Salmonella typhimurium and its host : host-pathogen cross-talk, immune evasion, and persistence

Diepen, A. van

chapter two

Gamma irradiation or $CD4^+$ T cell depletion causes reactivation of latent *Salmonella enterica* serovar Typhimurium infection in C3H/HeN mice

Angela van Diepen,[1] Joke S. van de Gevel,[1]
Margaretha M. Koudijs,[1] Ferry Ossendorp[2], Henry Beekhuizen,[1]
Riny Janssen,[1,†] and Jaap T. van Dissel[1,†]

[†]*equally contributed to this study*
Department of Infectious Diseases, Leiden University Medical Center, PO Box 9600, 2300 RC Leiden.[1]
Department of Immunohematology and Blood Transfusion, Leiden University Medical Center,
PO Box 9600, 2300 RC Leiden, The Netherlands.[2]

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Abstract

Salmonella is a gram-negative pathogen that can cause a range of diseases in both animals and man varying from mild diarrhea to severe disseminated infections like typhoid fever. Upon infection, a host develops an immune response to limit bacterial growth and kill and eliminate the pathogen. *Salmonella* has evolved mechanisms to evade the host immune system and remain dormant within the body, only to reappear (reactivate) at a later time point when the immune system is abated. The exact mechanism by which *Salmonella* is able to persist is unknown, but evading the immune response likely plays an important role. We have developed an in vivo model for studying reactivation of *Salmonella enterica* serovar Typhim*urium* infection in mice. Upon subcutaneous infection, C3H/HeN (Ity^r) mice showed an increase in bacterial numbers in livers and spleens reaching a peak on day 19. After full recovery of the infection, these mice were irradiated or depleted for $CD4^+$ T cells. The mice showed a decrease in numbers of leukocytes and $CD4^+$ T cells, respectively, and displayed a secondary infection peak in livers and spleens with a course similar to the primary infection. From this we concluded that $CD4^+$ T cells are involved in active suppression of *S. enterica* serovar Typhimurium during persistency.

The role of $CD4^+$ T cells during primary infection with *S. enterica* serovar Typhimurium is well established. This is the first study to describe a role of $CD4^+$ T cells during the phase of latent *S. enterica* serovar Typhimurium infection.

Introduction

Salmonellae are Gram-negative, facultative intracellular pathogens that can cause a range of diseases in both animals and man varying from mild diarrhea to severe disseminated infections such as typhoid fever. Like other intracellular bacteria, *Salmonella* has the capacity to adhere to host cells and induce its own uptake, even by nonprofessional phagocytes. Natural infection with *Salmonella* occurs through the intestinal tract. Once *Salmonella* has survived the antimicrobial mechanisms of the gastrointestinal tract, e.g. low pH, normal intestinal flora, intestinal mucus layer, and has reached the lining of the intestine, *Salmonella* preferentially penetrates the M cells that transport the bacteria from the lumen to the underlying Peyer's patches, or alternatively, *Salmonella* passes the epithelial cells (14, 30). After passing the epithelial barrier *Salmonella* is taken up by phagocytic cells, especially macrophages. These cells are important components of the host innate defense and are equipped with a multitude of antimicrobial mechanisms. Following ingestion by macrophages, bacteria reside within phagosomes that fuse with lysosomes to form phagolysosomes in which the bacteria can be rapidly killed due to acidification and bacteriolysis (reviewed in (10)). However, despite this multitude of antimicrobial mechanisms present as part of the innate defense of phagocytic cells, *Salmonella* is able to enter, survive and even replicate within these cells and can cause chronic or persistent infections by evasion or disturbance of the host defense (15). This ability of *Salmonella* to enter and replicate within phagocytic cells is essential for its survival, as mutants unable to do so are avirulent (5). Although the exact mechanisms for intracellular survival of *Salmonella* after phagocytosis are still unknown, it has become clear that *Salmonella* responds to the specific host environment by expressing factors crucial for intracellular survival and for circumvention of the host defense systems (3, 6, 7, 15, 23).

Upon infection, the host will try to develop an immune response to limit bacterial growth and to eventually kill and eliminate the pathogen. B cells, T cells and macrophages are important for host resistance and their protective effects are mediated by several cytokines such as IFNγ, IL-12, and TNFα (4, 16, 19-21, 26). This integrated response results in activation of macrophages that in turn kill *Salmonella*. Although the macrophages are the main host cells, necessary for survival and replication of *Salmonella* within the host, and mediate the *Salmonella*-induced pathology, these same macrophages also play a crucial role in host defense against *Salmonella* (31). They are necessary for the early local control of infection, and, subsequently, for the induction of acquired immunity (11, 18) as well as for restriction of bacterial growth in immune mice (31). During a *Salmonella* infection, macrophages are mainly activated via TLR4 signaling and via several cytokines produced by NK cells and T lymphocytes.

Following full activation of the macrophages and the acquired immune system, the host should be able to eliminate and clear *Salmonella* from the body. However, *Salmonella* has evolved mechanisms to evade the host immune system, to persist reside within the body, and to reappear (reactivate) at a later time point. Several studies and case reports

have shown that patients who underwent total body irradiation or received an organ transplant and were treated with glucocorticoids or other immunosuppressive therapy, as well as patients suffering from HIV infection (1, 9, 12) or Interleukin 12 receptor β1 deficiency (27) can suffer from recurrent (systemic) infections with a *Salmonella* strain that persisted within the host.

By investigating the possibility of *S. enterica* serovar Typhimurium to persist and reactivate after immune intervention in a mouse model of latent *S. enterica* serovar Typhimurium infection, we aim to gain insight into the mechanisms by which *S. enterica* serovar Typhimurium is able to persist and to reactivate at a later time point. This study shows that total body irradiation or selective $CD4^+$ T cell depletion in C3H/HeN (Ity^r) mice that were infected with *S. enterica* serovar Typhimurium and had apparently cleared the bacteria form the organs, leads to reactivation of the *S. enterica* serovar Typhimurium infection. In addition, this is the first study that describes the role of $CD4^+$ T cells during persistent *S. enterica* serovar Typhimurium infection in mice.

Materials and Methods

Mice. Six- to eight-week old female *Salmonella*-resistant (Ity^r) C3H/HeN mice were obtained from Harlan (Horst, The Netherlands). Mice were maintained according to the institutional guidelines. Water and food were given ad libitum. All studies involving animals were carried out in accordance with, and after approval of, the animal research ethics committee of the Leiden University Medical Center.

Bacteria. For in vivo infection experiments, single colonies of *S. enterica* serovar Typhimurium strain 14028s were grown overnight in Luria-Bertani (LB) medium (10 mg of tryptone, 5 mg of yeast extract, and 10 mg of NaCl/ml) at 37°C while being shaken (225 rpm). The overnight cultures were diluted in fresh LB medium and grown to the end of log phase and were then washed and diluted in sterile PBS. The CFU in the inoculum were determined by plating serial dilutions.

Antibodies. Monoclonal antibodies (mAbs) directed to mouse T cell surface antigen CD4 were obtained from the supernatant of cultured hybridoma GK1.5 (rat anti-mouse CD4, American Type Culture Collection (ATCC)). The hybridoma was cultured in protein-free hybridoma medium (Gibco). The supernatant was isolated and concentrated using a capillary dialyzer. The concentrated supernatant was filter-sterilized and, after determination of the protein concentration, stored at –20°C until use. FITC-conjugated rat anti-mouse CD4 (L3T4) and PE-conjugated rat anti-mouse CD8 (Ly-2) monoclonal antibodies were obtained from BD Biosciences.

***S. enterica* serovar Typhimurium infection.** Mice were inoculated subcutaneously in the flanks with 0.1 ml bacterial suspension containing 3×10^4 CFU *S. enterica* serovar Typhimurium 14028s. For each group on each time point 3-6 mice were used. Mice were sacrificed by carbon dioxide inhalation and blood was taken immediately by cardiac puncture. Part of the blood was prevented from coagulation by adding 40 U heparin/ml and another part was used to obtain serum. To determine the bacterial load within spleens, livers, and inguinal lymph nodes, these organs were aseptically removed and single cell suspensions were prepared by using sterile 70-µm-mesh-size cell strainers (Falcon). Cells were pelleted by centrifugation for 10 min and were lysed in distilled water. The bacterial number per organ was determined bacteriologically by plating serial dilutions. The lowest number of bacteria that can be detected in this way is 30 CFU for the spleens and lymph nodes and 50 CFU for the livers.

Leukocyte count and blood cell differentiation. Blood was obtained by cardiac puncture and 40 U heparin/ml was added to prevent coagulation. The number of leukocytes was determined by counting the number of nucleated cells in the blood and we used 5 µl blood to make blood smears for the differentiation of the blood cells. Blood smears were fixed in methanol and stained with Giemsa and were used to determine the relative percentages of the different types of cells in the blood. This, together with the total leukocyte numbers was used to calculate the number of lymphocytes, monocytes, and PMN present in the blood on the different time points. The number of lymphocytes was used together with the FACS analysis data, to calculate the number of $CD4^+$ and $CD8^+$ T cells in the blood on the different time points after infection.

Gamma irradiation and in vivo T cell depletion. Immune intervention was carried out either by sublethal total body gamma irradiation or in vivo depletion of $CD4^+$ T cells on a time point when bacteria could no longer be detected bacteriologically within livers, spleens, and inguinal lymph nodes. For total body gamma irradiation, mice were put in a small perspex box and were irradiated until a dose of 6 Gy was reached. $CD4^+$ T cells were depleted using 200 µg rat anti-CD4 GK1.5 antibody injected intraperitoneally. Mice received a second and a third injection with 100 µg of this antibody on day two and four after the first injection.

Flow Cytometry. To determine $CD4^+$ and $CD8^+$ T cell counts in blood, 100 µl heparinized blood was used. Before staining, erythrocytes were lysed on ice for 10 minutes using lysisbuffer (155 mM NH_4Cl, 100 mM $KHCO_3$, 10 mM EDTA). After washing with PBS, the cells were stained during 30 minutes with FITC-conjugated rat anti-mouse CD4 mAb and PE-conjugated rat anti-mouse CD8 mAb, respectively. Flow cytometry was performed on a FACSCaliber System (Becton Dickinson).

Detection of *S. enterica* serovar Typhimurium-specific antibodies. Induction of *S. enterica* serovar Typhimurium -specific antibodies was determined in a whole cell ELISA in which Maxisorp plates (Nunc) were coated with whole *S. enterica* serovar Typhimurium 14028s. Briefly, *S. enterica* serovar Typhimurium 14028s was grown ON in LB medium and after washing with PBS was resuspended in 0,5 mM Na_2HPO_4, pH 9.6. Maxisorp plates were filled with 100 μl of this suspension and were dried ON at 37°C. Non-specific binding was blocked by incubating the plates with 3% FCS in PBS for 2 h at 37°C. Serial dilutions of the sera were added to the wells. Sera from naïve mice were included as a control. The plates were incubated at 37°C for 2 h. After washing the plates four times with PBS-Tween (0.05%) per well 100 μl 1:10,000 diluted peroxidase-labeled goat-anti-mouse IgG antibodies (SouthernBiotech, Birmingham, AL35260, USA) were added and the plates were incubated at 37°C for another 2 h. Plates were washed again with PBS-Tween (0.05%) and substrate solution (0.11 M NaAc, 1 mg/ml TMB, 0.01% H_2O_2) was added to the wells. The coloring reaction was stopped by addition of H_2SO_4 and the wavelength absorbance was measured at 490 nm using an ELISA plate reader (VICTOR^2 1420 multilabel counter, PerkinElmer Life and Analytical Sciences). Titers are defined as the dilution for which:

$$OD_{450} \text{ (sample)} > OD_{450} \text{ (naïve serum)} + 2 \times \text{standard deviation.}$$

Calculation of bacterial growth. The growth rate was determined according to the equation: k = [ln N(t peak) – ln N(day 1)] / t, in which N(t peak) is the number of viable bacteria per organ on the peak of infection after time t (min) has elapsed, and N (day 1) represents the bacterial number on day 1 after infection.

Statistical analysis. For comparison between treatments we used Student's *t* tests and for correlation analysis a Pearson correlation analysis was performed. For both analyses, a P value <0.05 was considered significant.

Results

Replication of *S. enterica* serovar Typhimurium 14028s during a primary infection in C3H/HeN mice. Mice were inoculated subcutaneously in the flanks with 3×10^4 CFU *S. enterica* serovar Typhimurium 14028s. During the first 5 days after the infection, the body weight of the infected mice was comparable to that of the age-matched weight controls (Fig. 1A). The infected mice showed a decrease in body weight between days 12 and 19. During this period, some of the mice also showed signs of illness like ruffled fur and malaise. During the next week, the mice fully recovered and showed an increase in body weight. The infection with *S. enterica* serovar Typhimurium 14028s induced severe hepatosplenomegaly reaching a peak on day 19 (Fig. 1C and 1E). The isolated livers and spleens were weighed and were used to determine the pathology

induced by the *S. enterica* serovar Typhimurium infection. Pathology was calculated as the percentage organ weight of the total body weight as described before (28). The induction of pathology during the first 19 days after infection was similar for the spleens and livers as is shown by a statistically significant correlation between the induced liver and spleen pathology ($R=0.763$; $P<0.001$). The pathology induced by *S. enterica* serovar Typhimurium became less severe during the next week and eventually stabilized at 7.5% for the livers and 1-1.25% for the spleens.

Fig. 1B shows that *S. enterica* serovar Typhimurium 14028s was detectable in the lymph nodes already on day 1 after infection and was able to replicate reaching a peak on day 5 after infection. From there, the infection spread to the spleens and livers and within these organs bacterial loads up to 3×10^7 CFU per organ were reached (Fig. 1D and F). The bacterial loads were highest on day 19, which correlated with the induced pathology in the livers ($R=0.590$; $p=0.001$) and spleens ($R=0.577$; $P=0.002$). The bacterial loads eventually declined, which coincided with the increase in body weight and a reduction in spleen and liver pathology (Fig. 1A, 1C and 1E).

Reactivation of the *S. enterica* serovar Typhimurium infection by gamma irradiation. On day 41, when the bacterial loads in the organs were reduced below the detection limit, the mice received a sublethal total body irradiation (6 Gy). The infection control group was not treated. The irradiated mice showed signs of illness, like ruffled fur and malaise between day 54 and 61. Due to the irradiation, the spleenweight on average decreased by ~40% compared to the infection controls (0.18 g vs 0.33 g) and the liverweight showed ~8% reduction (1.86 g vs 2.03 g). The effects of the irradiation on the bacterial numbers in the organs are shown in Fig. 2. In the infection control group, 60% of the mice showed detectable amounts of bacteria in the livers (Fig. 2B), but the averages were around or below the detection limit and no increase in bacterial numbers could be observed. The bacterial numbers in the lymph nodes and spleens stayed below or around the detection limits (Fig. 2A and 2C).

In the irradiated mice population, on the other hand, we observed an increase in bacterial numbers of *S. enterica* serovar Typhimurium in the livers and spleens (Fig. 2B and 2C) upon immune intervention and in all the mice the infection peaked on day 54. This secondary infection (i.e. reactivation) was milder than the primary infection as is shown by lower maximal bacterial numbers in the organs reached, but otherwise followed a course that was similar to that of the primary infection peak (Table 2, Fig. 2). In the lymph nodes we also observed a slight increase in bacterial numbers on day 54 (Fig. 2A), although the growth rate differed from that of the primary infection.

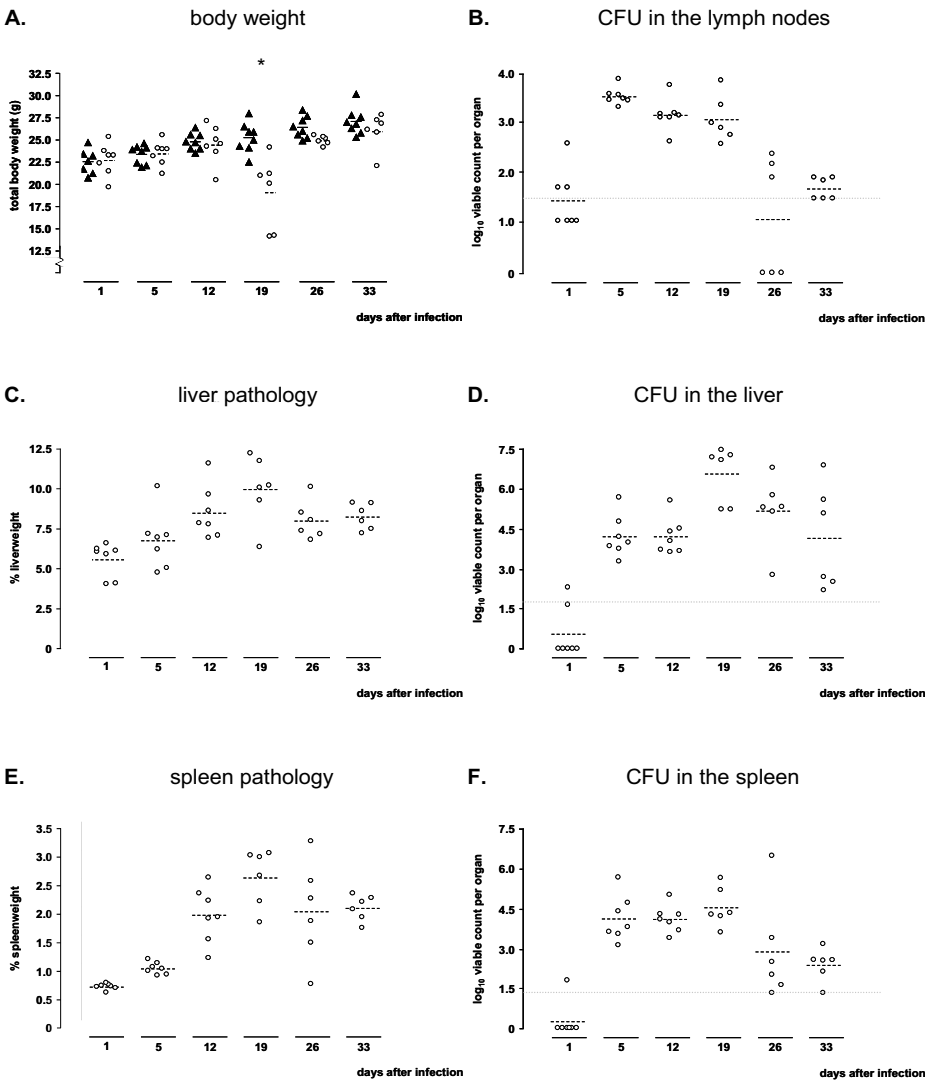

Figure 1. Total body weight (A), liver and spleen pathology (C and E) and bacterial numbers within the lymph nodes (B), livers (D), and spleens (F) of C3H/HeN (Ity^r) mice during a primary infection with *S. enterica* serovar Typhimurium 14028s (open circles). The crosses in A represent the total body weights of mice that were not infected with *S. enterica* serovar Typhimurium and that served as weight controls. Averages from 2 independently performed experiments are shown. On day 0, C3H/HeN (Ity^r) mice were infected subcutaneously in the flanks with ~3×10^4 CFU *S. enterica* serovar Typhimurium 14028s. The actual dose was confirmed by plating serial dilutions of the inoculum. At the indicated time point, the livers, spleens, and lymph nodes were aseptically removed and weighed. The pathology in the livers and spleens was calculated as the percentage organ weight of the total body weight. The viable count within the organs was determined by making lysates and plating serial dilutions of the lysates (n=6-8 per group). The results are expressed as $\log_{10}$ viable counts (means ± standard errors of the means). Asterisks indicate statistically significant differences ($P<0.05$) compared to the infection controls (Students *t* test) and the gray dashed lines represent the detection limit of the microbiological method.

Leukocyte counts in the blood. On each time point we determined the leukocyte counts in the blood for each mouse and differentiated the types of blood cells present at the different time points after infection (Table 1). During the growth phase in the primary infection, there was a statistically significant correlation between time and the total number of leukocytes (R=0.544; p<0.001), meaning that the number of leukocytes increased during the primary infection peak. The number of leukocytes did not change further, but stabilized up to the day of treatment on day 41 (Table 1). Giemsa stained blood smears were used to determine the percentages of the different types of cells in the blood on the different time points after infection. The observed increase in the number of leukocytes was mainly due to the increase in the number of granulocytes (Table 1) whereas the number of lymphocytes and monocytes did not change during the primary infection as well as in the infection control group on the later time points. FACS analysis on the lymphocyte population revealed no change in the number and ratio of $CD4^+$ and $CD8^+$ T cells during the primary infection peak (data not shown).

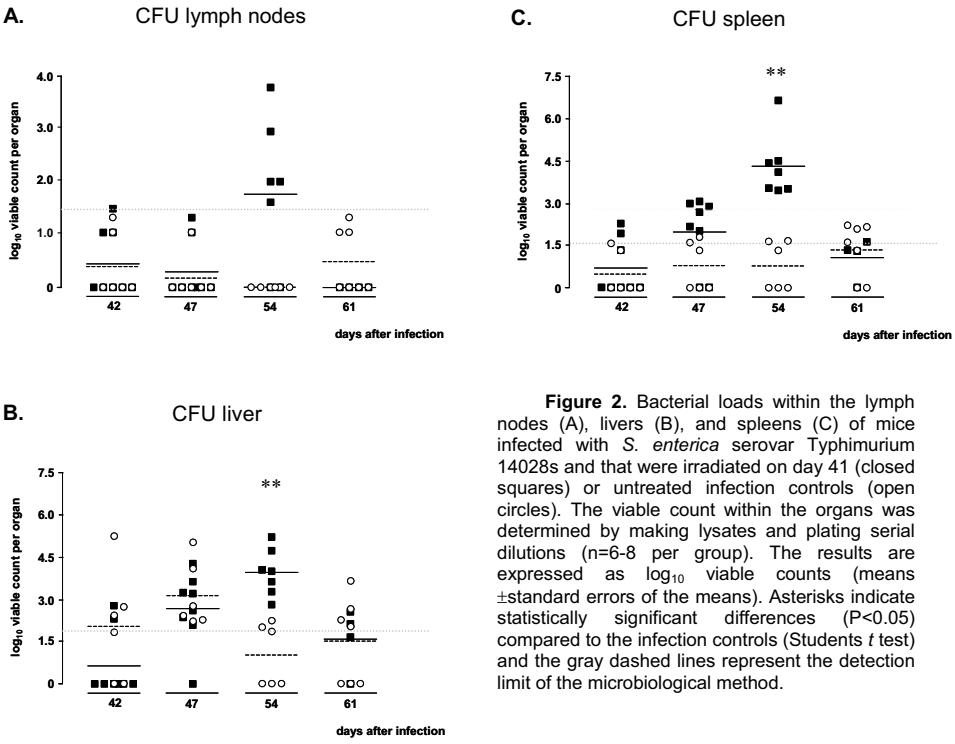

Figure 2. Bacterial loads within the lymph nodes (A), livers (B), and spleens (C) of mice infected with *S. enterica* serovar Typhimurium 14028s and that were irradiated on day 41 (closed squares) or untreated infection controls (open circles). The viable count within the organs was determined by making lysates and plating serial dilutions (n=6-8 per group). The results are expressed as $\log_{10}$ viable counts (means ±standard errors of the means). Asterisks indicate statistically significant differences (P<0.05) compared to the infection controls (Students *t* test) and the gray dashed lines represent the detection limit of the microbiological method.

Table 1. Number of leukocytes, lymphocytes, monocytes, and granulocytes in the blood[a]

day	treatment	leukocytes ($n \times 10^5$)	lymphocytes ($n \times 10^5$)	monocytes ($n \times 10^5$)	granulocytes ($n \times 10^5$)
1	---	54.7 ± 8.3	38.7 ± 12.9	1.1 ± 0.5	14.3 ± 8.9
5	---	85.1 ± 13.5	48.3 ± 9.4	1.8 ± 1.4	29.9 ± 7.3
12	---	77.3 ± 24.4	30.4 ± 12.7	0.8 ± 0.7	42.3 ± 13.6
19	---	100.7 ± 28.5	33.8 ± 13.8	2.8 ± 2.2	52.2 ± 13.1
26	---	78.0 ± 13.4	39.3 ± 5.6	1.2 ± 1.0	33.8 ± 11.7
33	---	110.8 ± 37.6	52.2 ± 10.3	1.8 ± 1.9	53.3 ± 29.2
42	---	86.2 ± 29.7	53.7 ± 18.0	0.7 ± 0.7	27.9 ± 14.2
42	irr[b]	23.5 ± 9.4	7.4 ± 3.8	0.0 ± 0.0	15.5 ± 6.3
43	depl[c]	50.2 ± 11.3	26.3 ± 6.9	1.1 ± 0.9	21.6 ± 13.0
47	---	87.8 ± 23.9	58.5 ± 20.6	1.0 ± 0.5	24.7 ± 7.6
47	irr	9.1 ± 4.4	5.1 ± 2.6	0.2 ± 0.2	3.1 ± 2.3
47	depl	61.1 ± 21.2	31.4 ± 11.8	1.5 ± 1.6	26.2 ± 17.1
54	---	50.8 ± 29.0	29.0 ± 22.4	0.6 ± 0.7	19.3 ± 10.2
54	irr	10.6 ± 5.1	5.8 ± 3.0	0.1 ± 0.1	2.8 ± 3.4
54	depl	89.6 ± 21.1	39.0 ± 16.2	2.1 ± 1.4	39.0 ± 16.2
61	---	94.6 ± 24.1	45.6 ± 14.3	1.0 ± 1.1	41.9 ± 27.4
61	irr	29.5 ± 7.6	8.7 ± 2.2	0.2 ± 0.2	15.8 ± 4.5
61	depl	55.0 ± 14.2	29.2 ± 8.5	0.7 ± 0.4	23.2 ± 11.9

[a] values are mean numbers/ml ± stdev
[b] mice that received a total body irradiation (6 Gy)
[c] mice that were depleted for CD4+ T cells by injection of anti-CD4 antibodies

The irradiated mice, however, showed reduced leukocyte counts in the blood already on day 1 after the irradiation (day 42) when compared to the untreated infection controls and the numbers remained lower up to day 61 after infection (Table 1). Table 1 also shows that the numbers of granulocytes declined in the irradiated mice and were lowest on day 47 and 54. On day 61 the number of granulocytes had not recovered fully yet, but showed an increase compared to day 54. The number of monocytes seemed to be decreased by the irradiation, but the numbers are too low to reach statistical significance. The number of lymphocytes, on the other hand, was reduced dramatically already on day 1 after the irradiation compared to the infection controls and remained significantly lower up until day 61. We used these lymphocyte numbers together with the FACS analysis data to calculate the number of $CD4^+$ and $CD8^+$ T cells and, as expected, observed that both the $CD4^+$ and

the $CD8^+$ T cells were reduced to the same extent in the irradiated mice compared to the infection controls (Fig. 3A and 3B). These data suggest that the observed reactivation of the *S. enterica* serovar Typhimurium infection upon irradiation could have been due to the reduction in the numbers of either granulocytes, $CD4^+$ T lymphocytes, $CD8^+$ T lymphocytes, or a combination of cells.

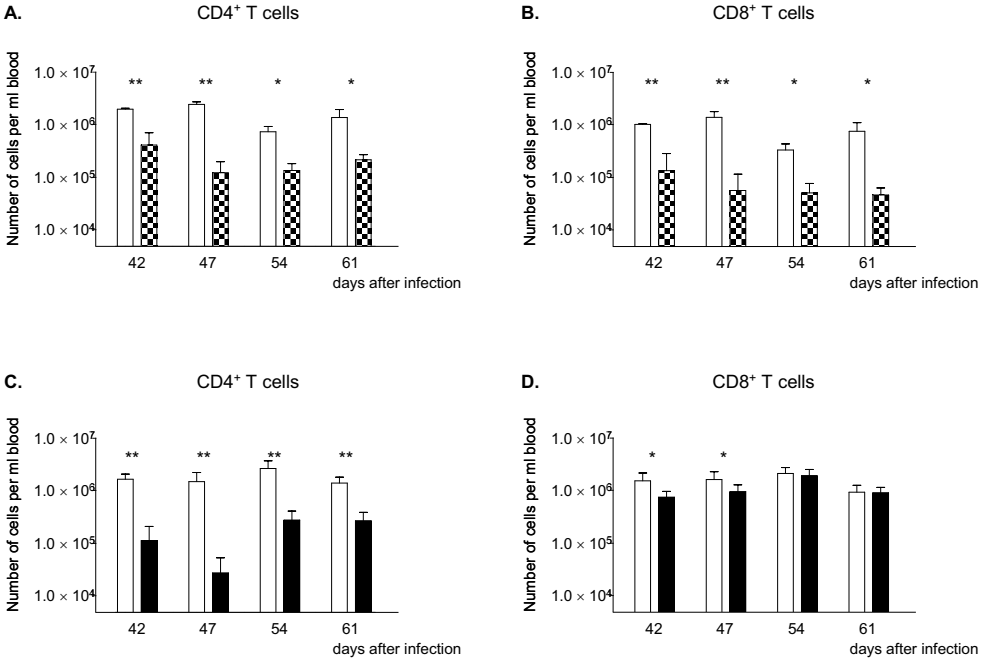

Figure 3. Number of $CD4^+$ T cells (A and C), and $CD8^+$ T cells (B and D) per ml blood of C3H/HeN mice infected with *S. enterica* serovar Typhimurium that received no further treatment (white bars), mice that received a total body irradiation (checkered bars), or mice that were depleted for T cells (black bars). The number of $CD4^+$ and $CD8^+$ T cells was calculated by combining data from leukocyte counts, cell differentiation, and FACS analysis.

Reactivation following T cell depletion. Reactivation of a latent *S. enterica* serovar Typhimurium infection in people has been described for patients suffering from AIDS. These patients showed reduced $CD4^+$ T cells counts and suffered from recurring infections with the same *S. enterica* serovar Typhimurium strain. This strongly suggests a role for the $CD4^+$ T cells in the suppression of *S. enterica* serovar Typhimurium during the persistence phase. Since the irradiated mice also showed a reduction in the numbers of $CD4^+$ T cells as well as the numbers of granulocytes and $CD8^+$ T cells, we wondered whether only reducing the number of $CD4^+$ T cells by in vivo depletion could also result in the reactivation of a latent *S. enterica* serovar Typhimurium infection in C3H/HeN mice. The primary infection and change in the number of leukocytes followed a course similar to that shown in Fig. 1 and Table 1, respectively (data not shown). In the infection control group

we observed no reactivation of the infection as the bacterial numbers stayed around or below the detection limits in all the organs up to day 61. In the lymph nodes of the mice that were depleted for $CD4^+$ T cells, we observed no detectable outgrowth of *S. enterica* serovar Typhimurium (Fig. 4A). In the livers and spleens, on the other hand, we observed an increase in bacterial numbers that was significantly different from those in the infection controls and this reactivation reached a peak on day 47 (Fig. 4B and 4C). As for the irradiated mice, this reactivation peak was lower than the peak observed for the primary infection, but followed a course that was similar to that of the primary infection peak, although the growth rate was slightly lower (Table 2). Of note, neither spleenweight nor liverweight significantly changed upon $CD4^+$ T cell depletion as the average spleen- and liverweights were 0.39 g and 2.03 g, respectively for the $CD4^+$ T cell depleted mice compared to 0.35 g and 2.10 g for the infection controls. Table 1 shows that the total number of leukocytes and lymphocytes slightly, though significantly, changed upon injection with the rat anti-CD4 antibody compared to the infection controls with an exception for day 54. FACS analysis on the lymphocyte population revealed a strong decrease in the number of $CD4^+$ T cells in the depleted mice indicating that the injection of the rat-anti CD4 antibody resulted in a successful depletion (~95% reduction on days 42 and 47) of the $CD4^+$ T cell population (Fig. 3C) and, as expected, had no effect on the number of $CD8^+$ T cells (Fig. 3D). From this we concluded that $CD4^+$ T cells are involved in the suppression of *S. enterica* serovar Typhimurium during persistency.

Table 2. Growth rates of *S. enterica* serovar Typhimurium in the spleen, liver, and inguinal lymph nodes[a]

	liver (hr^{-1})	spleen (hr^{-1})	lymph nodes (hr^{-1})
primary	0.030 ± 0.003	0.038 ± 0.006	0.048 ± 0.004
irradiation	0.026 ± 0.005	0.030 ± 0.005	0.012 ± 0.004
T cell depletion	0.017 ± 0.018	0.013 ± 0.007	ND

[a] values are growth rates ± stdev
ND, not done
Growth rate = k
k=[ln N(t) – ln N(t=0)] / t

Anti-*Salmonella* IgG antibodies in the serum of *S. enterica* serovar Typhimurium infected mice. To determine whether the mice had antibodies to the pathogen, serum was collected from the mice that were sacrificed on each time point and anti-*Salmonella* IgG antibodies were detected using a whole cell ELISA. The infected mice showed no antibodies to *S. enterica* serovar Typhimurium on days 1 and 5, but started producing antibodies between day 5 and 12, when the infection reached its peak in the lymph nodes and spleens.

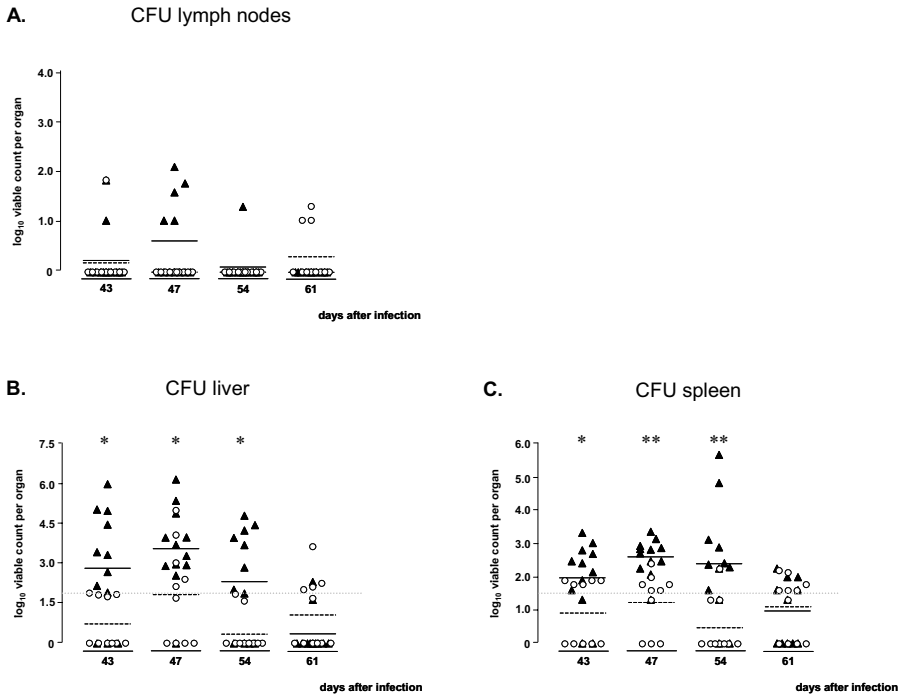

Figure 4. Bacterial loads within the lymph nodes (A), livers (B) , and spleens (C) of mice infected with *S. enterica* serovar Typhimurium 14028s and that were depleted for $CD4^+$ T cells (closed triangles) or untreated infection controls (open circles). On day 39 after infection, the mice were treated with 200 μg rat anti-CD4 GK1.5 and/or anti-CD8 2.43 antibodies injected intraperitoneally. Mice received a second and a third injection with 100 μg of these antibodies on day two and four after the first injection. The infection controls were injected intraperitoneally with an equal volume of PBS. At the indicated time points, livers, spleens, and lymph nodes were aseptically removed and weighed. The viable counts within the organs were determined by making lysates and plating serial dilutions of the lysates (n=6-8 per group) and are expressed as $\log_{10}$ viable counts (means ± standard errors of the means). Asterisks indicate statistically significant differences compared to the infection controls (Students *t* test).

The titers increased further until day 43 after infection and then remained around a $\log_3$ dilution factor of 8.5 (Fig. 5). The mice that were irradiated or depleted for $CD4^+$ T cells and that showed a reactivation of the *S. enterica* serovar Typhimurium infection had serum antibody levels that were similar to those of the infection controls. So despite the fact that these mice have serum antibodies to *S. enterica* serovar Typhimurium they still showed reactivation of the *S. enterica* serovar Typhimurium infection. To exclude the possibility that mice displaying the strongest reactivation of the *S. enterica* serovar Typhimurium infection showed lower serum levels of anti-*Salmonella* IgG, the serum antibody titers were plotted against the number of bacteria in the organs and showed no statistically significant correlation (data not shown). From this, we concluded that the observed reactivation of the *S. enterica* serovar Typhimurium infection was not due to a reduction in the serum antibody levels, but was due to the effects of the irradiation and depletion on the blood cells. The

observed reactivation of the *S. enterica* serovar Typhimurium infection in the irradiated mice might have been due to the reduction in the numbers of either granulocytes, $CD4^+$ T lymphocytes, $CD8^+$ T lymphocytes, or a combination of cells, while in the depleted mice, the reactivation was due to a reduction in the number of $CD4^+$ T cells in the blood.

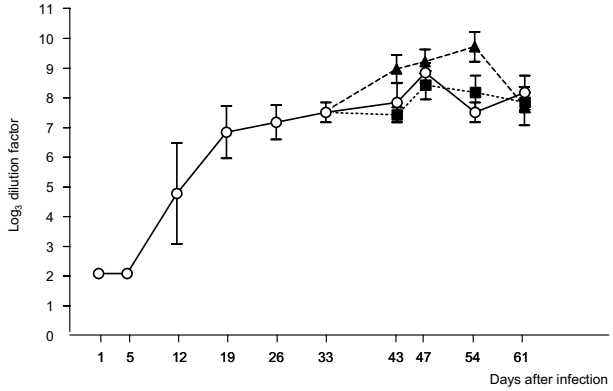

Figure 5. Anti-*Salmonella* IgG antibodies in the serum of *S. enterica* serovar Typhimurium infected mice that received no further treatment (open circles), received a total body irradiation (closed squares), or were depleted for $CD4^+$ T cells (closed triangles). Titers are defined as the OD_{450}(serum sample) > OD_{450}(naïve serum) + 2 × stdev.

Discussion

The main findings of the present study on reactivation of *Salmonella* after clearance of a primary systemic infection in C3H/HeN (Ity^r) mice are that after total body irradiation or selective $CD4^+$ T cell depletion, the number of bacteria in the livers and spleens increased at a rate identical to that in the primary infection. The main difference between the two outgrowth curves was that upon reactivation, the *S. enterica* serovar Typhimurium infection appeared to be controlled more rapidly than in the primary infection, i.e. by about two instead of three weeks. The presence of high levels of *Salmonella*-specific antibodies did not prevent the reactivation, but could play a role in the differences in outgrowth rate between the irradiated and $CD4^+$ T cell depleted mice, since antibodies might act as opsonins for granulocytes which were not affected by $CD4^+$ T cell depletion.

We have used subcutaneous *S. enterica* serovar Typhimurium infection of C3H/HeN (Ity^r) mice in the inguinal region to set up a model for reactivation of *S. enterica* serovar Typhimurium infection. By infecting subcutaneously, a reservoir is established near the draining lymph nodes from where *Salmonella* readily spreads via the lymph stream and becomes systemic, finally reaching the liver and spleen (2). This model gives rise to a more subtle infection than the intraperitoneal, intravenous, or oral models that result in peracute and overwhelming infections. Another advantage over oral infection is that the subcutaneously injected bacteria can be dosed precisely, while in an oral infection, the actual dose depends on the amount of bacteria that pass the stomach. Using this subcutaneous infection model, we have been able to set up a new in vivo model for reactivation of latent *S. enterica* serovar Typhimurium infection in which total body

irradiation or in vivo depletion of $CD4^+$ T cells in C3H/HeN (Ity^r) mice that fully recovered from a primary infection with *S. enterica* serovar Typhimurium resulted in the outgrowth of bacteria that resided within the body. In our reactivation model we have accepted that some of the control mice showed bacterial numbers in the organs that were slightly above the detection limits. Otherwise, we would have needed many more animals to find only a few in which *S. enterica* serovar Typhimurium persisted and reactivated upon irradiation or T cell depletion.

Upon total body irradiation, the mice showed a reduction in both granulocyte and $CD4^+$ and $CD8^+$ T cell numbers and, as a result, they showed a strong reactivation of the *S. enterica* serovar Typhimurium infection. Reactivation of a latent *S. enterica* serovar Typhimurium infection in humans has been mainly described for patients suffering from AIDS (1, 8, 9, 13). These patients all showed strongly reduced $CD4^+$ T cells counts and suffered from recurring infections with the same *S. enterica* serovar Typhimurium strain. This strongly suggests a role for the $CD4^+$ T cells in the suppression of *S. enterica* serovar Typhimurium during the persistence phase. The studies describing reactivation of *Salmonella* infections in HIV infected individuals are all studies that were done before the introduction of the highly active antiretroviral therapy (HAART) (1, 8, 13) or were done in developing countries where patients receive no such effective therapies (9). HAART therapy has been shown to be very effective in restoring the $CD4^+$ T cell population in patients with AIDS, thereby reconstituting immunity. Hung et al have shown that the risk of recurrent non-typhoidal *Salmonella* bacteremia has decreased significantly by the introduction of HAART therapy in Taiwan (12). In our in vivo reactivation model by irradiation, the mice showed reduced numbers of both granulocytes and $CD4^+$ and $CD8^+$ T lymphocytes. We investigated whether reducing the number of $CD4^+$ T cells, as occurs in HIV infected individuals, could also result in a reactivation of (a latent) *S. enterica* serovar Typhimurium infection C3H/HeN mice. Therefore, we performed another set of reactivation experiments in which we depleted the mice for $CD4^+$ T cells. Like for the irradiated mice, these $CD4^+$ T cell depleted mice showed a reactivation of the *S. enterica* serovar Typhimurium infection in both the livers and spleens. Strikingly, this reactivation took place despite the presence of high anti- *S. enterica* serovar Typhimurium antibody titers (Fig. 5). This is consistent with the observation that protection against *Salmonella* requires both immune serum and T cells (22). The reactivation peak followed a course similar to that of the primary infection, although slightly less severe. This could be due to the presence of *S. enterica* serovar Typhimurium -specific antibodies that might suppress extracellular bacterial growth, but cannot prevent the bacteria from reactivating. A more likely explanation could be that these antibodies act as opsonins for granulocytes thus promoting granulocyte-mediated uptake and killing of *S. enterica* serovar Typhimurium (29). This is also consistent with the observation that the growth rate upon irradiation was similar to that of the primary infection peak while after T cell depletion it was lower, especially in the spleen (Table 2). If the antibodies, which are highly abundant (Fig. 5), favor granulocyte-mediated uptake and killing of *S. enterica* serovar Typhimurium, this would indeed reduce the growth rate in $CD4^+$ T cell depleted mice as compared to the irradiated mice since the

granulocytes disappeared upon irradiation, but were still present in the CD4 depleted mice (Table 1). Furthermore, the pool of phagocytic cells in the spleens would be lower in the spleens of the irradiated mice as was evident from a reduction in spleenweight (~40% reduction compared to the infection controls), while in the $CD4^+$ T cell depleted mice no significant reduction in spleen weight was observed. Since $CD4^+$ T cell depletion still resulted in reactivation, apparently, granulocytes cannot completely compensate completely for the absence of $CD4^+$ T cells.

Very recently, Monack et al. have described a model for chronic carriage of *S. enterica* serovar Typhimurium in Ity^r mice (24). In contrast to our latent infection model in which bacteria could no longer be detected in the lymph nodes after 43 days, these mice show high numbers of bacteria in the mesenteric lymph nodes up until 268 days after oral infection and periodical fecal shedding as observed for chronic carriers of *S. enterica* serovar Typhi and *S. enterica* serovar Paratyphi in man and serves a good model for chronic carriage. They have shown that in their chronic carriers *S. enterica* serovar Typhimurium persisted within macrophages in the mesenteric lymph nodes. Apparently, these macrophages were not able to eliminate the pathogen. When IFNγ, the most potent activator of macrophages produced by T cells, was neutralized in this in vivo chronic carriage model, these mice showed reactivation of the *S. enterica* serovar Typhimurium infection (24). This indicates that IFNγ plays an essential role in the control of chronically persistent *S. enterica* serovar Typhimurium infection, but is not able to activate the macrophages in such a way that they can completely eliminate the pathogen as the bacteria persisted in the macrophages of the mesenteric lymph nodes from which they could spread to the environment. IFNγ is produced by NK cells and by activated $CD4^+$ and $CD8^+$ T cells in response to cytokines like IL-12 and IL-18 that are produced by infected macrophages (19, 20). IFNγ then acts together with bacterial components like LPS to activate the macrophages to display maximal capacity to kill the pathogen. In the reactivation model described by Monack et al. they have neutralized IFNγ and thereby have prevented the activation of the infected macrophages by all these types of IFNγ producing cells, resulting in the reactivation of the *S. enterica* serovar Typhimurium infection. In our reactivation model of latent infection, however, we have depleted the mice for the $CD4^+$ T cells and, as a result, have prevented the production of IFNγ by this type of cell. In our reactivation model upon $CD4^+$ T cell depletion, the IFNγ producing NK and $CD8^+$ T cells were still present (Fig. 3D). Apparently, the amounts of IFNγ produced by the $CD8^+$ T cells and by the NK cells are not sufficient to appropriately activate macrophages and prevent reactivation. Our data, together with those described by Monack et al., indicate that IFNγ produced by $CD4^+$ T cells is necessary to suppress bacterial growth during the persistence phase and suggests that IFNγ produced by NK cells and $CD8^+$ T cells do not play an important role in this process.

It has been known for several years that $CD4^+$ T cells play a very important role in the clearance of the bacteria during a primary infection with *S. enterica* serovar Typhimurium. Previous studies have shown that mice depleted for $CD4^+$ T cells on the day of infection are highly susceptible to *S. enterica* serovar Typhimurium and die due to the lack of $CD4^+$

T cell-mediated defense against *Salmonella* (25). This is the first study, however, that describes the role of $CD4^+$ T cells in preventing reactivation of the *S. enterica* serovar Typhimurium infection in Ity^r mice during the persistence phase. We have set up an in vivo reactivation mouse model that is very suitable for further studies on reactivating *S. enterica* serovar Typhimurium infections and might give further insights into the strategies that are used by *S. enterica* serovar Typhimurium to persist without being noticed by the host's immune system. Such knowledge might eventually lead to the development of new treatments to prevent recurrent infections with *S. enterica* serovar Typhimurium.

References

1. **Burckhardt, B., P. Sendi, D. Pfluger, W. Zimmerli, R. Nuesch, H. C. Bucher, J. Drewe, N. Gyr, and M. Battegay.** 1999. Rare AIDS-defining diseases in the Swiss HIV Cohort Study. Eur. J. Clin. Microbiol. Infect. Dis. **18:**399-402.
2. **Chen, Z. M. and M. K. Jenkins.** 1999. Clonal expansion of antigen-specific CD4 T cells following infection with *Salmonella typhimurium* is similar in susceptible (Ity^s) and resistant (Ity^r) BALB/c mice. Infect. Immun. **67:**2025-2029.
3. **Demple, B.** 1991. Regulation of bacterial oxidative stress genes. Annu.Rev. Genet. **25:**315-337.
4. **Everest, P., M. Roberts, and G. Dougan.** 1998. Susceptibility to *Salmonella* typhimurium infection and effectiveness of vaccination in mice deficient in the tumor necrosis factor alpha p55 receptor. Infect. Immun. **66:**3355-3364.
5. **Fields, P. I., R. V. Swanson, C. G. Haidaris, and F. Heffron.** 1986. Mutants of *Salmonella typhimurium* that cannot survive within the macrophage are avirulent. Proc. Natl. Acad. Sci. U.S.A. **83:**5189-5193.
6. **Finlay, B. B. and S. Falkow.** 1989. Common themes in microbial pathogenicity. Microbiol. Rev. **53:**210-230.
7. **Foster, J. W. and M. P. Spector.** 1995. How *Salmonella* survive against the odds. Annu. Rev. Microbiol. **49:**145-174.
8. **Glaser, J. B., L. Morton-Kute, S. R. Berger, J. Weber, F. P. Siegal, C. Lopez, W. Robbins, and S. H. Landesman.** 1985. Recurrent *Salmonella typhimurium* bacteremia associated with the acquired immunodeficiency syndrome. Ann. Intern. Med. **102:**189-193.
9. **Gordon, M. A., H. T. Banda, M. Gondwe, S. B. Gordon, M. J. Boeree, A. L. Walsh, J. E. Corkill, C. A. Hart, C. F. Gilks, and M. E. Molyneux.** 2002. Non-typhoidal *Salmonella* bacteraemia among HIV-infected Malawian adults: high mortality and frequent recrudescence. AIDS **16:**1633-1641.
10. **Holden, D. W.** 2002. Trafficking of the *Salmonella* vacuole in macrophages. Traffic. **3:**161-169.
11. **Hormaeche, C. E.** 1979. The natural resistance of radiation chimeras to *S. typhimurium* C5. Immunology **37:**329-332.
12. **Hung, C. C., S. M. Hsieh, C. F. Hsiao, M. Y. Chen, and W. H. Sheng.** 2001. Risk of recurrent non-typhoid *Salmonella* bacteraemia after early discontinuation of ciprofloxacin as secondary prophylaxis in AIDS patients in the era of highly active antiretroviral therapy. AIDS **15:**645-647.
13. **Jacobson, M. A., S. M. Hahn, J. L. Gerberding, B. Lee, and M. A. Sande.** 1989. Ciprofloxacin for *Salmonella* bacteremia in the acquired immunodeficiency syndrome (AIDS). Ann. Intern. Med. **110:**1027-1029.
14. **Jepson, M. A. and M. A. Clark.** 2001. The role of M cells in *Salmonella*. Microbes. Infect. **3:**1183-1190.
15. **Jones, B. D. and S. Falkow.** 1996. Salmonellosis: host immune responses and bacterial virulence determinants. Annu. Rev. Immunol. **14:**533-561.
16. **Jouanguy, E., R. Doffinger, S. Dupuis, A. Pallier, F. Altare, and J. L. Casanova.** 1999. IL-12 and IFN-gamma in host defense against mycobacteria and and *Salmonella* in mice and man. Curr. Opin. Immunol. **11:**346-351.
17. **Lo, W. F., H. Ong, E. S. Metcalf, and M. J. Soloski.** 1999. T cell responses to Gram-negative intracellular bacterial pathogens: a role for CD8+ T cells in immunity to *Salmonella* infection and the involvement of MHC class Ib molecules. J. Immunol. **162:**5398-5406.
18. **Maskell, D. J., C. E. Hormaeche, K. A. Harrington, H. S. Joysey, and F. Y. Liew.** 1987. The initial suppression of bacterial growth in a *Salmonella* infection is mediated by a localized rather than a systemic response. Microb. Pathog. **2:**295-305.
19. **Mastroeni, P., S. Clare, S. Khan, J. A. Harrison, C. E. Hormaeche, H. Okamura, M. Kurimoto, and G. Dougan.** 1999. Interleukin 18 contributes to host resistance and gamma interferon production in mice infected with virulent *Salmonella* typhimurium. Infect. Immun. **67:**478-483.
20. **Mastroeni, P., J. A. Harrison, J. A. Chabalgoity, and C. E. Hormaeche.** 1996. Effect of interleukin 12 neutralization on host resistance and gamma interferon production in mouse typhoid. Infect. Immun. **64:**189-196.
21. **Mastroeni, P., J. N. Skepper, and C. E. Hormaeche.** 1995. Effect of anti-tumor necrosis factor alpha antibodies on histopathology of primary *Salmonella* infections. Infect. Immun. **63:**3674-3682.

22. **Mastroeni, P., B. Villarreal-Ramos, and C. E. Hormaeche.** 1993. Adoptive transfer of immunity to oral challenge with virulent salmonellae in innately susceptible BALB/c mice requires both immune serum and T cells. Infect. Immun. **61:**3981-3984.
23. **Miller, R. A. and B. E. Britigan.** 1997. Role of oxidants in microbial pathophysiology. Clin. Microbiol. Rev. **10:**1-18.
24. **Monack, D. M., D. M. Bouley, and S. Falkow.** 2004. *Salmonella* typhimurium persists within macrophages in the mesenteric lymph nodes of chronically infected Nramp1$^{+/+}$ mice and can be reactivated by IFNγ neutralisation. J. Exp. Med. **199:**231-241.
25. **Nauciel, C.** 1990. Role of CD4+ T cells and T-independent mechanisms in acquired resistance to *Salmonella typhimurium* infection. J. Immunol. **145:**1265-1269.
26. **Nauciel, C. and F. Espinasse-Maes.** 1992. Role of gamma interferon and tumor necrosis factor alpha in resistance to *Salmonella typhimurium* infection. Infect. Immun. **60:**450-454.
27. **Staretz-Haham, O., R. Melamed, M. Lifshitz, N. Porat, C. Fieschi, J. L. Casanova, and J. Levy.** 2003. Interleukin-12 receptor beta1 deficiency presenting as recurrent *Salmonella* infection. Clin. Infect. Dis. **37:**137-140.
28. **Van Diepen, A., T. van der Straaten, S. M. Holland, R. Janssen, and J. T. van Dissel.** 2002. A superoxide-hypersusceptible *Salmonella enterica* serovar Typhimurium mutant is attenuated but regains virulence in p47$^{phox-/-}$ mice. Infect. Immun. **70:**2614-2621.
29. **Van Dissel, J. T., J. J. Stikkelbroeck, W. Sluiter, P. C. Leijh, and R. van Furth.** 1986. Differences in initial rate of intracellular killing of *Salmonella* typhimurium by granulocytes of *Salmonella*-susceptible C57BL/10 mice and *Salmonella*-resistant CBA mice. J. Immunol. **136:**1074-1080.
30. **Vazquez-Torres, A. and F. C. Fang.** 2000. Cellular routes of invasion by enteropathogens. Curr. Opin. Microbiol. **3:**54-59.
31. **Wijburg, O. L., C. P. Simmons, N. van Rooijen, and R. A. Strugnell.** 2000. Dual role for macrophages in vivo in pathogenesis and control of murine *Salmonella enterica* var. Typhimurium infections. Eur. J. Immunol. **30:**944-953