of ATP and induce necrosis, macrophages in glucose-free Ringer's buffer

were treated for 3 hrs with ionomycin (Calbiochem) and carbonylcyanide-m-chlorophenylhydrazone (Sigma) at concentrations of 5μM and 15μM, respectively (54). Caspase-1 was inhibited with acetyl-Tyr-Val-Ala-Asp-cholоromethyl ketone (ac-YVAD-cmk, Calbiochem, effective concentration 100 to 200mM) for one hour prior infection, or induction of apoptosis or necrosis. Glycine protection was tested in macrophages pretreated with 5mM glycine (Fisher Chemicals) for one hour (54, 242) prior to infection or induction of necrosis or apoptosis.

Invasion Protocol

Macrophages in supplemented DMEM were allowed to adhere and mature in vitro for 24h prior to infection. Peritoneal macrophages were cultured overnight in antibiotic-free media prior to infection. Using a previously described invasion protocol (25) bacteria were added to macrophages at the indicated multiplicity of infection (MOI) in antibiotic-free media and allowed to invade for two hours. After washing twice with HBSS, media containing 15μg/mL gentamicin was added to kill extracellular bacteria. The infected cells were allowed to incubate until timepoints specified and then evaluated experimentally. Macrophage infection was confirmed by microscopy or lysing infected macrophages and determining the number of gentamicin-protected intracellular bacteria. At an MOI of 10, multiple experiments demonstrated that 98% of macrophages were infected by SL1344 (average of 10 bacteria/macrophage), while 78% were infected by the *prgH1::TnphoA* derivative (average of 3 bacteria/macrophage).

TUNEL Staining

J774A.1 adherent to 12mm^2 glass coverslips were washed five times with PBS, fixed, permeabilized, and fluoresceinated nucleotide was incorporated enzymatically (In Situ

Death Kit, Boehringer Manheim) to label nicked DNA. Cellular fluorescence was evaluated by microscopy (40x objective) and images captured using a digital camera were equally adjusted for brightness and contrast and reduced in size for publication using Adobe Photoshop.

Flow Cytometry

Adherent J774A.1 cells were removed from 25mm^2 flasks using PBS/EDTA and pooled with any non-adherent cells in the culture media. Cells were analyzed for FITC-Annexin V and 7-aminoactinomycin D (7-AAD, Sigma) staining similarly as previously described (129). Cells washed twice in Annexin V binding buffer (10mM HEPES-Na, 133mM NaCl, 5.8mM KCl, 5mM glucose, 0.1% BSA, 2.5mM $CaCl_2$, pH 7.4) were resuspended to 1x10^7 cells/mL and 10^6 cells were incubated with 100nM AV-FITC for 15min. on ice. After two washes in binding buffer 7-AAD was added to 2μg/mL final concentration, and cells were immediately analyzed on a Coulter EPICS MCL flow cytometer.

Lactate Dehydrogenase Release

Supernatants from macrophages grown in 96-well plates in the presence of 5% FCS were evaluated for the presence of cytoplasmic enzyme lactate dehydrogenase (LDH) using the Cytotox 96 Kit (Promega). % Cytotoxicity = 100 x (Experimental LDH minus Spontaneous LDH)/ (Maximum LDH release minus Spontaneous LDH).

Caspase-3 Assays

Adherent macrophages removed from 25mm^2 flasks using PBS/EDTA and pooled with non-adherent cells from culture media were washed once in PBS/0.1% BSA, and resuspended to 10^7 cells/mL. Lysates from 10^6 cells were assayed for active caspase-3

using the substrate Aspartate-Glutamate-Valine-Aspartate-7-Amino-4-tri-Fluoromethyl Coumarin (DEVD-AFC, ApoAlert Caspase-3 Assay Kit, Clontech). AFC release was measured fluorometrically at 505nm. Fluorescent units (FU) Fold Induction was calculated as the fluorescence of experimental sample incubated with substrate divided by the fluorescence of the PBS-treated control sample incubated with substrate. Active caspase-3 could be detected in a population with a minimum of 25% apoptotic cells (data not shown).

PARP Immunoblot

Adherent J774A.1 cells were removed from 25mm^2 flasks using PBS/EDTA and pooled with non-adherent cells from culture media. Total protein from 10^6 cells washed once in PBS/0.1% BSA, were separated by 10% SDS-PAGE and PARP cleavage was assessed by Western blotting using anti-PARP monoclonal IgG1 antibody clone C-2-10, (Clontech).

RESULTS

DNA Fragmentation without Nuclear Condensation in Salmonella-*Infected Macrophages*

DNA cleavage, previously considered a hallmark of apoptotic nuclear collapse (138), can also occur during necrosis (45, 54, 171). The nicked DNA ends can be labeled using the TUNEL reaction (Terminal deoxynucleotidyl transferase-mediated dUTP-biotin Nick End-Label) and detected in cells by fluorescence microscopy. The condensed nuclear morphology typical of apoptotic cells was demonstrated by macrophages treated with gliotoxin (Figure 2.1B), a fungal toxin that induces apoptosis (240) and serves as a positive control (158) in our experiments. *Salmonella* infection also stimulated

macrophage DNA fragmentation (Figure 2.1C & D), in agreement with previous reports (25, 130, 158, 191). However, the diffuse staining of nicked chromatin in infected cells (Figure 2.1C & D) contrasts with the condensed pattern in gliotoxin treated macrophages (Figure 2.1B). Significantly, a *prgH* mutant failed to induce macrophage DNA fragmentation (Figure 2.1E & F) despite infecting the phagocytes at levels similar to the parent WT strain (see Experimental Procedures); therefore, bacterial infection per se is not sufficient to induce DNA cleavage. PrgH is an essential component of the *Salmonella* type-III secretion system (TTSS) (97, 177) encoded by *Salmonella* pathogenicity island 1 (SPI1) (148). This TTSS is responsible for exporting effector molecules (96), including the SipB protein (97, 107, 108, 177) that is required for cytotoxicity (25, 86). The SPI1 TTSS is therefore also required for *Salmonella* to induce macrophage DNA fragmentation that is morphologically distinguishable from that observed in apoptotic cells.

Salmonella *Infection of Macrophages Induces Rapid Membrane Damage*

Fluorescence microscopy is a powerful tool for discerning cellular morphology, but it does not necessarily reveal features of the entire host cell population because analyses are limited to relatively small numbers of adherent cells. To evaluate large numbers of *Salmonella*-infected macrophages, we used a flow cytometric assay that discriminates between apoptosis and necrosis: a fluorescently-labeled phospholipid-binding protein, annexin V, was used to specifically detect phosphatidylserine (PS), a membrane lipid which is normally localized to the inner leaflet of the cellular plasma membrane (234). Early apoptotic cells display PS in their outer leaflet while maintaining membrane integrity. In contrast, necrotic cells expose PS as a result of membrane damage and

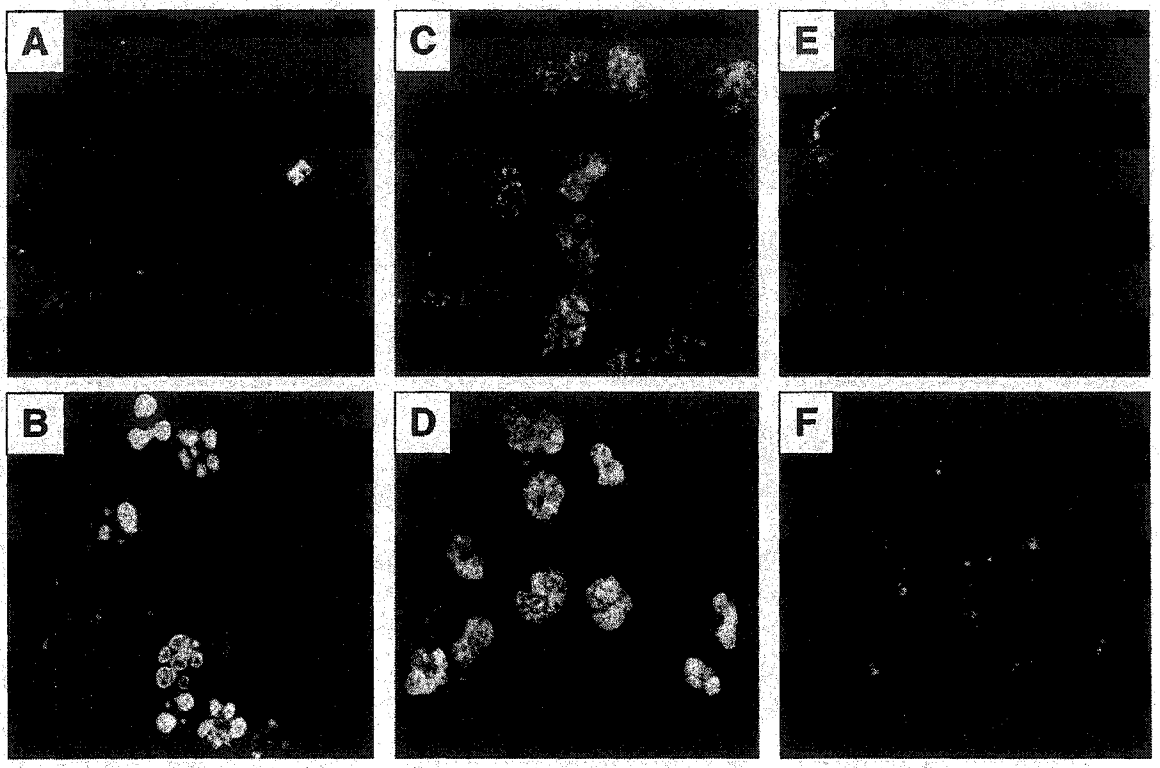


Figure 2.1 *S. typhimurium* infection stimulates macrophage DNA fragmentation without nuclear condensation. J774A.1 macrophages adherent to glass coverslips were uniformly infected with *S. typhimurium,* mock-infected, or treated with gliotoxin for 4h to induce apoptosis. Adherent cells were labeled with the TUNEL reaction, detected by fluorescence microscopy (40x objective) and imaged using a digital camera. PBS-treated cells (A), gliotoxin-treated cells (B), *S. typhimurium* SL1344 10:1 (C), SL1344 100:1 (D), SL1344 *prgH* 10:1 (E), SL1344 *prgH* 100:1 (F). A minimum of 200 cells were examined for each experimental condition in seven separate experiments; results from one representative experiment are shown.

therefore exhibit simultaneous uptake of membrane-impermeant dyes like 7-aminoactinomycin D (7-AAD) (234). Both *Salmonella* infection and gliotoxin treatment stimulated macrophage PS exposure to similar levels, as detected by staining with FITC-annexin V (Figure 2.2A). Unlike apoptotic macrophages, however, *Salmonella*-infected cells positive for annexin V-binding also stained with 7-AAD (Figure 2.2B), which indicates membrane disruption of the infected host cells. Examination of *Salmonella*-infected cells at earlier time points (1 hr, 2 hrs, & 3 hrs) following invasion did not reveal apoptotic populations (FITC-annexin V positive, 7-AAD negative, data not shown) and macrophages infected with a *prgH* mutant did not show Annexin V or 7-AAD staining (Figure 2.2).

Although membrane damage eventually typifies both apoptosis and necrosis *in vitro*, the temporal association of the stimulus initiating cell death and detectable membrane damage is distinct. Necrotic cells lose membrane integrity early after injury, while apoptotic cells delay this until later stages in their program of death. Indicative of membrane damage, infected and gliotoxin-treated macrophages released similar levels of the cytoplasmic enzyme lactate dehydrogenase (LDH) during a six-hour timecourse (Figure 2.3). Rapid LDH release by infected macrophages shows significant cytoplasmic leakage occurred within 2 hrs after bacterial invasion (Figure 2.3), and similar kinetics were observed using infected thioglycollate-elicited macrophages (data not shown). This was not simply a result of bacterial infection, as the *prgH* mutant failed to stimulate LDH release (Figure 2.3), which confirms the requirement of the SPI1 TTSS for *Salmonella* to cause macrophage death (25, 86). In contrast to infected macrophages, LDH release by apoptotic macrophages was markedly slower, and did not reach similar levels until 6 hrs

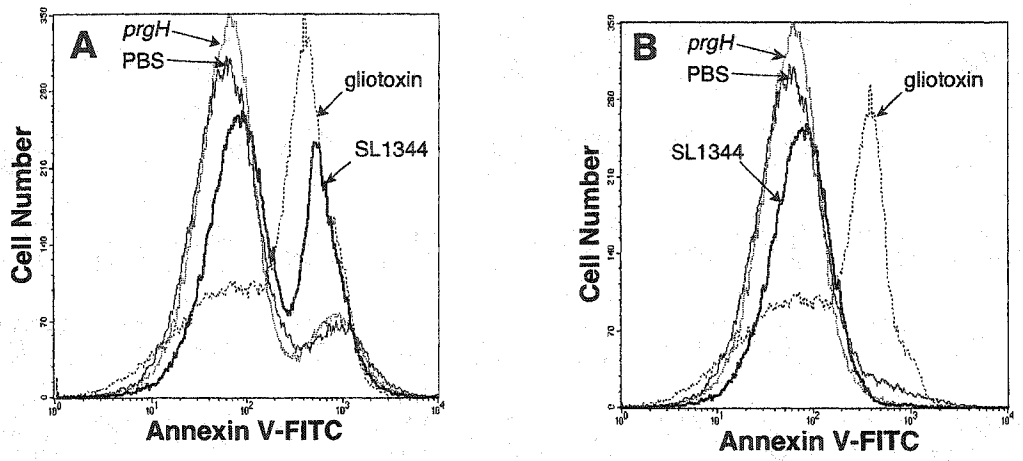


Figure 2.2 *S. typhimurium*-infected macrophages expose phosphatidylserine as a result of membrane damage. J774A.1 were infected with *S. typhimurium* strains at an MOI of 10:1, mock-infected, or treated with gliotoxin for 4h to induce apoptosis. Macrophages were then stained with phosphatidylserine-binding protein FITC-Annexin V and vital dye 7-aminoactinomycin D and immediately evaluated by flow cytometry. 20,000 cells at a minimum were evaluated per sample. A, FITC-Annexin V staining of all cells. B, FITC-Annexin V staining of cells with intact plasma membranes excluding vital dye (7-AAD negative cells). One of four representative experiments is shown.

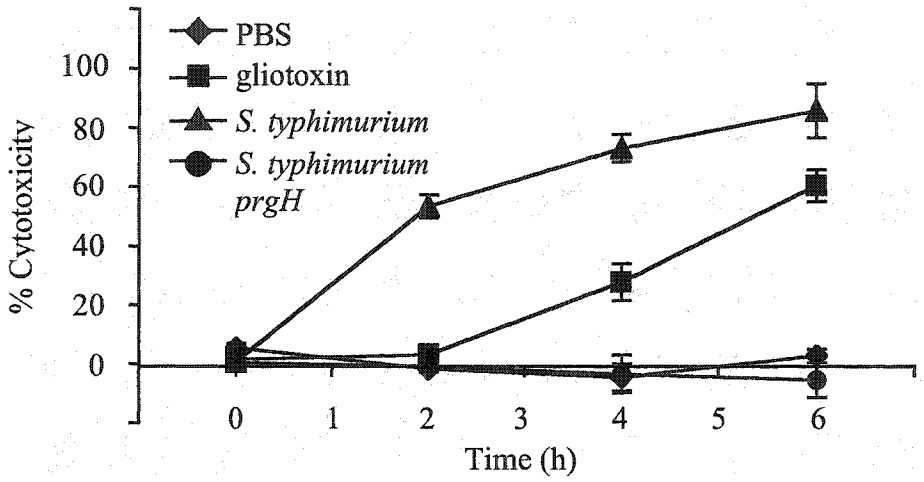


Figure 2.3. Salmonella infected macrophages rapidly lose membrane integrity. J774A.1 cells were infected with S. typhimurium strains at an MOI of 10:1, mock-infected, or treated with gliotoxin to induce apoptosis. Release of the cytoplasmic enzyme lactate dehydrogenase (LDH) was measured in supernatants from these macrophage cultures, and cytotoxicity was calculated as described in the Experimental Procedures. Each data point represents quadruplicate measurements +/- standard deviation. One of four representative experiments is shown.

after gliotoxin treatment (Figure 2.3). These observations further define cytotoxicity as a process characterized by diffusely distributed DNA fragmentation and rapid membrane damage that results in exposure of PS, uptake of vital dye, and release of cytoplasmic contents.

Host Cell Death is Independent of Apoptotic Caspases

Apoptosis and necrosis use different enzymatic pathways to execute death, and the caspase-3 protease activated during many apoptotic programs (182) is required for DNA fragmentation (101, 252). Because infection stimulated macrophage DNA cleavage (Figure 2.1), we examined the role of caspase-3 during *Salmonella*-induced cell death. In contrast to gliotoxin-treated apoptotic cells, infected macrophages did not activate caspase-3 at any time during a six-hour infection (Figure 2.4A). Similar results were obtained with infected peritoneal macrophages (data not shown). However, *Salmonella*-infected cells remained competent for caspase-3 upregulation by gliotoxin treatment (Figure 2.4B), demonstrating that infection neither inhibits activated caspase-3 nor obscures its experimental detection. This suggests that infection-stimulated DNA cleavage and cytotoxicity are caspase-3 independent events. As caspase-3 functions downstream of caspase-9, and is also required for caspase-2, -6, -8, and -10 activation during apoptosis (219), the absence of caspase-3 activation in infected macrophages strongly suggests that *Salmonella* induces cell death by a mechanism distinct from apoptosis.

During apoptosis, cleavage and inactivation of poly-ADP-ribose polymerase (PARP) by one or more caspases (194) is critical for preserving cellular ATP and therefore the energy required to complete programmed cell death (82). DNA damage, including strand

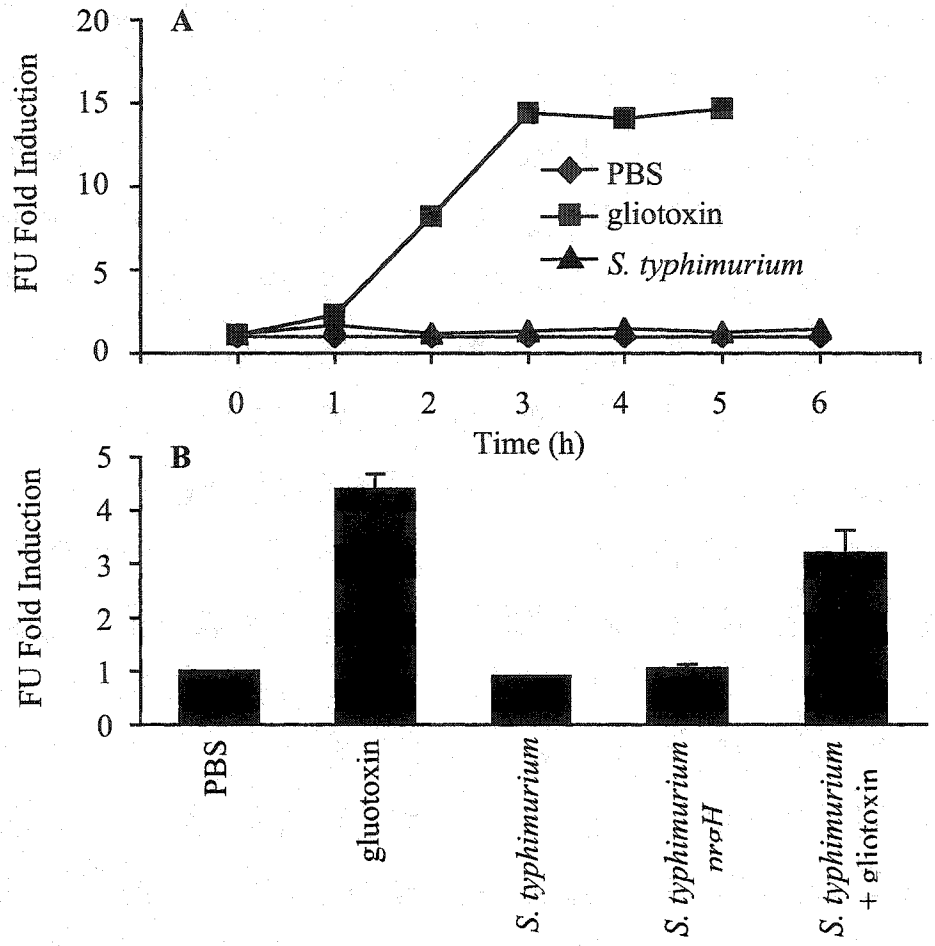


Figure 2.4 The apoptotic protease caspase-3 is not activated during *Salmonella*-induced cytotoxicity. J774A.1 macrophages were infected with *S. typhimurium* strains at an MOI of 10:1, mock-infected, or treated with gliotoxin to induce apoptosis. Active caspase-3 was detected using a fluorescent substrate as described in the Experimental Procedures. A, Time course of Caspase-3 activation. One of three representative experiments is shown. B, Infection does not inhibit macrophage caspase-3 activation by gliotoxin. J774A.1 cells infected with *S. typhimurium* at an MOI of 10:1 for two hours were washed and incubated with gentamicin-containing media, with or without gliotoxin, for an additional two hours before measuring caspase-3 activity. Data represent means ± SD from three separate experiments.

breakage, activates PARP to consume substrate NAD+ which leads to ATP depletion and cellular necrosis (82). In contrast to apoptotic macrophages, PARP remained in its active, uncleaved state in infected cells (Figure 2.5). This suggests that the apoptotic pathways leading to PARP inactivation do not function during *Salmonella*-induced macrophage death. Together with the kinetics of membrane damage and release of cytoplasmic contents, and the distribution of DNA fragmentation without detectable caspase-3 activation, the mechanism of *Salmonella*-induced cytotoxicity is most consistent with necrosis.

Salmonella-*Induced Macrophage Death is Prevented by Inhibition of either Caspase-1 or Non-Specific Ion Fluxes through the Plasma Membrane*

Necrotic cell death resulting from ATP depletion can be prevented by treatment with exogenous glycine (54), which blocks the formation of non-specific plasma membrane leaks for small ions like sodium (67). Glycine treatment also blocks necrosis induced by mitochondrial poisons, cold ischemia, and hypoxia (54, 67, 242). Our observations suggest the possibility that the attendant DNA damage of *Salmonella* infection activates PARP to deplete ATP and subsequently cause death by necrosis. Consistent with this hypothesis, glycine completely prevented cell death induced by *Salmonella* infection or ATP depletion, but negligibly affected apoptosis induced by gliotoxin (Figure 2.6).

Recent work has demonstrated that *Salmonella*-induced cytotoxicity requires active caspase-1 (86), which unlike other homologous caspases is not required for apoptosis (120, 127, 128). Pretreatment with a caspase-1 peptide inhibitor, YVAD, prevented infection-induced death of macrophages (Figure 2.6), including thioglycollate ellicited peritoneal macrophages (data not shown). This confirmed a caspase-1-dependent

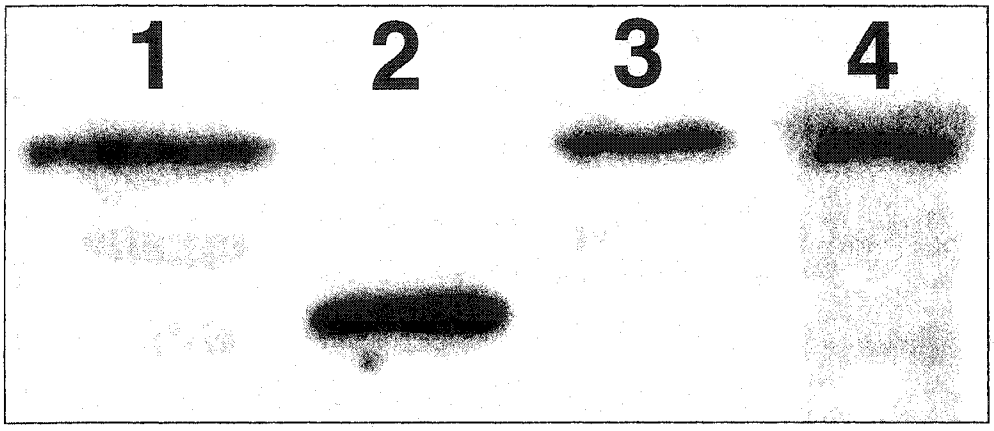


Figure 2.5 Poly-ADP-ribose polymerase (PARP), a mediator of necrotic cell death, remains in its active, uncleaved state in *Salmonella*-infected macrophages. J774A.1 were infected with *S. typhimurium* at an MOI of 10:1, mock-infected, or treated with gliotoxin for 4h to induce apoptosis. 10^6 cells were analyzed for PARP inactivation by cleavage using Western blotting with an anti-PARP monoclonal antibody. Lane 1 PBS, lane 2 gliotoxin, lane 3 *S. typhimurium* , lane 4 SL1344 *prgH*. Results from one of four representative experiments are shown.

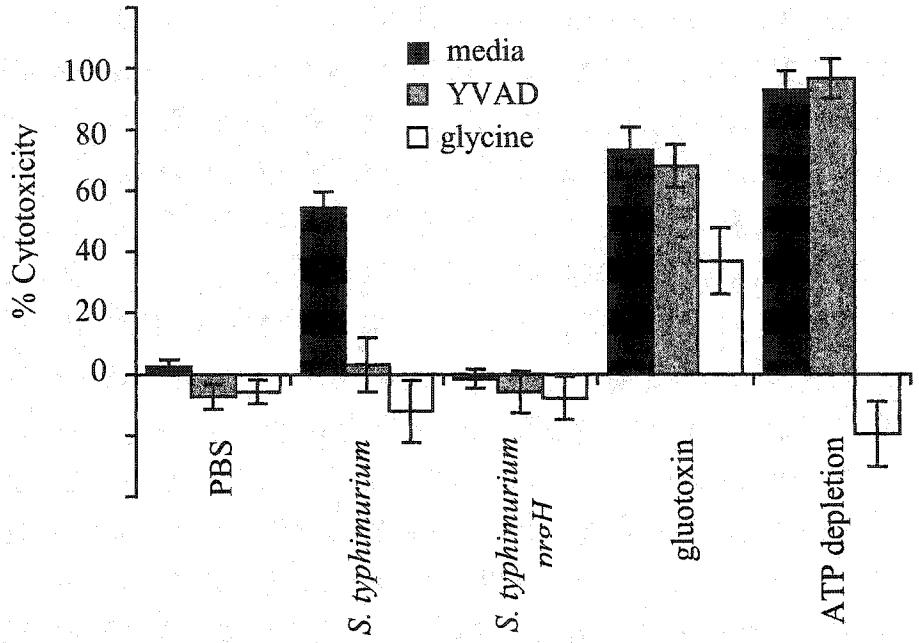


Figure 2.6 Inhibition of either non-specific ion fluxes or caspase-1 blocks *Salmonella*-induced cytotoxicity. J774A.1 cells were pretreated with 5mM glycine to prevent non-specific ion fluxes through the plasma membrane, or with 100μM YVAD, a peptide inhibitor of caspase-1. The pretreated macrophages were infected with *S. typhimurium* at an MOI of 10:1 for 2h, mock-infected, treated with gliotoxin for 4h to induce apoptosis, or induced to undergo necrosis as a result of ATP depletion as described in the Experimental Procedures. LDH released into the supernatant from one of three representative experiments is shown.

mechanism of cytotoxicity in our experimental system, which was previously observed by Hersh *et al.* using RAW 264.7 and peritoneal macrophages. Significantly, caspase-1 inhibition did not prevent experimentally induced apoptosis or necrosis (Figure 2.6). We conclude that infected macrophages release their cytoplasmic LDH and probably die as a result of non-specific leaks in their plasma membrane, which can be blocked by exogenous glycine. Unlike conventional necrosis, this process requires the IL-1-converting enzyme caspase-1, and thus *Salmonella* induces cytotoxicity by a unique mechanism.

DISCUSSION

We demonstrate that *Salmonella typhimurium* induces macrophage death via an unusual caspase-1-dependent mechanism of necrosis. The evidence supporting this conclusion are presented in Table 2.1. An intact SPI1 TTSS system, responsible for secreting SipB, was required for *Salmonella* to stimulate macrophage DNA fragmentation. Caspase-3, the protease required for DNA fragmentation during apoptosis, was not activated following *Salmonella* infection nor did infected macrophages display the condensed nuclei typical of apoptotic cells. *Salmonella*-infected macrophages demonstrated rapid loss of membrane integrity, release of cytoplasmic LDH, and uptake of vital dye concomitant with PS exposure. Infected cells retained PARP in its active uncleaved state, suggesting the possibility that PARP activation by damaged macrophage DNA caused ATP depletion and death by necrosis (82). Experimental ATP depletion causes necrotic membrane damage which is blocked by exogenous glycine (54), and glycine treatment also blocked *Salmonella*-induced cytotoxicity. Conversely, gliotoxin treatment of

Table 2.1 Comparison of apoptosis, necrosis, and *Salmonella*-induced pyrotposis

Death criteria	Apoptosis	Necrosis	*Salmonella*-induced cytotoxicity
DNA fragmentation	yes	yes	yes
nuclear condensation	yes	no	no
caspase-3 activity	yes	no	no
membrane damage	yes- late in death	yes- early in death	yes- early in death
phosphatidylserine exposure	yes	yes	yes
PARP cleavage	yes	no	no
blocked by glycine	no	yes	yes
caspase-1 activity	no	no	yes

macrophages induced *bona fide* apoptosis as demonstrated by DNA fragmentation, nuclear condensation, caspase-3 activity, PS exposure without membrane damage, and PARP proteolysis. Finally, *Salmonella*-induced cytotoxicity required active caspase-1, unlike apoptosis induced by gliotoxin or necrosis induced by ATP-depletion.

The mechanism of *Salmonella*-induced cytotoxicity was examined in a macrophage cell line and primary macrophages at several time points during infection using multiple criteria to distinguish between apoptosis and necrosis. Controls for both types of cell death were included, as were experiments with a non-cytotoxic bacterial mutant to exclude the possibility that our observations were simply the result of bacterial invasion or infection. Others have also observed features of *Salmonella*-infected macrophages which were consistent with apoptosis (25, 86, 130, 158, 191), such as DNA cleavage and caspase-1-dependent cell death; however, neither of these events are apoptosis-specific, as revealed in our investigation (Table 2.1) and supported by a growing body of evidence in the literature (45, 54, 120, 127, 128, 171). Further, it is unlikely that small populations of cells in early or late stages of apoptosis would go undetected in our experiments using sensitive methods such as flow cytometry (Figure 2.2) and western blotting for PARP cleavage (Figure 2.5). Thus, our results extend previous studies by demonstrating that infected macrophages die by a caspase-1-dependent mechanism of necrosis, a conclusion at odds with the current model of *Salmonella*-induced apoptosis.

Our data indicated the necrotic death of infected macrophages can be prevented by glycine (Figure 2.6) which blocks non-specific ion fluxes through the plasma membrane (67). We conclude that an unusual mechanism accounts for *Salmonella*-induced cytotoxicity: necrotic cell death experimentally induced by ATP-depletion was also

prevented by glycine but did not depend upon the activity of caspase-1 (Figure 2.6). Inflammation at sites of infection *in vivo* (104) may therefore result from both the production of IL-1β and IL-18 by caspase-1 and the release of cytoplasmic contents from infected, necrotic cells. Collectively these data suggest caspase-1 activation triggers non-specific plasma membrane leaks, possibly by utilizing effector pathways common to other forms of necrosis such as cold ischemia, hypoxia, mitochondrial poisons, and ATP depletion (54, 67, 242).

The requirement for other enzymes during *Salmonella*-induced macrophage death remains largely unknown. For example, are serine proteases required for infected cell death? DNA fragmentation during necrosis results from serine protease activation, in contrast to caspase (cysteine protease)-mediated DNA fragmentation during apoptosis (54). Interestingly, exogenous glycine also inhibits degradative intracellular proteolytic activity in necrotic cells during ATP depletion (51). PARP may also function during *Salmonella*-induced cytotoxicity. This polymerase acts in a polar manner during cell death: inactivation of PARP by cleavage preserves cellular energy levels and allows apoptosis to proceed, while damaged DNA activates uncleaved PARP to deplete ATP, leading to membrane damage and necrosis (82). The *Salmonella*-induced DNA damage may therefore activate PARP, resulting in ATP depletion and the formation of plasma membrane leaks that can be blocked by glycine (Figure 6). Fibroblasts from PARP-deficient mice are protected against ATP depletion and necrotic death, but remain sensitive to apoptotic stimuli (82). Thus, PARP-/- macrophages may also be resistant to *Salmonella*-induced cytotoxicity if death requires polymerase activity.

Numerous bacterial pathogens, including *Salmonella*, *Shigella*, and *Yersinia* spp. (149, 157, 158, 197, 262), have been described to induce apoptosis in eukaryotic cells (243). These pathogens utilize TTSS to kill macrophages *in vitro*: the secreted proteins SipB (*Salmonella*), IpaB (*Shigella*), YopJ (*Y. psuedotuberculosis*) and YopP (*Y. enterocolitica*) are required to induce death of infected cells (25, 149, 157, 261). Caspase-1 stimulation may be a common feature of TTSS-mediated cytotoxicity as both SipB and IpaB bind and activate caspase-1 (26, 86). These similarities suggest that *Shigella* also kills macrophages by a mechanism distinct from apoptosis: macrophages lacking the apoptosis effector proteins caspase-3, caspase-11, or p53, and macrophages overexpressing the cell death inhibitor proteins Bcl-2 or Bcl-X_L, remain susceptible to *Shigella*-induced cytotoxicity while caspase-1-deficient macrophages are resistant (89). While this manuscript was in preparation, *Salmonella* was shown to induce cytotoxicity of infected macrophages despite inhibition of caspase-3 (241). Collectively, these data and our observations suggest that these Gram negative bacteria induce cell death by a unique pro-inflammatory mechanism most closely resembling necrosis.

Infections with *Salmonella*, *Shigella*, and *Yersinia* result in macrophage death and extensive inflammation (243), and production of IL-1 and IL-18 by caspase-1 are required for host inflammatory responses (127). This suggests caspase-1-dependent cell death specifically leads to inflammation, which is central to the pathogenesis of these bacteria *in vivo*. Supporting this hypothesis, the pathophysiology of disease is altered in caspase-1 knockout mice infected with *Shigella* or *Salmonella*: the acute inflammatory response and macrophage death were reduced during *Shigella* infection (200), while *Salmonella* was unable to colonize tissues deeper than Peyer's Patches after oral infection (155). The strong correlation between bacteria-induced cytotoxicity and inflammation

supports our model of cell death, as conventional apoptosis does not stimulate an inflammatory response *in vivo* (189). This raises the question of how to describe bacterial-induced host cell death that appears inflammatory in nature. We propose that *Salmonella typhimurium* kills macrophages by an unusual mechanism: this pro-inflammatory process requiring the *Salmonella* SPI1 TTSS system and host caspase-1 ultimately gives rise to necrotic cell death resulting from pathological ion fluxes in the plasma membrane.

The influence of *Salmonella*-specific CD4+ T cell recognition upon *Salmonella*-induced macrophage death remains to be determined. Macrophage apoptosis can be prevented by interactions with activated $CD4^+$ T cells (60, 223), but no published reports exist that describe T cell-dependent attenuation of macrophage necrosis. Indeed the rapidity of *Salmonella*-induced macrophage death or other types of necrosis may preclude T cell recognition and help. As necrotic cells are immunologically potent and can induce innate and adaptive immune responses (202), we speculate that *Salmonella*-induced macrophage death may positively influence generation of *Salmonella*-specific CD4+ T cells. This hypothesis remains to be tested.

CHAPTER 3: DURING SALMONELLA INFECTION, HOST CD4+ T CELLS RECOGNIZE NATURAL ANTIGENS EXPRESSED IN BACTERIAL SURFACE ORGANELLES WITH TLR-STIMULATORY ACTIVITY

ABSTRACT

A better understanding of immunity to infection is revealed by the characteristics of the microbial antigens recognized by the host immune responses. We show that murine infection with the intracellular bacterium *Salmonella* generates CD4+ T cells that specifically recognize *Salmonella* proteins that are expressed in unique bacterial surface organelles. *Salmonella* antigens are ligands for Toll-like receptors (TLR) or avidly associated with the TLR ligands lipopolysaccharide (LPS) and lipoprotein. During intracellular bacterial growth, production of surface-exposed antigens is coordinately repressed by PhoP/PhoQ, the regulatory system that also regulates *Salmonella* virulence and remodeling of LPS to resist innate immunity. These data suggest that TLR recognition influences the antigenic specificity of CD4+ T cells responding to infection and that genetically coordinated surface modifications provide a growth advantage for *Salmonella* in host tissues by limiting both innate and adaptive immune recognition.

BACKGROUND

A successful defense against pathogenic microorganisms requires appropriate coordination between innate and adaptive immune responses. Initial recognition of microbes by dendritic cells, neutrophils and macrophages via Toll-like receptors (TLRs) leads to recruitment and activation of B and T lymphocytes, resulting in sterilization of infected tissue and long-lasting immunological memory (142). TLRs bind conserved microbial components (100), while the B and T cell receptors identify pathogen-specific molecules. Although distinct in temporal and functional aspects of host immunity, the connections between innate and adaptive immune responses are emerging, as recent work has identified signal transduction pathways required for both immune systems (209).

Murine infection with *Salmonella typhimurium* causes a typhoid-like disease where bacteria replicate in the intracellular vacuoles of professional phagocytes (104, 133). Salmonellae express classical inflammatory molecules like lipopolysaccharide (LPS) and lipoproteins in their outer membranes but resist innate immune recognition by modifying the bacterial envelope through processes controlled by the PhoP/PhoQ regulatory system (59). PhoP/PhoQ is required for *Salmonella* virulence in infected hosts: PhoP-null bacteria fail to cause disease in susceptible hosts, but instead induce protective immunity (64, 69, 91, 146, 235). CD4+ T cell responses are an essential component of immunity to *Salmonella* (137) and the major subunit protein of bacterial flagella, FliC, is a dominant antigen recognized by CD4+ T cells from both *Salmonella*-infected mice and humans (32, 140, 226). The innate immune response also recognizes FliC via TLR5 (85, 220).

We hypothesized that other natural *Salmonella* antigens recognized by CD4+ T cells are also localized in bacterial surface organelles (complex multi-component surface structures produced by multi-gene pathways). We identified secreted membrane vesicles as a unique source of natural antigens, and determined that FljB, the alternate flagellin molecule expressed by *Salmonella*, also stimulated CD4+ T cell responses. Natural antigens directly stimulated Toll-like receptors (TLRs) or were intimately associated with TLR ligands, suggesting that innate recognition biases T cell responses to *Salmonella*. Correspondingly, *Salmonella* repressed production of natural antigens inside macrophages via PhoP/PhoQ, the virulence regulon that controls bacterial resistance to innate immunity. These results suggest that coordinate regulation of antigen expression and bacterial membrane modifications contributes to *Salmonella* virulence, and supports the notion that microbial pathogenic strategies have co-evolved with the host immune system.

MATERIALS AND METHODS

Bacteria

Salmonella typhimurium strain SL3261 (SL1344 Δ*aroA*) (93) was used for oral immunization of mice. For antigen preparations, *S. typhimurium* strains ST14028 (ATCC), ST14028 *pho-24* (PhoP^c), ST14028 *phoP102*::Tn*10dCam* (PhoP-null) (116, 146, 147), ST14028 *phoP* phoQ*::Tn*10* (23) were obtained; ST14028 *fliC*^{i}::Tn*10* (BC118) and ST14028 Δ*fliC* Δ*fljB* (BC698) were constructed by generalized transduction via P22 phage (32). GFP reporter plasmids were constructed by cloning *gfp* (35) downstream of the *fliC* or *trc* promoters on plasmids with a pMB1 replicon. Organisms

were grown in LB or TSB (BD Diagnostic Systems, Sparks, MD); sodium acetate was added to 1% for growth of ST14028 *phoP* phoQ*::Tn*10*.

Mice and Immunizations

6-8 week-old female C3H/HeJ mice (Jackson Laboratory, Bar Harbor, ME) were used for immunization and splenocyte antigen-presenting cells (APC). Mice were immunized by oral gavage with 10^9 viable SL3261 bacteria (feeding needle no. 7920, Popper & Sons, Inc., New Hyde Park, NY).

Generation of Salmonella-*Specific T cells*

Mice immunized 90d prior were sacrificed, spleens removed and purified splenic lymphocytes stimulated with APC and *Salmonella* antigen to generate *Salmonella*-immune CD4+ T cell lines as previously described (32). T cells were grown in RPMI 1640 supplemented with L-glutamine, 50μM 2-ME, 10% FCS, with penicillin, streptomycin, and gentamicin. Antigen-specific T cell clones were isolated by limiting dilution, were protein-specific, CD4+ and restricted to MHC molecule A^k or E^k, as determined by 1) proliferative responses to antigen and A^k-expressing splenocyte APC from B10.4R mice (Jackson Laboratory, Bar Harbor, ME) or inhibition of proliferative responses to antigen and APC in the presence of blocking anti-E^k antibody (clone 14-4-4S, BD Biosciences Pharmingen, San Diego, CA) (data not shown). T cell responses to *Salmonella* antigens were not detected using purified splenic lymphocytes from naïve C3H/HeJ mice (data not shown).

Generation of Salmonella *Antigens*

Heat-killed *Salmonella* (HKST) were prepared from bacteria grown to stationary phase and incubated at 65°C for 1h. SDS-PAGE fractionated *Salmonella* was prepared from ST14028 bacteria separated on SDS-16.5% PAGE (205) as described (32); briefly, protein was eluted from gel sections, polyacrylamide removed by filtration (Spin-X, Corning Inc, Corning NY) and SDS-PAGE buffer replaced with PBS by diafiltration through Microcon 10,000 MWCO filtration units (Millipore, Bedford, MA). Proteinase K -treated *Salmonella* were obtained by treating whole bacteria with 0.1mg/mL proteinase K (Boehringer Mannheim Corp, Indianapolis, IN) at 37C° for 24h followed by enzyme inactivation at 65°C for 15 minutes; addition of 0.2% SDS during proteinase K treatment dissolved bacterial membranes, allowing complete proteolysis. To purify FliC and FljB flagellins, flagella from logarithmic phase bacteria expressing only FljB or FliC (32) were sheared off by blending (222) (Waring, East Windsor, NJ), depolymerized at 60°C for 20 minutes and passed through a Centricon 100,000 MWCO filtration unit (Millipore, Bedford, MA) to remove contaminating lipopolysaccharide. *In vivo*-expressed *Salmonella* antigens were generated by murine infection with virulent bacteria followed by isolation of total splenocytes (containing 0.6-4 intracellular bacteria/splenocyte); infected splenocytes were immediately used as APC for *Salmonella*-specific CD4+ T cells.

Membrane Vesicles and Size-exclusion Chromatography

ST14028 bacteria were grown in TSB to logarithmic phase and organisms removed from the culture by centrifugation. Culture supernatant was filtered through 0.22μm filter units (Corning, Inc, Corning, NY) and further concentrated by diafiltration through CentriconPlus 100,000 MWCO filtration units (Millipore, Bedford, MA), yielding

membrane vesicle preparations. Size-exclusion chromatography of membrane vesicles was performed with Sephacryl S-500 resin (Amersham Biosciences, Piscataway, NJ).

Proliferation Assays

T cell proliferation in response to APC plus antigen was assayed as previously described (32). Briefly, 10^5 T cells and 10^6 irradiated splenocytes plus antigen were combined in triplicate, (^{3}H)TdR added after 48h, DNA harvested after 16h, and incorporated ^{3}H measured using liquid scintillation spectrophotometry.

Immunoblots

Salmonella antigen preparations were separated by SDS-10%PAGE, transferred to nitrocellulose, probed with polyclonal sera specific to SecA, OmpA (kind gifts from Drs. Tina Guina and Samuel I. Miller), LPS (BD Diagnostic Systems, Sparks, MD) or FliC (Denka Seiken, Tokyo, Japan), followed by goat anti-rabbit IgG conjugated to horseradish peroxidase (HRP) (Santa Cruz Biotechnology, Santa Cruz, CA). Reactive HRP was detected by enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ).

Electron Microscopy

Membrane vesicles were negatively stained with 1% phosphotungstic acid (pH 7.0) and applied directly to 0.5% formvar-coated 300 mesh copper grids. Samples were observed with a JEM-1200EXII transmission electron microscope (JEOL). Micrographs were taken at an accelerating voltage of 80 kV.

Infection and Immunofluorescence Microscopy of Macrophages

RAW 264.7 macrophage-like cells on 12mm^2 cover slips were infected with stationary-phase *Salmonella* at a multiplicity of infection of 100:1, incubated for 45 minutes, washed, and media containing 15μg/mL gentamicin added. After 5h, media was removed, and cells were washed, fixed with 10% formalin, permeabilized with PBS containing 0.25% Triton X-100, washed, and incubated with polyclonal rabbit sera anti-O-antigen (BD Diagnostic Systems, Sparks, MD), followed by goat anti-rabbit-biotin and avidin-Texas Red (BD Biosciences Pharmingen, San Diego, CA). GFP and Texas Red fluorescence was visualized with a BioRad 1024 Scanning Confocal Microscope and images captured using a digital camera.

TLR2- and TLR5-dependent NF-κB activation

CHO K1 cells (ATCC, number CRL-9618) were grown in Hams F-12 medium supplemented with 10% FBS, L-glutamine, penicillin and streptomycin. CHO K1 cells were transfected by electroporation with 8μg of either murine TLR2 or TLR5 cloned into to the pEF6 V5/His TOPO expression vector (Invitrogen), 1μg of an NF-κB-dependent firefly luciferase reporter (ELAM 1 Luc) (208), and 0.1μg of a control *Renilla* luciferase reporter (pRL-TK, Promega) plasmids. Stable cell lines were selected with blasticidin (Calbiochem), and cloned by limiting dilution. Individual clones that demonstrated TLR2-specific recognition of synthetic bacterial lipopeptide, PAM_3CSK_4 (Roche Biochemicals), or TLR5-specific recognition of purified bacterial flagellin (85) were chosen, and used for the assays reported in this study. Clones were plated at approximately 2 x 10^4 cells per well in 96 well plates, and, after 48 hours, stimulated with bacterial products for 5 h at 37 °C 5% CO_2. Firefly and *Renilla* luciferase activities were measured using the Dual Luciferase Assay System (Promega). Luciferase activity was

calculated as a ratio of NF-κB-dependent ELAM firefly luciferase activity divided by control thymidine kinase *Renilla* luciferase activity (relative luciferase units).

RESULTS

Salmonella-*Specific T Cells from Immune Mice*

We orally infected mice (32) with *S. typhimurium* strain SL3261 (93) and generated *Salmonella*-specific CD4+ T cell lines (see Methods). SDS-PAGE-fractionated bacteria were used as a source of stimulatory antigen in proliferation assays, and the responses of the *Salmonella*-specific T cell line MAR.D demonstrated recognition of proteins of varying molecular weight (Figure 3.1). As previously described, FliC was an important stimulatory antigen for CD4+ T cells from infected mice (32, 140), and the second largest response was to an approximately 10kDa-protein (p10) (32) (Figure 3.1). p10-specific CD4+ T cell clones from a *Salmonella*-specific CD4+ T cell line (see methods) only responded to SDS-PAGE fraction 10 (Figure 3.1, T cell clone F4, one of 32 representative clones), recognized antigen in the context of the class II MHC molecule E^k, and were no longer stimulated by *Salmonella* antigen treated with proteinase K in the presence of detergent (see below).

Salmonella *Antigen p10 Localizes to Unique Membrane Vesicles*

The natural *Salmonella* antigen FliC is exported and polymerized on the bacterial surface as the flagellar appendage (2). We hypothesized that other natural antigens are also secreted proteins, and determined that bacteria-free supernatant from exponentially-growing *Salmonella* cultures contained stimulatory activity (data not shown). Interestingly, stimulatory antigen in supernatant was completely retained by diafiltration

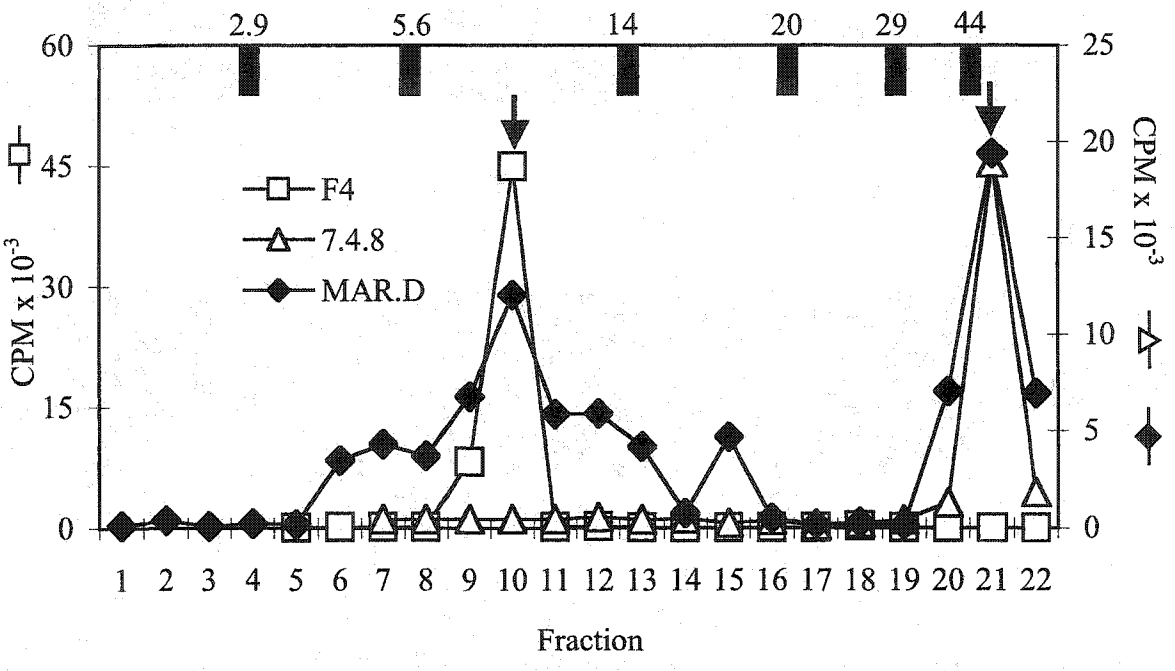
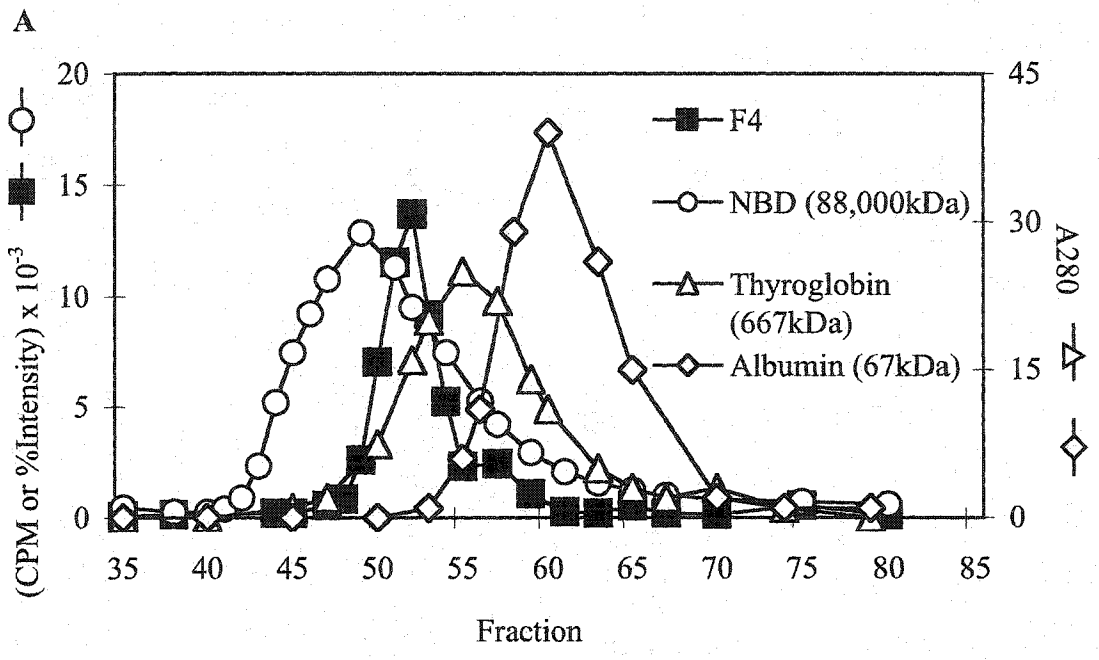
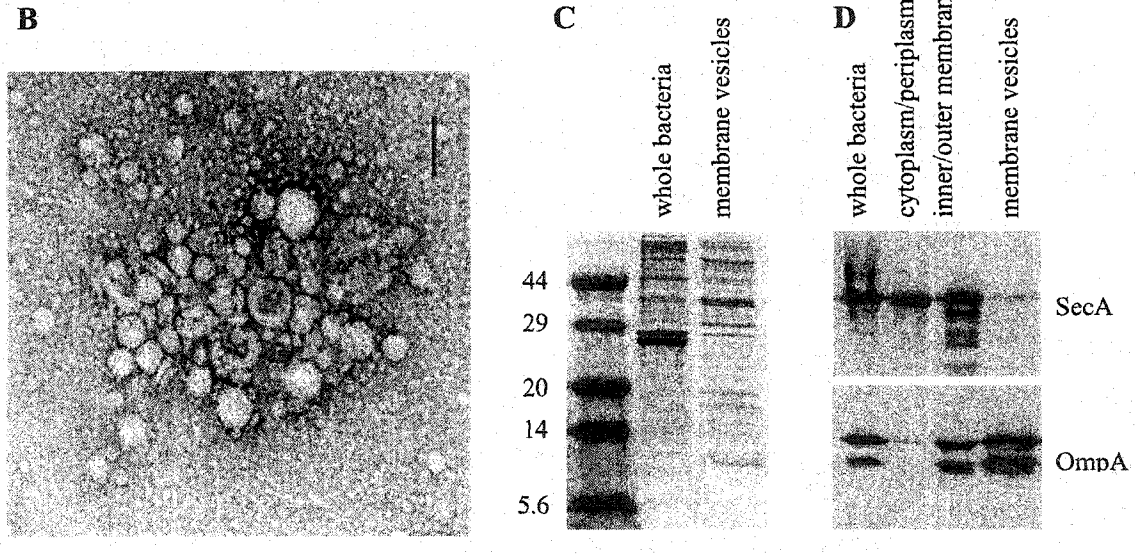


Figure 3.1 *Salmonella*-specific CD4+ T cells from orally immunized mice recognize bacterial antigens. The *Salmonella*-specific T cell line derived from immune mice (MAR.D) and p10-specific (F4) and the FliC-specific (7.4.8) CD4+ T cell clones were assayed for proliferative responses to SDS-PAGE-fractionated *Salmonella* (see Methods). Bars indicate molecular weight (x1000) of protein standards; arrows indicate responses to p10 and FliC (approximately 52,000Da). Data are representative of four experiments.

Figure 3.2 The natural antigen p10 is expressed in secreted membrane vesicles. Concentrated *S. typhimurium* culture supernatant was (A) separated by size exclusion chromatography and fractions were used as stimulatory antigen in proliferation assays with the p10-specific CD4+ T cell clone F4 (molecular weight standards: fluorescein-labeled NBD vesicles (% fluorescence intensity), thyroglobulin and albumin (A280)), and (B) examined by electron microscopy (bar = 100nm). Vesicles were compared to whole bacteria by (C) SDS-PAGE with Coomassie staining, and by (D) Western blot for the presence of SecA, a bacterial cytoplasm/inner membrane protein, and OmpA, an outer membrane protein. Isolated subcellular bacterial fractions containing cytoplasm/ periplasm and inner/outer membrane proteins are provided for comparison. Data are representative of three experiments.


A
20
15
10
5
0
45
30
15
0
F4
NBD (88,000kDa)
Thyroglobin (667kDa)
Albumin (67kDa)
A280
(CPM or %Intensity) x 10^{-3}
35 40 45 50 55 60 65 70 75 80 85
Fraction


B
C
whole bacteria
membrane vesicles
44
29
20
14
5.6
D
whole bacteria
cytoplasm/periplasm
inner/outer membranes
membrane vesicles
SecA
OmpA

membranes with high molecular weight cut-offs (e.g. 100,000) and size-exclusion chromatography identified native p10 in culture supernatants as a single species with an apparent molecular weight between 700,000 and 88,000,000 (Figure 3.2A). Electron microscopy of concentrated supernatant revealed membrane vesicles 100 to 200 nm in diameter (Figure 3.2B), that were clearly distinguishable from bacteria in size (Salmonellae are 0.5-1 x 2μm (167)) and in protein content. Vesicles displayed a distinct subset of proteins (Figure 3.2C) and lacked the cytoplasmic/inner membrane protein SecA (Figure 3.2D). Membrane vesicles are organelles that emanate from the surface of numerous bacteria (12); vesicles from Gram-negative bacteria like *Salmonella* often include components of the outer membrane, including LPS (12). In agreement with this, *Salmonella* membrane vesicles also contained the outer membrane protein OmpA (Figure 3.2D). Together these data exclude the possibility that vesicles are lysed bacteria, because vesicles are produced by growing *Salmonella* and have a unique protein composition. Like flagella, the stimulatory antigen p10 is also surface-exposed on the bacterial cell, as proteinase K treatment did not destroy stimulatory antigen (Figure 3.3A), but converted it to a 5kDa species (Figure 3.3A). p10 avidly associated with LPS, and resisted extraction from membrane vesicles by phenol (6) and urea (95) (data not shown), yet was completely degraded by proteinase K treatment in the presence of detergent (Figure 3.3B). Thus, membrane vesicles are a unique source of natural antigens for *Salmonella*-specific CD4+ T cells. Significantly, flagella and membrane vesicles are conserved structures expressed by most Gram-negative bacteria (12, 152), and vesicle production is triggered by *in vivo* growth of diverse intracellular pathogens, including *Salmonella* (72) and *Mycobacterium tuberculosis* (8, 169, 206).

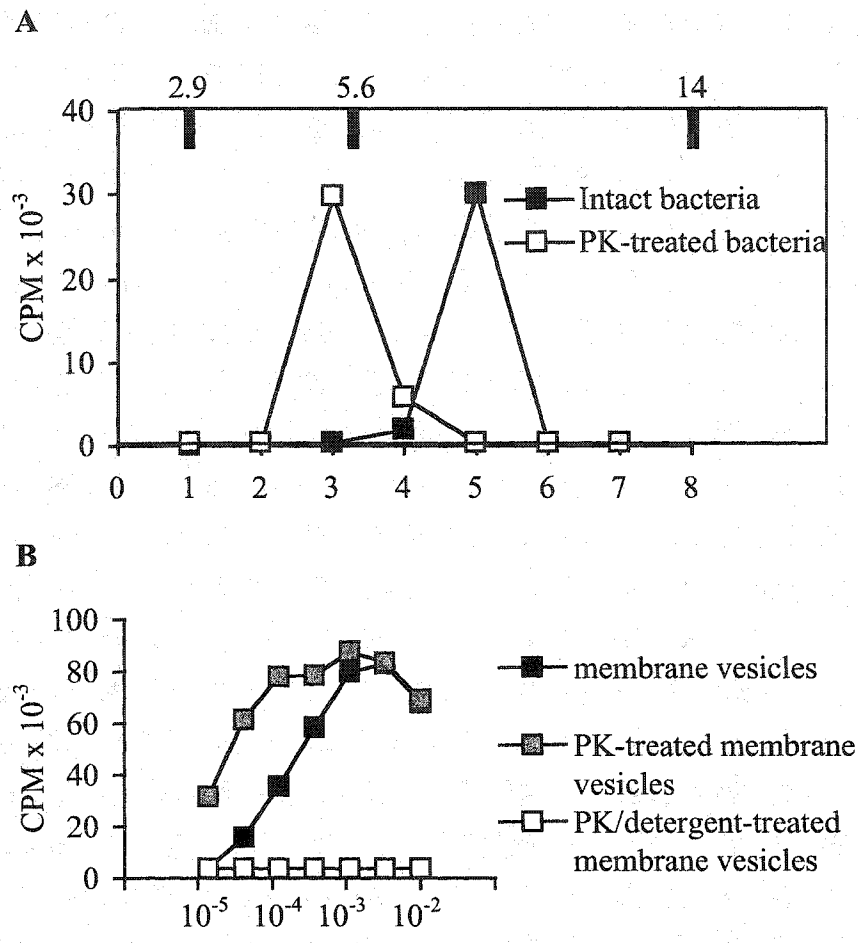


Figure 3.3 p10 is a surface-exposed antigen in bacterial membranes and membrane vesicles. Proliferative responses of the p10-specific CD4+ T cell clone F4 were used to assay for the presence of p10 in (A) SDS-PAGE fractions derived from *Salmonella* bacteria and bacteria pretreated with proteinase K, and in (B) membrane vesicles treated with or without proteinase K, in the presence or absence of detergent (enzyme inactivated by heat treatment and detergent removed by diafiltration prior to assay). Data are representative of three experiments.

Salmonella *Surface Organelles Contain Numerous Natural Antigens Stimulatory for Innate and Adaptive Immune Responses*

Conserved surface organelles like flagella and membrane vesicles are made by many bacteria (12, 152) and contain the TLR ligands FliC and LPS, respectively (85, 180). We hypothesized that other natural *Salmonella* antigens are contained in bacterial surface organelles that stimulate TLRs. To address this hypothesis, we used FliC-negative *Salmonella* to examine the antigenic specificity of CD4+ T cells in the absence of the dominant antigen FliC. 50% (12 of 24) of CD4+ T cell clones derived from *Salmonella*-infected mice proliferated in response to purified FljB (Figure 3.4A), the alternate flagellar subunit protein that can be expressed by *S. typhimurium* (2). The innate immune system also recognized FljB, and compared with the known ligand FliC (85), purified FljB stimulated similar levels of TLR5-dependent NF-κB activation in CHO cells (Figure 3.4C).

The remaining CD4+ T cell clones (n=12) proliferated in response to proteins expressed in membrane vesicles (Figure 3.4B). These clones recognized vesicle antigens with apparent molecular weights of 10, 12, and 18 KDa, and individual clones only responded to single fractions from SDS-fractionated bacteria (data not shown). Regardless of the identity of these particular *Salmonella* proteins, important evidence that membrane vesicles are a rich source of natural antigens for the adaptive immune response was obtained using splenocytes from infected mice as antigen-presenting cells (APC) directly *ex vivo* (Methods). Using these APC to stimulate splenic CD4+ T cells from immunized mice, 73% (41 of 56) of *Salmonella*-specific CD4+ T cell clones responded to vesicle proteins (Cummings and Cookson, in preparation). Correspondingly, *Salmonella* vesicles activated TLR2-dependent NF-κB transcription in CHO cells at levels comparable to

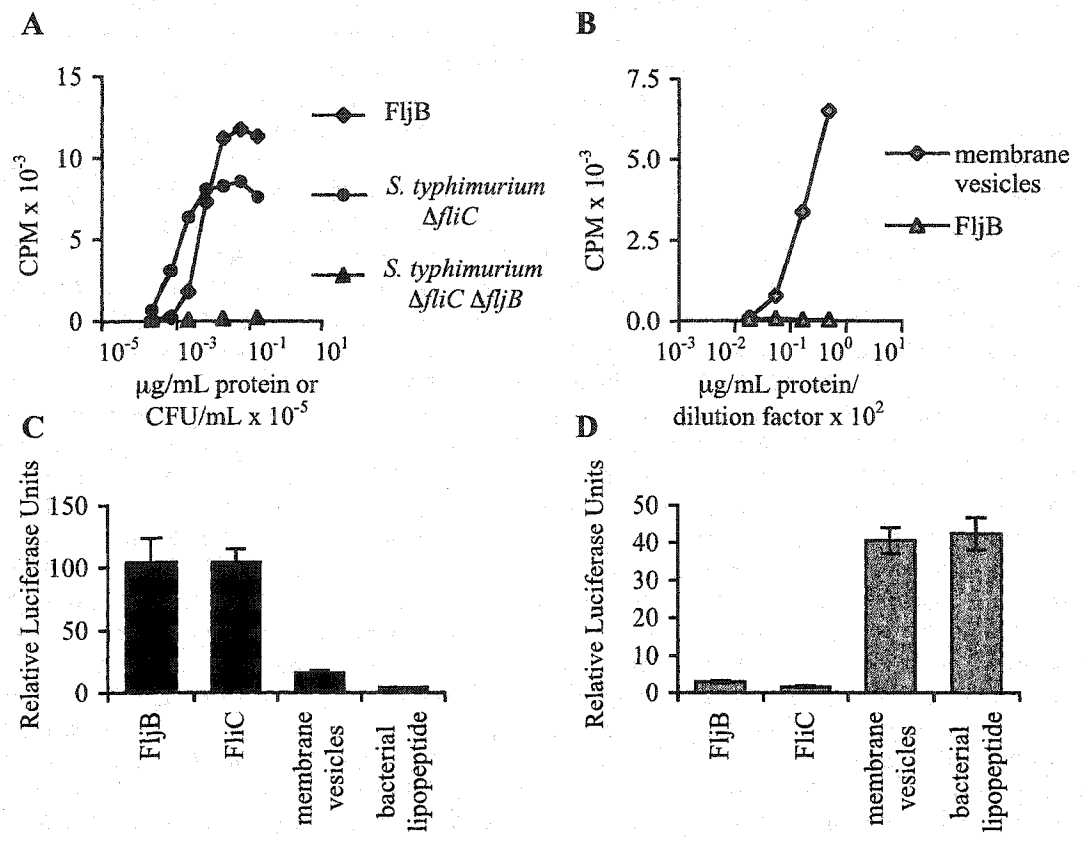


Figure 3.4 Antigens recognized by *Salmonella*-specific CD4+ T cells intimately associate with TLR ligands. CD4+ T cells, derived from *Salmonella*-infected mice in the absence of the dominant FliC Ag, recognize either the (A) alternate flagellin molecule FljB (n=12), or (B) membrane vesicles (n=12). Proliferative responses of representative T cell clones were assayed in the presence of purified FljB protein (μg/ml), FliC-null bacteria (CFU/ml), FliC-null FljB-null bacteria, or purified membrane vesicles as Ag. CHO cells transiently transfected with expression vectors for TLR5 (C) or TLR2 (D) together with an NFκB luciferase reporter, were used to assess the ability of purified FljB and membrane vesicles to serve as TLR ligands. Data are representative of three experiments.

control synthetic bacterial lipopeptide (Figure 3.4D), indicating that membrane vesicles contain TLR2 ligands like lipoproteins (4, 15), which are abundant in the bacterial envelope (98). *Salmonella* vesicles also contain the TLR4 ligand lipopolysaccharide (12, 180). Taken together, these results demonstrate that natural antigens recognized by *Salmonella*-specific CD4+ T cells are TLR5 ligands (FliC or FljB flagellin) or are intimately associated with TLR-2 and -4 ligands (lipoprotein and LPS) in membrane vesicles. This is the first demonstration of coordinated innate and adaptive immune recognition of specific bacterial antigens in a natural infection model system.

PhoP/PhoQ Controls Antigen Expression In Vivo

The *Salmonella* two-component regulatory system PhoP/PhoQ, composed of the sensor kinase PhoQ and the response regulator PhoP, both activates and represses bacterial gene expression in response to environmental signals (75). PhoP/PhoQ regulates virulence and increases resistance to innate immunity (59, 75). PhoP/PhoQ activation is responsible for bacterial envelope modifications that promote resistance to cationic anti-microbial peptides (64, 77, 80) and render LPS less immunostimulatory (79). Considering that PhoP/PhoQ can control bacterial surface modifications, and that bacterial surface organelles contain proteins recognized by T cells, we investigated the influence of PhoP/PhoQ on production of the natural antigens FliC and p10. Strains of *Salmonella* that constitutively express the active phosphorylated form of PhoP ($PhoP^c$ and PhoP*) (23, 78, 116, 147) provide a genetic means to examine antigen production by bacteria during PhoP/PhoQ activation. T cell proliferation assays indicate >100-fold reduction in p10 (Figure 3.5A) and >1000-fold decrease in FliC antigens (data not shown) by $PhoP^c$ and PhoP* *Salmonella* relative to PhoP-wildtype and PhoP-null strains. These data are confirmed by Western analysis of the same strains for FliC production (Figure 3.5B) as

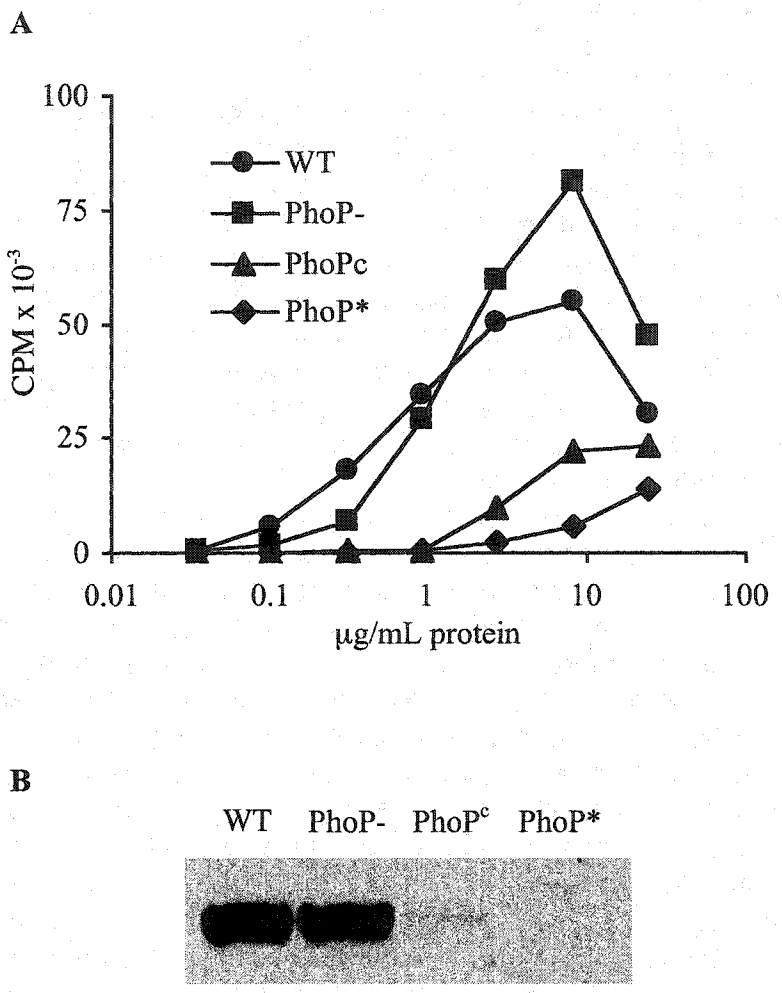


Figure 3.5 The two component regulatory system PhoP/PhoQ negatively regulates expression of natural *Salmonella* antigens p10 and FliC. Wild type *Salmonella* were compared with PhoP-null and PhoP-activated (PhoP^c, PhoP*) strains for expression of (A) p10 antigen by proliferation of the CD4+ T cell clone F4, and (B) FliC by Western blot analysis. Two of six representative experiments are shown.

well as other studies (1), which collectively exclude the possibility that altered antigen uptake, processing, or presentation accounts for the dramatic reduction of FliC-specific T cell proliferation in response to PhoP^c or PhoP* bacteria. Thus, active PhoP/PhoQ coordinately triggers surface modifications that help Salmonellae resist innate immune functions (59) and down-regulates production of antigens recognized by CD4+ T cells. This is the first demonstration that PhoP/PhoQ controls expression of ligands stimulatory for the adaptive immune system.

Because maximal PhoP/PhoQ activation occurs during bacterial growth in macrophage phagosomes (5), the above data predicts PhoP/PhoQ-dependent repression of FliC antigen production by *Salmonella* during intracellular growth. To test this hypothesis, GFP fluorescence was used to report transcription from the *fliC* promoter (P*fliC::gfp*) during *Salmonella* infection of macrophages. Bacteria grown *in vitro* displayed equivalent fluorescence from either P*fliC::gfp* or P*trc::gfp* (constitutive promoter) (Figure 3.6A-C). Wild-type *Salmonella* inside macrophages (Figure 3.6D,E,G,H) repressed transcription of P*fliC::gfp* (Figure 3.5E) while P*trc::gfp* was constitutively expressed (Figure 3.6D). In contrast, intracellular PhoP-null *Salmonella* (Figure 3.5I) failed to repress P*fliC::gfp* (Figure3.6F). Thus, PhoP/PhoQ-dependent repression of natural antigen production occurs *in vivo*.

DISCUSSION

During infection with *Salmonella*, we have shown that T cell responses are generated to natural antigens expressed in the bacterial surface organelles of flagella and in unique membrane vesicles. Antigens recognized by *Salmonella*-specific T cells directly

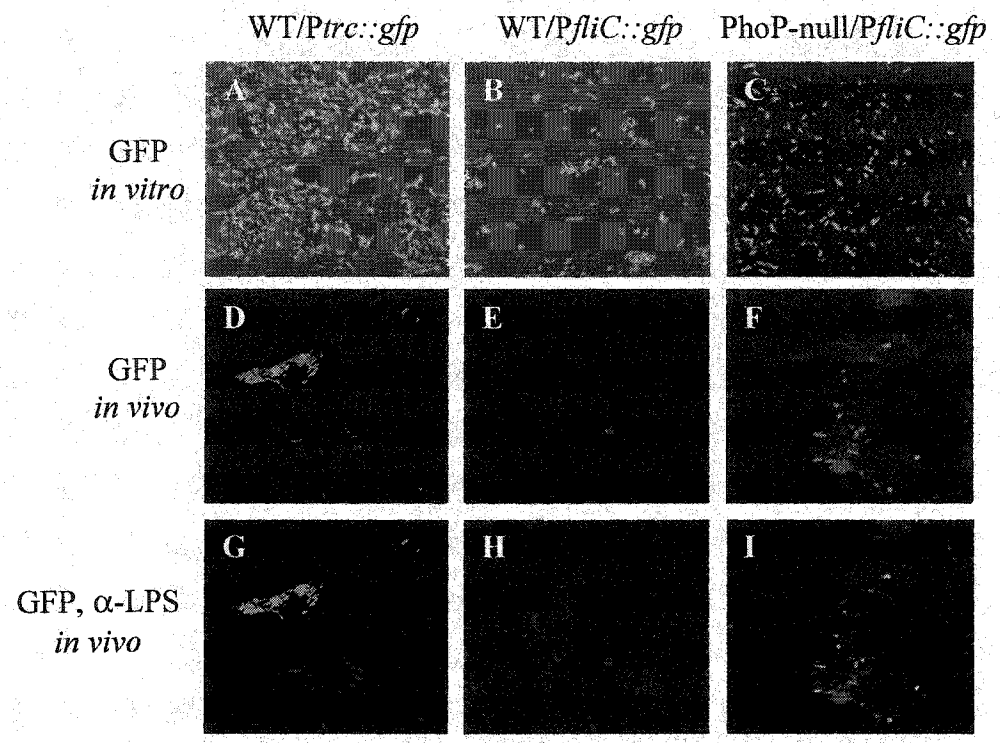


Figure 3.6 Transcription of the gene encoding the FliC antigen is repressed by a PhoP/PhoQ-dependent mechanism during *Salmonella* growth inside macrophages. PhoP-wildtype *Salmonella* expressing GFP under the control of the constitutive *trc* promoter (1st column) or the native *fliC* promoter (2nd column), and PhoP-null *Salmonella* expressing GFP from the native *fliC* promoter (3rd column), were evaluated by confocal microscopy for GFP fluorescence (to assess promoter activity) (green, A-F), or by indirect immunofluorescence for anti-LPS staining (to visualize bacteria inside macrophages) (red, G-I). Bacteria were grown *in vitro* (A-D), or inside RAW 264.7 macrophages (D-I). Data are representative of three experiments.

stimulated TLRs or were intrinsically associated with TLR ligands. *Salmonella* growing inside macrophages repressed antigen expression via PhoP/PhoQ, the two-component regulatory system that controls resistance to innate immunity. These results indicate that TLR recognition directs the antigenic specificity of CD4+ T cells responding to *Salmonella* infection, and suggests that genetically coordinated surface modifications enhance *Salmonella* growth *in vivo* by diminishing both innate and adaptive immune recognition.

The intimacy of CD4+ T cell antigenic specificity with innate immune ligands suggests the possibility that TLRs specifically deliver antigens to processing and presentation machinery. Indeed, other host receptors with affinity for antigen like Fcγ and complement receptors also capture antigen and augment presentation 10^3-10^4 fold (47, 123, 192). At least one TLR has been shown to direct T cell responses: compared to native model antigen, covalent linkage to the *Klebsiella pneumoniae* TLR2 ligand OmpA significantly increased antigen processing and presentation *in vitro* and *in vivo* (102). TLR recognition of bacterial ligands may serve a two-fold purpose in simultaneously initiating inflammatory responses with adjuvant effects and capturing antigens for presentation to T cells. Comprehensive identification of antigens from pathogens recognized by TLRs and T cells (34) may further solidify this intriguing connection between innate and adaptive immune recognition. We are currently employing a proteomic approach to identify *Salmonella* antigens, as their high affinity for LPS has challenged traditional protein purification methods. Irrespective of antigen identity, our results indicate that natural antigen localization in surface organelles and recognition by TLRs are important features that influence T cell recognition of antigen *in vivo*.

Many bacterial pathogens express surface organelles like flagella and membrane vesicles (12, 152). Recognition of such conserved surface structures is an obvious benefit to the host, but this receptor-ligand interaction may also be exploited by pathogens as virulence strategies. For intracellular pathogens like *Salmonella*, TLR-initiated innate immune responses could recruit cells permissive for bacterial replication (133) to the site of infection (105). Similarly, *Shigella* and *Mycobacteria*, which also infect and replicate within eukaryotic cells, actively secrete lipoproteins stimulatory for TLR2 (3, 15). Other pathogens may utilize TLR recognition of their surface organelles to deliver specific virulence factors. Delivery of heat labile enterotoxin by Enterotoxigenic *Escherichia coli* (ETEC) (95), and packaging of virulence proteins like the hemolysins and proteases expressed by *Pseudomonas aeruginosa*, both rely on membrane vesicles (106). Therefore, directly targeting eukaryotic cells without the need of dedicated toxin-specific host receptors, resulting in cellular intoxication or inappropriate stimulation of inflammatory responses, would favor bacterial colonization and replication *in vivo*. *Salmonella* employs a similar strategy by activating the caspase-1 protease in macrophages, initiating an inflammatory cell death (14) required for *Salmonella* infection *in vivo* (155). Thus, microbes appear to have evolved one sub-set of pathogenic mechanisms specifically to exploit host immune recognition.

However, in contrast to exploitation, avoiding immune responses also facilitates pathogen replication inside infected hosts. *P. aeruginosa* successfully colonizes the lungs of cystic fibrosis patients in part by growing as a biofilm, a virtual mat of microbes with increased resistance to opsonization and phagocytosis (37). During malarial infection, the host humoral immune response targets malarial antigens expressed at the surface of infected red blood cells; *Plasmodium* counters by expressing different alleles to generate antigenic

variants that exhibit minimal immunological cross-reactivity (122). CD8+ T cell responses are important for viral immunity, and herpes simplex for example, has accordingly evolved mechanisms to interfere with class I MHC antigen presentation by blocking peptide translocation into the endoplasmic reticulum (253). Regulation of antigen production therefore represents a new contribution to our understanding of the immune evasion mechanisms employed by microbial pathogens. Activation of the PhoP/PhoQ regulon represses expression of surface antigens recognized by *Salmonella*-specific CD4+ T cells. Thus, enhanced expression of FliC in PhoP-null *Salmonella* could explain why these mutant bacteria stimulate protective immunity rather than fatal infection in murine and human hosts (64, 69, 91, 146, 235). Conversely, PhoP/PhoQ-coordinated surface modifications by virulent bacteria may contribute to chronic *Salmonella* persistence in humans (125, 176), exemplified by Typhoid Mary (198). Genetically programmed responses by the pathogen to resist innate and adaptive immune recognition, together with our observation that TLR ligands appear to be selecting the antigens recognized by CD4+ T cells responding to infection, supports the proposal that microbial pathogenesis has been shaped by the host immune system. That is, a co-evolutionary dynamic where coordinated immune recognition of microbial antigens has selected for pathogens capable of exploiting, modulating, or evading that recognition to allow them to successfully colonize and infect their host.

CHAPTER 4: *SALMONELLA* FLAGELLIN CONTAINS MULTIPLE T CELL EPITOPES AND IS PREFERENTIALLY EXPRESSED *IN VIVO* TO STIMULATE MUCOSAL T CELL RESPONSES

ABSTRACT

The flagellin protein FliC is a natural antigen for CD4+ T cells from *Salmonella*-infected hosts. To further investigate T cell responses to FliC, we derived FliC-specific CD4+ T cell clones from *Salmonella*-infected mice of two different haplotypes. Mapping of antigenic activity confirmed two known FliC epitopes (339-350, 428-442), and identified two additional epitopes (72-97, 444-459) located in the regions of FliC sequence conserved between different bacterial flagellins. *In vitro* presentation of FliC by *Salmonella*-infected macrophages suggested a dominant epitope in each haplotype, but quantification of epitope-specific T cell frequencies during primary and secondary infection revealed a different hierarchy of epitope immunodominance. Surprisingly, dysregulation of FliC expression *in vivo* influenced FliC-specific immune responses. Salmonella expressing FliC later in infection (from an *in vivo*-activated promoter) primed fewer numbers of mucosal FliC-specific CD4+ T cells as compared to *Salmonella* expressing FliC early in infection (from the native promoter), indicating that that presence of FliC during mucosal infection is critical for appropriate FliC-specific immune recognition. Phenotypic analysis of these strains revealed that *Salmonella* regulated FliC expression at both transcriptional and post-transcriptional levels. These results highlight the differences between epitope selection during bacterial infection *in vitro* and *in vivo*, and reveal the importance of native expression of antigens for immune recognition *in vivo*.

BACKGROUND

Salmonella spp. are facultative intracellular pathogens capable of causing localized and systemic disease of significant morbidity and mortality. *S. typhi* causes typhoid fever in humans, and disease can be modeled by murine infection with *S. typhimurium*. Natural acquisition of *Salmonella* via contaminated food or water routes the bacteria to the gastrointestinal tract, where *Salmonella* invades M cells to colonize underlying mucosal tissue (105). *Salmonella* disseminates to deeper tissues of spleen and liver (22) and there preferentially infects macrophages and replicates inside phagosomes. Unchecked bacterial replication is fatal for the infected host.

Oral infection with viable, attenuated bacteria generates protective immunity against virulent *Salmonella* infection, and requires both humoral and cellular immune functions (137). Robust CD4+ responses result from the pathogen's phagosomal location, via T cell receptor engagement of pathogen-derived peptides complexed with MHC Class II on the surface of infected cells (104). The majority of *Salmonella*-specific CD4+ T cells recognize FliC, the major subunit protein of bacterial flagella (32, 140). Infected macrophages are also capable of processing and presenting FliC to activate FliC-specific CD4+ T cells, further illustrating the importance of both cell types for *Salmonella* immune responses (32, 140). FliC contains epitopes for CD4+ T cells from infected mice of both H-2^b and H-2^k haplotypes (32, 140), but the magnitude and breadth of the FliC-specific T cell response in mice of either haplotype is unknown, as is the immundominance of FliC epitopes during infection.

Typically, T cell responses to intracellular pathogens target antigens that are abundant, secreted or exposed on the bacterial surface, and contain multiple epitopes capable of binding MHC molecules (20). Bioavailability of natural antigens influences immune responses to other intracellular pathogens. *Mycobacterium* expressing surface-exposed recombinant antigen generated larger numbers of antigen-specific T cells as compared to bacteria expressing cytosolic antigen (74), and *Listeria* expressing non-secreted antigen failed to be recognized by antigen-specific T cells during infection (210, 258). CD4+ T cells preferentially target natural antigens expressed in *Salmonella* surface organelles: flagellins in the bacterial flagellar apparatus, and low-molecular weight proteins localized in secreted membrane vesicles (Chapter 3), suggesting that antigen localization during *Salmonella* infection is also critical for antigen-specific immune responses. However, the benefits of antigen bioavailability to the host are apparently reduced during infection, as *Salmonella* represses production of natural antigens during *in vivo* growth, via coordinated regulation of transcription of antigen-encoding genes (Chapter 3).

To better understand details of the host response to *Salmonella*, we identified new FliC epitopes recognized by CD4+ T cells responding to *Salmonella* infection. Surprisingly, T cell recognition of individual FliC epitopes differed during *in vitro* and *in vivo* infection, revealing an unexpected hierarchy of epitope immunodominance. To dissect bacterial features that influence host recognition, we created *Salmonella* strains that localize FliC in different bacterial compartments or express FliC during intracellular infection. Phenotypic analysis of strains with regulated FliC expression revealed that *Salmonella* controls FliC expression occurs both transcriptionally and post-transcriptionally. *In vivo,* regulated expression of FliC significantly influenced FliC-specific immune responses

during *Salmonella* infection, while compartmentalization minimally effected generation of FliC-specific memory T cells.

MATERIALS AND METHODS

Bacteria and Growth Conditions

Derivatives of *Salmonella enterica* serovar *typhimurium* were used for all experiments; see Table 2 for list of bacterial strains. *Salmonella* were grown in Luria broth (BD Diagnostic Systems, Sparks, MD) at 37°C to indicated growth phase; sodium acetate was added to 1% for growth of ST14028 *phoP* phoQ*::Tn*10*.

Plasmid Construction

Plasmids carrying mutant alleles encoding FliC with 31 amino acid insertions (*fliC::i31, fliC-wt, -in, -ex* genes) and phenotypic analysis of strains expressing mutant FliC proteins will be described elsewhere (Rassoulian Barrett and Cookson, in preparation). To replace the *fliC* promoter with an *in vivo* inducible promoter (*fliC-up*), we cloned and fused the *pagC* promoter DNA (92) to DNA that flanks the *fliC* promoter (upstream 0.7kb and downstream 1.5kb) and transferred the fusion into a pBR322 derivative to create plasmid p*pagC::fliC*. Removal of the 1.5kb *fliC* open-reading frame (orf) from p*pagC::fliC* and replacement with the 0.7kb *gst* orf created the plasmid p*pagC::gst*. Drs. Kelly Smith and Kelly Hughes generously provided FliC-encoding plasmids p*trc::fliC1* and p*trc::fliC2*, respectively; p*trc::fliC1* contains the *fliC* orf directly downstream of the *trc* promoter, while p*trc::fliC2* contains all 62bp of the 5'untranslated region (5'UTR, 207) upstream of the *fliC* orf. All plasmids were transferred to flagellin-negative SL1344 and SL3261 by electroporation to determine FliC expression and compartmentalization.

Table 4.1 Bacterial Strains. List of bacterial strains used in Chapter 4 studies.

Strain	Genotype	Phenotype	Source or Reference
S. typhimurium			
SL1344	*hisG, xyl, rpsL*	virulent	B. Stocker
SL3261	Δ*aroA, hisG, xyl, rpsL*	attenuated	B. Stocker
HB686	LT2 *fliC*::FRT•Kan•FRT	FliC- FljB+	K. T. Hughes
JG368	LT2 *fljB*::FRT•Kan•FRT	FliC+ FljB-	K. T. Hughes
PJOY1218	LT2/pCP20 (*flp* recombinase)	FLP+	K. T. Hughes
BC684	SL1344 Δ*fliC*	FliC- FljB+	this study
BC687	SL1344 Δ*fljB*	FliC+ FljB-	this study
BC696	SL1344 Δ*fliC* Δ*fljB*	FliC- FljB-	this study
BC685	SL3261 Δ*fliC*	FliC- FljB+	this study
BC688	SL3261 Δ*fljB*	FliC+ FljB-	this study
BC697	SL3261 Δ*fliC* Δ*fljB*	FliC- FljB-	this study
BC740	SL3261 Δ*fljB* $fliC_{287::i31}$	FliC-WT	this study
BC763	SL3261 Δ*fljB* $fliC_{\Delta 444\text{-}492::i31}$	FliC-IN	this study
BC742	SL3261 Δ*fljB* $fliC_{444::i31}$	FliC-EX	this study
BC1034	SL1344 Δ*fljB pagC::fliC*	FliC-UP	this study
ST14028	wild-type	$PhoP^{wt}$	ATCC
CS401	ST14028 *pho-24*	$PhoP^{c}$	(147)
EG10232	ST14028 *phoP* phoQ*::Tn*10*	PhoP*	(23)
S. paratyphi	clinical isolate	Flagellin+	UWMC
S. cholerasuis	clinical isolate	Flagellin+	UWMC
S. enteritidis	clinical isolate	Flagellin+	UWMC
Escherichia coli	clinical isolate	Flagellin+	UWMC
Serratia marcescens	clinical isolate	Flagellin+	UWMC
Pseudomonas aeruginosa PAO1	Type B	Flagellin+	S. Lory

To construct vectors carrying *fliC- wt, -in, -ex*, or *–up* genes for allelic exchange, DNA for each gene was cloned by PCR, sequence-confirmed and transferred to suicide vector pCVD442.

Allelic Exchange of Mutant fliC *Alleles*

Construction of Δ*fliC*/Δ*fljB* strains: *Salmonella* strains encoding the kanamycin resistance (Kan^R) gene in place of either the *fliC* or *fljB* orf (constructed by the method of Datsenko and Wanner (41)) were kindly provided by the laboratory of Dr. Kelly Hughes. Kan^R was transferred from HB686 (*fliC*::FRT•Kan•FRT) or JG368 (*fljB*::FRT•Kan•FRT) to SL1344 and SL3261 by generalized transduction, and subsequently the gene encoding Kan^R (flanked by FLP recognition target [FRT] sites) was excised by introduction of plasmid pCP20 (temperature-sensitive replicon, ampicillin-resistant Amp^R), which expresses the *flp* recombinase gene after thermal induction. Resultant Kan^S, Amp^S colonies were subsequently confirmed to be Δ*fliC* or Δ*fljB* by PCR screening. The same procedure was repeated to create strains containing both deletions, and strains lacking either one or both flagellin genes were confirmed to be motile or non-motile, respectively. Construction of FliC-WT, -IN, -EX, -UP strains: suicide vectors carrying mutant *fliC* alleles (see *Plasmid Construction*) and counterselectable markers were used to replace the wild-type *fliC* alleles in SL1344 and SL3261 similarly to a previously described approach (151). Briefly, plasmid pCVD442 (λpir-dependent replicon, Amp^R, *sacB* gene conferring sucrose sensitivity) carrying a mutant *fliC* allele was transferred to *Salmonella* strains by conjugation. The resulting Amp^R *Salmonella* were then grown in sucrose-containing media to select for loss of *sacB* gene and plasmid DNA. Individual Amp^S, $sucrose^R$ colonies were screened by PCR for identify the presence of the mutant allele, whereupon the predicted motility phenotype was confirmed.

Salmonella *Antigens*

Salmonella expression of recombinant FliC proteins was performed as described elsewhere (32). Heat-killed *Salmonella* antigen (HKST) was prepared from bacteria grown to stationary phase and incubated at 65°C for 1h. To purify FliC and FljB flagellins, flagella from logarithmic phase bacteria expressing only FljB or FliC were mechanically sheared from bacterial cells (222) (Waring, East Windsor, NJ), depolymerized at 60°C for 20 minutes, passed through a Centricon 100,000 MWCO filtration unit (Millipore, Bedford, MA) to remove high-molecular weight lipopolysaccharide, and resulting monomeric flagellin preparations were confirmed to be free of contaminating antigens (*Salmonella* proteins recognized by non-FliC-specific T cells). To fractionate *Salmonella* into extracellular and intracellular compartments, whole cultures of *Salmonella* grown to logarithmic phase were heated at 60°C for 20min. to depolymerize surface attached flagella, pelleted, separated into intracellular (pellet) and extracellular (supernatant) fractions. Synthetic peptides were constructed by Global Peptide Services, Ft. Collins, Colorado.

Immunoblots

Salmonella bacteria or bacterial fractions were separated by SDS-10%PAGE, transferred to nitrocellulose, probed with polyclonal anti-FliC (Denka Seiken, Tokyo, Japan, absorbed against flagellin-negative *Salmonella*), polyclonal anti-GST (Molecular Probes, Eugene, Oregon), or monoclonal anti-DnaK (StressGen, Victoria, British Columbia, Canada) followed by goat anti-rabbit or anti-mouse IgG conjugated to horseradish peroxidase (HRP) (Santa Cruz Biotechnology, Santa Cruz, CA). Reactive HRP was detected by enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ).

RT-PCR

Total RNA was isolated from *Salmonella* grown to stationary phase with Trizol reagent (Invitrogen, Carlsbad, CA) and contaminating DNA removed with DNA-*free* (Ambion, Austin, Texas). cDNA was synthesized from DNA-free RNA using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) according to manufacturer's instructions, and used as template in PCR reactions; control reactions lacking reverse transcriptase were also included. The following primers were used to amplify a portion of the coding sequences of *fliC*, *gst*, and *dnaK* (5' to 3' sequence indicated): fliCRT1 (ATGGCA CAAGTCATTAATACAAAC), fliCRT2 (AGCAGACTGAACCGCCAGTTCACG) , gstRT1 (ATGTCCCCTATACTAGGTTATTGG), gstRT2 (AGGAACATTGGGAAA CTCCAAACC), dnaKRT1 (ATGGGTAAAATTATTGGTATCGAC), dnaKRT2 (TCACCATCCTGGCGATAAGCAATG) (Invitrogen, Carlsbad, California). Amplified products were visualized following agarose gel electrophoresis and ethidium bromide staining.

Mice and Oral Infections

4-5 week-old female C3H/HeJ (Jackson Laboratory, Bar Harbor, ME) and C57BL/6 (National Cancer Institute, Bethesda MA) were used for oral infections and splenocyte antigen-presenting cells (APC). Mice were inoculated by oral gavage (feeding needle no. 7920, Popper & Sons, Inc., New Hyde Park, NY) with 10^9 viable attenuated bacteria (SL3261 background) or 10^7 viable virulent bacteria (SL1344 background).

Eukaryotic Cell Culture and Infections In Vitro

All eukaryotic cells were maintained in RPMI 1640 supplemented with L-glutamine, 50μM 2-ME, 10% FCS, with penicillin, streptomycin, and gentamicin and incubated at

37°C/5% CO_2. Every 14-17d, T cell lines and clones were restimulated with irradiated syngeneic splenocyte APC and antigen for 48h, followed by dilution into supplemented media containing IL-2 (T-STIM, BD Discovery Labware, San Diego, CA) and methyl-α-D-mannopyranoside (Calbiochem, San Diego, CA). Elicited peritoneal macrophages were obtained three days after intraperitoneal injection of 0.1mL sterile Brewer's thioglycollate (BD Diagnostic Systems, Sparks, MD) and 48h culture in media containing 50u/mL IFNγ (R&D Systems, Minneapolis, MN). To generate infected macrophages for antigen-presenting cells, bacteria from stationary phase cultures were added to macrophages at the indicated multiplicity of infection (MOI) in antibiotic-free media, incubated for 2h, cells were washed twice with HBSS, and media replaced with that containing 15μg/mL gentamicin to kill extracellular bacteria. After 2h, the cells were washed extensively, fixed with 0.2% paraformaldehyde/HBSS, washed extensively, and after replacing the media, fixed cells were used as APC for T cells in proliferation assays.

Generation of FliC-Specific CD4+ T Cell Clones

T cells were generated as described previously (32). Briefly, mice immunized 90d prior were sacrificed, spleens removed and nylon-wool purified splenic lymphocytes stimulated with irradiated syngeneic splenocyte APC and $5x10^6$ cfu/mL heat-killed *Salmonella* antigen to generate *Salmonella*-immune CD4+ T cell lines. After three rounds of restimulation, FliC-specific T cell clones were isolated from lines by limiting dilution using 10μg/mL purified FliC as stimulatory antigen (see *Generation of* Salmonella *Antigens*). Clones were specific to protein antigens and CD4+; those clones from C3H/HeJ mice were restricted to MHC molecule A^k or E^k, as determined by 1) proliferative responses to antigen and A^k-expressing splenocyte APC from B10.4R mice (Jackson Laboratory, Bar Harbor, ME) or inhibition of proliferative responses to antigen

and APC in the presence of blocking anti-E^k antibody (clone 14-4-4S, BD Biosciences Pharmingen, San Diego, CA) (data not shown), while those clones from C57BL/6 were assumed to be restricted to MHC molecule A^b (genes encoding the α chain of E^b molecule are inactivated in this strain). TCR Vβ expression was determined using the Mouse TCR Vβ Screening Panel (BD Biosciences Pharmingen, San Diego, CA) and a FACScan (BD Biosciences Immunocytometry Systems, San Jose, CA).

Proliferation Assays

T cell proliferation in response to APC plus antigen was assayed as previously described (32). Briefly, 10^5 T cells and 10^6 irradiated syngeneic splenocyte APC plus $5x10^6$ HKST or 0.1-1.0μg/mL antigen were combined in triplicate, (^{3}H)TdR added after 48h, DNA harvested after 16h, and incorporated ^{3}H was measured using liquid scintillation spectrophotometry.

IFNγ ELISpot Assays

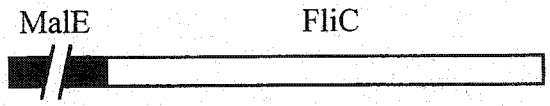
The frequency of *Salmonella*-specific and FliC-specific IFNγ-producing T cells generated after *Salmonella* infection was determined by the ELISPOT assay (227). C3H/HeJ or C57BL/6 mice were infected orally with *Salmonella* for indicated time periods, then lymphoid organs removed, and CD4+ T cells were purified by positive selection using anti-CD4 magnetic beads according to the manufacturer's instructions (Miltenyi Biotech). After two serial passages through magnetized columns, populations were typically 97–99% CD4+ cells as determined by flow cytometry. $2.5x10^6$ purified CD4+ T cells were combined with $2.5x10^6$ irradiated syngeneic splenocyte APC and either HKST ($5x10^7$/ml), purified FliC or peptide (0.1μg/ml). After 48h at 37°C/5%CO_2, cells were harvested, counted, and added to wells of 96-well antibody-coated IFNγ ELISpot plates

(BD Biosciences, San Diego, CA) in serial dilutions starting with 10^5 viable cells/well. After 18h at 37°C/5%CO_2 cells were removed and foci of IFNγ-secreting cells ("spots") were detected according to manufacturer's instructions (BD Biosciences, San Diego, CA). Spot-containing wells were removed from the plate, scanned into Adobe Photoshop (Adobe, San Jose, CA), and spots were counted by an investigator blind to the experimental protocol.

RESULTS

FliC-Specific CD4+ T cells from Salmonella-*Immune Mice*

We orally infected C3H/HeJ (H-2^k) and C57BL/6 (H-2^b) mice with *S. typhimurium* strain SL3261 (93) and generated *Salmonella*-specific CD4+ T cells as described previously (32). Using purified FliC (see Material and Methods) as stimulatory antigen, we isolated 37 CD4+ FliC-specific T cell clones restricted to E^k, A^k, or A^b MHC Class II molecules with variable TCR Vβ usage (each clone expressed a single TCR Vβ chain) (Figure 4.1). Using recombinant MalEFliC fusion proteins with sequential C-terminal deletions (32) as stimulatory antigen, we identified four FliC regions containing antigenic activity: amino acids 89-344, 345-400, 401-460, and 461-494 (Figure 4.1). As FliC amino acid residues 1-170 and 404-494 are nearly identical between FliC and FljB, the alternate flagellin subunit expressed by *S. typhimurium* (199), we hypothesized that T cell epitopes present within FliC residues 89-344 and 401-494 may also be present in FljB. Purified flagellins and *Salmonella* bacteria expressing either flagllin were used as stimulatory antigen to determine that clones represented by HeJ.F8, B6.2D4, and B6.2A4 all recognize an epitope common to both FliC and FljB, while HeJ.3A7 and similar clones are FliC-specific (Figure 4.2). These results demonstrate that FliC-specific CD4+ T cells from



Mouse Strain	Total # Clones	MHC Restriction	TCR V_ Expression	(in FliC)	1-88	1-344	1-400	1-460	1-494 (all FliC)	Representative Clones	Reference
C3H/HeJ	5/21	E^k	6	ø	ø	+	+	+	+	HeJ.F8	This study
C3H/HeJ	16/21	A^k	2, 4, 7, 8.1/8.2	ø	ø	ø	+	+	+	HeJ.3A7	This study
C57BL/6	4/16	A^b	8.1/8.2, 9	ø	ø	ø	ø	+	+	B6.2D4	This study
C57BL/6	12/16	A^b	8.3, 4	ø	ø	ø	ø	ø	+	B6.2A4	This study
C3H/HeJ	1	A^k	4	ø	ø	ø	+	+	+	7.4.8	(32)
C57BL/6	26	A^b	2, 3.4, 15	ø	ø	ø	ø	+	+	CN.B1	(140)

Figure 4.1 Summary of FliC-specific CD4+ T cell clones from *Salmonella*-immune C3H/HeJ and C57BL/6 mice. 37 FliC-specific T cell clones were derived from protectively immunized mice, confirmed CD4+ and subgrouped based on MHC restriction, TCR Vβ expression and epitope mapping. Recombinant MalEFliC fusion proteins with sequential C-terminal deletions (FliC residues in each recombinant protein indicated) were used as stimulatory antigen to map epitopes recognized by the 37 clones; responses of representative clones are shown as + (≥10,000cpm with background ≤200cpm) or ø (same as background). Shown for comparison are characteristics of previously described FliC-specific T cell clones.

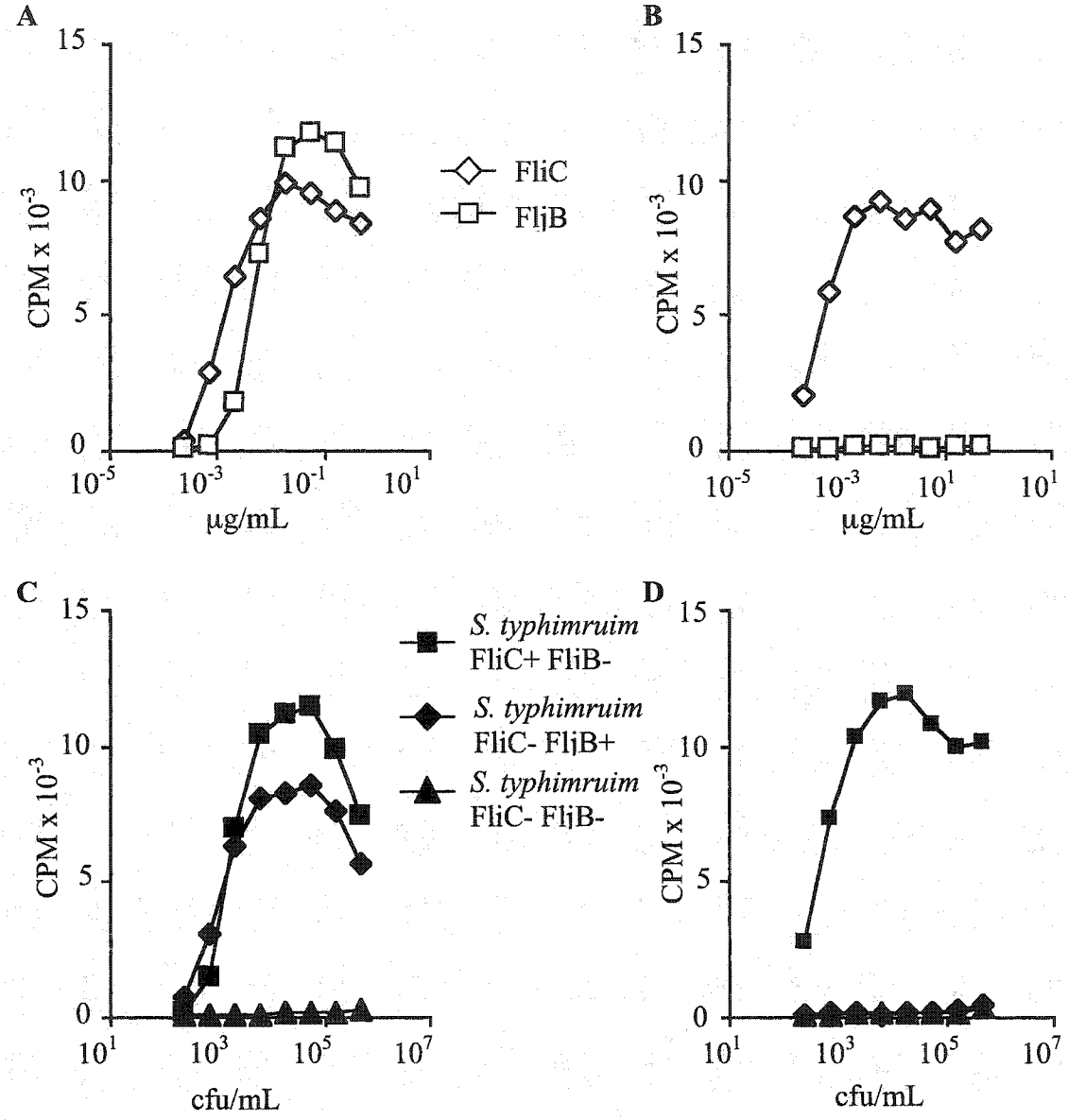


Figure 4.2 FliC-specific CD4+ T cell clones recognize both *Salmonella* flagellins. Proliferative responses of representative FliC-specific T cell clones HeJ.F8 (A, C) and HeJ.3A7 (B, D) to purified FliC or FljB (A, B) or *Salmonella* expressing FliC or FljB (heat-killed bacteria) (C, D) were measured. Like HeJ.F8, all A^b-restricted clones (B6) responded to both FliC and FljB (data not shown). A, B share same legend; C, D share same legend.

Salmonella-immune mice recognize four different epitopes, some of which are present in both *Salmonella* flagellin proteins.

Mapping and Identification of FliC Epitopes

The two known FliC epitopes, FliC 339-350 (A^k-restricted) and FliC 428-442 (A^b-restricted), juxtapose or map near the regions of FliC containing stimulatory activity for clones HeJ.3A7 and B6.2D4 (Figure 4.1. We determined that all A^k-restricted clones, like the previously described 7.4.8 clone (32), recognized the FliC 339-350 epitope (Figure 4.3A). Indicating the difference in TCR receptors used by FliC 339-350-specific clones (Figure 4.1), residues K348-T350 were nonessential for stimulation of HeJ.3A7 (Figure 4.3A). The four A^b-restricted clones represented by B6.2D4 responded to a peptide containing FliC 428-442 (Figure 4.3B), similar to previously described FliC-specific A^b-restricted CD4+ T cells (140), but expressed different TCR Vβ receptors than published clones.

The E^k-restricted and remaining A^b-restricted clones (representative clones HeJ.F8 B6.2A4), which comprise 24% and 75% respectively of total FliC-specific CD4+ T cells from *Salmonella*-immune mice, did not respond to either peptides FliC 339-350 or FliC 428-442 (Figure 4.3A,B). To define the epitopes recognized by these two classes of clones, we assayed for the presence of stimulatory antigen in various flagellin proteins: recombinant FliC proteins (MalEFliC deletions, FliC with insertions of 31 amino acids) and flagellins from different bacterial species. Stimulatory activity for HeJ.F8 mapped between residues 77-92 and was present in flagellins expressed by some *Enterobacteriaceae* (Figure 4.4A), confirming epitope location within the conserved N-terminal region of FliC. Sequence analysis of different flagellin proteins containing or

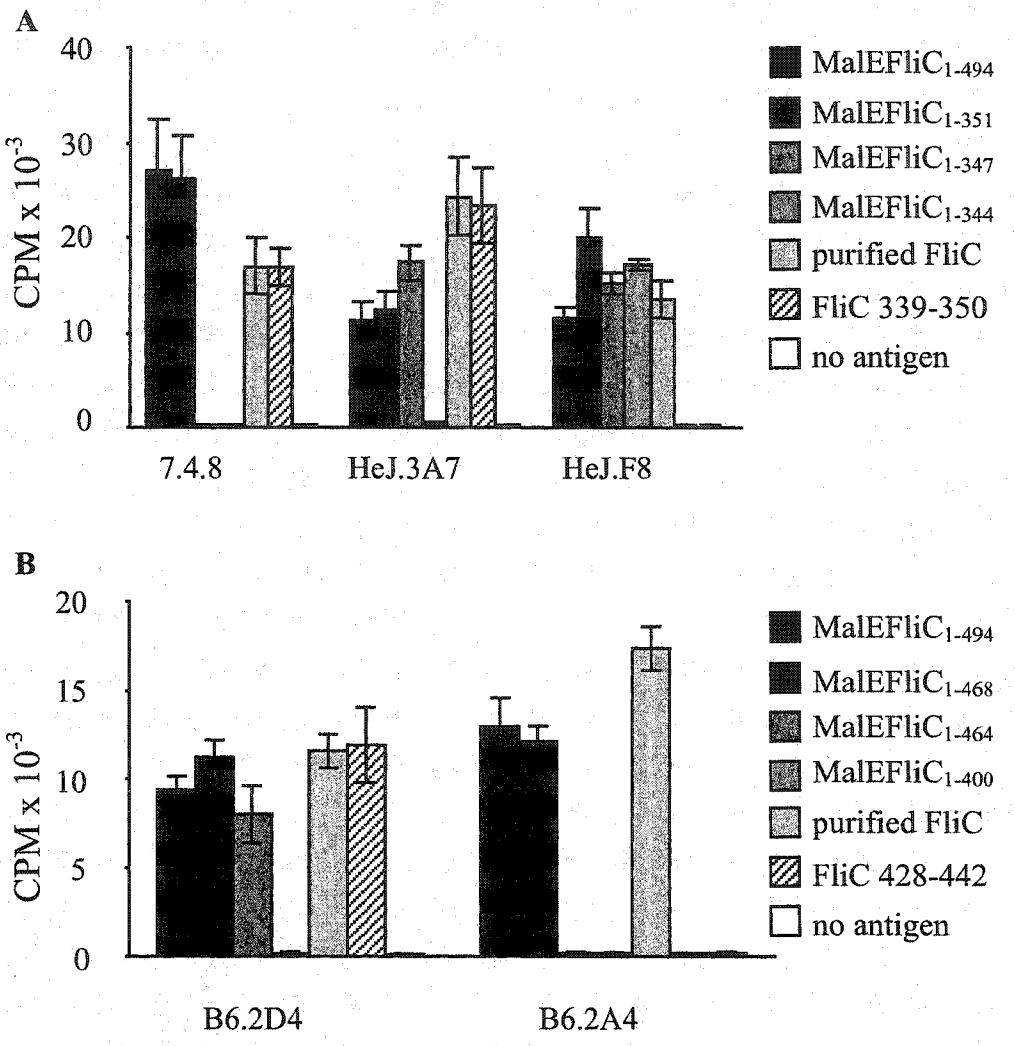


Figure 4.3 FliC-specific CD4+ T cell clones that respond to known FliC epitopes 339-350 and 428-444. Representative FliC-specific CD4+ T cell clones were assayed for proliferative responses to flagellin-negative *Salmonella* expressing recombinant MalEFliC deletion proteins, purified FliC protein, and A^k-restricted FliC 339-350 peptide (32) (A) or A^b-restricted FliC 428-442 peptide (140) (B). The presence of FliC in all antigen preparations was confirmed by Western blot (data not shown).

Figure 4.4 Identification of new FliC epitopes recognized by FliC-specific CD4+ T cells clones. Sequences of flagellin proteins containing or lacking stimulatory antigen for representative FliC-specific CD4+ T cell clones. Antigens used were flagellin-negative *Salmonella* expressing recombinant MalEFliC or FliC containing 31 extra amino acids (first and last two residues of insertion sequence in brackets), other flagellin-positive bacteria (residues non-identical to FliC marked in bold), or synthetic peptides. E^k-restricted (**A**) and A^b-restricted (**B**) epitopes are indicated. Responses are represented as in Figure 1. All antigens were confirmed FliC-positive by Western blot or were prepared from motile bacteria (data not shown).

A

	80					85					90					95		HeJ.F8 Respons
***S. typhimurium* FliC, FljB**	T	E	G	A	L	N	E	I	N	N	N	L	Q	R	V	R	E	L	A	V	+
MalEFliC$_{1\text{-}92}$	T	E	G	A	L	N	E	I	N	N	N	L	Q	R	V	R	*G*	*P*	*		+
MalEFliC$_{1\text{-}88}$	T	E	G	A	L	N	E	I	N	N	N	L	*G*	*P*	*						ø
FliC$_{78}$	T	E | G	A	L	N	E	I	N	N	N	L	Q	R	V	R	E	L	A	V		+
	A---TE																				
FliC$_{93}$	T	E	G	A	L	N	E	I	N	N	N	L	Q	R	V	R	E | L	A	V			+/-
																P---RE					
FliC$_{95}$	T	E	G	A	L	N	E	I	N	N	N	L	Q	R	V	R	E	L	A | V			+
																			A---LA		
S. typhi, S. paratyphi, S. choleraesuis	T	E	G	A	L	N	E	I	N	N	N	L	Q	R	V	R	E	L	A	V	+
S. enteritidis	T	E	G	A	L	N	E	I	N	N	N	L	Q	R	V	R	E	L	**S**	V	+
Escherichia coli	T	E	G	A	L	**S**	E	I	N	N	N	L	Q	R	**I**	R	E	L	**T**	V	+
Serratia marcescens	T	E	G	A	L	N	E	**V**	N	**D**	N	L	Q	**N**	**I**	R	**R**	L	**T**	V	ø
Pseudomonas aeruginosa	**A**	E	G	A	L	**Q**	**Q**	**S**	**T**	N	**I**	L	Q	R	**I**	R	**D**	L	A	**L**	ø
FliC peptide 77-91	T	E	G	A	L	N	E	I	N	N	N	L	Q	R	V						ø
FliC peptide 80-94				A	L	N	E	I	N	N	N	L	Q	R	V	R	E	L			+
Predicted E^{k} Binding Motif					L	N	E	I	N	N	N	L	Q								

B

	455					460					465					470		B6.2A4 Respons
***S. typhimurium* FliC, FljB**	R	I	E	D	S	D	Y	A	T	E	V	S	N	M	S	R	A	Q	I	L	+
MalEFliC$_{1\text{-}468}$	R	I	E	D	S	D	Y	A	T	E	V	S	N	M	S	R	A	*P*	*		+
MalEFliC$_{1\text{-}464}$	R	I	E	D	S	D	Y	A	T	E	V	S	N	*I*	*I*	*N*	*C*	*Q*	*A*	*L*	ø
S. typhi, S. choleraesuis, S. paratyphi	R	I	E	D	S	D	Y	A	T	E	V	S	N	M	S	R	A	Q	I	L	+
S. enteriditis	R	I	E	D	S	D	Y	A	T	E	V	S	N	M	S	**K**	A	Q	I	L	+
Escherichia coli	R	I	**Q**	D	**A**	D	Y	A	T	E	V	S	N	M	S	**K**	A	Q	I	L	+
Serratia marcescens	R	I	**Q**	D	**A**	D	Y	A	T	E	V	S	N	M	S	R	A	**N**	I	L	+
Pseudomonas aeroginosa	R	I	**K**	D	**T**	D	**F**	A	**A**	E	**T**	**A**	**A**	**L**	S	**K**	**N**	Q	**V**	L	ø
FliC peptide 452-471		I	E	D	S	D	Y	A	T	E	V	S	N	M	S	R	A	Q	I		+
FliC peptide 455-469				D	S	D	Y	A	T	E	V	S	N	M	S	R	A	Q			+
Predicted A^{b} Binding Motif							Y	A	T	E	V	S	N	M	S						

lacking stimulatory activity for HeJ.F8, along with analysis of the E^k binding motif (187), predicted two potential epitopes within the 77-92 amino acid sequence. Overlapping synthetic peptides FliC 78-92 and 82-96 both contained, while FliC 77-91 peptide lacked, stimulatory antigen for HeJ.F8, identifying FliC 82-92 as the E^k-restricted epitope (Figure 4.4A). For B6.2A4, stimulatory activity mapped upstream of residue 469 and was also present in flagellins expressed by different bacteria (Figure 4.4B), confirming an epitope located within the conserved C-terminal region of FliC. Sequence analysis of bacterial flagellins containing or lacking stimulatory activity for B6.2A4, along with identification of the A^b binding motif (132), predicted that FliC residues 458-466 comprise part of the epitope. Synthetic peptides FliC 452-471 and 455-469 both contained stimulatory antigen for clone B6.2A4, identifying FliC 455-469 as the second epitope recognized by A^b-restricted FliC-specific T cells (Figure 4.4B).

Relationship Between Epitope Location and FliC Protein Domains

The recently solved crystal structure of *Salmonella* FliC flagellin (199) revealed four major domains within the protein: D0 (aa1-55, 451-494), D1 (aa56-176, 402-450), D2 (aa177-189, 284-401) and D3 (aa190-283). The flagellin monomer is shaped like a hairpin and polymerizes into a right-handed helix, with exposed D2/D3 loops outside and buried D0/D1 straight arms inside the filament polymer. D2/D3 comprise the hyper-variable domains used for serological typing of *Salmonella* and are dispensable for function (motility) (255), while monomer secretion and polymerization into filaments requires the highly conserved D0/D1 regions (94). The conserved nature of D0/D1 amino acid sequences may target flagellin for immune recognition, as three of the FliC epitopes described here map within the D0 and D1 regions (Figure 4.5A, B) and the innate

A

AQVINTNSLSLLTQNNLNKSQSALGTAIERLSSGLRINSAKDDAA 45

GQAIANRFTANIKGLTQASRNANDGISIAQTTEGALNEINNNLQR 90
E^k

VRELAVQSANSTNSQSDLDSIQAEITQRLNEIDRVSGQTQFNGVK 135

VLAQDNTLTIQVGANDGETIDIDLKQINSQTLGLDTLNVQQKYKV 180

SDTAATVTGYADTTIALDNSTFKASATGLGGTDQKIDGDLKFDDT 225

TGKYYAKVTVTGGTGKDGYYEVSVDKTNGEVTLAGGATSPLTGGL 270

PATATEDVKNVQVANADLTEAKAALTAAGVTGTASVVKMSYTDNN 315

GKTIDGGLAVKVGDDYYSATQNKDGSISINTTKYTADDGTSKTAL 360
A^k

NKLGGADGKTEVVSIGGKTYAASKAEGHNFKAQPDLAEAAATTTE 405

NPLQKIDAALAQVDTLRSDLGAVQNRFNSAITNLGNTVNNLTSAR 450
A^b

SRIEDSDYATEVSNMSRAQILQQAGTSVLAQANQVPQNVLSLLR* 494
A^b

B

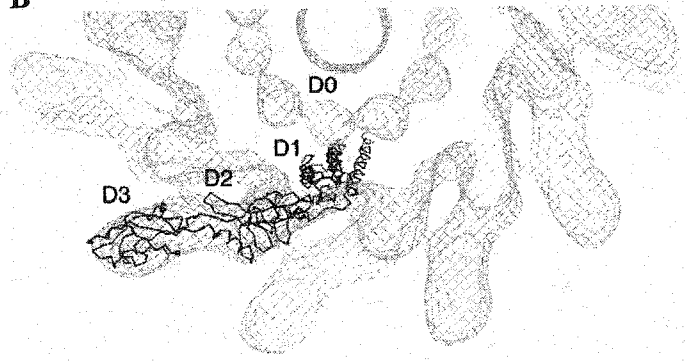


Figure 4.5 Location of FliC epitopes in FliC primary amino acid sequence and secondary structure. Shown in (A) is the amino acid sequence of native FliC (mature protein lacks initiating methionine and contains only 494 amino acids); residues marked in bold indicate sequence conserved between different bacterial flagellins (D0 and D1 domains, see text for details). Epitope-containing sequence is underlined; MHC restriction is listed below each epitope. Shown in (B) is the location of D0 and D1 regions in the teritiary structure of FliC monomer as overlaid on a filament density map (adapted from Samatey *et. al.* 2001 (199)).

immune receptor TLR5 recognizes bacterial flagellin (85) via the D0/D1 domains (55, 57).

Selective Presentation of FliC Epitopes by Salmonella*-Infected Macrophages*

While *Salmonella* infection of macrophages enables bacterial replication *in vivo,* macrophages activated by T cell cytokines like IFNγ can destroy *Salmonella* and are required for bacterial clearance. To determine if *Salmonella*-infected macrophages process and present FliC epitopes to epitope-specific T cells, we infected primary macrophages *in vitro* and used them as stimulatory APC in proliferation assays. We confirmed that A^k-restricted FliC 339-350 is presented by infected macrophages, as clone HeJ.3A7, like a previously described 339-350-specific hybridoma (32), was stimulated by these APC (Figure 4.6A) comparable to control splenocyte APC pulsed with antigen (Figure 4.6E). However, the same population of infected macrophages was not capable of stimulating 82-92 -specific E^k-restricted clone HeJ.E8 (Figure 4.6B) as compared to control APC (Figure 4.6F), suggesting that epitope 82-92 is less efficiently processed and presented during *Salmonella* infection. Differential presentation of A^b-restricted epitopes by infected macrophages was also observed. *Salmonella*-infected macrophages and control APC were both recognized by the 455-469-specific clone B6.2A4 (Figure 4.6C, G), while FliC 428-442 was poorly processed and presented by infected macrophages, as 428-442-specific clone B6.2D4 failed to proliferate in response to infected macrophage (Figure 4.6D) but did respond to control APC (Figure 4.6H). To exclude the possibility that the observed proliferative responses were unique to individual clones, we confirmed that other epitope-specific clones in each subgroup demonstrated the same response to *Salmonella*-infected macrophages (data not shown). Thus, these results demonstrate that

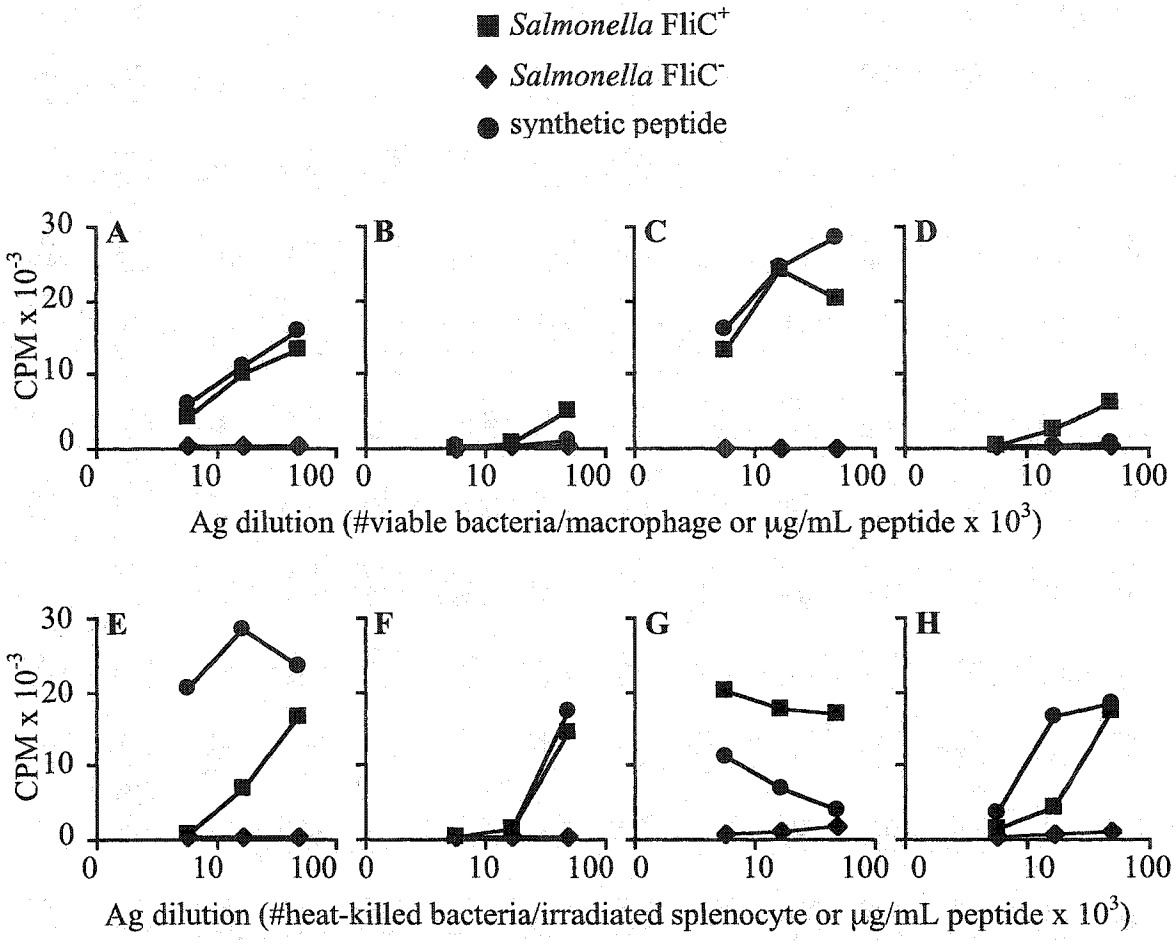


Figure 4.6 FliC epitopes are selectively presented by *Salmonella*-infected macrophages. Syngeneic macrophages were infected with *Salmonella*, at a starting concentration of 100:1 bacteria to macrophage, or incubated with peptides, at a starting concentration of 0.1μg/mL (A-D). Control antigen-presenting cells were syngeneic irradiated splenocytes incubated with heat-killed *Salmonella* bacteria or peptide at the same concentrations (E-H). The FliC-specific CD4+ T cells were used to assess epitope presentation (listed by epitope specificity): 339-350 (A, E), 82-92 (B, F), 455-469 (C, G), and 428-444 (D, H). See Methods for macrophage infection conditions.

FliC epitopes 339-350 and 455-469, rather than 81-94 and 428-442, are selectively processed and presented during *Salmonella* infection of macrophages.

Dominance of FliC Epitopes during Primary and Secondary Salmonella *Infection*

The selective presentation of FliC epitopes 339-350 and 455-469 by *Salmonella*-infected macrophages (Figure 4.6), coupled with the observation that 339-350 and 455-469-epitope specific clones were isolated in higher numbers (Figure 4.1), predicts that the same epitope dominance would manifest during *Salmonella* infection *in vivo*. To test this hypothesis, we orally infected mice with *Salmonella* and used the IFNγ ELISpot assay to quantify the frequency of epitope-specific CD4+ specific T cells during primary and secondary infection. In *Salmonella*-infected C57BL/6 mice (H-2^b haplotype), epitope dominance was dependent on the phase of infection: during primary infection (18d post-infection of naïve mice with attenuated *Salmonella*), 428-442 and 455-469-specific T cell responses were equivalent, each comprising approximately 50% of the total FliC-specific response. During secondary infection (14d post-infection of immune mice with virulent *Salmonella*), 428-442-specific T cells outnumbered 455-469-specific T cells by two-fold, revealing an inverted hierarchy of epitope-specific T cell responses than predicted by *in vitro* experiments. Surprisingly, the frequency of FliC-specific T cells was significantly lower than previously reported (140), comprising 7% and 17% of the total *Salmonella*-specific CD4+ T cells during primary and secondary infection, respectively.

Similarly in *Salmonella*-infected C3H/HeJ (H-2^k haplotype), FliC-specific T cells constituted a minor fraction of total *Salmonella*-specific CD4+ T cells generated during both primary and secondary infection (13% and 4.3%, respectively). The frequency of 339-350-epitope-specific T cells was essentially the same as background regardless of the

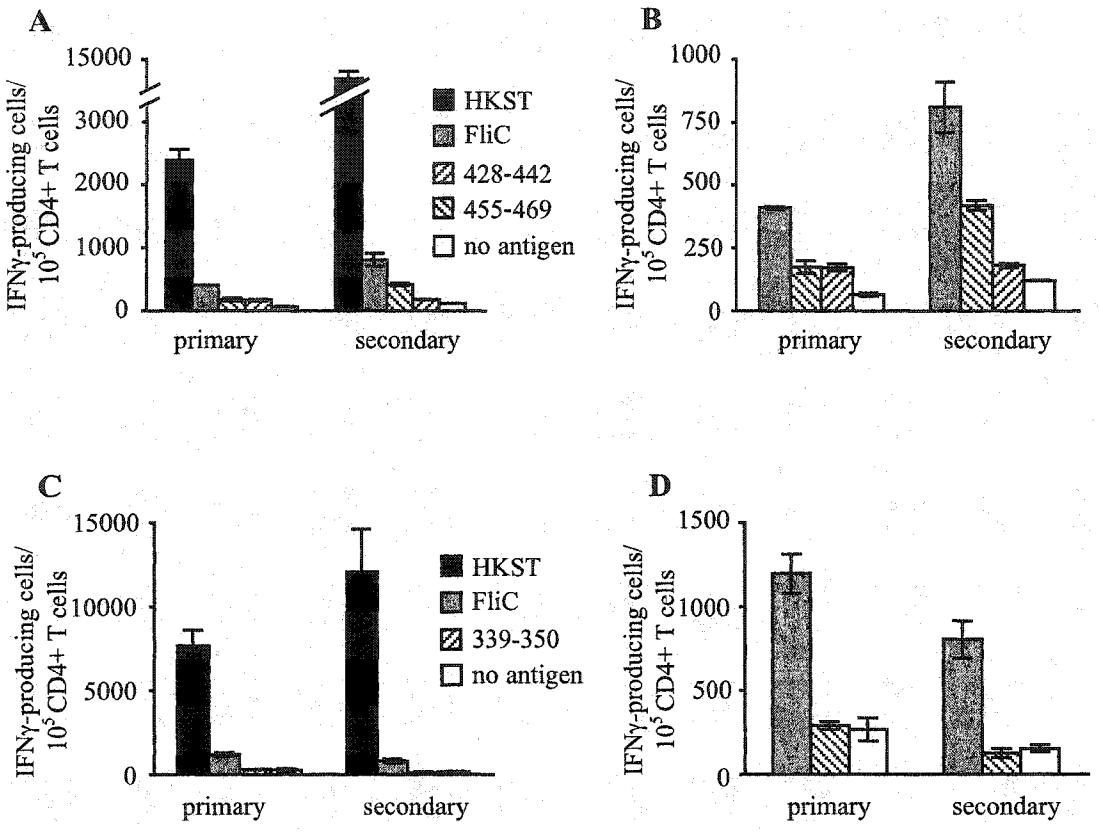


Figure 4.7 FliC-specific CD4+ T cells are differentially generated during primary and secondary infection. The number of IFNγ-secreting splenic CD4+ T cells from *Salmonella*-infected C57BL6 (A, B) and C3H/HeJ (C, D) mice were measured during primary (post-SL3261 immunization) or secondary (post-SL1344 infection of SL3261 immune mice). Responses of all antigens (A, C) are shown; responses to FliC and FliC epitopes (B, D) are magnified for better comparison. Mean results ± SD of two pooled, infected spleens per group assayed in triplicate.

infection stage, suggesting that other FliC epitopes are more dominant *in vivo* (synthetic peptide 82-92 was not available at the time of analysis). Collectively, these results demonstrate that CD4+ T cell responses to specific FliC epitopes are generated during presented by *Salmonella*-infected cells *in vitro* and *in vivo*.

Salmonella *Mutant Strains that Express FliC in Discrete Bacterial Compartments or under Differentially-Regulated Promoters*

FliC normally localizes outside *Salmonella*, in surface-attached polymeric flagella. We engineered *Salmonella* strains that localize FliC as extracellular polymer (FliC-WT) or as extracellular (FliC-EX) or intracellular (FliC-IN) monomer (Table 4.1), using FliC insertion mutants that will be described elsewhere (Rassoulian Barrett and Cookson, in preparation). Immunoblots of total FliC protein from log-phase *Salmonella* cultures demonstrate equivalent amounts of FliC are expressed by the three strains (Figure 4.8A, D, lanes 3-5). Phenotypic analysis of FliC expression in *Salmonella* confirmed that the FliC-IN strain contains cytosolic antigen (Figure 4.8B, lane 4), FliC-WT and FliC-EX produce both cytosolic and secreted antigen (Figure 48C, lanes 3, 5), but only FliC-WT is motile (Figure 4.8), indicating that FliC secreted by this strain polymerizes into functional flagella. Thus, FliC-WT, -IN and -EX *Salmonella* strains express FliC in discrete compartments.

FliC is expressed during *Salmonella* growth outside eukaryotic cells or in mucosal tissue, then repressed during intracellular or systemic infection; transcriptional repression is mediated by the PhoP/PhoQ two-component regulatory system (Chapter 3). Conversely, the PhoP/PhoQ-activated gene *pagC* is induced during *Salmonella* replication in macrophages and *in vivo*, and expression of model antigens from the *pagC* promoter can

fliC allele	*fliC* (wt)	null	*fliC::* 287	*fliC::* Δ444-492	*fliC::* 444
Localization Phenotype	FliC-WT	FliC-NEG	FliC-WT	FliC-IN	FliC-EX
Motility Phenotype	+	ø	+	ø	ø
Lane	1	2	3	4	5

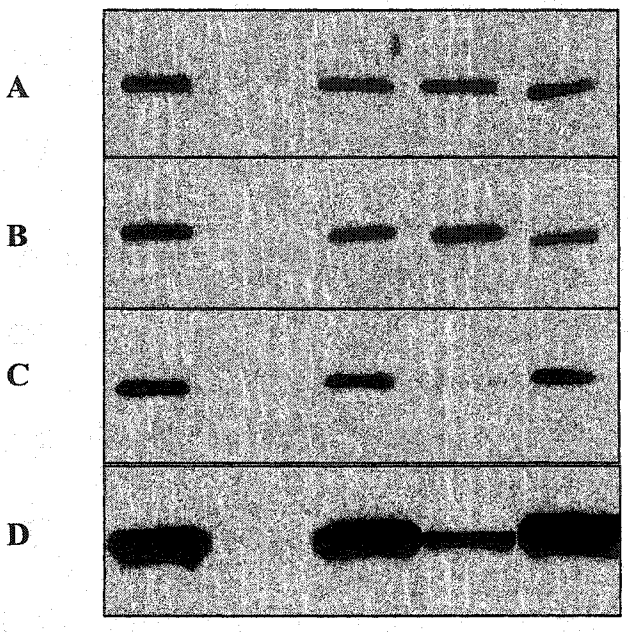
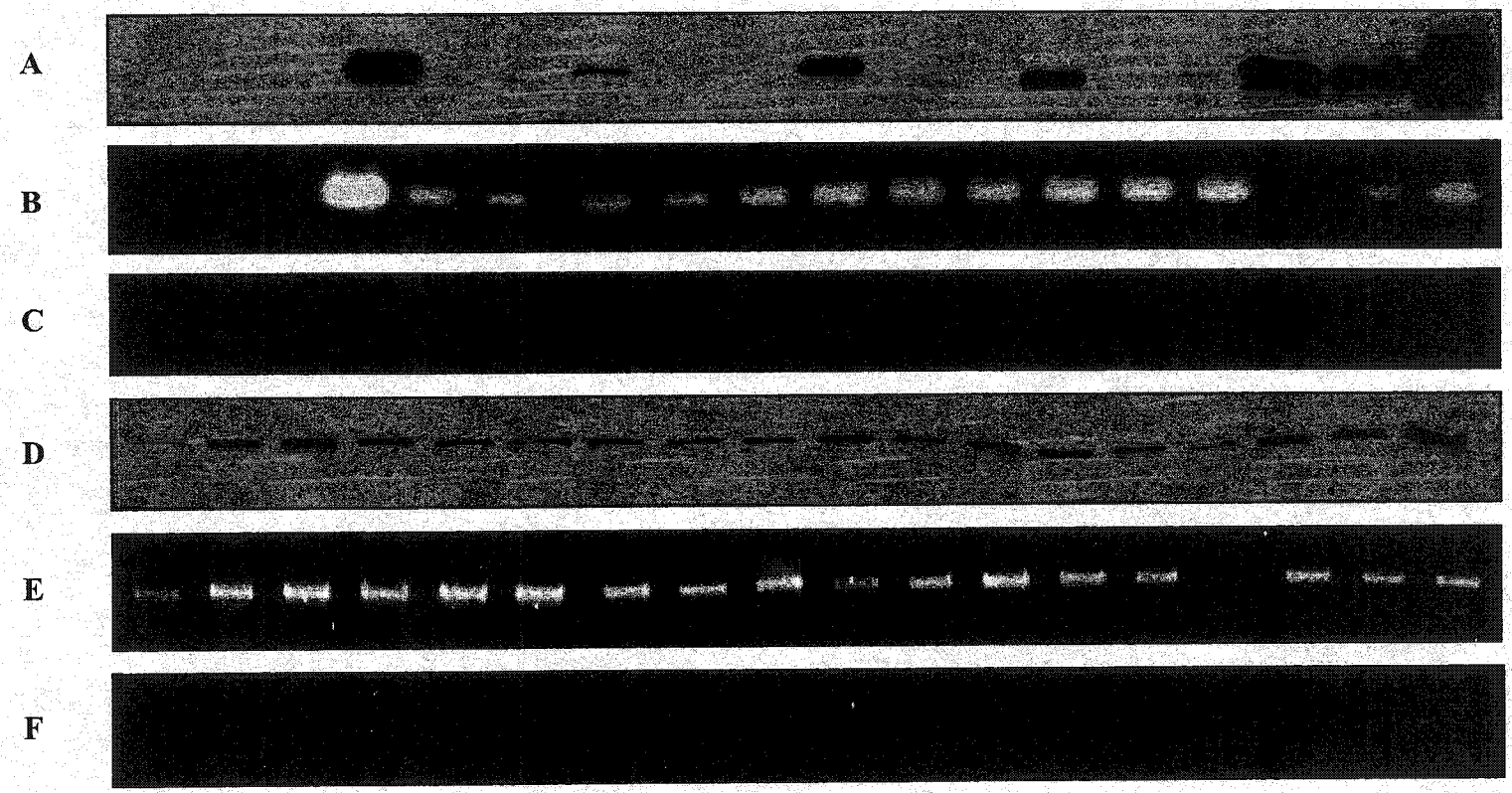


Figure 4.8 Phenotypic analyses of *Salmonella* strains that express FliC in different bacterial compartments. Constructs that encode wildtype FliC or FliC with an extra 31 amino acids were generated and transferred to FliC-negative *Salmonella*. FliC immunoblot and motility assay were used to determine FliC compartmentalization and function, respectively. Top panel indicates the expressed FliC protein and motility phenotype; A-D shows FliC immunoblots of log-phase *Salmonella* expressing plasmid-encoded FliC separated into total (A), intracellular (B) and extracellular (C) fractions (see Materials and Methods), or total FliC expressed by log-phase *Salmonella* with chromosomal-encoded FliC (D).

enhance antigen-specific immune responses (16, 18, 24, 56, 92). To construct a *Salmonella* strain that expresses FliC during systemic infection (FliC-UP), we first cloned the *fliC* gene downstream of the *pagC* promoter. To confirm *in vivo*-inducible expression from P*pagC::fliC*, we transferred the construct into *Salmonella* strains that genetically recapitulate the intracellular growth state via expression of the active phosphorylated form of PhoP ($PhoP^c$ and PhoP*) (23, 78, 116, 147). Surprisingly, $PhoP^c$ and PhoP* *Salmonella* carrying P*pagC::fliC* actually produced less FliC than $PhoP^{wt}$ bacteria (Figure 4.9A, lanes 13-15). Expression of control protein glutathione *S*-transferase (GST) under the *pagC* promoter was enhanced when PhoP was activated (Figure 4.9A, lanes 6-18), indicating that the cloned *pagC* promoter was functional and PhoP-inducible. Repression was independent of the promoter upstream of *fliC*, as *Salmonella* encoding FliC under the native *fliC* or heterologous *trc* promoter also repressed FliC production during PhoP activation (Figure 4.9A, lanes 4-12); comparison of P*trc::fliC1* and P*trc::fliC2* (Figure 4.9A, lanes 7, 10) indicates that the presence of the 5' untranslated region (5'UTR, see Materials and Methods for details) upstream of *fliC* is positively correlated with FliC expression (Figure 4.9A, lanes 4-12). RT-PCR analysis revealed that each promoter was transcriptionally active in *Salmonella* (Figure 4.9B, lanes 4-18), and transcript from the wild-type P*fliC::fliC* was repressed during PhoP activation (Figure 4.9B, lanes 4-6), confirming previous observations (Chapter 3). Immunoblot and RT-PCR for DnaK expression confirmed the presence of bacterial protein and RNA in all samples (Figure 4.9D, E). These results indicate that PhoP/PhoQ represses FliC expression at the post-transcriptional level, which is a novel finding. Regardless, FliC expression from P*pagC::fliC* (FliC-UP) may be enhanced *in vivo* relative to the native P*fliC::fliC* (FliC-WT), as the *pagC* promoter is activated *in vivo* (18), while the *fliC* promoter is completely shut off during systemic *Salmonella* infection (Wilkerson and Cookson, in

Figure 4.9 Phenotypic analyses of *Salmonella* strains encoding FliC under non-native promoters reveals PhoP/PhoQ-dependent post-transcriptional regulation of FliC. Constructs that encode FliC under the native *fliC* promoter or the heterologous *trc* promoter (1 = without *fliC* 5'UTR, 2 = with *fliC* 5'UTR, 5'UTR = 62bp upstream of *fliC* orf), or encode FliC or control protein GST under the *in vivo*-inducible *pagC* promoter were generated and transferred to wildtype *Salmonella* or *Salmonella* expressing constitutively active PhoP ($PhoP^c$ and PhoP*). Immunoblot and RT-PCR were used to determine allelic expression from stationary-phase *Salmonella*. Top panel indicates *fliC* genotype and PhoP phenotype of the different strains that were assayed for FliC or GST (A) or DnaK (D) protein by immunoblot, and for *fliC* or *gst* (B) or *dnaK* (E) transcript by RT-PCR (no RT controls for *fliC* or *gst* (C) and *dnaK* (F) shown).

fliC allele	null			P*fliC::fliC*			P*trc::fliC1*			P*trc::fliC2*			P*pagC::fliC*			P*pagC::gst*		
PhoP Phenotype	WT	PhoP^c	PhoP*	WT	PhoP^c	PhoP*	WT	PhoP^c	PhoP*	WT	PhoP^c	PhoP*	WT	PhoP^c	PhoP*	WT	PhoP^c	PhoP*
Lane	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18



preparation). Additionally, factors influencing *pagC* promoter activity *in vivo* may not be fully recapitulated by the PhoPc or PhoP* strains, such that increased expression of FliC from the *pagC* promoter *in vivo* may overcome any post-transcriptional repression effects.

Influence of FliC Compartmentalization and Regulated Expression upon Generation of FliC Immune Responses

To evaluate the influence of FliC localization and regulated expression upon generation of FliC-specific immune responses during *Salmonella* infection, we constructed strains encoding FliC-WT, -IN, -EX, and -UP in single copy on the *Salmonella* chromosome, orally infected mice with the different strains and measured the ensuing FliC immune response. In convalescent C3H/HeJ mice (d100 post-immunization), the percentage of FliC-specific CD4+ T cells present was very low (1.5% of total *Salmonella*-specific CD4+ T cells) and was similar regardless of immunizing strain (Figure 4.10A). The low percentage of FliC-specific CD4+ T cells is surprising, as previous studies suggested that FliC is a dominant recall antigen for memory CD4+ T cells. These results suggest that FliC is not a dominant recall antigen for memory CD4+ T cells, although alternative explanations are possible (see discussion). Experiments are currently underway to measure FliC-specific responses sooner after immunization (day 21), as the bacterial burden in C3H/HeJ mice infected with attenuated *Salmonella* is maximal at two weeks post infection (data not shown) such that the number of FliC and *Salmonella*-specific T cells should be accordingly higher.

Altered FliC regulation did influence generation of FliC-specific CD4+ T cell responses during immunization in C57BL/6 mice. Oral infection with FliC-WT *Salmonella*

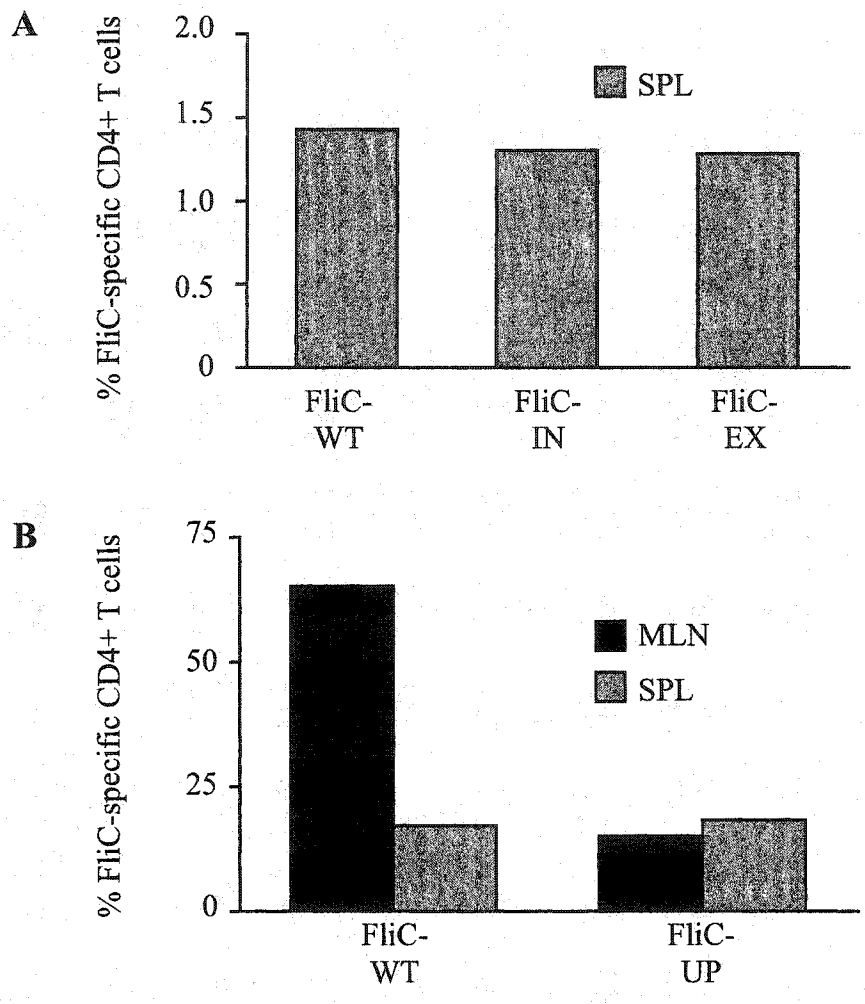


Figure 4.10 Regulated expression of FliC, but not compartmentalization, influences immune recognition of FliC during *Salmonella* infection. The %FliC-specific CD4+ T cells generated in C3H/HeJ (A) or C57BL/6 (B) following immunization with FliC-WT, -IN, -EX, or -UP strains in the indicated lymphoid organs were determined. %FliC-specific CD4+ T cells = [#FliC-specific CD4+ T cells/#HKST-specific CD4+ T cells] x 100). Responses were determined in C3H/HeJ at d100 and in C57BL/6 at d18 post-*Salmonella* infection.

generated FliC-specific T cells in both mucosal (mesenteric lymph nodes, MLN) and systemic (splenic) lymphoid organs, but at varying frequencies: 65% of *Salmonella*-specific T cells in the MLN were FliC-specific, as compared to 17% in the spleen (Figure 4.10B). The bias of MLN over splenic FliC-specific responses supports our observation that FliC is expressed in mucosal but not systemic lymphoid tissue after oral *Salmonella* infection (Wilkerson and Cookson, in preparation).

Demonstrating that FliC-UP *Salmonella* can express FliC *in vivo*, oral infection generated FliC-specific T cells comprising 15% and 18% of total *Salmonella*-specific T cells in MLN and spleen. However, the absence of increased frequencies of FliC-specific T cells in either organ during FliC-UP (*Salmonella* P*pagC::fliC*) as compared to FliC-WT (*Salmonella* P*fliC::fliC*) infection is surprising, as we predicted that FliC-UP would express larger amounts of FliC than FliC-WT and generate larger FliC-specific CD4+ T cell responses. Previous studies supported our initial hypothesis, as the *pagC* promoter is strongly transcribed by *Salmonella* in mucosal and systemic lymphoid tissue when green fluorescent protein (GFP) is used as a reporter (19), and we have observed the *fliC* promoter is strongly repressed during systemic infection using the same GFP reporter. Additionally, the *pagC* promoter has been used to drive *in vivo* expression of other non-*Salmonella* antigens to enhance antigen-specific immune responses. Several explanations exist for the inability of FliC-UP strain to enhance FliC expression *in vivo* and thus increased the frequencies of FliC-specific CD4+ T cells (see discussion). Future efforts to dysregulate FliC expression will utilize a tetracycline-inducible promoter, which will allow tighter control of FliC expression *in vivo*.

DISCUSSION

Here we describe host and microbial factors influencing immune recognition of the natural *Salmonella* antigen FliC. We identified two new FliC epitopes recognized by CD4+ T cells responding to *Salmonella* infection, and demonstrate that FliC epitope dominance *in vivo* is opposite that predicted by epitope prevalence during *in vitro* infection. Phenotypic analysis of *Salmonella* mutant strains revealed that FliC can be localized in various bacterial compartments, but that FliC expression is repressed both at transcriptional and post-transcriptional levels during *in vivo* growth conditions. Dysregulation of FliC expression via an *in vivo*-inducible promoter did not increase the number of FliC-specific CD4+ T cells generated during *Salmonella* infection, suggesting that post-transcriptional repression of FliC may also occur *in vivo*. Compartmentalization of FliC during infection had minimal influence upon the generation of FliC-specific memory CD4+ T cells.

Previous studies of *Salmonella*-specific CD4+ T cells identified FliC as a major target of the T cell response. Our examination of CD4+ T cells from *Salmonella*-immune mice confirmed that FliC is a natural antigen in two different haplotypes. Using FliC-specific T cell clones from immune mice as reagents, we confirmed the existence of two known FliC epitopes (339-350, 428-442) and identified two additional epitopes (82-92, 455-469). Multiple epitopes are often present in natural antigens. For example, the listeriolysin O antigen of *Listeria monocytogenes* contains at least 8 epitopes recognized by T cells from infected mice (73), and the ESAT6 antigen of *Mycobacterium tuberculosis* contains 2-6 epitopes recognized by CD4+ T cells from human tuberculosis patients (112). The presence of multiple epitopes in one antigen often results in epitope dominance, that is, epitopes are processed and presented more or less efficiently to

generate varying numbers of epitope-specific T cells (50). For FliC epitopes, two experiments suggested a particular epitope dominance: firstly, the ratios of epitope-specific CD4+ T cell clones generated following *in vitro* passage, secondly, the variable processing/presentation of epitopes by macrophages infected with *Salmonella in vitro*. Using the sensitive IFNγ ELISpot assay to quantify epitope-specific CD4+ T cells directly *ex vivo*, however, we observed a different epitope dominance predicted by *in vitro* studies. This discrepancy between epitope prevalence *in vitro* and epitope dominance *in vivo* has been observed before. *Listeria monocytogenes* infection generates MHC Class I-restricted CD8+ T cell responses to epitopes derived from listerial antigens (20). Using *Listeria*-infected cultured macrophages and epitope-specific T cell clones *in vitro*, it was determined that three epitopes were presented by infected macrophages in markedly different amounts, demonstrating a hierarchy of epitope prevalence. However, ELISpot analysis of epitope-specific T cell responses after *in vivo* infection revealed that epitope dominance was exactly the opposite observed during *in vitro* infection. These results suggest that antigen processing and presentation pathways used by infected macrophages to stimulate epitope-specific clones *in vitro* differ from pathways used by antigen-presenting cells to activate epitope-specific T cells *in vivo*. This could be due to additional environmental signals (e.g. cytokines) provided by the *in vivo* milieu, or use of different antigen-presenting cells stimulating CD4+ T cells *in vitro* (macrophages) versus *in vivo* (likely dendritic cells).

Identification of FliC epitopes and demonstration of epitope immunodominance provides a glimpse of the host's view of *Salmonella*. We speculated that *Salmonella* influences that view, by engendering microbial proteins with attributes that influence antigen-specific immune responses. Correlative evidence supports this hypothesis: natural *Salmonella*

antigens are both localized in surface organelles and repressed during bacterial growth *in vivo*. We tested the hypothesis that antigenic features influence antigen recognition by first altering either compartmentalization or regulated expression of the natural antigen FliC, then determining if FliC-specific immune responses were correspondingly changed during *Salmonella* infection. Others have attempted to address the issue of antigen compartmentalization in *Salmonella*. One study infected macrophages *in vitro* with *Salmonella* expressing model (non-native) antigen and observed no effect of antigen localization upon antigen-specific T cell stimulation (249). However, our results suggest that *in vitro* infection does not recapitulate antigen processing and presentation *in vivo*. A second study found that *Salmonella* expressing secreted listerial antigen provided protection against virulent *Listeria* challenge, while *Salmonella* expressing non-secreted listerial antigen did not (87). Interpretation of these results is complicated by the use of non-native antigen and *Listeria* as the challenge organism for readout of immune recognition. We designed our experiments such that *Salmonella* expressed the natural antigen FliC in different compartments, and quantified FliC-specific memory responses after *in vivo* immunization. Unfortunately, the frequency of FliC-specific CD4+ T cells in the memory subset was too low to permit comparison between mice. We plan to examine the impact of FliC compartmentalization upon FliC-specific T cell responses sooner after immunization (during the activation phase, rather than the memory phase), such that total FliC-specific responses will be greater and permit better comparison between mice immunized with the different strains.

We observed a low percentage of FliC-specific CD4+ T cells in most of our experiments, in two different haplotypes of mice and at different times after *Salmonella* infection. These results are surprising, as prior studies suggested that FliC is a dominant recall

antigen for CD4+ T cells responding to *Salmonella* infection. Our group has previously shown that in splenic CD4+ T cell lines from *Salmonella*-immune C3H/HeJ mice (derived by several rounds of *in vitro* restimulation), approximately 50% of the T cell response is directed to FliC (32, 140) (Chapter 3, Figure 1). Another group used ELISpot to quantify splenic CD4+ T cell frequencies directly *ex vivo* (i.e. with minimal *in vitro* restimulation) and observed that T cells responding to FliC comprised nearly 100% of the total *Salmonella*-specific CD4+ T cell population at d28 post-infection (32, 140). Several explanations may reconcile the contradiction between prior studies and results presented here. To explain the discrepancy in FliC-specific responses from C3H/HeJ mice, we speculate that the relative abundance of individual antigens in a given restimulating antigen will affect the population dynamics of *Salmonella*-specific CD4+ T cells during restimulation *in vitro*, such that the frequencies of antigen-specific CD4+ T cells will be different after multiple rounds of restimulation as compared to directly *ex vivo*. We typically use heat-killed *Salmonella typhimurium* (HKST), prepared from bacteria grown in rich media (LB), as restimulating antigen. HKST has copious amounts of FliC (data not shown) but may lack other antigens expressed solely *in vivo*, such that FliC-specific CD4+ T cells are preferentially selected during restimulation over CD4+ T cells specific to non-FliC antigens. Restimulating antigen prepared from *Salmonella* grown under "*in vivo*" conditions (i.e. growth in magnesium-limiting media, or PhoP^c bacteria) should allow *in vitro* propagation of CD4+ T cells specific to *Salmonella* antigens expressed preferentially during *in vivo* growth. To explain the discrepancy observed in the C57BL/6 background, we speculate that the presence of contaminating natural antigens in flagellin preparations used by McSorley and colleagues may have boosted the number of "FliC"-specific T cell responses to artificially high levels. We have observed that flagellin preparations can contain small amounts of contaminating LPS-associated proteins (likely

in membrane vesicles, Chapter 3) that are stimulatory for *Salmonella*-specific CD4+ T cells, such that non-FliC-specific T cells will respond to flagellin preparations, and consequently ensured the flagellin preparations used in our studies purified away from LPS. Thus we propose that T cells specific to non-FliC *Salmonella* antigens dominate the splenic CD4+ T cell response to *Salmonella*; current efforts are aimed at identification of additional non-FliC natural *Salmonella* antigens recognized by splenic CD4+ T cells. Our observations of large percentages of FliC-specific CD4+ T cells in the mesenteric lymph nodes of immunized mice suggests that the population dynamics can also vary between lymphoid organs.

Dysregulation of FliC expression did influence immune recognition of FliC, although not as expected. As we initially believed repression of FliC expression occurred only at the transcriptional level (Chapter 3), we circumvented the transcriptional block by replacement of the native "*in vivo*-repressible" *fliC* promoter with the well-defined *in vivo*-inducible *pagC* promoter, to create a *Salmonella* strain capable of increased FliC expression *in vivo*. Surprisingly, C57BL/6 mice infected with *Salmonella* carrying P*pagC* (FliC-UP) generated reduced or equivalent FliC T cell responses as compared wild-type *Salmonella* (FliC-WT). Firstly, the observed post-transcriptional repression of FliC production (Figure 4.9, discussed below) could be occurring *in vivo*, such that increased transcription of *pagC::fliC in vivo* does not result in increased FliC protein *in vivo*. Secondly, the FliC-UP strain does not express surface flagella and is non-motile while the FliC-WT strain does express functional FliC (data not shown), suggesting the possibility that the presence of FliC on bacteria in the immunizing inoculum is required for generation of FliC-specific CD4+ T cells. This hypothesis will be tested by adding exogenous flagella to non-flagellated bacteria and determining if the FliC-specific

immune response is increased. Thirdly, the FliC-UP strain is mildly defective for splenic colonization (10^2-fold fewer bacteria in spleen compared to parent strain, data not shown), which could explain the low frequency of FliC-specific CD4+ T cells in the spleen (i.e. fewer bacteria expressing FliC in this organ to drive FliC-specific T cell proliferation). Fourthly, it is possible that the *fliC* promoter is actually stronger than the *pagC* promoter during *Salmonella* mucosal infection, such that the FliC-WT strain expresses more FliC and primes more FliC-specific T cells in the MLN than does the FliC-UP strain; this hypothesis will be tested by directly comparing P*pagC::gfp* and P*fliC::gfp* reporters in *Salmonella* during infection of the mucosa. Finally, *Salmonella* may support overexpression of model or heterologous but not natural (i.e. FliC) antigens *in vivo* to enhance antigen-specific immune responses, as prior studies that used *pagC* to enhance antigen expression *in vivo* utilized non-natural *Salmonella* antigens like alkaline phosphatase, ovalbumin, and *E. coli* fimbrial protein. Future efforts to dysregulate FliC expression *in vivo* will utilize a tetracycline-inducible *fliC* allele to more precisely control FliC production during the different temporal and spatial stages of *Salmonella* infection.

Repression of FliC production is controlled by PhoP/PhoQ *in vivo* occurs not only transcriptionally but also post-transcriptionally, demonstrating that *Salmonella* has evolved multiple strategies control antigen production. Post-transcriptional control of expression of other flagellar apparatus components has been recently observed, demonstrating the promiscuity of this type of regulation in *Salmonella* (13, 109).
The observation that attenuated *Salmonella* expressing FliC normally (FliC-WT) primed larger FliC-specific T cell responses in the mesenteric lymph nodes than in the spleen is intriguing, as bacterial colonization is at least one log greater in the spleen than in the MLN at time of measurement (d21, data not shown). This result supports our hypothesis

that FliC is "on" during *Salmonella* infection of the gastrointestinal tract and mucosal lymphoid tissue but shut "off" during/after dissemination to systemic tissue, such that FliC is processed and presented in mucosal but not splenic tissue. Localized antigen expression and presentation has recently been demonstrated in the murine model of herpes simplex virus infection. Mueller and colleagues showed that a burst of early viral antigen expression and subsequent antigen capture at the local infection site was followed by antigen presentation in the draining lymph nodes to rapidly activate virus-specific T cell responses (160). Interestingly, the antigen (glycoprotein B, or gB) is a classic "late" viral gene product, such that maximal expression occurs much later after activation of gB-specific responses. We speculate that antigens like FliC and gB serve as decoy antigens: expressed early to prime antigen-specific immune responses at the initial infection site, then repressed to prevent further immune recognition and allow pathogen dissemination and colonization. Collectively, our results demonstrate that host T cell responses target the natural antigen FliC via recognition of multiple FliC epitopes, and suggest that *Salmonella* has correspondingly evolved the strategy of regulated FliC expression to escape host FliC-specific immune responses and more successfully colonize host tissue.

CHAPTER 5: CONCLUSIONS AND FUTURE DIRECTIONS

The overall theme of my thesis project was host-pathogen interactions. My examination of interactions at the cellular level showed that macrophages infected with *Salmonella* died by a unique pro-inflammatory mechanism distinct from necrosis and apoptosis. Peering through the window of host immunity revealed that innate immune recognition of *Salmonella* surface organelles directed adaptive immune responses to specific natural *Salmonella* antigens. Surprisingly, *Salmonella* inhibited antigen-specific T cell recognition by repressing antigen production *in vivo* at the transcriptional and post-transcriptional level. Finally, I found that the natural *Salmonella* antigen FliC contains multiple epitopes for FliC-specific T cells, and that mucosal immune responses to FliC are disrupted by dysregulation of FliC expression during oral *Salmonella* infection.

Publications describing *Salmonella*-induced macrophage apoptosis were abundant when I started this project. I initially hypothesized that *Salmonella*-specific CD4+ T cells might contribute to immunity by helping infected macrophages avoid apoptosis, as this phenomenon had been observed in other model systems of macrophage apoptosis. I began studying *Salmonella* infection of macrophages, with the eventual goal of examining the tripartite interaction of bacteria, macrophage, and T cells. Surprisingly, I consistently observed that *Salmonella*-infected macrophages displayed features of death distinct from apoptotic macrophages (summarized in Table 2.1): diffuse rather than condensed patterns of nuclear fragmentation and absence of pro-apoptotic enzyme caspase-3 activity (Figures 2.1, 2.4). Like necrotic cells, *Salmonella*-infected macrophages had damaged membranes (Figures 2.2, 2.3), retained the pro-necrotic enzyme PARP in its active uncleaved state (Figure 2.5), and lethal ion fluxes contributed

to cell death (Figure 2.6). Indicating a unique mechanism of death, *Salmonella*-induced cytotoxicity, but not apoptosis or necrosis, required caspase-1 activity (Figure 2.6). We have proposed that caspase-1 dependent necrosis be termed pyroptosis (Greek *pyro*, fire or fever, *ptosis* a falling) to indicate the screaming, alarm-ringing pro-inflammatory nature of the mechanism of cytotoxicity (33). The discovery of pyroptosis has significant implications for the field of bacterial pathogenesis. Publications describing microbe-induced cytotoxicity abound, almost uniformly reporting DNA fragmentation and concluding apoptosis. The trend is puzzling, given that DNA fragmentation can occur during necrosis, and the inflammatory consequences of apoptosis (usually none) and microbial infection (significant). *Shigella* was the first pathogen described to induce macrophage apoptosis, but *Shigella*-induced cell death resembles that induced by *Salmonella*, and shigellosis *in vivo* is marked by extensive inflammation. Our results suggest that careful and prudent examination of microbe-induced cytotoxicity should precede definitive assignment of the mechanism of death.

Currently, explorations of *Salmonella*-induced cytotoxicity are focused the mechanistic and phenotypic hallmarks of death, discovery of caspase-1 substrates, and elucidation of the consequences of pyroptosis during *in vivo* infection. As caspase-1-dependent pathophysiological cell death occurs in the immune, central nervous and cardiovascular systems (68, 118, 211), *Salmonella*-induced macrophage death provides a good model for dissecting mechanisms downstream of casspase-1 activation. Future experiments may also address the relationship between macrophage pyroptosis and microbial immune responses. Suggesting a positive correlation between *Salmonella*-induced macrophage death and immune recognition of *Salmonella*, it was recently shown that dendritic cells phagocytose dying *Salmonella*-infected macrophages and cross-present model antigens to

stimulate antigen-specific T cells *in vitro* (256). However, immunization with *Salmonella* expressing model antigens primed antigen-CD8+ T cells independently of *Salmonella* SPI1-induced cell death (250), suggesting pyroptosis is not necessary for induction of specific CD8+ T cell responses. It is unknown if *Salmonella*-induced pyroptosis is required for generation of *Salmonella*-specific CD4+ T cells *in vivo*. A combination protocol of *in vitro* macrophage infection followed by *in vivo* injection of dying cells and measurement of ensuing immune responses may reveal if SPI1-dependent macrophage death is sufficient to cross-prime T cell responses.

The studies of CD4+ T cell responses to specific *Salmonella* antigens have been both fruitful and intriguing. Characterization of antigenic specificities of CD4+ T cells from *Salmonella*-immune mice (Figure 3.1) revealed novel features of natural *Salmonella* antigens. Firstly, antigens were shown to localize in special bacterial surface organelles, flagella and membrane vesicles (Figures 3.2, 3.3, 3.6). Expression of antigens FliC and p10 was repressed during *Salmonella* growth *in vivo* via PhoP/PhoQ (Figures 3.4, 3.5), the two component regulatory system that also controls bacterial surface modifications engendering innate immune resistance. Finally, natural antigens were shown to be recognized by TLRs or intimately associated with TLR ligands (Figures 3.3, 3.6), suggesting that innate recognition of surface organelles directs antigen-specific T cell responses.

Identification of membrane vesicles as a rich source of natural antigens for *Salmonella*-specific CD4+ T cells has generated several new questions. Firstly, what are these antigens? We can distinguish individual antigens by apparent molecular weight (p10, p12, p14, p15, p18, p35) using one-dimensional SDS-PAGE. However, the complexity of

species in 1D SDS-PAGE fractions has prevented accurate identification; moreover, the high affinity of antigens for LPS has challenged traditional protein purification methods (data not shown). Current efforts are using 2D gel electrophoresis to determine the pI and molecular weight of *Salmonella* membrane proteins; spots containing stimulatory antigen for *Salmonella*-specific CD4+ T cells will be subjected to liquid chromatography-mass spectrometry for amino acid sequence determination. Comparative proteomics and bioinformatics may also facilitate antigen identification, as numerous members of *Enterobacteriaceae* express some of these antigens (data not shown) and also possess completely sequenced genomes. The conserved nature of these antigens begs the question: why do non-*Salmonella* bacteria express antigens recognized by CD4+ T cells from *Salmonella*-immune mice? As the conserved antigens localize in membrane vesicles, it may be that membrane vesicle production pathways are conserved amongst different bacteria, such that homologous bacterial proteins route to these organelles. Future studies of membrane vesicles will seek to identify factors mediating membrane vesicle biogenesis via genome-wide mutagenesis coupled with high-throughput screens for vesicles (LPS) in bacterial culture supernatants. Finally, is CD4+ T cell recognition of vesicular antigens unique to *Salmonella* infection? Preliminary investigations of *Yersinia enterocolitica*-infected mice revealed CD4+ T cells responded to heat-killed but not vesicular yersinial antigen (data not shown). This observation may reflect a requirement for membrane vesicle production within infected antigen-presenting cells, as Yersiniae reside extracellularly during infection, while Salmonellae and Mycobacteriae both replicate and produce membrane vesicles during intracellular infection (8, 72, 169, 206).

My examination of the CD4+ T cell response to the natural *Salmonella* antigen FliC (Figure 4.1) confirmed the existence of known epitopes (339-350, 428-442) and

identified new epitopes (82-92, 455-469) (Figures 4.3, 4.4), in regions of amino acid sequence unique and common to different bacterial flagellins (Figures 4.2, 4.5). Epitope-specific T cell responses differed between infection *in vitro* and *in vivo* (Figures 4.6, 4.7), allowing determination of FliC epitope immunodominance. Immunization with *Salmonella* localizing FliC in different bacterial compartments (Figure 4.8) had minimal influence on maintenance of FliC-specific memory CD4+ T cells in the convalescent host (Figure 4.10). Dysregulation of FliC expression revealed that antigen production is controlled both transcriptionally and post-transcriptionally (Figure 4.9), and that mucosal immune responses to FliC are highly dependent on wild-type expression during oral immunization (Figure 4.10).

One obvious question arises from the study of CD4+ T cell responses to FliC: how are these responses primed, if FliC expression is repressed *in vivo*? From our studies of mice orally infected with *Salmonella* carrying P*fliC::gfp*, we know that bacteria express GFP in Peyer's Patches and somewhat in mesenteric lymph nodes, but are non-fluorescent once established in the spleen (Wilkerson and Cookson, in preparation). Intraperitoneal (i.p.) injection of *Salmonella* to directly produce systemic infection also provides the necessary signals for repression of FliC production, as *Salmonella* from spleens of i.p.-infected mice do not express FliC (Cummings and Cookson, in preparation). Additionally, activation of FliC-specific T cells occurs only in mucosal but not systemic tissue, despite the presence of bacteria in both areas (139). Collectively, these investigations suggest that *Salmonella* senses signals first during gastrointestinal colonization and then during dissemination to alternatively activate and repress FliC production. This hypothesis could be tested by via i.p. infection to circumvent gastrointestinal signals, and determination of mucosal versus systemic FliC-specific

CD4+ T cells as compared to those generated following oral immunization. Alternatively, one could eliminate bacterial detection of signals to repress FliC expression by infecting with PhoP-null bacteria (PhoP-null *Salmonella* are incapable of repressing FliC expression during intracellular growth, Figure 3.5), and determine if FliC-specific immune responses are increased. Enhanced expression of FliC in PhoP-null *Salmonella* could explain why these mutant bacteria stimulate protective immunity rather than fatal infection in murine and human hosts (64, 69, 91, 146, 235). Others have suggested that *Salmonella*-specific T cell responses are primed to antigen from dead *Salmonella*, as histological examination of infected Peyer's Patches revealed that intact *Salmonella* cells do not colocalize with antigen-specific CD4+ T cells (adoptively transferred into mice prior to oral immunization) (17). FliC stimulation of intestinal epithelial cells recruits dendritic cells (214), allowing them to penetrate gut epithelial monolayers and sample lumenal bacteria (190), suggesting that intestinal dendritic cells might capture and present flagellin to prime FliC-specific CD4+ T cells. To test the hypothesis that antigen-specific CD4+ T cells are primed in response to "carrier" antigen and not endogenously expressed antigen, one could orally immunize mice with a mixture of flagellin-negative *Salmonella* plus exogenous purified FliC and evaluate generation of FliC-specific T cells, comparing to control mice immunized with flagellin-positive or flagellin-negative *Salmonella*.

The realization that FliC-specific T cells comprise only a small fraction of splenic CD4+ T cells responding to *Salmonella* infection suggests that other antigen-specific T cells dominate this response. Preliminary experiments using *Salmonella* membrane vesicles as stimulatory antigen to quantify antigen-specific CD4+ T cells directly *ex vivo* suggest that the majority of *Salmonella*-specific T cells from spleens of immune mice respond to vesicular antigens (data not shown). Identification and purification of non-flagellin

antigens will allow more accurate assignments of antigen immunodominance during *Salmonella* infection; a combination of bioinformatics and proteomics is currently being utilized to identify those antigens found in membrane vesicles. Additionally, elucidation of antigens expressed during the splenic phase of *Salmonella* infection should help focus vaccine efforts. Expression of different antigens subsets by *Salmonella* during different phases of infection may explain the observed chronic *Salmonella* persistence in humans (125, 176), exemplified by Typhoid Mary (198). As *Salmonella* spp. continue to plague humankind, rational vaccine design becomes even more important.

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ABSTRACTS

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