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Salmonella Host Specificity and Role of Macrophages *in vivo*

A Dissertation submitted in partial satisfaction of the requirements for the degree
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in

Biology

by

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DEDICATION

to

my parents

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PUBLICATIONS and PRESENTATIONS

1. The use of bacterial minicells to transfer plasmid DNA to eukaryotic cells. Giacalone MJ, Gentile AM, Lovitt BT, Xu T, Surber MW and Sabbadini RA. Cell Microbiol. 2006 Oct; 8(10): 1624-33.

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ABSTRACT OF THE DISSERTATION

Salmonella Host Specificity and Role of Macrophages *in vivo*

by

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According to their host range, *Salmonella enterica* can be divided into generalists, host-adapted, and host-specific serovars. The generalist *Salmonella enterica* serovar Typhimurium causes disease in many animal species but host specific serovars cause disease only in one host. In this study, the hypothesis that macrophages contribute to *Salmonella* host specificity *in vivo* was tested in mice. The survival of Typhimurium and the host specific *Salmonella enterica* serovar Typhi were compared in mouse macrophage cell lines (RAW 264.7 and J774A.1), and murine peritoneal, spleen and bone marrow derived macrophages *in vitro*. Differences in survival

observed for the two serovars in these *in vitro* studies could not distinguish host specific from generalist serovars. When BALB/c mice were infected *in vivo* i.p., equivalent levels of Typhimurium could be found in the peritoneum 0.5 and 4 hr post-infection but Typhi decreased drastically between these two time points. Both Typhi and Typhimurium induced neutrophil influx into the peritoneum and macrophages disappeared with both serovars. With either serovar, macrophages were shown to be the major cell type containing internalized bacteria after both 0.5 and 4 hr of infection. However, significantly lower numbers of viable Typhi were recovered from macrophages infected *in vivo* than Typhimurium. These data showed that macrophages were able to distinguish Typhi from Typhimurium when infected *in vivo,* suggesting that the *in vivo* environment is important for macrophages to play an important role in *Salmonella* host specificity.

General Introduction

Salmonella is a Gram-negative facultative rod-shaped bacterium. Currently over 2500 *Salmonella* serovars belonging to six subspecies are recognized, but only about 50 serotypes have been isolated and are considered as human and animal pathogens (46, 160). They all belong to *Salmonella* enterica subspecies (S. enterica). Subspecies are subdivided into serovars according to their flagella (H), somatic (O) and capsular (Vi) antigens. S. enterica species are usually oral pathogens and cause salmonellosis in the form of enterocolitis/diarrhea, enteric fever (typhoid), septicemia, abortion or chronic asymptomatic carriage. The severity of the diseases depends on both host susceptibility and the infectious serovar involved (46).

Salmonella **host specificity**

According to their host range, *Salmonella* serotypes are divided into three groups: host generalist, host adapted and host specific. Based on the systemic disease caused by the organism, host specific serotypes typically cause systemic disease in a specific host. For example, Typhi is exclusively associated with systemic disease in humans. Host adapted serotypes generally cause disease in one animal species and may also cause disease infrequently in other hosts, such as Dublin. Host generalists have a wide range of host animals, such as Typhimurium and Enteritidis (160). Investigations on bacteria-special nutritional requirements, bacterial and host genetic

background, pathogen-host interaction, et al. indicated that all of them might get involved in determining S*almonella* host-specificity.

Salmonella host specificity and special nutritional requirements have been paid attention to in the past. *Salmonella* strains have been divided into “ammonium strong” and “ammonium weak” strains depending on their ability to assimilate nitrogen from ammonia in a defined media that contains simple carbon compounds such as citrate (Simmons citrate agar) (175). Host generalists were usually found to be “ammonium strong”, while some host specific or host adapted serovars have been found to be “ammonium weak”. For example, several Dublin strains were unable to grow in Simmons citrate agar (45). Host generalists seem easier to grow in simple media, which might be the reason that they can survive and cause disease in many different hosts. In contrast, host specific serovars might have special requirements on specific substances, such as certain amino acids, which can only be obtained from limited host species.

Bacterial genetic determinants have been studied *via* genetic, molecular, and genomic approaches. One strategy is to isolate mutants with altered host-specificity. For example, an insertional mutagenesis system using transposons was developed to identify bacterial virulence genes (63). Another approach is to construct hybrid strains carrying heterologous host-specificity determinants (179). *In vivo* expression technology has also been developed to detect *Salmonella* genes that are specifically induced in host tissues (99). Over 200 virulence genes have been identified and some of them, e.g. in *Salmonella* pathogen island (SPI)-1 and SPI-2, will be described in detail later.

The *rpoS* (aka *katF*) gene encodes an alternative sigma factor for RNA polymerase that functions as a global stationary phase regulator and controls important aspects of *Salmonella* virulence (42). The *rpoS* gene from *Salmonella* Typhimurium was cloned and its sequence was highly conserved relative to the *E. coli rpoS* gene (134). RpoS is involved in general stress resistance such as nutrition deprivation, acid stress and DNA damage (141). In *Salmonella* Typhimurium, it is showed that RpoS influences the expression of *Salmonella spv* plasmid virulence genes during bacterial starvation. The *spv* genes encoded on these plasmids are essential for the virulence of Typhimurium in systemically infected mice, hence *rpoS* mutants in *Salmonella* Typhimurium have attenuated virulence in susceptible mice (62).

In contrast, serotype Typhi does not have the Spv plasmid or the *spv* genes, and the role of *rpoS* in the virulence of Typhi is still not tested (141). It has been suggested that RpoS might also contribute to the virulence of Typhi because *rpoS* mutants of Typhi are less cytotoxic than wild type strain (87). However, the Typhi *rpoS* mutant survived better inside resting THP-1 macrophages. In addition, RpoS plays a role in regulation of synthesis of Vi polysaccharide, which is a major virulence determinant in Typhi (145).

Relative levels of RpoS can be measured by monitoring β-galactosidase expression from a *katE-lac* fusion (15, 31).There is considerable evidence that *rpoS* mutations which affect RpoS levels accumulate when bacteria are stored on laboratory media ((88, 125)). This may explain why different strains of *Salmonella enterica*, even strains of the same serovar and multiple strains of the same original isolate, have

different levels of RpoS expression. The Typhi Ty2 strains used in this study have "intermediate" levels of RpoS expression (April Stanley, unpublished results).

Host genetic background also contributes to the risk of infection and disease severity. In human tuberculosis studies, a racial difference in disease susceptibility was reported (1, 6, 57). Studies using large-scale family-based genome scans indicated a higher concordance on monozygotic *vs* dizygotic twins. Congenic mouse strains are used to study the interaction between host and *Salmonella* (149). According to their susceptibility to *Salmonella* infection, classical inbred mice strains can be classified into three distinct groups: 129S6/SvEvTac mice are extremely resistant to infection. A/J mice show an intermediate susceptibility phenotype and the survival time of the infected mice increases with decreasing infectious dose. Other strains, such as C57BL/6, BALB/c and C3H/HeJ, are extremely susceptible to infection and all succumb within the first week after infection. *Salmonella* resistance genes, such as the natural resistance-associated macrophage protein gene (Nramp1) and Toll-Like Receptor 4 (TLR4) have been well investigated and will be described below.

Both innate and acquired immunity play important roles in *Salmonella* pathogenesis at different stages of infection with potential involvement in the development of host specificity. This will be discussed in detail in the following pages. The two serovars focused on in this study are *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) and *Salmonella enterica* serovar Typhi. (*S.* Typhi). *S.* Typhimurium is a host generalist and causes a typhoid-like disease in mice. In humans *S.* Typhimurium does not cause as severe systemic disease and is not normally fatal. The disease is characterized by diarrhea, abdominal cramps, vomiting, and nausea and

generally lasts up to 7 days. But in immunocompromized people, *Salmonella* infections are often fatal if they are not treated with antibiotics. *S.* Typhi is a host specific serovar to human causing typhoid fever. The symptoms of typhoid fever include nausea, vomiting and fever. It will cause death in the worst cases. It is important to note that Typhi will not cause disease in mice, even at very high doses.

Typhoid fever

Human typhoid fever is caused by *S.* Typhi and *S.* Paratyphi infection. It is a disease occurring more commonly among people after travel to, or residence in, developing countries. The morbidity of typhoid fever is more severe among infected immunosuppressed patients (71). Most of the infections occur through ingestion of contaminated food or water. Besides the systemic symptoms such as fever, headache, or splenomegaly, extra-intestinal infectious complications sometimes also happen in many other systems, for example in the central nervous and cardiovascular systems.

S. Typhi is serologically positive for lipopolysaccharide antigens O9 and O12, protein flagellar antigen Hd and polysaccharide capsular antigen Vi. The Vi capsular antigen is largely restricted to Typhi. The virulence of *S.* Typhi is dependent on its ability to invade cells, which is in turn dependent upon its possession of a complete lipopolysaccharides (LPS) coat, the presence of the Vi antigen, and the production and excretion of a protein known as invasin. It was shown that Typhi and Typhimurium used similar mechanisms of invasion and intracellular trafficking in human epithelial cells (112). The expression of Vi antigen has been determined to be crucial for Typhi to survive in mouse and human macrophage cell lines (66). Because of its host

restriction characteristics, most of the pathogenesis studies have been based on Typhimurium infection in susceptible mice as indicated and in vaccine development (39). Similar to Typhimurium infection in mouse, once the intestinal epithelial layer is crossed, Typhi enters the blood stream, surviving within macrophages, and disseminates to the liver, spleen, bone marrow and other organs rich in phagocytic cells. In contrast, Typhimurium infection in human is usually localized to grastrointestinal tract with enteritis symptoms including diarrhea, vomiting and abdominal pain (100, 181).

***In vitro vs in vivo* models**

As described above, *Salmonella* species infect a broad range of animals and can cause different diseases among the hosts, which makes the interaction between the host and pathogen more complex. Both *in vitro* tissue culture and *in vivo* animal models have been widely used to investigate the bacterial virulence factors, the host response against infection, and the mechanisms that lead to host specificity.

In vitro (tissue culture) for *Salmonella* Pathogenesis

Much has been learned about the interaction between *Salmonella* and host using cultured mammalian cells. The advantages of the *in vitro* studies are that they are more consistent and the cell lines are relatively easy to handle. Epithelial cell lines and macrophage cell lines are the most commonly used and have provided enormous amounts of information about how *Salmonella* interacts with them, much of it at the molecular level. *Salmonella* adherence and invasion processes have been well

investigated in epithelial cell culture. For example, unlike some other pathogenic bacteria that use adhesion-receptor interactions for their uptake, *Salmonella* use a Type Three Secretion System (TTSS) to cause host epithelial cell ruffling and thus drive their internalization. The molecular genetic analysis has revealed that TTSS mediates the export and/or translocation of putative signaling proteins into the host cell (24). Rearrangements of the actin cytoskeleton have been shown to be crucial to invasion. Involved effectors in this signaling pathway have been identified on Henle-407 epithelial cells (61). However, the ability of *Salmonella* to survive and replicate within macrophages has been shown to be closely related to the virulence, which makes macrophage cell lines the most useful model to discover the molecular mechanisms. This will be discussed in detail later.

Despite those advantages of the *in vitro* models, it is obvious that they also have their limitations and problems (48). For example, cultured cells are usually immortalized and normal disease processes in cultured cells are affected. Also because these cells are studied in an isolated status, it is not possible to study their interaction with other cells and factors that are present *in vivo*. Some cell types, such as M cells, which play a major role in *Salmonella* penetration across the intestinal barrier, are difficult to study *in vitro* because it's still impossible to grow them in tissue culture. During culturing, mammalian cells sometimes lose important features seen *in vivo*. It is a great challenge to test whether the events studied in tissue culture actually occur during disease *in vivo*. Combining tissue culture and animal studies and comparing the results obtained from different models is apparently important.

Animal Models for *Salmonella* Pathogenesis

Various animal models are available to study both intestinal enteritis and systemic salmonellosis. It is important to choose proper animal models to study the particular bacterial effectors or host mechanisms. *S*. Typhimurium infection on susceptible mice is a crucial model for systemic disease because it shares similar symptoms with human typhoid fever. Livestock, such as cattle or chickens, are also widely used since they are a major source of human infection especially for enteric disease. For example, different roles of *Salmonella* pathogenicity island 1 (SPI-1) and SPI-2 have been investigated using newly hatched chicks (80, 81, 173). A calf ileal-loop model of enterocolitis was selected to demonstrate the induction of massive neutrophil influx into intestine by *S.* Typhimurium but not by *S.* Typhi (135). Recently, a new model of *Salmonella*-induced enterocolitis has been developed using streptomycin-pretreated mice, which made it possible to explore the factors of *Salmonella* that induce acute intestinal inflammation *in vivo* (4). Combining this new mouse model and a calf ileal-loop model, a novel role of *Salmonella* enterica serovar Typhimurium TTSS in intestinal disease has been discovered *in vivo* (27).

Disparity between *in vitro* and *in vivo* was found using animal models and this emphasizes the importance to understand pathogenesis i*n vivo*. For example, in a study on how *S.* Typhimurium impacts immunity *in vivo*, Duncan Maskell and Pietro Mastroeni found that in contrast to infections in tissue culture, where *Salmonella* grow to high numbers within macrophages, only one or two bacteria grew per phagocyte *in vivo* (152). The growth of *Salmonella* in the liver results in the spread of the microorganisms to new foci of infection rather than simply in the expansion of the

initial foci. These foci were associated with independently segregating bacterial populations and with low numbers of bacteria in each infected phagocyte.

The *Salmonella* mouse typhoid model has been extensively used to study the mechanisms of pathogenesis and immunity in typhoid salmonellosis. *S*. typhi is a host specific serotype because it exhibits limited host range to humans and chimpanzees. The infections are usually caused by contaminated water, animal products, or close contact with an infected individual or carrier (68, 69). Since the severity and outcome of *Salmonella* infections in mice depend on several variables, including the virulence of the infecting strains, infectious dose, route of infection, genetic background, and immunological status of the host, most of the research on the pathogenesis of typhoid fever is based on the infection of susceptible mouse strains (e.g., Balb/c) with *S.* typhimurium. Following oral inoculation, virulent *S.* Typhimurium survives the non-specific host defense, such as gastric acidity and intestinal mucus, competing with the normal gut microflora (8, 23, 154). After colonization in the ileum and cecum, it penetrates the intestinal epithelium and enters the Peyer's patches (18, 25). This process is via M cells, a specialized cell population overlaying the Peyer's patches (79, 123). Some of the bacteria then move into the mesenteric lymph nodes and gain access to the host circulation via the efferent lymph, leading to transient bacteremia (113). They are rapidly cleared from the blood by phagocytes in spleen and liver and a large portion of them are killed by these cells (21, 34, 35, 140). The remaining *Salmonella* reach and multiply within the intracellular location of the reticuloendothelial system such as spleen and liver. This is also the phase where extraintesinal infection (e.g. intraperitoneal infection) starts. In the reticuloendothelia system, the bacteria

associate with phagocytic cell populations including macrophages, neutrophils and dendritic cells as well as non-professional phagocytes in those organs (140, 178). In mice, approximately 10^8 bacteria are thought to be the critical load for survival, and if bacterial titers reach this threshold, the animal is no longer able to contain the infection. As a consequence, secondary bacteremia, endotoxic shock and rapid death happen (113).

During non-fatal infection, mice restrict bacterial growth at a certain level. Depending on the mouse strain and the strain of *S.* Typhimurium used, disease lasts from one to several weeks characterized by splenomegaly, general macrophage-mediated immune suppression and a plateau level of bacterial load (113). Similarly, human typhoid patients can become asymptomatic carriers for months or years (23). At the end, an acquired immune response will be generated against reinfection. How long this protection will last is not quite clear. However, from the vaccine development studies indicates the protective immunity produced by vaccination might prevent *Salmonella* infection for years (107, 156).

Host immune response to *Salmonella* infection

Infection of susceptible mice with *S.* Typhimurium results in a systemic infection similar to Typhi infection in human. Both innate immunity and acquired immune responses are important to control the primary infection and protection against secondary infection.

Innate Immunity to *Salmonella*

The innate immune defense system consists of many different components that act coordinately to respond rapidly and hopefully eliminate, or at least contain, the infection. Epithelial cell layers of the skin, intestine or respiratory tract are the first protection barriers provided by the innate immune system. They not only provide a physical barrier but also have bacterial killing capacity through antimicrobial substances such as defensins (54). A diverse array of cell types including neutrophils, macrophages, dendritic cells (DCs), natural killer (NK) cells and NKT cells, are the most important cell populations of this system. Other granulocytes such as mast cells and eosinophils can also have a role in innate defense mechanisms. Besides these cellular components, numerous soluble products such as cytokines, chemokines, complement components, and secreted antibodies are also essential parts of the host innate immune system (103, 128, 172).

After oral ingestion of *Salmonella*, the intestinal epithelium is the initial place where the host and bacteria interact and initiate the innate immune response. *In vitro* studies have shown that *Salmonella* interaction with epithelial cells results in a proinflammatory response characterized by the release of several cytokines and chemokines (135, 180). IL-8 is in one of the best investigated. Activation of the nuclear factor-kappa B (NF-κB) signal transduction pathway is central in mediating the epithelial proinflammtory response, including IL-8 release (56). *Salmonella*-induced cytokines and chemokines attract neutrophils migrating through the epithelial layer into the intestinal lumen (108).

As indicated, various cell populations get involved during the early stage of infection. Understanding the coordination and function of those cells, as well as

identifying the cell types that contain bacteria, provide valuable information on the innate immune response to *Salmonella*.

Dynamics of cell populations in different organs have been studied and proved useful in providing a clearer picture of their role during *Salmonella* infection. Flow cytometry and fluorescence microscopy have been used to analyze the cell surface molecules for identification purposes. Among those cell populations, neutrophils and macrophages are the ones that have been focused on to elucidate their involvement and contribution to the innate response during the early stages of infection. Neutrophils are recruited rapidly after injection of bacteria into experimental models (47). Changes that occur in innate cell populations in the spleen during the early stages of oral *Salmonella* infection have also been quantitatively investigated (90). In this study, macrophages and neutrophils were tested by the expression of their surface markers through flow cytometry analysis. These cell populations expanded 3- and 10-fold, respectively, in mouse spleen five days after oral infection. Since neutrophils were rare in the spleen of naïve mice, this increase resulted in approximately equal number of macrophages and neutrophils in the spleen. Other cell types like NK cells and NKT cells were also analyzed in the same study. In contrast to the phagocytes, immune animals had a modest reduction in NK cells, and no change NKT cells. DCs are also an important cell type increased in the infection and are poised to initiate the adaptive immune response with their antigen presenting function. Similar to other cell types, DCs also contain different subsets. The localization, number, and function of differential DC subsets were defined during acute *S.* Typhimurium infection (89).

However, despite the increase in DC numbers in infected mice relative to naïve animals, they remain less abundant than neutrophils or macrophages (172).

Not only changes in the cell number of each type were studied, but also the functions of each cell population have been investigated. One of the important functions for those cells is their cytokine secretion ability. Two approaches have been used to study the role of specific cytokines in the pathogenesis of *Salmonella* infection. One is focused on the interactions of bacteria with specific cell types in cell culture (37). The advantage for this approach is that it allows the identification of the cellular mechanisms by which *Salmonella* enters that defined cell type and the corresponding cellular responses. The second approach is to characterize the role of certain cytokines during the course of infection, and the consequence of altering cytokine levels for infection *in vivo* (37). These are usually done using mouse models and may explain the physiological interaction in the infection with limitation on identifying the related cell types (37). During the early stage of the infection, *Salmonella* components, such as LPS of the cell wall and lipoproteins induce a massive inflammatory response in the surrounding tissue, resulting in the expression of inflammatory cytokines and chemokines that recruit cells of the immune system to these sites (36, 83). All cell types involved in innate immunity have been studied using these approaches, including intestinal epithelial cells, macrophages, neutrophils, DCs, NK cells and NKT cells. A range of cytokines, such as IFNγ, TNFα, IL-1, IL-6, IL-12, IL-18, and chemokines, has been detected. It has been shown that some cytokines could be expressed by different cell types. For example, IL-1, IL-6, IL-12 and IL-18 are made by both macrophages and DCs (102, 105, 106, 147). IFNγ is probably the

most studied cytokine in host defense against *Salmonella*. It is expressed mainly by NK cells (136, 146). But additional potential sources for it are macrophages, B cells, and specialized T cell populations, such as NK T cells. Those cells are able to recognize conserved structural patterns of bacteria (40, 120, 177). IL-18 and IL-12 are important regulators to enhance IFNγ expression (105, 106). Macrophages and DCs are major sources of these cytokines as indicated, and the expression of IL-12 is further enhanced by IFNγ through a feedback loop. IL-12 is also important for the polarization of T helper cells toward the Th1 pole, characterized by the expression of proinflammatory cytokines (113).

In summary, the initial stages of *Salmonella* infection are characterized by effective recruitment and activation of phagocytes. Large amounts of IFNγ are produced by a variety of cells and this causes inflammation in the infected tissue. Large numbers of bacteria are then eliminated and the host is able to contain *Salmonella* infection to a certain degree.

Acquired Immune Response to *Salmonella*

The host innate immune response is effective in restricting initial growth of bacteria, but it fails to completely kill the invading pathogen. The acquired immunity is developed during a later stage of the infection and is required for eradication of the infection.

Although there is no doubt of the importance of T cells for both primary and secondary immune response, the function of different T cells subsets in each infection stage is not quite clear. $CD4^+$ T cells were found to have a more pronounced effect on

control of primary *Salmonella* infection and protection induced by vaccination with attenuated strains, based on their cytokine production ability (104). Production of macrophage-activating cytokines, particularly IFNγ, has an important role in inducing a Th1 dominant response (85). *In vivo* studies using either a INFγ receptor-deficient mouse strain (65) or antibodies to neutralize its activity (121) confirmed that depleting IFNγ activity caused mice to be highly susceptible to *Salmonella* infection. However, it was also shown that INFγ is only critical during the initial phase of the infection as described in innate immunity, but has less function at later stages (130). Therefore, additional INFγ-independent mechanisms must be involved for $CD4^+$ T cell-mediated bacterial clearance, including production of other cytokines (e.g. TNFα is one protective cytokine) or regulation on B cells. Interestingly, there are also reports indicating that under certain conditions infection with attenuated *Salmonella* can induce Th2 responses, characterized by the production of IL-4 (153). Basically IL-4 production was not shown to be protective, but rather impairs control of infection (41).

There is also evidence for $CD8^+$ T cells in protection against *Salmonella* (65). $CD8^+$ T cells can differentiate into cytolytic T cells (CTL) whose main task is antigen-specific target cell lysis. Because *Salmonella* is a facultative intracellular bacterium, the lysis of infected cells by CTL might release bacteria from their protective *Salmonella* containing vacuole (SCV) and make them accessible for activated phagocyte killing. Granules in CTLs also contain granulysin, a protein that expresses direct antibacterial activity against a wide range of bacteria (155). Although evidence on the mechanisms is still lacking, based on the characteristics of CTLs $CD8^+$ T cells

are anticipated to be able to lyse infected cells and release bacteria from their protective environment as well as to directly participate in bacterial killing.

T cells have an important function in regulating antigen-specific B cell activation and maturation, either by direct cell-cell contact or by cytokine production (23). It is known that T cell help is essential for B cell differentiation such as isotype switching and affinity maturation. B cells are the only cell type that can produce antibody. The role of antibodies during different stages of infection and in protection is not clear. It is known that in the intestinal lumen, antibodies (particularly IgM and IgA) could block penetration of *Salmonella* into deeper tissues (110), which was probably mediated by the inhibition of bacterial adhesion to epithelial cells and M cells (111). While in resistant mice antibodies alone are sufficient for control of virulent bacteria, in susceptible mice antibodies participate in control but protection depends on additional mechanisms (113).

Cytokines and Chemokines in *Salmonella* Infection

As described above, cytokines and chemokines play a critical role in initiating and regulating innate and acquired immune responses during the infection. Both pro- and anti-inflammatory cytokines are induced through a variety of signaling pathways. The balance between them is essential to control the infection. Cytokines and chemokines are expressed by many different cell types, such as epithelial cells, macrophages, DCs and lymphocytes as described. While IFNγ, IL-12, IL-15, TNFα, IL-18 and TGFβ have protective functions during the infection, the anti-inflammatory cytokines IL-4 and IL-10 interfere with host defenses in animal models (37).

Salmonella **and its macrophage host**

The innate immune system allows a general and rapid response to pathogen. Phagocytes including both macrophages and neutrophils play critical roles during the early stages of *Salmonella* infection (16, 43, 44, 164). Macrophages normally reside in tissues and beneath mucosal surfaces, but they can also infiltrate infected tissue in large numbers and migrate to central sites, such as lymph nodes, to interact with other cells. Unstimulated macrophages constitutively express unique receptor repertoires, such as toll-like receptors, complement receptors and scavenger receptor, to detect bacteria rapidly and trigger cell signaling. Inflammatory stimuli such as cytokines can further enhance these responses. Signal transduction facilitates the cytoskeletal rearrangements and membrane trafficking events that are responsible for the phagocytosis and trafficking of bacteria (67). Thus macrophage roles include: ingestion of bacteria by phagocytosis; destruction of bacteria within the phagolysosome; and recruitment of inflammatory cells to the site of infection, using chemokines and acute-phase proteins. After *S.* Typhimurium is phagocytized by macrophages, the pathogen modifies and resides inside SCV (33). Bacterial uptake is not a simple one-way process directed by the macrophage alone. By means of the specialized TTSS, bacterial proteins are injected into the host cells (127, 151). These bacterial proteins interfere with the signaling machinery of macrophages and thus either promote or destroy the intracellular survival of the pathogen. They can also cause apoptosis of the macrophage host (72). The mechanisms used to allow

intracellular survival of the pathogen or, on the other hand, enable macrophages to kill the bacteria are not yet understood. The cytokines such as IFN-γ or TNF-α that activate macrophages might play important roles in the induction of a bactericidal mechanism (60, 167). Bacterial cell wall components of *Salmonella* such as LPS and some lipoproteins induce a massive inflammatory response, resulting in the expression of these cytokines. Thus, the interaction between *Salmonella* and macrophages, and the outcome of the interaction, depend on (1) the phagocytosis process during which TTSS and other bacterial regulatory systems such as PhoP-PhoQ, which will be described below, are induced; (2) the functional status of Nramp1 in macrophages; and (3) the activation status of the macrophages.

Salmonella Virulence Systems and SCV

After phagocytosis by the macrophage, wildtype *S.* Typhimurium remains segregated from early lysosomes and/or late endosomes as measured by confocal microscopy using endocytic pathway markers such as cation-independent mannose 6-phsphate receptor and the hydrolytic enzymes cathepsin L (138). SCV rapidly acquire and retain the lysosomal membrane glycoproteins and are acidified to pH 4.0-5.0 (139). The translocon protein secretion encoded by SPI-2, which plays important roles in intracellular replication of *Salmonella*, is induced by this low pH (124). As indicated above, maturation and trafficking of SCV depend on the functions of genes from two multifunctional *Salmonella* virulence systems, the SPI-2 TTSS and the PhoP-PhoQ regulon.

TTSS is a specialized organelle of gram-negative bacterial that deliver effector proteins to host cells. The TTSS apparatus is a needle-like structure. It spans the bacterial inner and outer membrane and secretes translocon and effector proteins. Translocon proteins allow access of effector proteins to the eukaryotic cells, probably by forming pores in the host cell membrane and in, some cases, a connecting channel between the bacterium and host cell membrane. There are two distinct TTSS within SPI. The SPI-1 TTSS encodes effector proteins that are important for bacterial invasion into epithelial cells, while the TTSS of SPI-2 plays important roles in bacterial intracellular survival and multiplication. SPI-2 is about 40 kb in size and the genes encoding for the secretion system are localized to a region of approximately 26 kb. These genes encode components of the secretion system apparatus, secretion system effectors, chaperones and one two-component regulatory system SsrAB (151). Some of these proteins, such as SpiC and SifA, are well investigated and proved to have important role in SCV formation and trafficking. Located within SPI-2, s*piC* encodes an inhibitor of phagosome-lysosome and phagosome-endosome fusion and affects SCV trafficking in macrophages (159). Unlike *spiC, sifA* is located outside of SPI-2 but it is tightly regulated by the SsrAB system. Its product has an N-terminal sequence similar to that which is required for translocation of other SPI-2 effector proteins (7). It is shown that SifA is required to maintain the integrity of the SCV membrane. The SCV membrane is also affected by another activity of the SPI-2. At late stages of infection, an F-actin meshwork forms around growing bacterial microcolonies. Actin remodeling was found to be dependent upon the *Salmonella* SPI-2 TTSS (109).

PhoP-PhoQ is a two-component regulatory system that controls the transcription of over 40 genes in *S.* Typhimurium (58). PhoQ is an inner membrane sensor-kinase domain that phosphorylates its cognate transcription factor, PhoP, upon perception of specific environmental signals. This system responds to changes in the concentration of divalent cations such as Mg^{2+}. *Salmonella* determines its subcellular location (at least in part) by examining the Mg^{2+} levels in its surroundings: a low Mg^{2+} concentration is an indication of an intracellular environment, whereas a high concentration indicates an extracellular environment (58). Then it activates or represses transcription of a wide range of genes including antimicrobial peptide resistance or bacterial outer membrane modification genes, thus contributing to bacterial intracellular survival (13, 14). The regulon controlled by the PhoP/Q two-component system also makes a major contribution to trafficking of the SCV in macrophages by inhibition of interactions between SCVs and late endosomes to hide the intracellular *Salmonella* (55).

Nramp1

Nramp1 was first called Ity (immunity to Typhimurium) in 1976 (131). In that study, eight murine strains were examined for *S.* Typhimurium C5 sensitivity. Four mouse strains were resistant while four were susceptible. In later studies, by using distinguishable phenotypes as chromosome markers, *Ity* was located to mouse chromosome 1 by the same group of researchers (132). Two other host resistance loci for two unrelated pathogens, *Mycobacterium bovis (Bcg)* and *Leishmania donovani (Lsh)* were discovered as well (10, 51). In 1993, Nramp was named in a study on a

candidate gene for the *Bcg* locus (168). The fact that *Nramp1, Bcg, Ity* and *Lsh* were identical was confirmed by targeted disruption of *Nramp1* in mice and phenotypic comparison among different genotypes of mice infected by *S.* Typhimurium, *M. bovis* and *L. donovani* (167). It is now known that Nramp1 encodes for a highly hydrophobic 56 kDa protein, which possesses 12 transmembrane domains and a glycosylated exracytoplasmic loop (50). It is expressed in the membrane fraction of macrophages and neutrophils as a phosphoglycoprotein of 90-100 kDa. During *Salmonella* infection, phagocytes ingest the bacteria into SCV and Nramp1 is recruited to the membrane of the phagosome and remains associated with this structure during its maturation to phagolysome. Nramp1 plays an important role in SCV maturation and thus controls the replication of intracellular pathogen survival. SCV maturation was compared in *Nramp1* mutant RAW macrophage cells and RAW cells transfected with *Nramp1,* as well as in primary macrophages from isogenic *Nram1*$^{+}$ and *Nram1*$^{-}$ mice (30). In Nramp1^{+} cells, SCV showed a much higher association with mannose 6-phosphage receptor, and were more accessible to incoming endocytic traffic. This result suggested that Nramp1 antagonises the ability of *Salmonella* to modify its vacuole and increases the fusogenicity of phagosones with late degradative compartments. Mechanisms of Nramp1 to kill the intracellular bacteria might be due to its function as a proton/divalent cation transporter dependent on pH (76, 168). Divalent cations like Mg^{2+} are important for the survival of pathogens, and removal of these from the phagosome probably results in enhanced bactericidal activity and hence in increased resistance to intracellular pathogens. But how this regulates SCV maturation and the interaction with the endocytic pathways are still not known. Mice

carrying two copies of the mutant Nramp1 allele are significantly less resistant to lethal *S.* typhimurium infections than mice that harbor one wild-type Nramp1 allele. The role for human Nramp in human typhoid fever has been difficult to establish and is still not well understood (50).

Salmonella Sensitivity and Resistance to reactive oxygen and nitrogen species

S. Typhimurium has several enzymes protecting it from reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI) stress *in vitro* and in murine infection.(98). The bacterial flavohemoglobin, hmp, protects *Salmonella* from NO *in vitro* (29). SodC I, one of the periplasmic superoxide dismutases, is most effective at protecting *Salmonella* from the combined effects of reactive nitrogen and oxygen species (32). recA and recBC DNA repair systems are also important for virulence (17).

Virulence genes encoded by SPI-2 can interfere with the trafficking of the respiratory burst oxidase to the SCV. The increased susceptibility of SPI-2 mutants to the macrophage ROI indicated that the low levels of SPI-2 gene expression induced early after phagocytosis of wild-type *Salmonella* is sufficient to confer protection (165). SPI-2 also plays a role in controlling the bacteriostatic effects mediated **by** inductible nitric oxide synthase (iNOS). Although an SPI-2 mutant was no more sensitive than wild-type to a range of nitrosative stresses *in vitro*, its growth rate was increased much more dramatically than that of the wild-type during infection of RAW macrophage cells (20).

Salmonella and Macrophage Apoptosis

During the evolution process, microbial pathogens have developed mechanisms to modulate or even exploit a variety of host cellular processes for their own survival and replication (53). Cellular processes that are used by the host to detect and eliminate invading pathogens are manipulated by pathogen adaptation systems. *Salmonella* have not only obtained mechanisms to modulate macrophage cell functions, such as vesicular trafficking or antimicrobial response (142), but also evolved the ability to trigger programmed cell death in its macrophage host (122). Pathogen-induced macrophage death has been observed in various microbial pathogen infections *via* different mechanisms and results in different pathology changes. Macrophage death caused by *Salmonella* has been the subject of some confusion and controversy (9, 26, 92). The questions remaining are: 1) at which stage cell death occurs; 2) what the mechanisms are; and 3) why cell death is important. Some groups reported that, upon infection, macrophage death was induced at a very early stage (within a couple of hours) (22, 117), while others observed the cell death in later stages, up to 24 hours post-infection (59, 97). In further studies, some researches showed that *Salmonella*-induced cell death is more like apoptosis with typical signs such as chromatin fragmentation (22, 117) and caspase-3 activation (77) while others reported features more consistent with necrosis, such as lack of caspase-3 activation and loss of membrane integrity (12, 170). These contradictory observations are likely the results of different experiment conditions including variations in macrophage cell lines, *Salmonella* strains and MOIs that have been used.

Except those controversies, *Salmonella* virulence systems SPI-1 and SPI-2 have been shown to have important roles inducing host cell death during infection. SPI-1 TTSS delivers effector proteins required for intestinal invasion and the production of enteritis (101). The SPI-1 effector SipB activates caspase-1 in macrophages, releasing IL-1β and IL-18 and inducing rapid cell death by a mechanism that has features of both apoptosis and necrosis (22, 59, 64, 117). Caspase-1 is required for *Salmonella* to infect Peyer's patches and disseminate to systemic tissues in mice and thus plays an important role in the innate immunity of the gastrointestinal tract to *Salmonella* infection. This has been proved in caspase-1 knock out mice (115). Progressive *Salmonella* infection in mice requires the SPI-2 TTSS and associated effector proteins as well as the SpvB cytotoxin (96). SPI-1 has no effect on the ability to induce cell death during later times (97). Furthermore, the delayed macrophage cell death is also accompanied by a delayed release of IL-1β (114, 116). Activation of TLR-4 by LPS may be required for this process as well (70).

It is well recognized that *Salmonella*-induced cell death displays characteristics of both necrosis and apoptosis. Even though the rapid macrophage death resembles necrosis, the caspase-1 dependence of this cytotoxicity distinguishes it from any usual form of accidental cell death. It is more considered as a programmed event and thus has been termed as “pytoptosis” (26). The unique caspase-1 dependent mechanism followed by pro-inflammatory cytokine IL-1β and IL-18 production has been given quite a bit of attention. One role of these pro-inflammatory cytokines is to augment the induction of IFN-γ, which in turn activate macrophages. It is proposed that rather than trying to kill the macrophages, the induction of the inflammatory response is the

primary goal of some microbial pathogens to disseminate from the gastrointestinal tract in the early stage (9).

Still little is known about the significance of the different types of macrophage cell death induced by *Salmonella* infection as well as their relative contribution to pathogenesis or host defence. Whether macrophage cell death is triggered by *Salmonella* to counteract host defense mechanisms or if it constitutes a host response to halt bacterial replication is still not clear. Since both rapid and delayed macrophage death was observed to occur at distinct times and conditions, a model for *Salmonella* – induced macrophage death *in vivo* has been described (116). The SPI-1 TTSS is important during the early, intestinal stage of infection and SPI-2 is required for the later systemic phase of infection. Rapid cell death was induced in macrophages in Payer's Patches via a caspase-1 dependent pathway with release of mature IL-1β and IL-18. These proinflammatory cytokines attract more macrophages and neutrophils to the site of infection. The surviving intracellular bacteria spread to lymphatics and the bloodstream mainly by macrophages. During the systemic phase of infection, SPI-2 TTSS secretion proteins are exported and induce delayed macrophage death in liver or spleen. Apoptotic cells are then phagocytosed by neighboring cells and this leads to further spread of pathogens.

In summary, the above distinct qualities that macrophages have make them the first line of defense during *Salmonella* infection. Many pathogens have evolved strategies to target macrophages that prevent them from elimination by the host and an ability to establish a permissive niche for survival and replication. To protect the host from infection, macrophages also have obtained the ability to rapidly recognize,

internalize and degrade bacterial pathogens to control the infection, and at the same time, recruit the appropriate types of immune cells to fight against the infection. Numerous signal transduction pathways are activated in both the pathogen and macrophages in this process. The balance between the macrophage's ability to contain the infection and the pathogen's ability to manipulate the macrophage host determines the outcome. Understanding of the interplay between a bacterial pathogen and macrophages is essential to understand the pathogenesis of many infectious diseases.

Hypothesis

Macrophages have been shown to play important role in *Salmonella* pathogenesis. Their function in *Salmonella* host-specificity however, still lacks evidence from *in vivo* study. In this work, the host generalist Typhimurium and the host specific Typhi were compared in a mouse model by intra-peritoneal infection. Both mouse peritoneal cell dynamics and bacteria clearance have been analyzed to identify a role for macrophages in *Salmonella* host specificity *in vivo*.

Materials and Methods

Cells and tissue culture

The murine macrophage cell lines RAW-264.7 (ATCC# TIB-71) and J774A.1 (ATCC# TIB-67), obtained from the American Type Culture Collection (ATCC, Rockford, MD), were grown in complete media (RPMI 1640 (Mediatech, Inc. Herndon, VA) containing penicillin and streptomycin supplemented with 10% fetal bovine serum and 300 mg/L L-glutamine). These cell lines were both isolated from BALB/c mice. Cells were maintained in a 37°C, 5% CO_2 incubator. Bone marrow-derived macrophages were derived from bone marrow that was extracted from the femurs of mice and grown in complete media containing 7 μg/ml MCSF (Sigma, St. Louis, MO). Cells were incubated for 5-7 days until uniform monolayers of macrophages were established (5). Mouse peritoneal macrophages were elicited by thioglycollate (11). Briefly, four days after mice were injected i.p. with 1 ml of 4% thioglycollate, cells were isolated, resuspended in complete media and plated on 24-well plates at 3×10^5/ml. After 4 hr, non-adherent cells were removed by washing with pre-warmed PBS and the adherent macrophages were cultured. Mouse primary peritoneal macrophages were isolated as above without thioglycollate treatment. Mouse splenic, macrophage-rich cell populations were also prepared as follows: Splenocytes were isolated, and incubated on tissue culture plates for 4 hr in complete media. Unattached cells were then removed by washing the plates with PBS. Cells that remained attached were designated as a macrophage-rich population (93).

Mice and *in vivo* infection

8-12 week old female BALB/c mice were purchased from Harlan-Sprague (Indianapolis, IN, USA). BALB/c DBA2 mice were kindly provided from Dr. Fierer (University of California, San Diego). These mice are identical with the BALB/c strain, except for a 28- to 30-cM segment of chromosome 1 that contains the *Ity* locus and the *Nramp* allele associated with resistance to *Salmonella* infection (36). Mice were infected i.p. with 2×10^5 Typhi or Typhimurium and after 0.5 hr or 4 hr infection were sacrificed by isoflurane inhalation. Bacteria and peritoneal cells were extracted as described before (38). Mouse peritoneal cells were spun down at 1000 rpm for 5 min at 4°C and resuspended in ammonium chloride lysing solution for 5 min to disrupt the RBC. The cells were subsequently washed in PBS to remove the lysing solution. All experiments were approved and performed following the rules of the Institutional Animal Care and Use Committees at San Diego State University and/or the University of California, San Diego.

Salmonella strains, plasmid and culture conditions

Typhimurium ATCC 14028s (TYT3236) and Typhi Ty2 (TYT1484) strains are wild-type pathogenic strains. The plasmid pPhoP-GFP Amp^R (38, 161, 162) contains a fusion between the PhoP promoter and the GFP gene, placing the expression of GFP under the control of the PhoP promoter. PhoP is required for

Salmonella virulence and its expression is specifically induced inside phagocytic cells by the acidic condition of the phagolysosome (161). TYT3236 and TYT1484 were transformed with this plasmid by electroporation, to create TYT4452 and TYT4455, respectively. Both wildtype *Salmonella* strains were routinely grown overnight at 37°C, with aeration, in Luria-Bertani (LB) broth. Another plasmid pRE233 was constructed by digesting pDS-Red (which encodes a red fluorescent protein), purchased from Clontech Labs Inc (Palo Alto, CA), with XbaI and cloning the resulting 719 bp fragment into XbaI digested pSU18, a derivative of pACYC184 which contains the alpha complementing fragment of lacZ. This cloning restores the appropriate reading frame of the lacZ-rfp fusion, and hence, the lac promoter on the plasmid controls transcription of DSRed. Colonies were screened for clones by their color because the red fluorescent protein gives bacterial colonies a pink hue (38, 161). This was confirmed by fluorescent microscopy. This plasmid was also used to transform the *Salmonella* serovars TYT 3236 and TYT1484, respectively, by electroporation, to create TYT4451 and TYT4454. All strains used in the infection assays were grown to stationary phase at 37°C, with aeration, in Luria-Bertani (LB) broth with or without corresponding antibiotics. Heat-killed bacteria were prepared by incubation at 70°C for 30 min (84).

Gentamycin protection assays

In vitro infection of macrophages with *Salmonella* was performed as described previously (38). Briefly, macrophages were cultured in complete media without antibiotics for 24 hr and seeded in 24-well plates at 3×10^5 cells/well. Macrophages

were infected with Typhimurium or Typhi at the multiplicity of infection (MOI) indicated. The bacteria used for infection were not opsonized by complement. After 1 hr, cells were washed with warm PBS and 50 μg/ml gentamycin was added to the media for 0.5 hr to kill the extracellular bacteria. This was considered the 0 hr time point in all studies. The gentamycin concentration was reduced to 12.5 μg/ml for further incubation. Before lysis of the macrophages, warm PBS was used to remove gentamycin. Macrophages were lysed in 500 μl 1% Triton X-100 and another 500 μl PBS was added and mixed. Serial dilutions were plated and incubated at 37°C overnight and the *Salmonella* CFUs seen on LB plates were counted.

***m*Abs, cell preparation, flow cytometry analyses and confocal microscopy**

The mAbs anti-mouse CD11b (M1/70), anti-F4/80 (BM8), anti-Ly6G (RB6-8C5), rat IgG2b (eB149/10H5), and affinity purified anti-mouse CD16/32 (93), were APC-conjugated and purchased from eBioscience (San Diego, CA) for flow cytometry. Mouse peritoneal cells were isolated, counted and adjusted to a concentration of 2×10^7/ml in staining buffer (PBS containing 1% FBS). Cell suspensions were incubated with anti-CD16/32 (FcγIII/II) for 10 min on ice to block Fc binding and APC-conjugated IgG2b, anti-CD11b, anti-F4/80 and anti-Ly6G were then added at the optimal concentration for each mAb (0.1, 0.6, 0.125 and 0.125 μg per million cells, respectively). The cells were incubated with the antibodies on ice for 20 min, then washed twice in staining buffer before being fixed in 400 μl fixing buffer

(PBS containing 0.5% formaldehyde). Cells were kept in the dark and on ice at all times during the analyses. All data were acquired using a FACSAria flow cytometer (BD Biosciences, San Jose, CA) and were analyzed using FACSDiva *software (BD Biosciences).* Stained samples were also analyzed by confocal microscopy, using an Leica TCS SP2 confocal microscope, in the studies presented in figure 5b.

Apoptosis assay

Caspase3 activities were measured by an Apo-ONETM homogeneous caspase3 assay (Promega) on 96-well plate. BALB/c mice were infected with Typhimurim or Typhi i.p., after 0.5 and 4 hr, peritoneal cells were isolated. 2×10^5 cells were loaded in each well and an equal volume of lysis buffer containing the caspase substrate benzyloxycarbonyl-DEVD-R100 was added and incubated at room temperature for 1 h. Fluorescence (RFU) was read at an excitation wavelength of 485 nm and an emission wavelength of 535 nm using erkinElmer Life Sciences HTS 7000 plus BioAssay Reader.

Real-Time PCR analysis of cytokine expression

SyBr green real-time PCR analysis for cytokine expression was performed on an IQ-Cycler (Bio-Rad). L32 and TNFα PCR conditions were 95°C for 3 min, followed by 40 cycles of 95°C for 10 sec, 65°C for 60 sec, and 72°C for 30 sec. Mouse IL-1β, IL-6 and IL-1α PCR conditions were 95°C for 3 min, followed by 40 cycles of 95°C for 10 sec, 60.7°C for 45 sec, and 72°C for 30 sec. All PCR analyses were run in

triplicate with a standard curve and melting curve analysis. Data were normalized to the internal L32 control. PCR primers for L-32 were (5'-3'): AAG TTC ATC AGG CAC CAG (forward), GGC TTT TCG GTT CTT AGA G (reverse); for TNFα were: CAT CTT CTC AAA ATT CGA GTG ACA A (forward), TGG GAG TAG ACA AGG TAC AAC CC (reverse); IL-1β were: AGG CAG GCA GTA TCA CTC ATT GT (forward), GGA AGG TCC ACG GGA AAG A (reverse); IL-6 were: CTT CAC AAG TCG GAG GCT TAA TTA C (forward), 5'AGA ATT GCC ATT GCA CAA CTC TT (reverse); and IL-1α were: AAG TTT GTC ATG AAT GAT TCC CTC (forward), GTC TCA CTA CCT GTG ATG AGT (reverse).

Statistical analyses

Statistical analyses were performed using two-tailed Student's t test. All values are reported as the mean ± one S.D.

Acknowledgement

Part of this section will be in the manuscript "Macrophages influence *Salmonella* host specificity *in vivo* " and will be submitted to Infection and Immunity. We thank for Dr. Constantine Tsoukas at San Diego State University, and Drs. Michael David and Joshua Fierer at the University of California, San Diego for their support throughout this research. We also thank for Dr. Robert Edwards for the GFP and DS-Red plasmids and Dennis Young at the Moores UCSD Cancer Center for

advice on flow cytometry. We thank Alejandra Gutierrez, Carl Gunderson, Juris Grasis and Matthew Giacalone for assistance with techniques.

Results

Survival of Typhimurium *vs* Typhi in murine macrophages *in vitro*

Previous *in vitro* studies have suggested that macrophages may contribute to *Salmonella* host specificity (74, 75, 148, 169). However, other studies dispute this conclusion (19). To test whether host specific *Salmonella* serovars differentially survive within macrophages *in vitro*, wild-type Typhimurium 14028 and Typhi Ty2 were compared for their ability to survive in the murine macrophage-like cell lines RAW264.7 and J774.A. They were also tested for their ability to survive in primary peritoneal macrophages, either resting or elicitated by thioglycolate, and in splenic and bone marrow-derived macrophages. Intracellular bacteria were counted at 0, 2, 8, and 24 hr after infection using a gentamicin protection assay.

Results showed that both serovars Typhi and Typhimurium invaded all of the macrophage cell types efficiently **(Figure 1; 0 hr)**. Typhi appeared to invade the macrophages more efficiently than Typhimurium, about 10-fold more at an MOI of 10. The difference observed was reproducible and statistically significant in all cell types. The number of intracellular bacteria of both serovars reached a similar level at 8 hr post infection. No significant differences were seen between Typhimurium and Typhi after 24 hr in any of the cell types tested. Mouse peritoneal residential macrophages (isolated without thiogylcolate stimulation) and mouse splenic macrophages were also tested and the numbers of intracellular bacteria from these two cell types were similar to what was observed with bone marrow-derived macrophages

(data not shown). These data suggested that differences in survival in macrophages *in vitro* were not correlated with the host specificity of these two serovars *in vivo*.

It is known that Typhimurium can have a cytotoxic effect on host cells (12, 117). Therefore, it is possible that a MOI of 10 may not have revealed differences in survival between the two serovars. To determine if different MOIs affect the survival and replication of intracellular Typhimurium *vs* Typhi, peritoneal macrophages were infected at MOIs of 10, 1, and 0.1 **(Figure 2)**. With decreasing MOIs, the number of intracellular bacteria also reduced by approximately the same ratio at initial time points but, once again, invasion by Typhi was about 10 fold higher than the invasion observed for Typhimurium. Both of the serovars persisted at equivalent levels at 24 hr of incubation. This indicates that the MOI was not responsible for the fact that no differences were observed in the intracellular survival of Typhimurium and Typhi in murine macrophages in the studies shown in Figure 1. Similar results were obtained from many natural isolates of both serovars (A. Zeituni, S. Maloy, unpublished data). These data strongly suggest that survival in macrophages *in vitro* can not explain the host specificity differences observed for the two serovars.

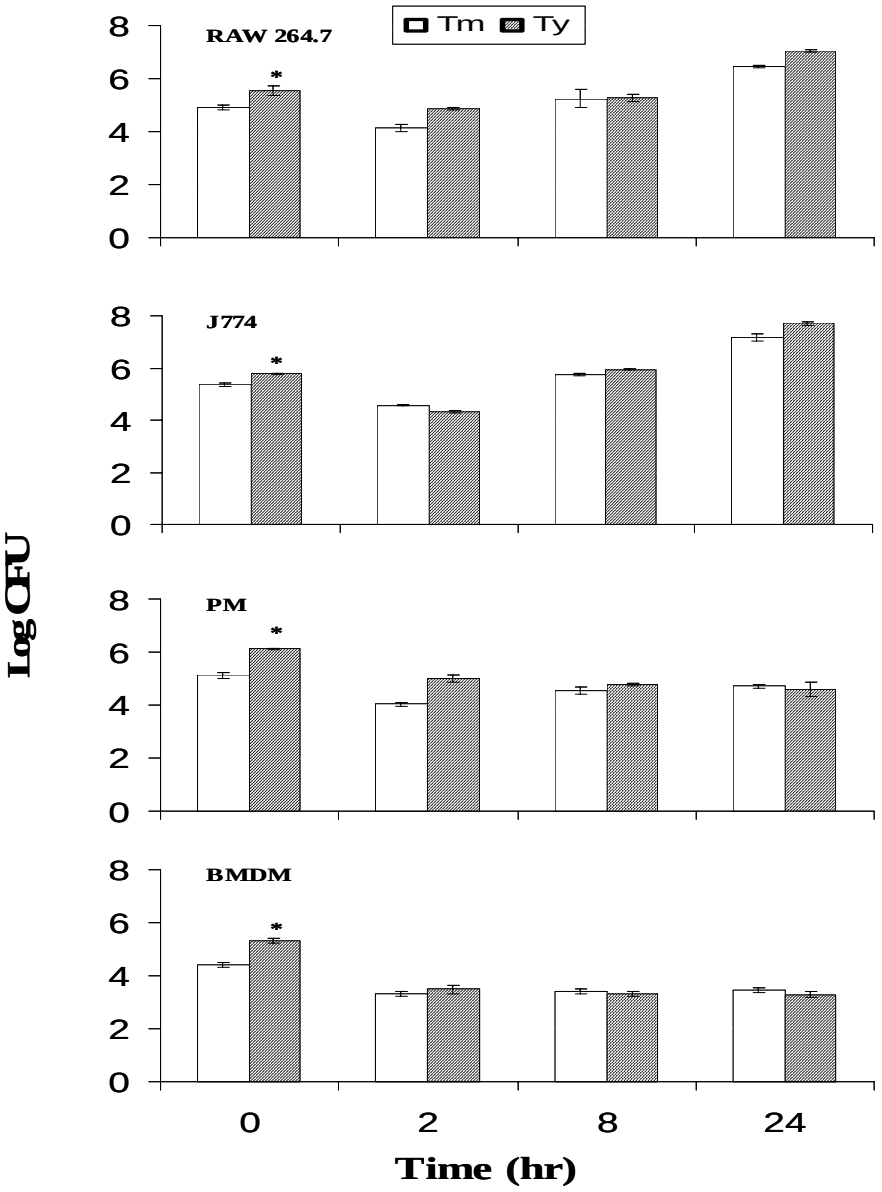


Figure 1. Survival of Typhimurium *vs* Typhi in murine macrophages *in vitro*. RAW 264.7, J774.A, peritoneal macrophages (PM) and bone marrow derived macrophages (BMDM) were infected with Typhimurium (Tm) or Typhi (Ty) at an MOI=10. The number of intracellular CFU were determined by plating serial dilutions and presented as Log CFU per 3×10^5 macrophage cells (Y axis). Time shown on the X-axis is after the addition of gentamycin. All values are the means ± standard deviations of at least three independent experiments. (∗, $p<0.05$).

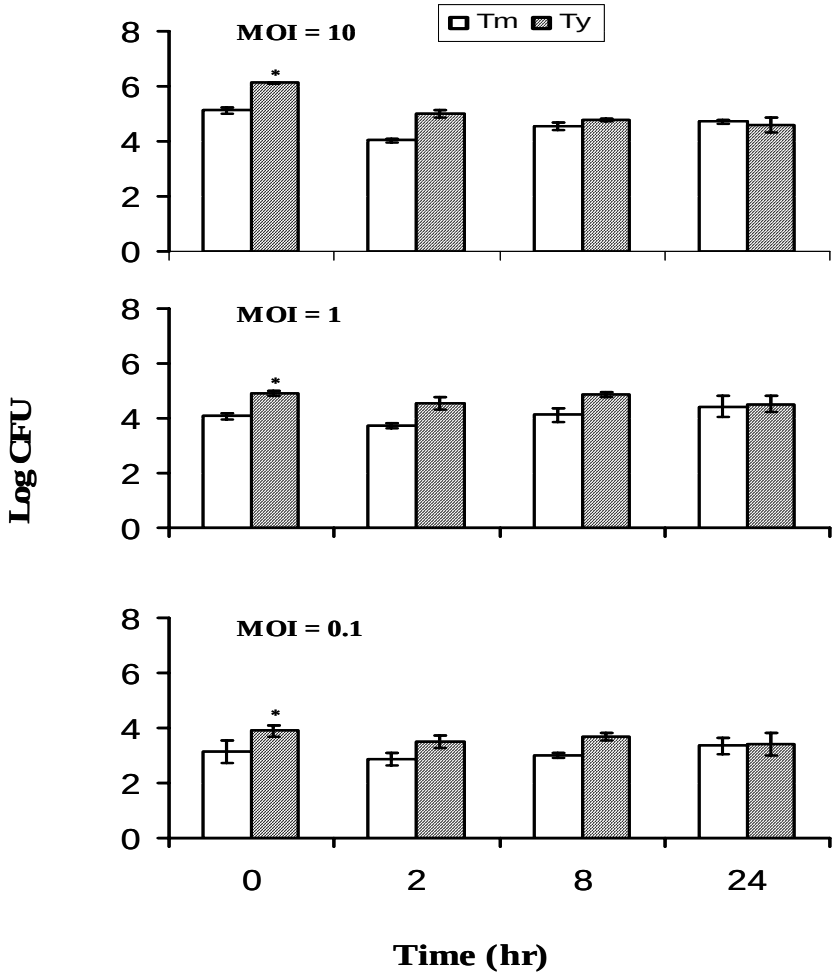


Figure 2. Survival of Typhimurium *vs* Typhi in macrophages at different MOIs *in vitro*.
Peritoneal macrophages were infected with Typhimurium and Typhi at the indicated MOI and the intracellular number of CFUs were determined as described in Figure 1. All values are the means ± standard deviations of at least three independent experiments. (∗, $p<0.05$).

Survival of Typhimurium and Typhi *in vivo*

Although the survival of Typhi and Typhimurium does not differ in macrophages *in vitro,* this may not reflect what occurs *in vivo*. Therefore, survival of Typhimurium and Typhi survival was tested following i.p. infection of BALB/c mice. After 0.5 and 4 hr post-infection, mice were sacrificed and the total number of CFU from the peritoneum was determined. At 0.5 hr post-infection, the recovery of Typhimurium and Typhi was similar **(Figure 3).** However, at 4 hr, many fewer Typhi were recovered than Typhimurium. Similar results were obtained when Nramp1 wildtype BALB/c mice were used (not shown). These results indicated that Typhi disappeared from the peritoneum much faster than Typhimurium during *in vivo* infection.

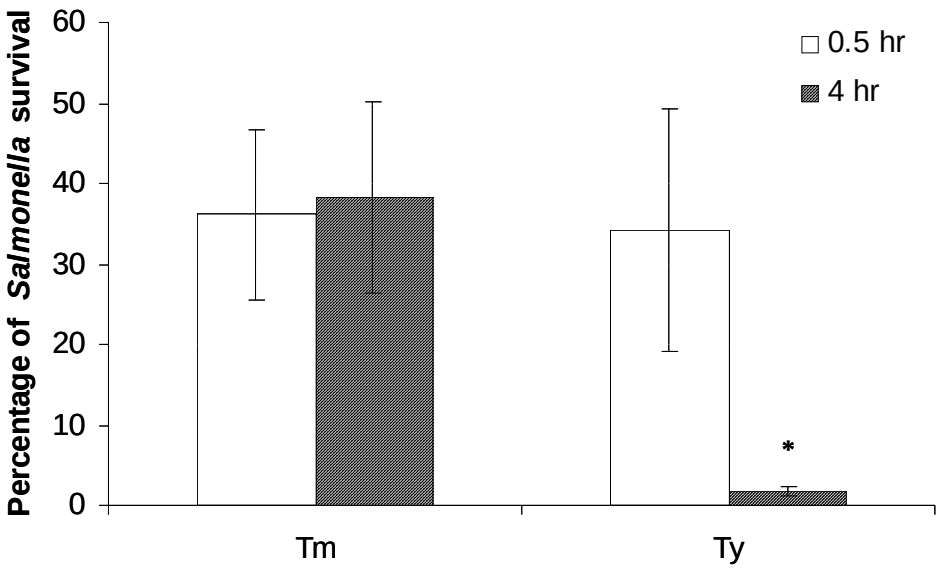


Figure 3. Survival of Typhimurium and Typhi *in vivo*.
BALB/c mice were infected i.p. with 2×10^5 Typhimurium or Typhi. The total number of bacteria recovered from the peritoneum was counted to determine the percent recovered. Shown are the results obtained from three independent experiments on 6 mice in each group. Data presented are the mean ± SD. (∗, $p<0.01$).

Cell types found in the peritoneum before and after *Salmonella* infection

The differences observed between Typhi and Typhimurium *in vivo* could be due to differences in survival inside phagocytes. Because macrophages and neutrophils have been shown to play important roles in *Salmonella* pathogenesis, flow cytometry was used to analyze the dynamics of macrophages and neutrophils in the peritoneal cavity before and after infection. Peritoneal cells were stained using antibodies to CD11b, F4/80, and Ly6G. CD11b is highly expressed in mature macrophages, but less so in other white blood cell types (144). F4/80 is a marker for mature macrophages and Ly6G is a marker for neutrophils (49, 94). Mice injected with PBS or the same amount of heat killed (HK) Typhimurium were used as controls. 3.6 ± 0.93 and 3.9 ± 1.65 million peritoneal cells were isolated per mouse before and after infection, respectively. Due to the large variation, the changes were not significant. The percentages of different cell populations from three different experiments are shown in **Table I** and representative data are shown in **Figure 4**.

Before infection, the mouse peritoneum contained approximately 27% F4/80+ macrophages and 6% Ly6G+ neutrophils. After infection with either Typhimurium or Typhi, the number of macrophages rapidly decreased to ~8%. This was most likely not due to the increase of neutrophils, because the total cell number did not change substantially (above). Nor is this decrease in cell number likely to be the result of marker down-regulation because the cells that remained in the peritoneum maintained

their high surface levels of F4/80 **(Figure 4)** and CD11b (not shown). These data strongly suggested that macrophages were dying, leaving the peritoneum after infection, or becoming adherent and no longer easily isolated. When mice were injected with HK-Typhimurium, the F4/80 positive macrophages remained in the peritoneum even after 4 hr. These data imply that the disappearance of peritoneal macrophages after infection required live bacteria and was thus not simply due to the presence of *Salmonella* LPS or other inflammatory mediators. These data also indicated that differences in macrophage number in the peritoneal cavity after infection were not correlated with the host specificity differences of Typhimurium and Typhi.

One possible explanation for the disappearance of macrophages is that the infection might induce cell death. To determine whether apoptosis occurred during the infection in the *in vivo* model presented here, a caspase3 assay was done on mouse peritoneal cells isolated 0.5 and 4 hr after Typhimurium or Typhi infection as described. As shown in **Figure 7**, in preliminary experiments, caspase3 activities did not show an increase in any of the infections compared to uninfected cells. However, because the total cell population was used in this assay, it is possible that the fraction of apoptotic cells was too small to be detected. To eliminate that possibility, other more sensitive apoptotic assays could be used, such as the Tunnel assay (118). Analyzed by flow cytometry, Tunnel assays might give more accurate analysis at a single cell level. It is also important to point out, however, that because the macrophages are already gone by 0.5 hr of infection it may be difficult to see apoptosis *in vitro* in this system.

Caspase3 activation is one of the characteristics for typical apoptosis. But whether it is activated in *Salmonella* infected macrophages is still not clear (12, 77, 170). Necrosis is generally an accidental cell death caused by physical damage and accompanied by a proinflammatory response. This differs from that observed with DNA-damaging agents which results in apoptotic or programmed cell death and is dependent on the activation of caspases. *Salmonella* induced cell death seems to share aspects of both apoptosis and necrosis, as it is both caspase dependent and proinflammatory, thus it has recently been termed as "pyroptosis" (23). Caspase1 was required during this process. In contrast to caspase3, caspase1 is the only caspase that can cleave the pro-forms of the inflammatory cytokines to produce proinflammatory cytokines IL-1β and IL-18 (52, 64). It has also been proposed that the induction of the inflammatory response is the primary goal of the pathogen rather than to kill macrophages (9). Therefore, macrophage death might be the result of the active product produced by caspase1 rather than caspase1 functioning as a death protease. Thus, understanding whether caspase1 is activated and the causing production of IL-1β and IL-18 will probably provide a better understanding on *Salmonella* pathogenesis, especially *in vivo*.

When Ly6G and CD11b were used to analyze the neutrophil population, a significant increase of cells in the peritoneum was observed after infection with either serovar. Prior to infection, only ~6% of the peritoneal cells were neutrophils but 50-60% of the cells were neutrophils after infection with either Typhimurium or Typhi, which is consistent with previous report on neutrophils influx during Typhimurium infection (176). No significant difference in the changes of neutrophils before and

post-infection was observed between the two serovars, suggesting that differences in the attraction of neutrophils were not responsible for the host-specificity of these two serovars. The injection of HK-Typhimurium also induced the influx of neutrophils, showing that live bacteria were not needed for this response.

Table 1. Peritoneal cell types before and after *Salmonella* infection

	Percentage of Gated Peritoneal Cells					
	PBS control	Tm 0.5 hr	Ty 0.5 hr	Tm 4 hr	Ty 4 hr	HK Tm 4 hr
CD11b (macrophages, monocyte, neutrophils)	48 ± 4.7[a]	35 ± 1.9	39 ± 5	61 ± 10.7	65 ± 3.9	82 ± 0.7
F4/80 (macrophages)	27 ± 2.1	**8** ± 1.8	**8** ± 2.3	**10** ± 1.5	**8** ± 2.4	23 ± 2.5
Ly6G (neutrophils)	6 ± 0.9	5 ± 0.1	9 ± 3.7	**53** ± 5.7	**58** ± 2.8	**61** ± 2.6

[a]Shown here are the means ± 1 SD. At least three independent experiments were averaged using two mice in each group.

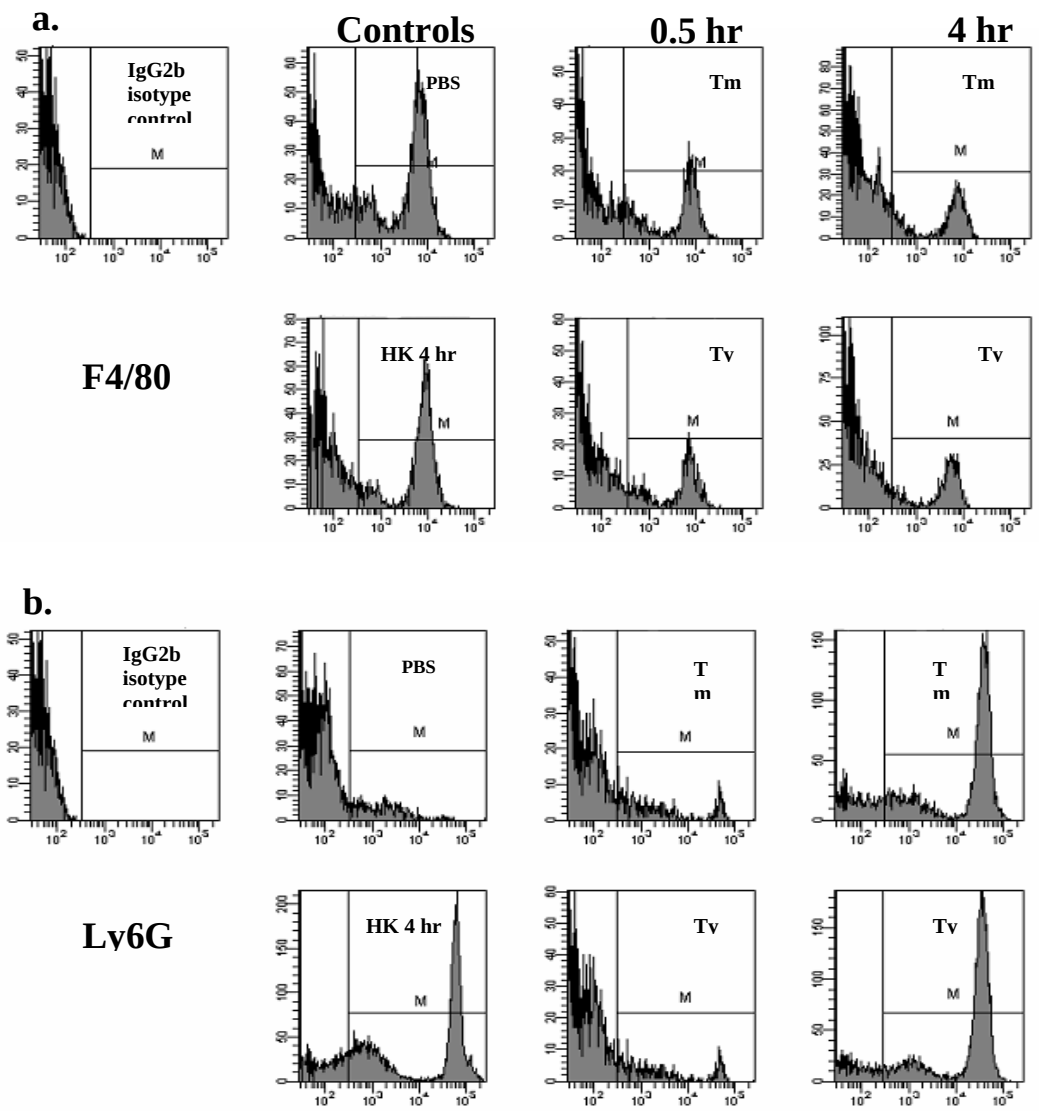


Figure 4. Dynamics of cell types found in the peritoneum before and after *Salmonella* infection.

BALB/c mice were infected i.p. with 2×10^5 Typhimurium or Typhi, injected with PBS (uninfected), or HK bacteria. Peritoneal cells were isolated after 0.5 and 4 hr of infection and stained for F4/80 (a) and Ly6G (b). In each staining, an APC-conjugated IgG2b was used as the isotype control (an example from uninfected mice is shown). Mice injected with HK bacteria were analyzed after 4 hr. Cells from two mice were combined for each condition to obtain adequate cell numbers from staining. The X-axis represents fluorescence intensity and the Y-axis indicates the cell counts. Shown here is one representative from three independent experiments

Bacterial internalization *in vivo*

To identify cell types that internalize the bacteria *in vivo*, BALB/c mice were infected with Typhimurium and Typhi carrying the pPhoP-GFP plasmid (see methods). Several characteristics of these two strains have been verified by comparing them to their wild-type strains. The growth curves of these fluorescent derivative strains are essentially identical to their wild-type counterparts (data not shown). Gentamycin protection assays done on macrophage cell lines also indicated that these two strains have the same susceptibilities to gentamycin killing and that the phagocytosis and intracellular survival rates were also comparable to the wild-type strains.

At 0.5 and 4 hr, peritoneal cells were isolated from mice infected with the pPhoP-GFP plasmid-carrying strains, stained with CD11b, F4/80, and Ly6G mAbs and analyzed by FACS. Representative analyses are shown in **Figure 5a.** These studies show that at 0.5 hr, all GFP-positive cells were CD11b high and F4/80 positive. These data indicated that macrophages phagocytosed the bacteria during the initial infection with either serovar. It is important to note that almost all of the F4/80 positive cells were also GFP positive, indicating that most macrophages in the peritoneum contained intracellular *Salmonella* early after infection. The mean fluorescence intensity of GFP did not differ between Typhimurium and Typhi (**Table II**), suggesting there were similar levels of intracellular bacteria after infection with either serovar. Similar to what was detected in figure 4, no residential neutrophils were detected at 0.5 hr in the mouse peritoneum.

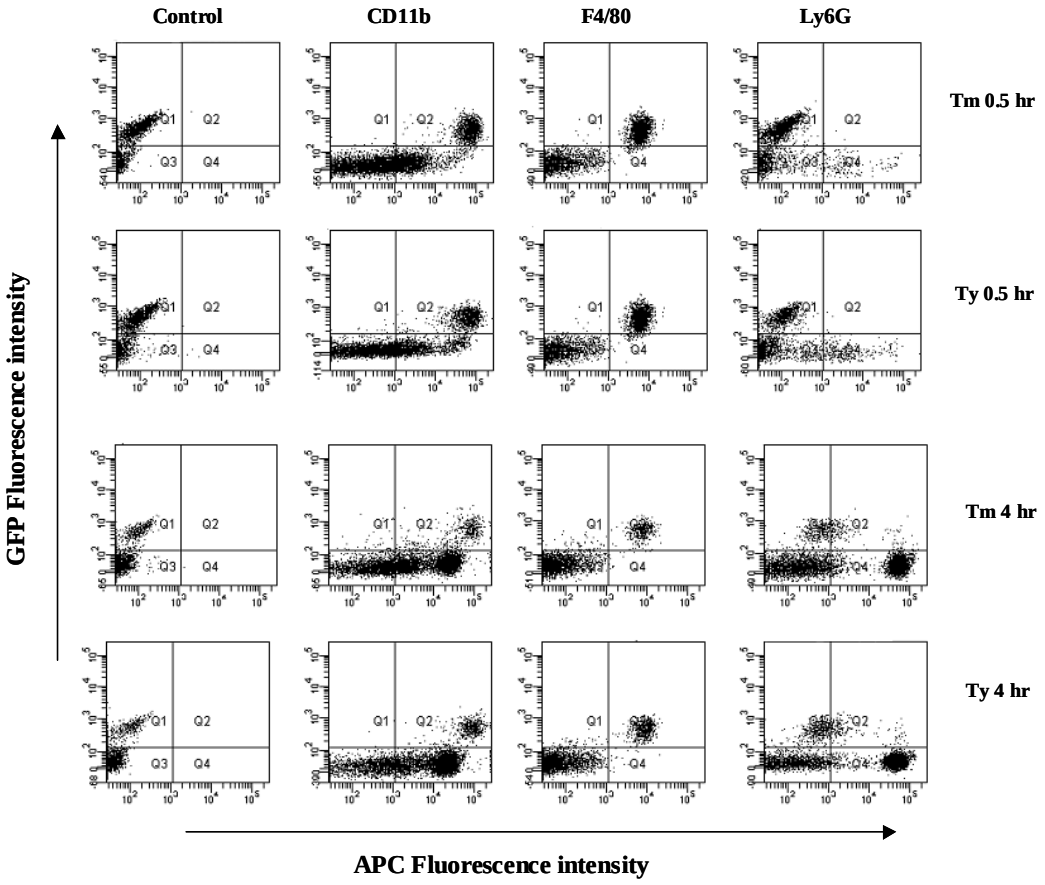
At 4 hr after infection, GFP-expressing bacteria were still mainly associated with CD11b high and F4/80 positive macrophages (**Figure 5a**). The mean GFP fluorescence in Typhi infection was slightly but significantly lower than that observed with Typhimurium (**Table II**), which might imply that Typhi was being cleared from the macrophages faster than Typhimurium. If so, this could contribute to the differences in host specificity observed for these two strains of *Salmonella*. Although large numbers of neutrophils were attracted to the peritoneum in both Typhimurium and Typhi infection at 4 hr, they rarely contained GFP-positive *Salmonella* **(Figure 5a)**.

To verify the results obtained from GFP-expressing *Salmonella*, mice were also infected with Typhimurium and Typhi constitutively expressing DSRed, a highly stable fluorescent protein (166). The results obtained for both macrophages and neutrophils were essentially identical to those shown in **Figure 5a**. Confocal microscopy was used to identify the intracellular bacteria and further confirmed the data obtained from flow cytometry analysis. A representative picture is shown in **Figure 5b**. The red fluorescence seen there is from APC-conjugated to anti-CD11b, F4/80 or Ly6G specific mAb as indicated. The green dots are GFP expressing *Salmonella*. GFP-positive bacteria were detected to be associated with most $F4/80^{+}$ and CD11b bright cells as was seen in **Figure 5a**.

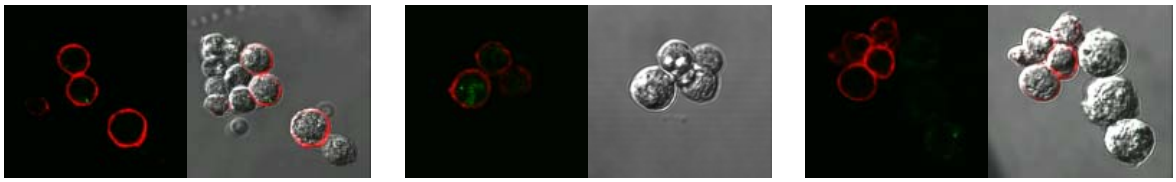
To determine if the absence of Nramp1 affects the observations reported to this point, the BALB/c DBA2 mouse strain, which has a wild-type Nramp1 gene, was also infected with the GFP-expressing *Salmonella* serovars. At 0.5 hr and 4 hr post-infection, mice were sacrificed and the peritoneal cells were isolated, stained and

analyzed by flow cytometry as described above. The results obtained were virtually identical to those shown in figure 5a. These results strongly indicate that the absence of a functional Nramp1 gene does not effect macrophage uptake of the two serovars *in vivo*. Thus it is unlikely Nramp1 is involved in macrophage differentiation of Typhimurium and Typhi *in vivo*.

a.



b.



CD11b **F4/80** **Ly6G**

Figure 5. Internalization of *Salmonella in vivo.*

a. Staining was carried out as described for Figure 4. Infection was carried out using *Salmonella*-expressing GFP after internalization. Shown here is one representative of three independent experiments.

b. Samples for flow cytometry analysis were also analyzed using confocal microscopy. Shown here is one representative of Typhimurium infection at 4 hr stained. The cells were stained with APC-conjugated anti-CD11b, anti-F4/80 and anti-Ly6G mAb, respectively (red color). Green indicates the presence of intracellular GFP-expressing bacteria.

Table 2. Percentage of GFP expressing, CD11b^{high} or F4/80 positive cells in *Salmonella* infection

	CD11b ^{high} and GFP positive cells	F4/80 and GFP positive cells	GFP mean fluorescence intensity	*p* value
Tm 0.5 hr	21 ± 7.1[a]	21 ± 6.2[a]	533 ± 43.9	
Ty 0.5 hr	20 ± 4.5	20 ± 5.1	549 ± 28.2	0.26[b]
Tm 4 hr	11 ± 2.7	10 ± 3.7	488 ± 25.3	
Ty 4 hr	8 ± 3.1	9 ± 3.5	441 ± 12.9	0.04[c]

[a] Shown are the means ± 1 SD. At least three independent experiments were averaged from different days. Two mice were used in each group.
[b] Comparison of Typhimurium infection with that from Typhi at 0.5 hr
[c] Comparison of Typhimurium infection with that from Typhi at 4 hr

Clearance of Typhi *vs* Typhimurium *in vivo*

Quantitation of live intracellular bacteria using the mean fluorescent intensity of GFP`**(Figure 5a and Table II)** was not possible due to of the long half life time of GFP (28). Therefore, to compare the survival of intracellular Typhimurium and Typhi in macrophages *in vivo*, the number of *Salmonella* CFU was determined after lysing the peritoneal cells isolated post infection. As shown in **Figure 6a**, intracellular Typhi decreased dramatically to 0.05% at 4 hr after infection. This is in contrast to Typhimurium, which did not change much between 0.5 and 4 hr post infection. To determine if the survival of Typhi would improve if the macrophages were cultured *in vitro* instead of remaining in the mouse (*in vivo*), cells isolated from the peritoneum 0.5 hr post-infection were cultured for 4 hr with 12.5 μg/ml gentamycin. Both intracellular Typhimurium and Typhi decreased but the number of viable intracellular Typhi decreased more dramatically than Typhimurium (**Figure 6b**). While this might be due to the addition of gentamycin, Typhimurium appeared to be killed more effectively when macrophages were incubated *in vitro* than *in vivo*. These results were in sharp contrast to the results in macrophages that were isolated and infected *in vitro* (**Figure 1**). Clearly macrophages infected *in vivo* were better equipped to destroy Typhi than macrophages infected *in vitro*. In addition, once infected *in vivo* macrophages retained their ability to clear Typhi better than Typhimurium even during subsequent *in vitro* culture.

BALB/c Nramp1 wildtype mice were also analyzed using this same assay. No difference was seen in survival of Typhimurium and Typhi between Nramp wildtype

and mutant mice at 0.5 and 4 hr post infection. Together these data strongly support the conclusion that mouse macrophages infected *in vivo* acquire the ability to more efficiently clear Typhi than Typhimurium. Therefore, macrophages regulated by infection *in vivo* may be responsible for many of the host specificity differences observed between Typhimurium and Typhi.

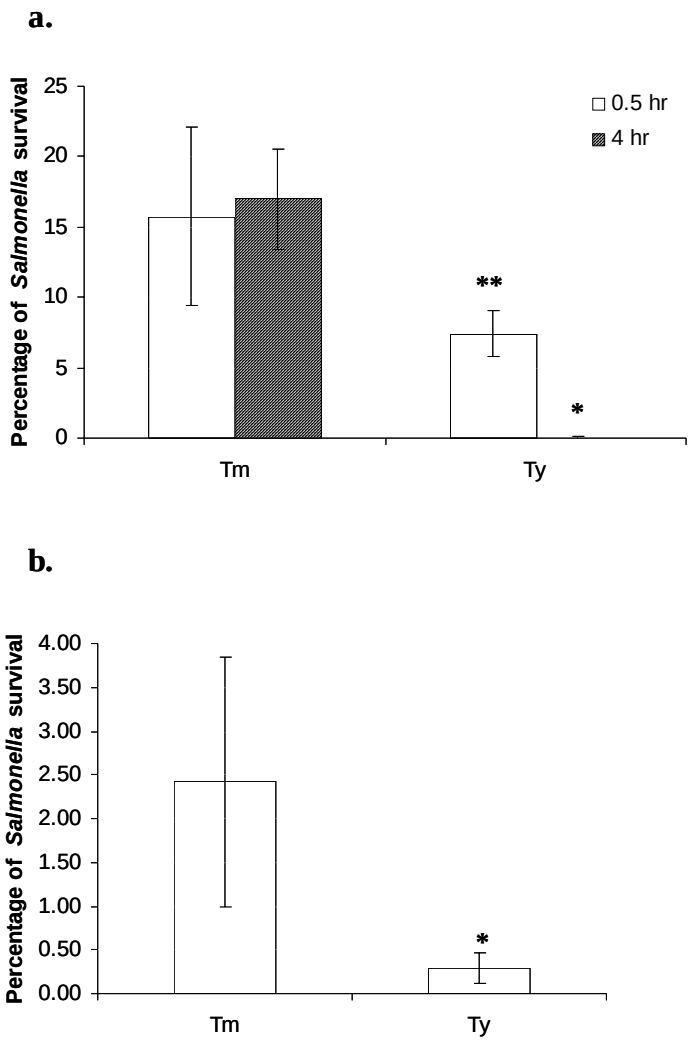


Figure 6. Clearance of intracellular Typhi *vs* Typhimurium *in vivo*.
BALB/c mice were infected i.p. with Typhi *vs* Typhimurium and after 0.5 and 4 hr infection, and peritoneal cells were isolated and counted. a. The cells were lysed and the CFU of intracellular *Salmonella* per two million peritoneal cells were calculated and then divided by total number of injected bacteria (2×10^5) to get the percentage of *Salmonella* survival. Three independent experiments were done. ** ($p<0.05$) shows the comparison between Typhimurium and Typhi at 0.5 hr. * ($p<0.01$) shows the comparison between Typhimurium and Typhi at 4 hr.
b. Two million cells isolated after 0.5 hr of infection were incubated for another 4 hr *in vitro* with gentamycin to kill the extracellular bacteria. Cells were washed and lysed. CFU of intracellular bacteria were divided by intracellular CFU isolated from 0.5 hr to get the percentage of *Salmonella* survival. Three independent experiments were done. (*, $p<0.01$).

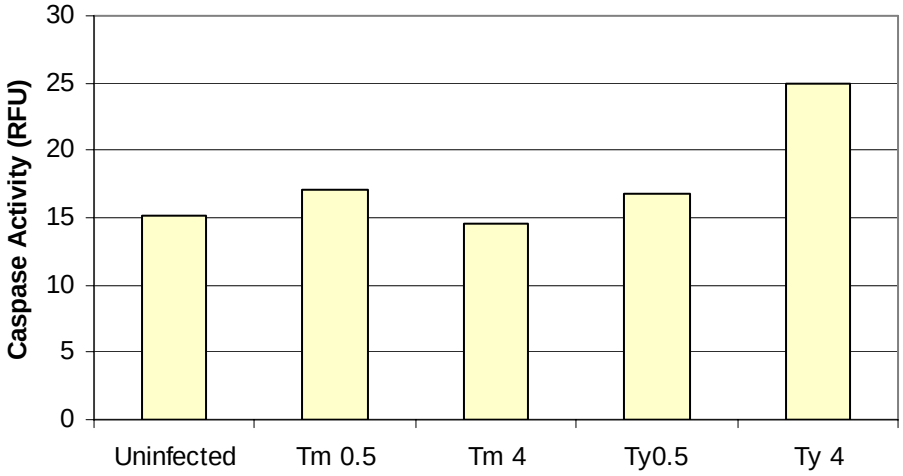


Figure 7. Caspase3 assay from peritoneal cells derived from BALB/c mice infected with *Salmonella*

Peritoneal cells were isolated from mice infected by Typhimurium *vs* Typhi. Caspase3 activities were detected as described. The results shown here are the preliminary data from single experiment.

Cytokine expression pattern on mouse peritoneal cells in the infection by Typhimurium *vs* Typhi.

The ribonuclease protection assays (RPA) were used to detect IL-12, IL-10, IL-1-α, IL-1β, IL-1R, IL-18, IL-6, IFNγ, MIF, TNF-α, IL-4, IL-5, IL-2, and IL-3 expression (data not shown) on peritoneal cell RNAs isolated from mice infected i.p. by Typhimurium *vs* Typhi *in vivo* as indicated above. Uninfected mice were injected with saline as the control. Large amount of TNF-α, IL-1α, IL-1β and IL-6 were detected induced from *S*. Typhimurium infection and *S*. Typhi infection. Real-time PCR was then used to confirm and accurately quantitate the expression of those cytokines (**Figure 8)**. Briefly, RNAs were isolated from mouse peritoneal cells with Typhimurium or Typhi at 0.5 and 4 hr post i.p. infection. Reserve transcription reactions were done to get cDNA templates for each sample. Real-time PCRs were performed to quantitate the cDNA for those cytokines. Slight more TNF-α and IL-6 were detected at 4 hr after infection in Typhimurium than Typhi.

Acknowledgement

Part of this section will be in the manuscript "Macrophages influence *Salmonella* host specificity *in vivo* " and will be submitted to Infection and Immunity. We thank for Dr. Constantine Tsoukas at San Diego State University, and Drs. Michael David and Joshua Fierer at the University of California, San Diego for their support throughout this research. We also thank for Dr. Robert Edwards for the GFP and DS-Red plasmids and Dennis Young at the Moores UCSD Cancer Center for

advice on flow cytometry. We thank Alejandra Gutierrez, Carl Gunderson, Juris Grasis and Matthew Giacalone for assistance with techniques.

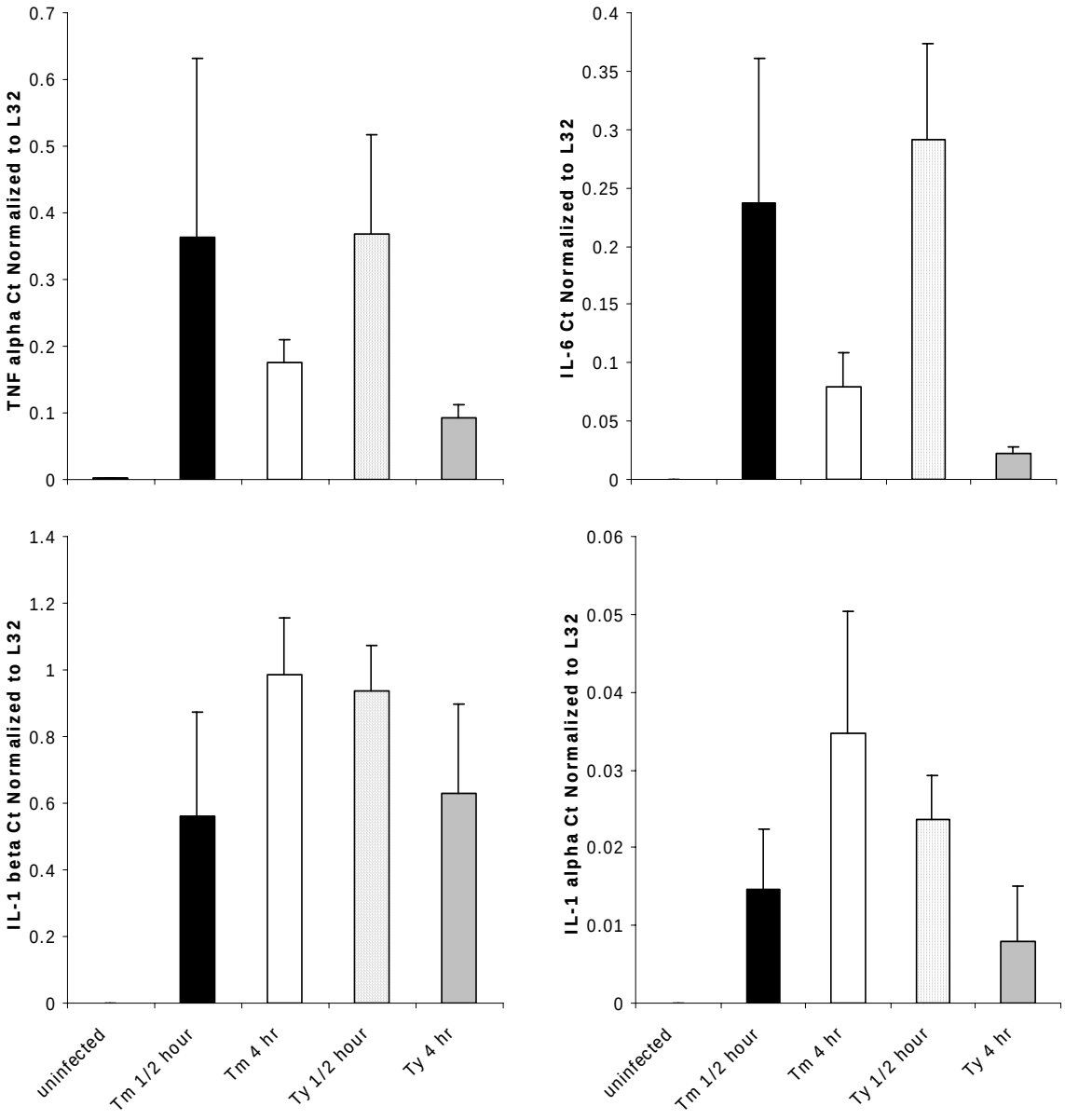


Figure 8. Real-time PCR to Detect TNF-α, IL-1β, IL-1α and IL-6 expression from mouse peritoneal cells

Two independent experiments were done on 6 mice (3 per group). Peritoneal cells were isolated at 0.5 and 4 hr post infection from mice infected with Typhimurium and Typhi as indicated. Results shown are average ratio between the amount of cytokine mRNA to L32 mRNA ± 1 SD.

Discussion

Salmonella enterica serovar Typhi, a host specific serovar to humans, is unable to cause disease in mice (126) while Typhimurium, a generalist, causes severe systemic infection in certain strains. The bacterial and host factors responsible for restricting the host range are unknown. Studies shown here demonstrate that macrophages infected *in vitro* are not able to explain the significantly different intracellular survival ability between host generalist and specific *Salmonella* serovars. However, when infected *in vivo* murine macrophages can recognize and clear Typhi much more efficiently than Typhimurium. These observations strongly support the conclusion that the *in vivo* environment contributes to the role ofmacrophages in *Salmonella* host specificity.

The survival of *Salmonella* inside macrophages is essential for *Salmonella* virulence (43, 103). Genes that are needed for Typhimurium intracellular replication have been studied by transposon mutation and tested in various cell lines including epithelial and macrophage cell lines. This research strongly suggested that the intracellular replication of bacteria is essential for its virulence (95). More research has been done to study the localization of Typhimurium in spleens of infected mice and splenic macrophages were found to be major cell type in which bacteria reside(143). *In vivo* studies comparing the infection of Gallinarum, Dublin, and Typhimurium in chickens and mice showed that although all three *Salmonella* serotypes could be isolated from the intestinal mucosa of both animals following oral infection, only Gallinarum, a chicken host specific serovar, propagated in deeper chicken tissues and

could be isolated from internal organs such as liver and spleen (3). Because macrophages are the main cell type in the mononuclear phagocyte system in the liver and spleen, these studies imply that macrophages can play an important role in determining *Salmonella* host specificity.

Several *in vitro* studies designed to provide direct evidence on how macrophages from various sources differentiate *Salmonella* serovars according to their host range have been done. Some of these studies using Typhimurium and Typhi support the hypothesis that *Salmonella* survival in macrophages determines host specificity. Mouse and human macrophage cell lines and primary macrophages have been compared. Vladoianu and colleagues (169) observed that Typhi persisted in human monocyte-derived macrophages, but not in murine macrophages, while Typhimurium was able to grow in both mouse and human cells. Similar results were obtained by Ishibashi and Arai on human derived macrophages (74, 75). Alpuche-Aranda et al. observed significant differences in survival between these two serovars at early times after infection of primary mouse bone marrow-derived macrophages (2). Although similar conclusions have been drawn from these previous works, there are contradictory data in the literature. For example, both Vladoianu et al (169). and Schawanet al (148). analyzed Typhimuium and Typhi survival in the mouse macrophage cell line J774.A. One study detected a large difference in survival between the two serovars while the other did not. In another study a similar growth rate was observed for Typhimurium and Typhi in the RAW264.7 mouse macrophage cell line even though they observed a different initial phagocytosis rate (129). Another published report demonstrated a lack of correlation between the animal species from

which macrophages were isolated and the ability of host specific *vs.* host generalist *Salmonella* to survive within these macrophages (19). The conflicting results may be due to differences in *Salmonella* strains, macrophage cell lines or approaches used. To compare with the previous reports, gentamycin protection assays were carried out here to measure the survival of Typhimurium and Typhi in both macrophage-like cell lines and primary macrophages from BALB/c mice (see Figure 1). Results show that at 24 hr Typhi and Typhimurium persist at similar levels in macrophages *in vitro* and the differences observed between the two serovars at early time points are insufficient to explain the rapid clearance of Typhi *in vivo*.

In the *in vivo* infection model used here, Typhi is cleared much more rapidly than Typhimurium from the peritoneum (Figure 3). Using GFP-expressing strains, these studies have shown that macrophages were still the major cell type in the peritoneum internalizing both Typhimurium and Typhi (seen in Figure 5). The fact that the total number of bacteria isolated from the peritoneum (shown in Figure 3) is higher than the intracelluar number of bacteria (shown in Figure 6) suggested there should be a portion of bacteria that remains extracellular. Thus, the number of intracellular bacteria was further determined from the macrophages that were infected in *vivo*. These studies still showed that, in contrast to what was seen *in vitro*, significant differences in survival were observed between these two serovars *in vivo* (Figure 6). These data strongly support the conclusion that macrophages do play an important role in *Salmonella* host specificity *in vivo*.

Internalization of live bacteria, but not heat-killed organisms, resulted in a decreased number of macrophages to be isolated from the peritoneum after infection

with both serovars. This conclusion is based on the disappearance of cells that were both F4/80 positive and CD11b high. Possible explanations for this decrease include apoptosis of infected cells, migration of infected cells out of the peritoneum, or an adherence to tissues such that the cells could no longer be easily isolated from the peritoneum (12, 22, 114, 117, 171). Which is the cause for the decreased number of macrophages isolated is still unknown and requires further investigation. However, it is clear from these studies that whatever causes the decreased number of macrophages, it does not differ between the two serovars.

Several studies have demonstrated that a gene product called Nramp1 plays an important role in resistance to *Salmonella* (131). Nramp1 was first described as Ity (immunity to Typhimurium) in 1976 (131). It is now known that Nramp1 encodes for a highly hydrophobic 56 kDa protein, which possesses 12 transmembrane domains and a glycosylated exracytoplasmic loop. It is expressed in the membrane fraction of macrophages and neutrophils as a phosphoglycoprotein of 90-100 kDa (50). Nramp1 plays an important role in *Salmonella*-containing vacuole maturation after phagocyte internalization of the bacteria and thus controls the replication of intracellular pathogen survival (30). The ability of Nramp1 to enhance the killing of the bacteria might due to its function as a proton/divalent cation transporter dependent on pH (168),(76). CORRECT CITATIONSHowever, studies presented here using BALB/c DBA2 mice, which are identical with the BALB/c strain except that they contain functional Nramp1, demonstrated that the presence of Nramp1 does not alter the phagocytosis of Typhimurium or Typhi after infection *in vivo*. Macrophages were still the major cell type to phagocytose the bateria in the model used, and neutrophils were

still attracted in large numbers. Although intracellular killing by resident peritoneal macrophages has been proved to be more efficient in this mouse strain even for Typhimurium (163), the Nramp1 wildtype BALB/c mice used in this study did not show much difference from the BALB/c mutant mice. It appears that Nramp1 can not help macrophages differentiate Typhimurium from Typhi at this early time point. The difference between Typhimurium and Typhi was significant in Nramp1 wildtype mice, similar to what was observed in the Nramp1 mutant mice. Therefore, it is unlikely that Nramp 1 plays an important role in the differentiation of Typhimurium from Typhi.

The role of neutrophils has also been investigated in *Salmonella* infection (44, 164). Neutrophils are attracted rapidly to the site of infections by chemoattractants such as chemokines (44, 158). The main mechanism for neutrophils to protect against infection is to kill the invading bacteria but few studies have analyzed how neutrophils interact with macrophages and other components of host innate immunity to determine *Salmonella* host specificity. In the studies presented here, because there are few residential neutrophils in mouse peritoneum, the bacteria were mainly phagocytosed by macrophages at 0.5 hr of infection. Neutrophils were attracted to the peritoneum by the presence of either heat-killed or living bacteria, but internalization of bacteria by these cells was still much less than that in macrophages at 4 hr after infection. This may be because the MOI used is very low and free bacteria may be rare in the peritoneum after neutrophil influx has occurred. These results do not in any way exclude a role for neutrophils in the protection of animals from infection. They also could not rule out two other possibilities: that induction of GFP in the phagocytosed bacteria either does not occur in neutrophils or that the killing by neutrophils was so

fast that there was not enough time for it. The studies presented here using strains that constitutively express dsRed suggested that the latter possibility is not the reason no bacteria were seen in neutrophils, however.

It is still not clear what causes macrophages to clear Typhi more efficiently than Typhimurium *in vivo*. It seems likely that another cell type and/or factor regulates the macrophages *in vivo* to more efficiently clear Typhi. Neutrophils, dendritic cells , NK cells and NKT cells are all possible candidates. Both cell function and changes of cell numbers have been investigated in *Salmonella* pathogenesis (78, 172). It will be interesting to further examine their interactions with, and regulatory effects on, macrophages *in vivo* to determine if they relate to host specificity. Cytokine expression and function may also contribute to differences in host specificity (37). Complement opsonization is another possible difference; *in vitro* research has indicated that it might be one possible mechanism causing a significant difference in intracellular survival between Typhimurium and Typhi (73), It is unlikely that complement alone, however, is responsible since uptake of the two serovars appears to be equivalent at early time points (0.5 hr) after infection *in vivo*. Mucin has also been shown to enhance Typhi virulence in mice, possibly due to an increase in bacterial intracellular survival inside peritoneal macrophages (133, 150). These data support the notion of the role of macrophages in host specificity determination. Unfortunately, studies attempting to eliminate macrophages *in vivo* have indicated a dual role for macrophages in *Salmonella* pathogenesis; i.e. both killing and protection of *Salmonella* (174). Therefore, it is very difficult to address this question directly *in vivo*.

Proinflammatory cytokines have been observed to play important roles in all stages of *Salmonella* infection. Studies on the role of cytokines in the pathogenesis of *Salmonella* generally have addressed two questions: 1) Which cytokines are expressed in various tissues during the course of infection; and 2) What are the consequences of altering cytokine levels for the course and severity of infection. To determine if different *Salmonella* serovars induce differential cytokine expression in the model system used here, real-time PCR was done to assay TNF-α, IL-1α, IL-1β and IL-6. RNA levels from cells isolated from the peritoneum after 4 hr showed slight difference in TNF-α and IL-6 between Typhimurium and Typhi. How this relates to host specificity is still not clear.

TNF-α production during *Salmonella* infection is controversial. Plasma TNF-α release in response to *Salmonella* infection has not been detected in some studies, while others have (82). TNF-α level was detected to rise in response to endotoxin or LPS injection. The important role of TNF-α in host defense against salmonellosis was displayed by evidence from exogenous TNF-α study, which showed that TNF-α together with IL-1 could increase resistance to Typhimurium infection in mice (119). Other study using anti--TNF-α antibodies decreased mouse resistance to *Salmonella* infection (157). The mechanism might be related to its cytotoxic activity against bacteria-infected cells (91).

IL-6 was first described as a B-cell terminal differentiation inducer. It plays important role in inflammatory response and mucosal antibody production (137). IL-6 might play roles in anti-*Salmonella* infection because typhoid fever patients present

increased IL-6 level in the serum (86). Besides this evidence for TNF and IL-6, however, whether they play roles in *Salmonella* host specificity is still unknown.

Cytokines are expressed by many different cell types, and they act on various cells. To determine which cell types express cytokines, intracellular cytokine staining could be used to assay cytokine expression for each cell type. Each cytokine can be tested *in vitro* in tissue culture of macrophages or administrated *in vivo* individually or together with other cytokines to figure out their possible role. Peritoneal fluids or supernatants isolated from *Salmonella* infection *in vivo* can also be tested in *in vitro* macrophage cultures to determine if this will allow them to distinguish the two serovars.

In conclusion, the hypothesis that macrophages contribute to *Salmonella* host specificity *in vivo* was tested in mice. When BALB/c mice were infected *in vivo* i.p., similar levels of Typhimurium could be found in the peritoneum 0.5 and 4 hr post-infection, which are very early time points after post infection. Typhi, on the other hand, decreased drastically by 4 hours post-infection. In both Typhi and Typhimurium infection macrophages were shown to be the major cell type containing internalized bacteria. However, significantly lower numbers of viable Typhi were recovered from macrophages infected *in vivo* than Typhimurium. These data showed that macrophages were able to distinguish Typhi from Typhimurium when infected *in vivo* rapidly at the early stage of infection, suggesting that the *in vivo* environment is required for macrophages to play an important role in *Salmonella* host specificity.

In vitro tissue culture cells provided useful information on pathogen host interactions. However, because of the complexity of the hose immune system, the

natural interactions between *Salmonella* and the immune system are not easily studied *in vitro*. To identify what actually occurs *in vivo* and how these events lead to disease is one of the biggest challenges facing the field. Understanding how host cell populations coordinate responses to infection as well as which cell types contain bacteria will provide valuable information on the innate response to infection. Using the mouse model described here, the role of macrophages has been investigated. But the cellular and molecular mechanisms of the host response are still not clear. It is most likely that more than one mechanism is involved in the differential survival of *Salmonella* serovars *in vivo* that leads to host specificity. The *in vivo* model used in this research provides direct evidence of the role of macrophage-*Salmonella* interactions in the initial phase of infection and contributes to the current understanding of this intriguing question on *Salmonella* host specificity.

Acknowledgement

Part of this section will be in the manuscript "Macrophages influence *Salmonella* host specificity *in vivo* " and will be submitted to Infection and Immunity. We thank for Dr. Constantine Tsoukas at San Diego State University, and Drs. Michael David and Joshua Fierer at the University of California, San Diego for their support throughout this research. We also thank for Dr. Robert Edwards for the GFP and DS-Red plasmids and Dennis Young at the Moores UCSD Cancer Center for advice on flow cytometry. We thank Alejandra Gutierrez, Carl Gunderson, Juris Grasis and Matthew Giacalone for assistance with techniques.

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