Gamma Irradiation or CD4+-T-Cell Depletion Causes Reactivation of Latent *Salmonella enterica* Serovar Typhimurium Infection in C3H/HeN Mice

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Upon infection with *Salmonella*, a host develops an immune response to limit bacterial growth and kill and eliminate the pathogen. *Salmonella* has evolved mechanisms to remain dormant within the body, only to reappear (reactivate) at a later time when the immune system is abated. 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, which reached a peak on day 19. After full recovery from the infection, these mice were irradiated or depleted of CD4+ T cells. The mice displayed a secondary infection peak in livers and spleens with a course similar to that of the primary infection. We concluded that CD4+ T cells are involved in active suppression of *S. enterica* serovar Typhimurium during latency. 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 latent phase of *S. enterica* serovar Typhimurium infection.

Salmonellae are gram-negative, facultative intracellular pathogens that can cause a range of diseases in both animals and humans that vary from mild diarrhea to severe infections such as typhoid fever. It predominantly invades mononuclear phagocytes, and despite antimicrobial mechanisms present in phagocytic cells, *Salmonella* is able not only to enter but to survive and even replicate within these cells. The bacterium can cause chronic or persistent infection by evasion of the host defense (13). This ability of *Salmonella* to 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 uncertain, it is clear that *Salmonella* responds to the specific host environment by expressing factors crucial for intracellular survival (3, 6, 7, 13, 20).

Upon infection, the host mounts 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 cytokines such as gamma interferon (IFN-γ), interleukin-12, and tumor necrosis factor alpha (4, 14, 16–18, 23). This integrated response results in activation of macrophages, which in turn kill the *Salmonella*. Although the macrophages are the main host cells, are necessary for survival and replication of *Salmonella* within the host, and mediate the *Salmonella*-induced pathology, macrophages also play a crucial role in host defense against *Salmonella* (27). They are necessary for the early local control of infection and, subsequently, for the induction of acquired immunity (10, 15), as well as for restriction of bacterial growth in immune mice (27).

Even in the presence of an acquired immune response, *Salmonella* has evolved mechanisms to persist within the body and reappear (reactivate) at a later time. Several studies and case reports have shown that patients who underwent total-body irradiation or received an organ transplant and were treated with glucocorticosteroids or other immunosuppressive drugs, as well as patients suffering from human immunodeficiency virus infection (11) or interleukin-12 receptor β1 deficiency (24), can suffer from recurrent infections with a *Salmonella* strain that persists within the host.

By investigating the possibility that *S. enterica* serovar Typhimurium persists and reactivates after immune intervention in a mouse model of latent *S. enterica* serovar Typhimurium infection, we aimed to gain insight into the mechanisms by which the host continually suppresses *Salmonella* from reactivating at a later time.

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 institutional guidelines with water and food ad libitum in filter top cages which were opened only inside a laminar flow cabinet. Studies 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 *S. enterica* serovar Typhimurium strain 14028s (50% lethal doses after intraperitoneal injection, 5×10^3 bacteria for Ity^r mice and $<10^2$ for Ity^s mice) was grown to the end of the log phase and then washed and diluted in sterile phosphate-buffered saline (PBS). The number of CFU in the inoculum was determined microbiologically.

Antibodies. Monoclonal antibodies (mAbs) directed to mouse T-cell surface antigen CD4 were obtained from supernatant of cultured hybridoma GK1.5 (rat anti-mouse CD4; American Type Culture Collection). The hybridoma was cul-

Irradiated

CD4+ T cell depleted

A. CFU lymph nodes

D. CFU lymph nodes

B. CFU liver

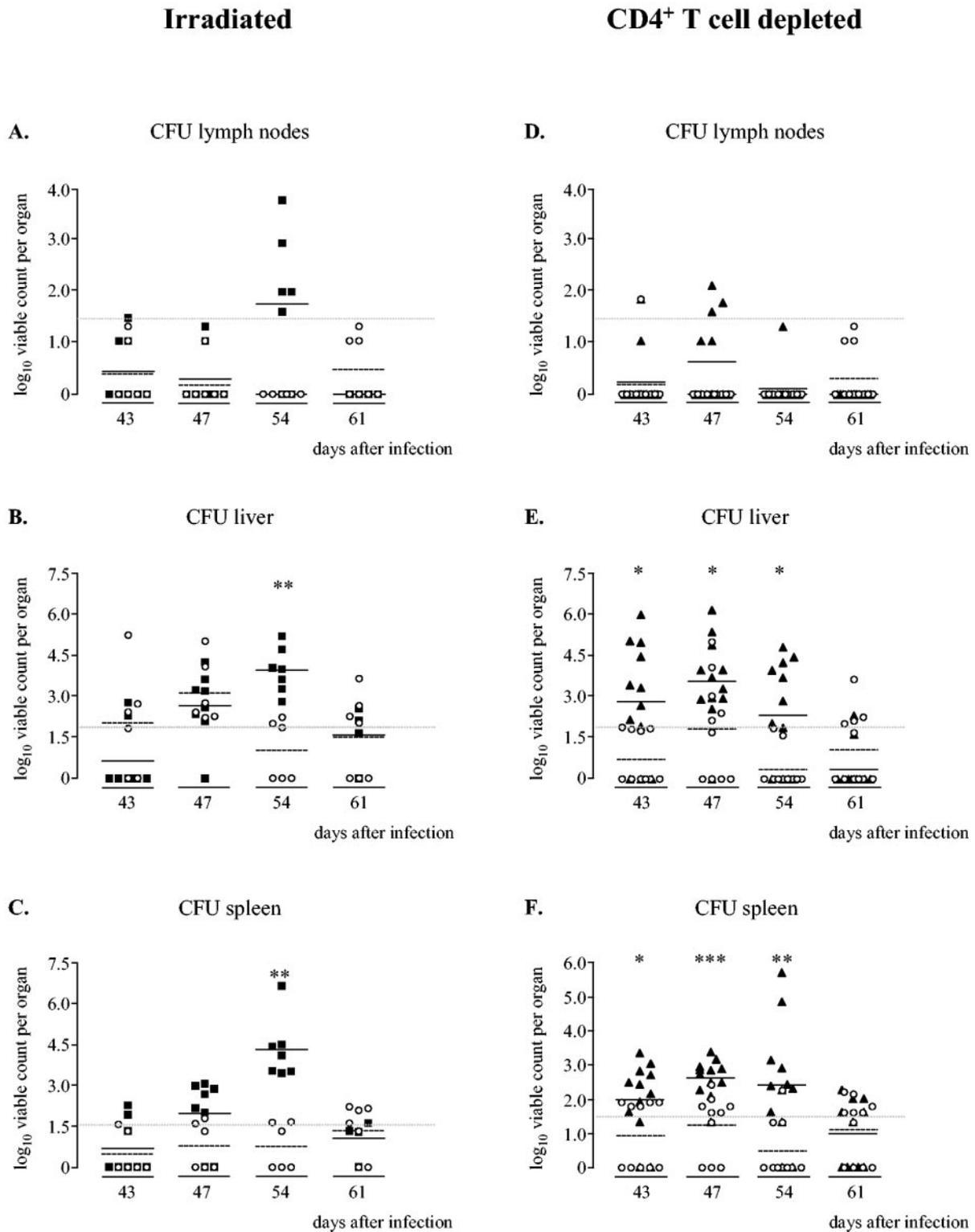**E.** CFU liver

C. CFU spleen

F. CFU spleen

FIG. 1. Bacterial loads within the lymph nodes (A), livers (B), and spleens (C) of mice infected with *S. enterica* serovar Typhimurium 14028s that were irradiated on day 41 (■) and untreated infection controls (○) and bacterial loads within the lymph nodes (D), livers (E), and spleens (F) of mice depleted of CD4+ T cells (▲) and infection controls (○). On days 39, 41, and 43 after infection, the mice were injected i.p. with 200 μg, 100 μg, and 100 μg of rat anti-CD4 GK1.5 antibody, respectively. The infection controls were injected i.p. with an equal volume of PBS. At different times, livers, spleens, and lymph nodes were aseptically removed, and cell lysates were made. The viable counts in the organs were determined by plating serial dilutions of the cell lysates and are expressed as $\log_{10}$ viable counts (means ± standard errors of the means). Data from two independently performed experiments are shown. Asterisks indicate statistically significant differences compared to the infection controls (one asterisk, $P < 0.05$; two asterisks, $P < 0.005$; Mann-Whitney rank order test), and the gray dashed lines indicate the detection limits of the microbiological method (50 CFU for the livers and 30 CFU for the spleens and lymph nodes).

TABLE 1. Numbers of leukocytes, lymphocytes, monocytes, and granulocytes in the blood[a]

Day	Treatment	No. (10^5) of cells/ml			
		Leukocytes	Lymphocytes	Monocytes	Granulocytes
1	None	54.7 ± 8.3	38.7 ± 12.9	1.1 ± 0.5	14.3 ± 8.9
5	None	85.1 ± 13.5	48.3 ± 9.4	1.8 ± 1.4	29.9 ± 7.3
12	None	77.3 ± 24.4	30.4 ± 12.7	0.8 ± 0.7	42.3 ± 13.6
19	None	100.7 ± 28.5	33.8 ± 13.8	2.8 ± 2.2	52.2 ± 13.1
26	None	78.0 ± 13.4	39.3 ± 5.6	1.2 ± 1.0	33.8 ± 11.7
33	None	110.8 ± 37.6	52.2 ± 10.3	1.8 ± 1.9	53.3 ± 29.2
42	None	86.2 ± 29.7	53.7 ± 18.0	0.7 ± 0.7	27.9 ± 14.2
	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	None	87.8 ± 23.9	58.5 ± 20.6	1.0 ± 0.5	24.7 ± 7.6
	irr	9.1 ± 4.4	5.1 ± 2.6	0.2 ± 0.2	3.1 ± 2.3
	depl	61.1 ± 21.2	31.4 ± 11.8	1.5 ± 1.6	26.2 ± 17.1
54	None	50.8 ± 29.0	29.0 ± 22.4	0.6 ± 0.7	19.3 ± 10.2
	irr	10.6 ± 5.1	5.8 ± 3.0	0.1 ± 0.1	2.8 ± 3.4
	depl	89.6 ± 21.1	39.0 ± 16.2	2.1 ± 1.4	39.0 ± 16.2
61	None	94.6 ± 24.1	45.6 ± 14.3	1.0 ± 1.1	41.9 ± 27.4
	irr	29.5 ± 7.6	8.7 ± 2.2	0.2 ± 0.2	15.8 ± 4.5
	depl	55.0 ± 14.2	29.2 ± 8.5	0.7 ± 0.4	23.2 ± 11.9

[a] Values are means ± standard deviations.
[b] irr, mice received total-body irradiation (6 Gy).
[c] depl, mice were depleted of CD4^{+} T cells by injection of anti-CD4 antibodies.

tured in protein-free medium (Gibco), and supernatant was concentrated with a capillary dialyzer, filter sterilized, and stored at −20°C. Fluorescein isothiocyanate-conjugated rat anti-mouse CD4 (L3T4) and phycoerythrin-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 of a bacterial suspension containing 3×10^4 CFU of *S. enterica* serovar Typhimurium 14028s. For each group at each time three or four mice were used. Mice were sacrificed by carbon dioxide inhalation, and blood was taken by cardiac puncture. Spleens, livers, and inguinal lymph nodes were removed, single-cell suspensions were prepared by using sterile 70-μm-mesh-size cell strainers (Falcon), and lysates were made. The number of bacteria per organ was determined microbiologically by plating serial dilutions of the lysates. The lowest numbers of bacteria that could be detected in this way were 30 CFU for the spleens and lymph nodes and 50 CFU for the livers.

Leukocyte count and blood cell differentiation. Leukocyte numbers were determined by counting the number of nucleated cells in heparinized blood and by making blood smears for the differentiation of the blood cells. Blood smears were fixed in methanol and stained with Giemsa stain, and relative percentages of the different types of blood cells were determined. By combining the data from leukocyte counts and blood smears, we calculated the numbers of lymphocytes, monocytes, and polymorphonuclear leukocytes present in the blood at the different times. The number of lymphocytes was used together with fluorescence-activated cell sorter (FACS) analysis data to calculate the number of CD4^{+} and CD8^{+} T cells.

Gamma irradiation and in vivo T-cell depletion. For total-body gamma irradiation, mice were put in a small perspex box and were irradiated until a dose of 6 Gy was reached. For depletion of CD4^{+} T cells, mice were injected intraperitoneally (i.p.) with 200 μg of rat anti-CD4 GK1.5 antibody. Mice received second and third injections of 100 μg of this antibody on days 2 and 4 after the first injection. Infection controls were injected i.p. with an equal volume of PBS.

Flow cytometry. To determine CD4^{+}- and CD8^{+}-T-cell counts in blood, 100 μl of heparinized blood was used. After the erythrocytes were lysed and washed with PBS, the cells were labeled for 30 min with fluorescein isothiocyanate-conjugated rat anti-mouse CD4 mAb and phycoerythrin-conjugated rat anti-mouse CD8 mAb. Flow cytometry was performed with a FACSCaliber system (Becton Dickinson).

Detection of *S. enterica* serovar Typhimurium-specific antibodies. Induction of *S. enterica* serovar Typhimurium-specific antibodies was determined by a whole-cell enzyme-linked immunosorbent assay (ELISA) (25). Maxisorp plates (Nunc) were coated with *S. enterica* serovar Typhimurium 14028s, and serial dilutions of the sera were added after nonspecific binding was blocked. Sera from naïve mice were included as a control. Wavelength absorbance was measured at 490 nm using an ELISA plate reader (VICTOR[2] 1420 multilabel counter; Perkin-Elmer Life and Analytical Sciences). The titer was defined as the dilution for which the optical density at 450 (OD_{450}) of the sample was more than the OD_{450} of the naïve serum plus 2 standard deviations.

Statistical analysis. For comparison between treatments we used Student's *t* tests for CD4^{+}- and CD8^{+}-T-cell counts and Mann-Whitney rank order tests for CFU counts. For all analyses, a *P* value of <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 of *S. enterica* serovar Typhimurium 14028s and showed an infection comparable to that described previously (26). *S. enterica* serovar Typhimurium 14028s was detectable in the lymph nodes on day 1 after infection; from there the infection spread to the spleens and livers, and within these organs bacterial loads of up to 3×10^7 CFU per organ were reached. The bacterial loads were highest on day 19. The bacterial loads eventually declined, which coincided with reductions in the spleen and liver weights (data not shown).

Reactivation of the *S. enterica* serovar Typhimurium infection by gamma irradiation. On day 41, when the bacterial loads in the organs were below the detection limit, the mice received sublethal total-body irradiation (6 Gy). Infection controls were not treated. The irradiated mice showed signs of illness, like ruffled fur and malaise, between days 54 and 61. The effects of the irradiation on the bacterial numbers in the organs are shown in Fig. 1. In the infection control group, 60% of the mice showed detectable amounts of bacteria in the livers (Fig. 1B), 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. 1A and C). In the irradiated mouse population, on the other hand, we observed increases in the numbers of *S. enterica* serovar Typhimurium cells in the livers and spleens (Fig. 1B and C) 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 shown by the lower maximal bacterial numbers in the organs reached, but otherwise it followed a course that was similar to that of the primary infection peak (Fig. 1). The irradiated mice showed reduced leukocyte counts in the blood as soon as day 1 after the irradiation (day 42) compared to the untreated infection controls, and the numbers remained lower up to day 61 after infection (Table 1). Table 1 and Fig. 2A and B also show that the numbers of granulocytes and CD4^{+} and CD8^{+} T cells declined in the irradiated mice.

These results 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.

Reactivation following T-cell depletion. Reactivation of a latent *S. enterica* serovar Typhimurium infection in people has

A.   CD4+ T cells

B.   CD8+ T cells

C.   CD4+ T cells
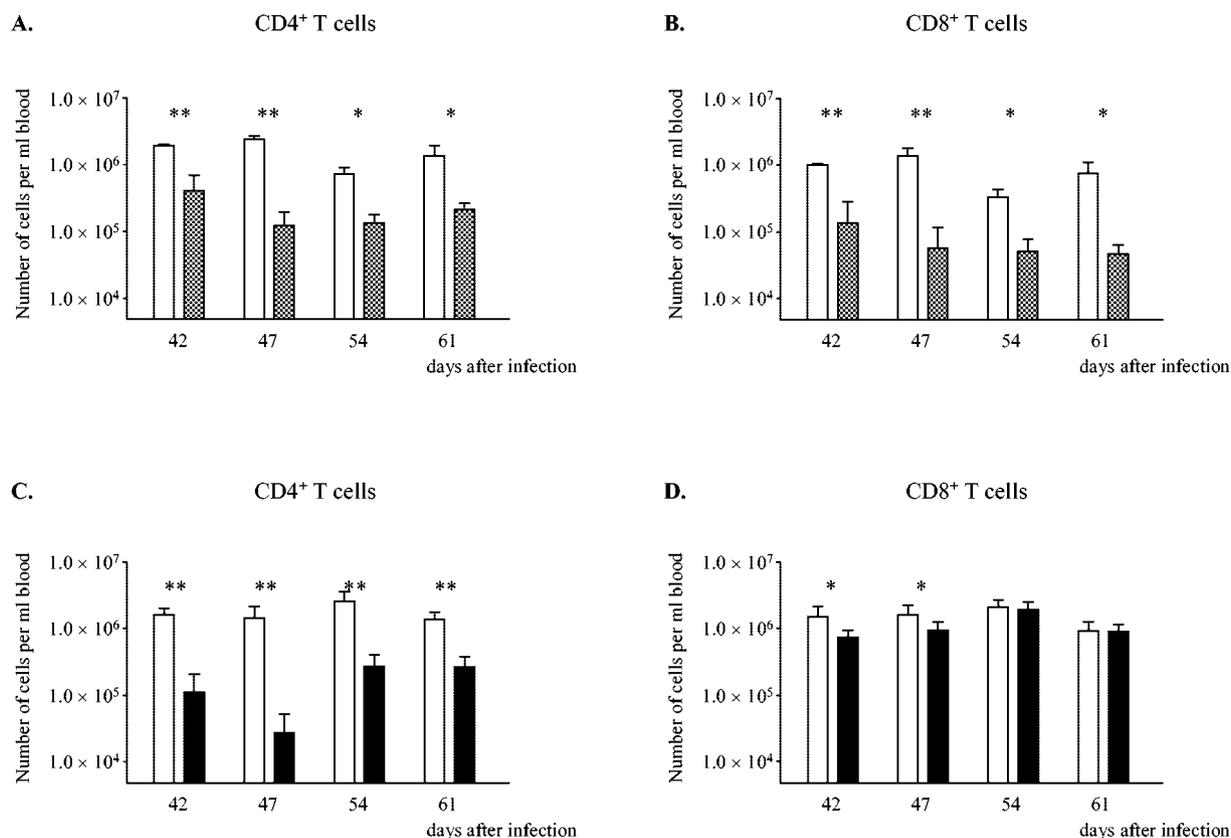

D.   CD8+ T cells

FIG. 2. Numbers of CD4+ T cells (A and C) and CD8+ T cells (B and D) per ml of blood of C3H/HeN control mice infected with *S. enterica* serovar Typhimurium (open bars), mice that received total-body irradiation (cross-hatched bars), and mice that were depleted of CD4+ T cells (solid bars). The numbers of CD4+ and CD8+ T cells were calculated by combining data from leukocyte counts, cell differentiation analysis, and FACS analysis. Two asterisks indicate that the P value is <0.005, and one asterisk indicates that the P value is <0.05.

been described for patients suffering from AIDS. 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 granulocytes and CD8+ T cells, we wondered whether reducing the number of CD4+ T cells alone by in vivo depletion could also result in the reactivation of a latent *S. enterica* serovar Typhimurium infection in C3H/HeN mice. 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 of CD4+ T cells, we observed no detectable outgrowth of *S. enterica* serovar Typhimurium (Fig. 1D). In the livers and spleens, on the other hand, we observed increases in bacterial numbers that were significantly different from those in the infection controls, and the reactivation reached a peak on day 47 (Fig. 1E and F). As observed for the irradiated mice, this reactivation peak was lower than the peak observed for the primary infection, but it followed a course that was similar to that of the primary infection. FACS analysis of 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 successful depletion of the CD4+-T-cell population (Fig. 2C) and, as expected, had little effect on the number of CD8+ T cells (Fig. 2D).

Anti-*Salmonella* immunoglobulin G (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 at each time, and anti-*Salmonella* IgG antibodies were detected using a whole-cell ELISA. The infected mice started to produce antibodies to *S. enterica* serovar Typhimurium 14028s between days 5 and 12, when the infection reached its peak in the lymph nodes and spleens. The titers increased further until day 43 after infection and then remained around a $\log_3$ dilution factor of 8.5 (Fig. 3). The mice that were irradiated or depleted of CD4+ T cells and that showed reactivation of the *S. enterica* serovar Typhimurium infection had serum antibody levels that were similar to those of the infection controls. Thus, despite the fact that these mice had serum antibodies to *S. enterica* serovar Typhimurium, they still showed reactivation of the *S. enterica* serovar Typhimurium infection.

DISCUSSION

The main finding of the present study of reactivation of *Salmonella* after clearance of a primary systemic infection in C3H/HeN (Ity^r) mice is that after total-body irradiation or selective CD4+-T-cell depletion, the numbers of bacteria in livers and spleens increased at a rate identical to that in the
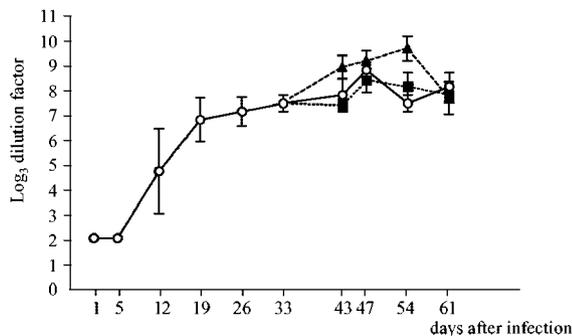
FIG. 3. Anti-*Salmonella* IgG antibodies in the serum of *S. enterica* serovar Typhimurium-infected mice that received no further treatment (○), received total-body irradiation (■), or were depleted of CD4$^+$ T cells (▲). The titer was defined as the dilution for which the optical density at 450 (OD$_{450}$) of the sample was more than the OD$_{450}$ of the naïve serum plus 2 standard deviations.

primary infection. The only difference between the outgrowth curves was that upon reactivation, *S. enterica* serovar Typhimurium infection was controlled more rapidly than it was in the primary infection (i.e., by about 2 instead of 3 weeks). The presence of *Salmonella*-specific antibodies did not prevent reactivation but may help explain the difference in outgrowth rates between irradiated and CD4$^+$-T-cell-depleted mice, since antibodies act as opsonins for uptake by granulocytes which are not affected by CD4$^+$-T-cell depletion.

We 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 draining lymph nodes, from which *Salmonella* spreads via the lymph and becomes systemic, reaching the liver and spleen (2). This model gives rise to a more subtle infection than the intraperitoneal or intravenous models that result in peracute and overwhelming infections. An advantage over oral infection is that subcutaneously injected bacteria can be dosed precisely, while in an oral infection the actual dose depends on the number of bacteria that pass through the stomach and cross the intestinal mucosa. Using subcutaneous infection, we 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 had fully recovered from a primary infection with *S. enterica* serovar Typhimurium resulted in outgrowth of bacteria that persisted within the body. In our reactivation model we accepted that some of the control mice still showed some low number of bacteria in the organs, just above the limit of detection. 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.

Reactivation of a latent *S. enterica* serovar Typhimurium infection in humans has been described in human immunodeficiency virus/AIDS patients (9), which (1, 8, 12) suggests a role for CD4$^+$ T cells in the suppression of *S. enterica* serovar Typhimurium during persistence. This is supported by a study of Hung et al. in Taiwan showing that the risk of recurrent nontyphoidal *Salmonella* bacteremia decreased dramatically after the introduction of highly active antiretroviral therapy

and coincides with recovery of CD4$^+$-T-cell counts and reconstitution of immunity (11).

We investigated whether reducing the number of CD4$^+$ T cells could result in reactivation of a (latent) *S. enterica* serovar Typhimurium infection. Like irradiated mice, CD4$^+$-T-cell-depleted mice showed reactivation of *S. enterica* serovar Typhimurium infection in both livers and spleens. This reactivation occurred despite the presence of high titers of anti-*S. enterica* serovar Typhimurium antibodies (Fig. 3). This is consistent with the observation that protection against *Salmonella* requires both immune serum and T cells (19).

Recently, Monack et al. described a model for chronic carriage of *S. enterica* serovar Typhimurium in Ity^r mice (21). In contrast to our latent infection model in which bacteria could no longer be detected in the lymph nodes after 43 days, these mice showed high numbers of bacteria in the mesenteric lymph nodes up until 268 days after oral infection and periodic fecal shedding, as observed for chronic carriers of *S. enterica* serovar Typhi and *S. enterica* serovar Paratyphi in humans. Monack et al. showed that IFN-γ plays an essential role in the control of chronically persistent *S. enterica* serovar Typhimurium infection, since neutralization resulted in reactivation (21). Neutralization of IFN-γ precludes activation of infected macrophages by all types of IFN-γ-producing cells and results in reactivation of *S. enterica* serovar Typhimurium infection. In our reactivation model of latent infection, however, we depleted mice of CD4$^+$ T cells, precluding the production of IFN-γ by this type of cell, but the IFN-γ-producing NK and CD8$^+$ T cells were still present (Fig. 2D). Apparently, the amounts of IFN-γ produced by these cells are not sufficient to appropriately activate macrophages and prevent reactivation. Our data, together with those described by Monack et al., indicate that IFN-γ produced by CD4$^+$ T cells is necessary to suppress bacterial growth during the persistence phase and suggest that IFN-γ produced by NK cells and CD8$^+$ T cells does not play a pivotal role in this respect.

It is generally accepted that CD4$^+$ T cells play an important role in the clearance of bacteria during a primary infection with *S. enterica* serovar Typhimurium. Mice depleted of CD4$^+$ T cells on the day of infection are highly susceptible to *S. enterica* serovar Typhimurium and rapidly die due to the lack of CD4$^+$-T-cell-mediated defense against *Salmonella* (22). Our study is the first study that describes a role of CD4$^+$ T cells in preventing reactivation of *S. enterica* serovar Typhimurium infection in Ity^r mice during the persistence phase. The in vivo reactivation mouse model is suitable for further studies on reactivating *S. enterica* serovar Typhimurium infections and might provide insight into bacterial strategies that *S. enterica* serovar Typhimurium uses to persist in its host. Detailed knowledge of these mechanisms is necessary to develop new approaches for preventing relapsing infections with salmonellae in immunocompromised hosts.

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DIEPEN ET AL.

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