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Role of Neutrophils in Murine Salmonellosis

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Gastrointestinal infections with *Salmonella enterica* serovars have different clinical outcomes that range from localized inflammation to a life-threatening systemic disease in the case of typhoid fever. Using a mouse model of systemic salmonellosis, we investigated the contribution of neutrophils to the innate immune defense against *Salmonella* after oral infection. Neutrophil infiltration was dependent on the bacterial burden in various infected organs (Peyer's patches, mesenteric lymph nodes, spleen, and liver). However, the massive infiltration of neutrophils did not allow clearance of an infection with wild-type *Salmonella*, presumably due to protection of intracellular *Salmonella* against neutrophil activities. A *Salmonella* mutant strain deficient in *Salmonella* pathogenicity island 2 (SPI2) was able to infect systemic sites, but its replication was highly restricted and it did not cause detectable attraction of neutrophils. Neutrophil depletion by antibody treatment of mice did not restore the virulence of SPI2 or auxotrophic mutant strains, supporting the hypothesis that attenuation of the strains is not due to greater susceptibility to neutrophil killing. Our observations reveal that neutrophils have completely different roles during systemic salmonellosis and localized gastrointestinal infections. In the latter conditions, rapid neutrophil attraction efficiently prevents the spread of the pathogen, whereas the neutrophil influx is delayed during systemic infections and cannot protect against lethal bacteremia.

The innate immune system has a crucial function in protection of naive organisms against colonization by pathogenic microbes. This function has special relevance for defense at the intestinal mucosa, where only a single layer of cells separates sterile host tissue from the rich microbial population of the gut and from food-borne pathogens. Although several lines of innate immune defense are present at this border, various bacterial pathogens have evolved strategies to inactivate or escape these defenses (reviewed in reference 18).

Salmonella enterica is an important gastrointestinal pathogen that causes diseases ranging from gastroenteritis accompanied by local inflammation and diarrhea to typhoid fever, a live-threatening systemic bacteremia caused by *S. enterica* serovar Typhi. The latter condition can be studied by using the infection model consisting of *S. enterica* serovar Typhimurium and susceptible (Nramp$^{-/-}$) mice, while infection of humans with this pathogen results in a self-limiting gastroenteritis.

Successful colonization of murine hosts by *S. enterica* serovar Typhimurium depends on the functions of a large number of virulence factors. In addition to metabolic flexibility that allows adaptation to nutritionally limited sites, serum resistance, and the ability to repair damage inflicted by host defense mechanisms, *S. enterica* serovar Typhimurium has evolved specific and complex virulence traits (reviewed in reference 12). Genes required for invasion of nonphagocytic cells by *S. enterica* serovar Typhimurium are clustered in a chromosomal region termed *Salmonella* pathogenicity island 1 (SPI1). A second locus, SPI2, harbors genes that are required for intracellular replication of *Salmonella* and the ability to cause a systemic infection. Mutant strains deficient in SPI1 function are moderately attenuated in systemic virulence after oral infection of mice but not after intraperitoneal or intravenous infection of mice. In contrast, SPI2-deficient mutant strains are highly attenuated after oral infection, as well as after intraperitoneal infection. Both SPI1 and SPI2 encode type III secretion systems that translocate sets of bacterial virulence proteins into eukaryotic host cells. SPI2 functions are required to influence the intracellular fate of *S. enterica* serovar Typhimurium, and a role for protection of intracellular *Salmonella* against antimicrobial activities of the host cell involving reactive oxygen intermediates (29) and reactive nitrogen intermediates (4) has been observed. Thus, SPI2 functions appear to interfere with the innate immune defense against *Salmonella*. The function of *spv* genes located on the *Salmonella* virulence plasmid (SPV) is less well understood. Mutant strains deficient in *spv* genes exhibit reduced replication in the organs of infected mice (13).

Localized intestinal infections by *S. enterica* serovars lead to a strong inflammatory response that is accompanied by a massive influx of neutrophils to the site of infection (see references 10 and 30 for reviews). Cell culture models (21) and the bovine model of *Salmonella*-induced gastroenteritis (19) indicate that epithelial cells can respond to *Salmonella* infection with the release of proinflammatory cytokines. Neutrophils attracted by these signals can migrate through the epithelium and inactivate the pathogen within the intestinal lumen. In the murine model of systemic salmonellosis, intestinal inflammation is not observed, and bacteria rapidly infect Peyer's patches (PP) via M cells. The subsequent pathogenesis is characterized by uptake of *Salmonella* by phagocytic cells, an intracellular lifestyle, and ultimately systemic spread.

Although the roles of several virulence factors in the interaction with epithelial cells and macrophages have been analyzed in detail, there is only limited information concerning the contribution of neutrophils to immunity against *Salmonella*

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TABLE 1. *S. enterica* serovar Typhimurium strains used in this study

Strain	Relevant characteristics	Source or reference
NCTC 12023	Wild type	NCTC[a]
HH104	*sseC*::*aphT*, Kan^r	16
P5D10	*spvA*::mTn*5*, Kan^r	15
P7B12	*sipC*::mTn*5*, Kan^r	9
P10H1	*purD*::mTn*5*, Kan^r	15

[a] NCTC, National Collection of Type Cultures, Colindale, United Kingdom.

infections. Furthermore, the role of virulence factors during the interaction of *Salmonella* and neutrophils is not well understood.

In general, neutrophils are not considered effective in the control of intracellular pathogens. However, the role of neutrophils in murine salmonellosis is controversial, as recent studies reported that neutrophils contribute to the control of infection (7, 27) but that neutrophils cannot prevent colonization of systemic sites (14).

As neutrophils are an important part of innate immunity, we set out to analyze the contribution of neutrophils to protection against systemic murine salmonellosis after oral uptake of the pathogen. We investigated the cellular compositions of various infected lymphoid organs and the role of *Salmonella* virulence factors SPI1, SPI2, and SPV in the cellular response to infection. Special emphasis was put on a mutant strain deficient in SPI2, since the immune response to this highly attenuated strain is not fully understood. The data presented here provide a comprehensive view of the effect of oral *Salmonella* infection on neutrophil recruitment.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *S. enterica* serovar Typhimurium strains used in this study are listed in Table 1. The bacteria were grown overnight in Luria broth (LB) at 37°C. If required for selection of strains, kanamycin and nalidixic acid were added at concentrations of 50 and 100 μg/ml, respectively. For enumeration of CFU, suspensions were plated on Muller-Hinton plates or LB agar plates containing appropriate antibiotics.

Infection model. Female C57BL/6 mice were purchased from Charles River Breeders (Sulzfeld, Germany). All mice were kept at the animal unit of the Institut für Klinische Mikrobiologie, Immunologie, und Hygiene (Erlangen, Germany) under specific-pathogen-free conditions. The mice used for the experiments were between 8 and 12 weeks old. All experiments were conducted in accordance with the ethical guidelines of the University of Erlangen-Nürnberg.

For infection, bacterial cultures were harvested and washed in phosphate-buffered saline (PBS) (Biochrom), and the concentration was adjusted to the desired value in 300 μl of PBS. The mice were infected by gavage with 5×10^7, 5×10^9, or 10^{11} CFU. For intraperitoneal infection, an inoculum containing 5×10^5 CFU was used. The actual number of viable bacteria in the inoculum was always verified by plating serial dilutions onto LB agar plates in the absence and presence of the appropriate antibiotic.

CI model. As previously described (16), mice were infected with a mixture containing 5×10^9 CFU of the *S. enterica* serovar Typhimurium wild type (*Salmonella* WT) and 5×10^9 CFU of a mutant strain. On days 1 and 3 after infection, mice were sacrificed, and various organs were removed and homogenized in PBS. The bacterial loads of the organs were quantified by plating serial dilutions of the homogenates onto agar plates containing nalidixic acid and agar plates containing kanamycin for enumeration of the wild-type strain CFU and the mutant strain CFU, respectively. The competitive index (CI) was the ratio of the mutant strain to the wild-type bacteria recovered from organs (output) divided by the ratio of the mutant strain to the wild-type strain in the inoculum (input).

Neutropenic mouse model. In this study, we used a mouse model in which neutrophil depletion is induced by application of RB6-8C5 monoclonal antibodies. Briefly, monoclonal antibodies were prepared from supernatants of the RB6-8C5 hybridoma clone by purification on a HiTrap rProtein A FF column (Amersham Pharmacia). The eluate containing antibody was dialyzed against PBS and sterilized by passage through 0.22-μm-pore-size membrane filters. The protein concentration was determined by the BCA protein assay (Pierce); the concentration was adjusted to 1.5 mg/ml with sterile PBS, and the preparation was stored at 4°C. One day before infection, 300 μg of RB6-8C5 or PBS (control) was injected into mice. As previously reported (27), this treatment resulted in neutrophil depletion in normal and infected mice for at least 3 days. Neither circulating nor resident populations of macrophages and dendritic cells (DC) were affected by this treatment.

Analysis of neutrophil influx by FACS analysis. At different times after infection, various organs were removed; one-half of each organ was used for verification of the infection course, and the remainder was processed for fluorescence-activated cell sorting (FACS) analysis. In case of the PP and mesenteric lymph nodes (MLN), cell suspensions were prepared by cutting tissues in pieces and then incubating the pieces in RPMI containing 1 mg of collagenase D per ml at 37°C. The suspensions were passed through cell strainers (pore size, 100 μm; Falcon) in order to remove debris. For preparation of spleen cell suspensions, pieces of tissue were processed directly by gentle passage through cell strainers. Subsequently, the cells were washed and fixed with 0.4% formaldehyde in PBS. After blocking with Fc block (Becton Dickinson) in PBS containing 10% fetal calf serum and 1% bovine serum albumin, the cells were stained with anti-CD11c–phycoerythrin (BD), anti-CD11b–fluorescein isothiocyanate (BD), or anti-Gr1–biotin (BD) as the primary antibody conjugate. Biotinylated antibodies were detected by incubation with streptavidin-allophycocyanin (BD). About 10^5 cells were analyzed by flow cytometry with a FACSCalibur.

Analysis of neutrophil influx by confocal microscopy. The half of the organs remaining after CFU quantification were embedded in Tissue Tek (Dia Tec) and frozen in liquid nitrogen. Cryotome sections were fixed with acetone-methanol, blocked, and stained for *Salmonella*, neutrophils, and DC, respectively, by using the following combinations: rabbit anti-*Salmonella* antibody (Difco) and fluorescein-conjugated donkey anti-rabbit antibody (Dianova); rat anti-Gr1 antibody (BD) and rhodamine red X-conjugated donkey anti-rat antibody (Dianova); and Armenian hamster anti-CD11c antibody (BD) and Cy5-conjugated goat anti-Armenian hamster antibody (Dianova). Labeling was performed overnight at 4°C for the primary antibodies and for 1 h at room temperature for the secondary antibodies. The sections were mounted in Mowiol/DABCO and analyzed by confocal laser scanning microcopy (Leica).

Statistical analysis. The means for the different groups were compared by one-way analysis of variance (Prism 3). In most cases the significance of the difference between a group and the control group was tested with Dunnett's multiple-comparison *t* test for unpaired values; the exception was the analysis of neutrophil infiltration in the intraperitoneal infection model, in which the values for the mutant groups were compared to the mean obtained for the wild-type strain. Statistical significance was defined as a P value of <0.05.

RESULTS

Neutrophil influx after oral infection with *Salmonella*. The dynamics in various cell populations were quantified after oral infection of mice with *Salmonella* WT and a mutant strain deficient in SPI2. In accord with previous data (3, 6, 26), oral infection of C57BL/6 mice with *Salmonella* WT resulted in rapid bacterial replication in PP, MLN, livers, and spleens (Fig. 1). Mice infected with *Salmonella* WT were moribund on day 5 or 6 after oral infection. Similar progressions of disease were observed after infection with 5×10^9 CFU and after infection with 10^{11} CFU. Oral infection with the SPI2-deficient strain also resulted in the presence of viable bacteria in various organs; however, the number of bacteria remained constant for as long as 20 days after infection.

Organs from mice in these infection experiments were analyzed to determine the compositions of various cell populations as described in Materials and Methods. A significant change in the neutrophil population was detected 3 days after infection with *Salmonella* WT in PP, MLN, and spleens, suggesting that there was an influx of neutrophils after infection of these or-

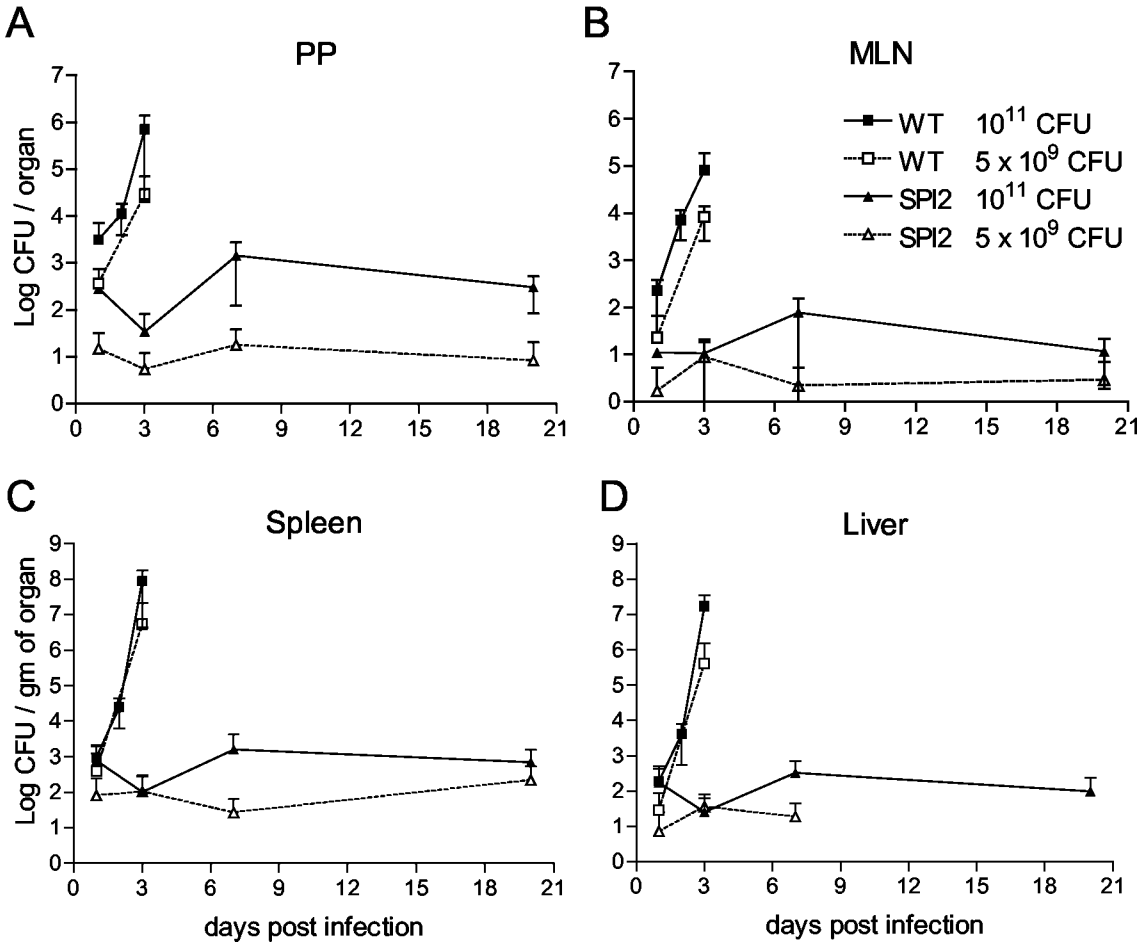

FIG. 1. Course of infection after oral application of *Salmonella* WT or an SPI2-deficient mutant strain. At various times after oral infection of mice with *Salmonella* WT and a mutant strain deficient in SPI2 (SPI2), the mean bacterial loads of various organs were determined. The kinetics of bacterial replication are shown for PP (A), MLN (B), spleens (C), and livers (D).

gans (Fig. 2). In contrast, no differences were detected in the populations of DC (marker $CD11c^+$) or macrophages (markers $CD11b^+$, $Gr1^{low}$, and $CD11c^-$) (data not shown). After infection with the SPI2-deficient strain, no increase in the neutrophil population was observed in PP, MLN, or spleens on day 1, 3, or 7 after infection (Fig. 2). Under these conditions, the populations of DC or macrophages also remained unchanged (data not shown).

For a more detailed characterization of the Gr1-positive population, the expression of CD11b was analyzed. The majority of the Gr1-positive population expressed CD11b (Fig. 2B), indicating that most of the cellular influx consisted of activated neutrophils.

These observations were confirmed by analyses of histological sections of MLN by laser scanning confocal microscopy (Fig. 3). On day 1 of infection and prior to neutrophil influx, about 16% of the *Salmonella* WT bacteria colocalized with Gr1-positive cells that probably represented the resident population. On days 2 and 3 after infection, neutrophil influx occurred in the interfollicular region, where replication of *Salmonella* WT resulted in the formation of clusters of bacteria. In histological sections from mice infected with the SPI2-deficient mutant strain, we never observed any alteration in the neutrophil population (data not shown).

Neutrophil influx after infection with mutant strains deficient in SPI1 or SPV. To investigate if the absence of neutrophil influx is a specific feature of infection by an SPI2-deficient mutant strain, we performed infection experiments with isogenic strains deficient in *invG* of SPI1 or *spvA* of SPV and analyzed the dynamics of cell populations under same infection conditions. Although the organ load of an SPI1-deficent strain in the spleen was lower on day 3 after infection, infected animals also developed a fatal systemic salmonellosis with a delay of 2 or 3 days compared to mice infected by *Salmonella* WT. The SPV-deficient mutant strain exhibited a highly reduced rate of replication in spleens; however, the rate of bacterial replication was still higher than the rate for the SPI2-deficient strain (Fig. 4A).

The results for the $Gr1^{high}$ population in spleens of infected mice (Fig. 4B) indicate that infection with an SPI1-deficient mutant strain induced the same neutrophil influx on day 3 after infection as *Salmonella* WT induced. Similar results were obtained for the $Gr1^{high}$ population in PP and MLN (data not shown). Infection with the SPV-deficient mutant strain did not

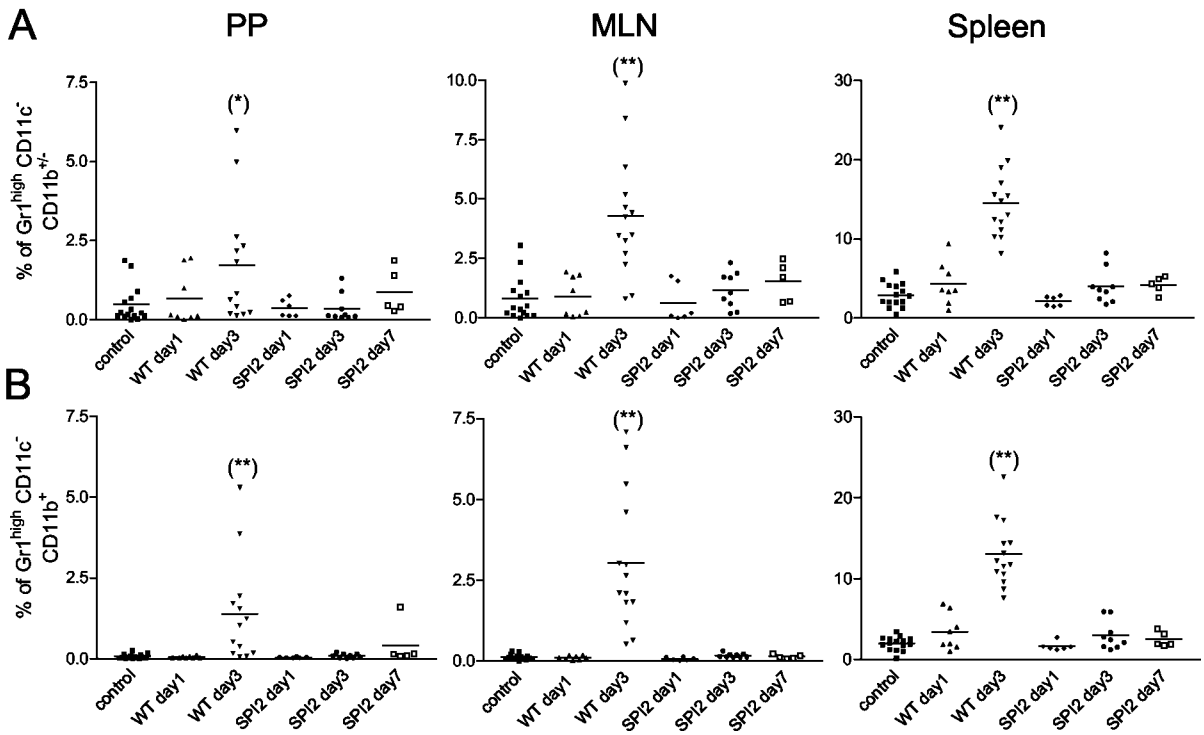

FIG. 2. Neutrophil influx after a single oral dose of *Salmonella* WT or a mutant strain deficient in SPI2. The data for infection studies performed with a dose of 5×10^9 CFU represent the data from four different experiments. Experiments with control, *Salmonella* WT, and SPI2-deficient (SPI2) groups were always performed in parallel, and each symbol indicates the data for one mouse. For panel A, the means (bars) of the relative neutrophil population levels were analyzed in PP, MLN, and spleens. For panel B, the $CD11b^+$ neutrophil subpopulation levels were analyzed. Figure 1 shows the organ loads in these experiments after infection with an dose of 5×10^9 CFU. One asterisk, $P < 0.05$; two asterisks, $P < 0.01$.

induce significant neutrophil influx on day 3 after infection; however, on day 7 after infection the neutrophil population in the spleen was comparable to the population observed on day 3 after infection with *Salmonella* WT or the SPI1-deficient mutant strain. This delayed influx of neutrophils was correlated with the reduced rate of replication of the SPV-deficient mutant strain in the spleen (Fig. 4) and other infected organs (data not shown).

In order to further analyze if neutrophil attraction was dependent on the phenotypes of the *Salmonella* strains used in this study, we used an infection model that did not result in colonization of host organs by the bacteria. *Salmonella* WT or mutant strains deficient in SPI1, SPI2, or SPV were injected into the peritoneal cavity, and 4 h after infection the influx of neutrophils was quantified by flow cytometry (Fig. 5). During this time, the numbers of bacteria remained similar for *Salmonella* WT and various mutant strains. Infection with *Salmonella* WT or various mutant strains induced neutrophil influx to similar extents. Based on these observations, we concluded that *Salmonella* WT and the mutant strains with defects in SPI1, SPI2, or SPV used in this study are equally capable of inducing an inflammatory response and neutrophil migration.

Neutrophil influx is dependent on the bacterial burden in infected organs. The observations described above indicate that the neutrophil influx could mainly be a function of the dose of bacteria in an infected organ rather than a function of the virulence factors. In order to establish this possible correlation in the oral infection model, we performed infection studies with different doses of *Salmonella* WT and mutant strains in order to compensate for the different rates of replication of the strains. Groups of mice were infected orally with an inoculum of 10^{11} CFU of the SPI2-deficient mutant strain or with 5×10^7 or 5×10^9 CFU of *Salmonella* WT. On day 3 after infection, the organ load was determined (Fig. 6A and C) and the neutrophil influx was analyzed (Fig. 6B and D). In MLN, similar organ loads (about 5×10^2 CFU per organ) were detected after infection with 10^{11} CFU of the SPI2-deficient mutant strain and after infection with 5×10^7 CFU of *Salmonella* WT. At these organ loads, no neutrophil influx was detected for either strain. Infection with 5×10^9 CFU of *Salmonella* WT resulted in a higher bacterial burden (10^4 CFU per organ), and a significant neutrophil influx was detected. Analyses of spleens of the same infected animals indicated that infection with 5×10^7 CFU of *Salmonella* WT led to a higher bacterial burden than infection with 10^{11} CFU of the SPI2-deficient mutant strain. This indicates that the spleen is more permissive for *Salmonella* replication than MLN. Again, the extent of the neutrophil population was dependent on the bacterial burden in the spleen (Fig. 6C and D).

After intraperitoneal infection, similar bacterial organ burdens of *Salmonella* WT and the SPI2-deficient strain were observed on day 1 after infection (Fig. 6D), indicating that SPI2-dependent bacterial replication had not started. Infection of the spleen by both strains resulted in similar infiltration of

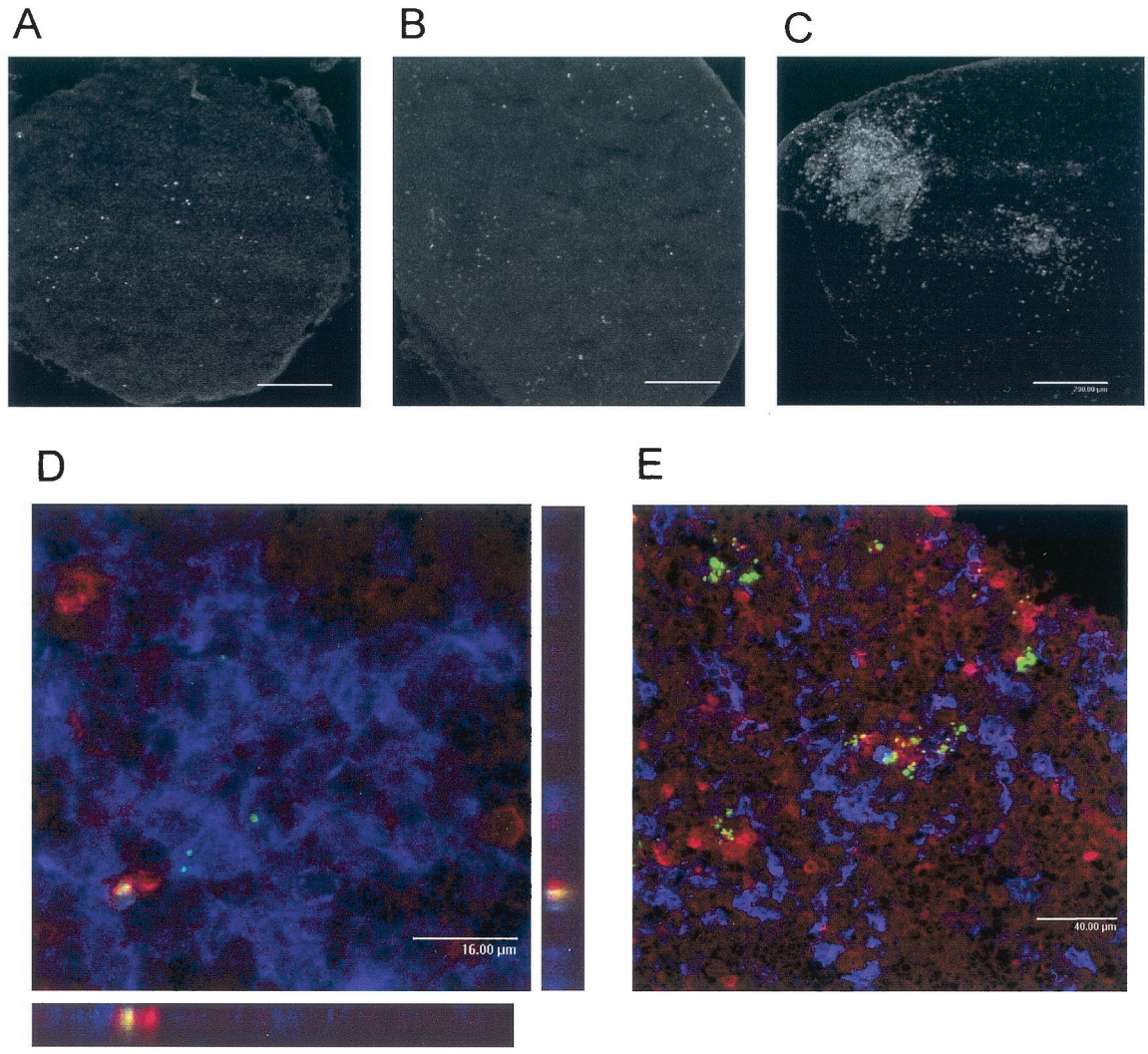

FIG. 3. Histological analysis of neutrophil influx during infection with a single oral dose of 10^{11} CFU of *Salmonella* WT by confocal microscopy. (A, B, and C) Fluorescence observed in the red channel after staining for Gr1. Scale bars = 200 μm. (A) Representative image obtained for MLN of the control mice. (B and C) Representative sections of infected MLN (positive for *Salmonella* not shown), indicating the neutrophil influx 1 day (B) or 2 days (C) after infection. (D and E) Localization of bacteria (green), neutrophils (red), and DC (blue) for MLN on day 1 after infection (D) (scale bar = 16 μm) and on day 3 after infection (E) (scale bar = 40 μm). Panel D also shows *xz* and *yz* sections of an area of colocalization of neutrophils and *Salmonella* WT. Figure 1 shows the organ loads in these experiments for a dose of 10^{11} CFU.

neutrophils (Fig. 6F). At later times, mice infected with *Salmonella* WT developed a fatal systemic salmonellosis, while the bacterial organ burdens of mice infected with the SPI2-deficient strain and the number of infiltrated neutrophils increased only slightly. The observations made in the intraperitoneal infection experiment are in line with the data shown in Fig. 5 and indicate that *Salmonella* WT and the SPI2-deficient strain induced the same extent of neutrophil infiltration.

Effect of neutropenia on the course of systemic infection. In order to investigate the role of neutrophils in controlling the progression of murine salmonellosis after oral infection, neutrophils were depleted prior to infection. Depletion by treatment with monoclonal antibody RB6-8C5 was performed since this model appears to be more reproducible than pharmacologically induced neutropenia (14, 27). The antibody-induced neutropenia was controlled by analysis of the Gr1-positive populations in blood and various organs. The effect of neutropenia on bacterial replication was analyzed by using the CI model. The CI model allows sensitive comparisons of the virulence of two bacterial strains in a low number of infected animals (2).

Because the conflicting results obtained previously for *Salmonella* WT and strains deficient in SPV could have been due to the method of neutrophil depletion, as well as to the infection model, we repeated a control with an auxotrophic strain used previously (27). We used a mutant strain of *S. enterica* serovar Typhimurium deficient in *purD* that was isogenic with other strains used in this study. In PP and spleens of neutropenic mice, the bacterial burden was higher in infected mice than in control mice (Fig. 7), and similar results were obtained for MLN and livers (data not shown). The replication rate of *Salmonella* WT in neutropenic mice was increased. However, the CI for WT versus the *purD* mutant strain in neutropenic

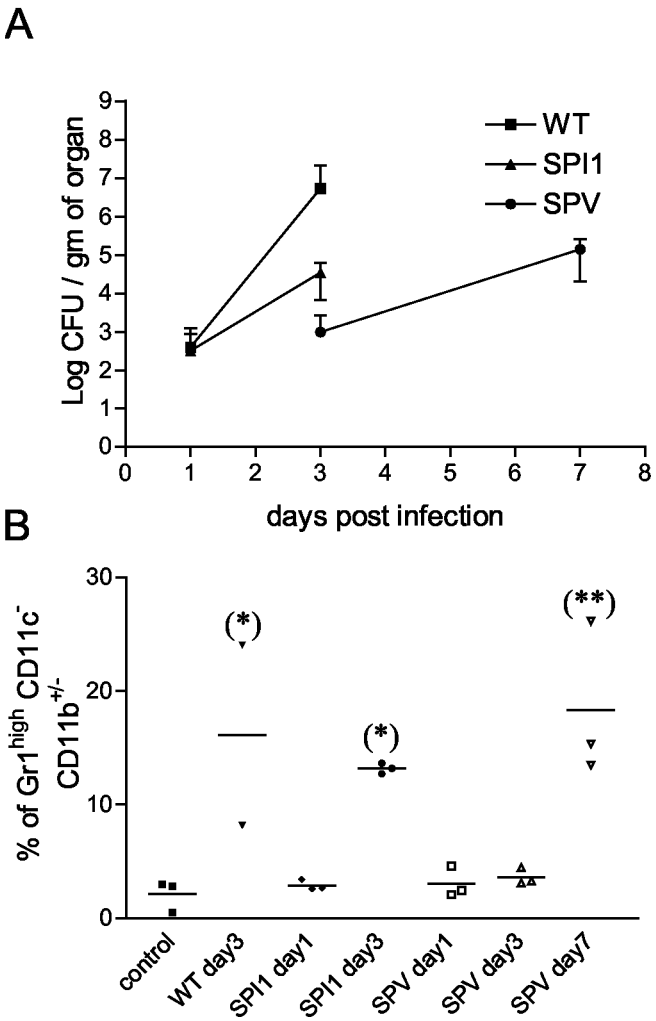

FIG. 4. Neutrophil influx and bacterial burden after infection with a single oral dose of *Salmonella* WT or a mutant strain deficient in SPI1 (SPI1) or SPV (SPV). (A) Bacterial burden in the spleens of infected mice analyzed by plating serial dilutions for determination of the number of CFU. (B) Neutrophil influx in the spleens of mice infected with *Salmonella* WT or a mutant strain deficient in SPI1 or SPV at a dose of 5×10^9 CFU. Each symbol indicates the data for one mouse from a representative experiment. One asterisk, $P < 0.05$; two asterisks, $P < 0.01$.

mice was not increased in PP, MLN, livers, or spleens (Table 2). As previously reported for a systemic infection model, the antibody-induced neutropenia did not restore the virulence of the *purD*-deficient strain (Fig. 7) in the oral model.

Finally, mixed-infection experiments were performed with neutropenic and control mice by using an inoculum consisting of a mixture of *Salmonella* WT and the SPI2-deficient strain. Three days after oral infection, bacterial counts were determined in various organs of both strains, and the CI were calculated (Table 3). The virulence of the SPI2-deficient strain was not restored in neutropenic mice, and the CI in all organs was higher than the CI in control mice. The susceptibilities of *Salmonella* WT and the SPI2-deficient strain were analyzed in vitro as previously described (10) by using neutrophils isolated from murine peritoneal exudates 1 day after injection of a

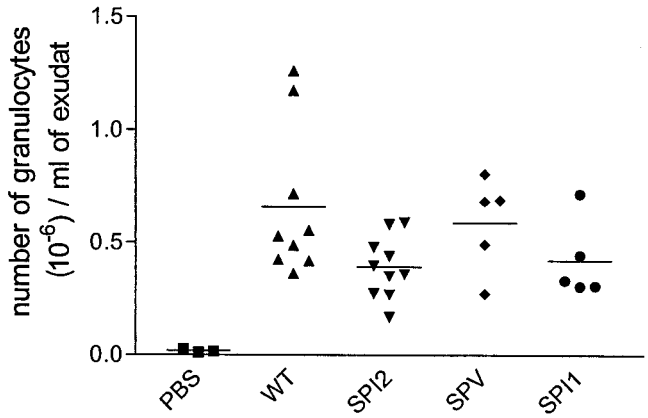

FIG. 5. Neutrophil infiltration in the peritoneal cavity in the intraperitoneal infection model. Four hours after injection of PBS or PBS containing 10^4 CFU of *Salmonella* WT or a mutant deficient in SPI2 (SPI2), SPV (SPV), or SPI1 (SPI1), the cavity was washed with 3 ml of RPMI. The cells present in the exudates were counted and analyzed for Gr1 staining by FACS. The data are data from two independent experiments.

thioglycolate solution. Both strains were killed to the same extent by murine neutrophils, but they were less susceptible to killing than a laboratory strain of *Escherichia coli* was (data not shown).

These observations indicated that depletion of neutrophils was not sufficient to restore the systemic virulence of highly attenuated mutant strains that were deficient either in metabolic functions (*purD*) or in a virulence factor important for intracellular survival and replication (SPI2).

DISCUSSION

In this study, we analyzed the contribution of neutrophils to the control of systemic murine salmonellosis after oral infection. Using confocal microscopy, we observed that 16% of the bacteria were associated with neutrophils on day 1 after infection. This population was also affected by antibody-mediated neutropenia, which increased the bacterial burden on day 1 after infection. Thus, the resident neutrophil population contributes to the early killing of *Salmonella* after infection. We observed that in various organs infected by *Salmonella* an influx of neutrophils was induced and that the dynamics of neutrophil infiltration were similar in various infected organs. The extent of infiltration of neutrophils was largely dependent on the bacterial loads in infected organs. However, the attracted neutrophils were not able to control the massive replication of *Salmonella* WT or a mutant strain deficient in SPI1. In this infection model, neutropenia was not sufficient to restore the virulence of highly attenuated strains harboring mutations causing auxotrophy or defects in SPI2 functions.

We propose that the inability of neutrophils to control *Salmonella* replication and systemic spread is a consequence of the intracellular lifestyle of *Salmonella*. In vitro experiments indicated that *Salmonella* WT and various mutant strains are equally susceptible to killing by neutrophils. However, during host infection, the bacteria rapidly enter safe sites, such as intracellular localizations in epithelial cells, macrophages, and

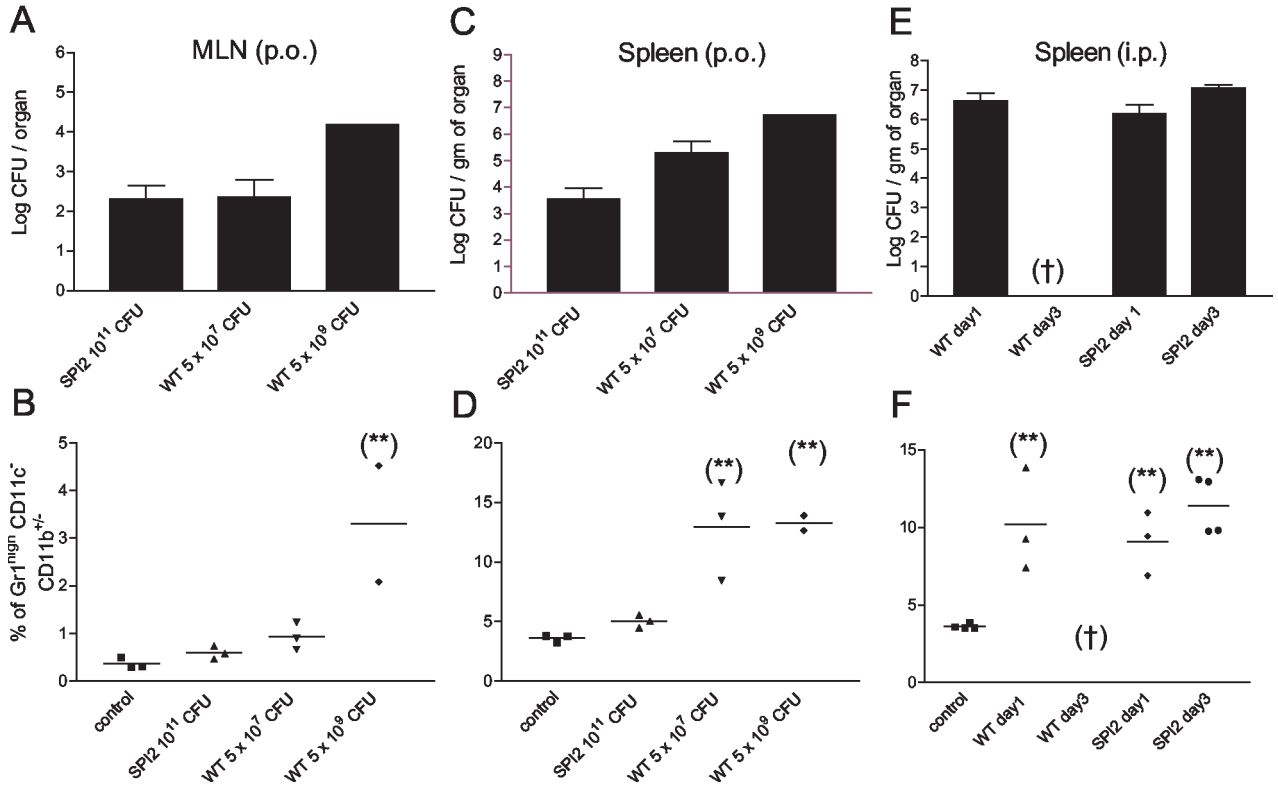

FIG. 6. Influence of bacterial load on the neutrophil influx after oral infection and intraperitoneal infection. The means of CFU counts in MLN (A) and spleens (C) were compared for three different groups of orally (p.o.) infected mice. One group was infected with 10^{11} CFU of SPI2-deficient bacteria (SPI2), the second group was infected with 5×10^7 CFU of *Salmonella* WT, and a dose of 5×10^9 CFU of *Salmonella* WT was used as a positive control for neutrophil infiltration. The neutrophil influx in MLN (B) and spleens (D) was quantified. Furthermore, the bacterial burden of the spleen (E) and the neutrophil infiltration (F) were determined on days 1 and 3 after intraperitoneal (i.p.) infection with 5×10^5 CFU of *Salmonella* WT or SPI2-deficient bacteria. Mice infected intraperitoneally with *Salmonella* WT were moribund between days 2 and 3 after infection and were not analyzed further. Each symbol indicates the data for one mouse from one representative experiment, and the means (bars) were compared to the mean for the uninfected group (two asterisks, $P < 0.01$).

DC, and the intracellular population appears to be protected from killing by neutrophils and other immune responses. It was recently observed that in murine DC, the *Salmonella* population is a static population that can survive for several days (5; C. Cheminay, J. Jantsch, and M. Hensel, unpublished observations). We found that two-thirds of *Salmonella* WT bacteria were associated with DC in MLN on day 1 after oral infection (data not shown). The intracellular location could also be a requirement for systemic spread. Previous observations indicated that migration to and survival in systemic sites is not dependent on the function of the SPI2 system (26). As survival of *Salmonella* in DC is independent of the function of various virulence factors (22), this cell population might be involved in the systemic spread of the pathogen.

Salmonella strains deficient in SPI2 exhibit highly reduced colonization of PP, and a similar defect was observed in MLN, livers, and spleens. However, under our experimental conditions, mutant strains deficient in SPI2 function were able to infect various organs after oral infection, indicating that the SPI2 function is not essential to initiate systemic spread of *Salmonella*. In addition, there is a cell population in organs like PP, MLN, livers, and spleens that is permissive for SPI2-dependent bacterial replication. Previous studies have shown that *Salmonella* is associated with macrophages in livers and spleens (24, 25). Furthermore, cell culture assays revealed that SPI2-deficient strains exhibit highly reduced intracellular replication in macrophages (6, 16, 23). We suggest that in the various organs colonized by *Salmonella* during systemic infection, macrophages are the main cellular population that promotes bacterial replication. Defects in bacterial replication in macrophages due to mutations in SPI2 result in highly reduced bacterial burdens in various organs, thereby reducing the virulence in the mouse model.

Our data do not support the hypothesis that SPI2 has a specific function in the interference with neutrophil functions. Depletion of neutrophils was not sufficient to restore the defect of an SPI2-deficient mutant strain in replication within the host. This is in contrast to the functions of SPI2 in *Salmonella* within macrophages, such as protection against antimicrobial activities involving reactive oxygen intermediates, as well as reactive nitrogen intermediates (4, 29). These antimicrobial activities are also generated by neutrophils, but they are mainly directed against extracellular bacteria. We observed that SPI2 functions cannot prevent inactivation of *Salmonella* by neutrophils in vitro.

With a mutant deficient in SPI1-dependent invasion the onset of systemic infection was delayed; a lower number of SPI1-deficient *Salmonella* cells were able to penetrate the in-

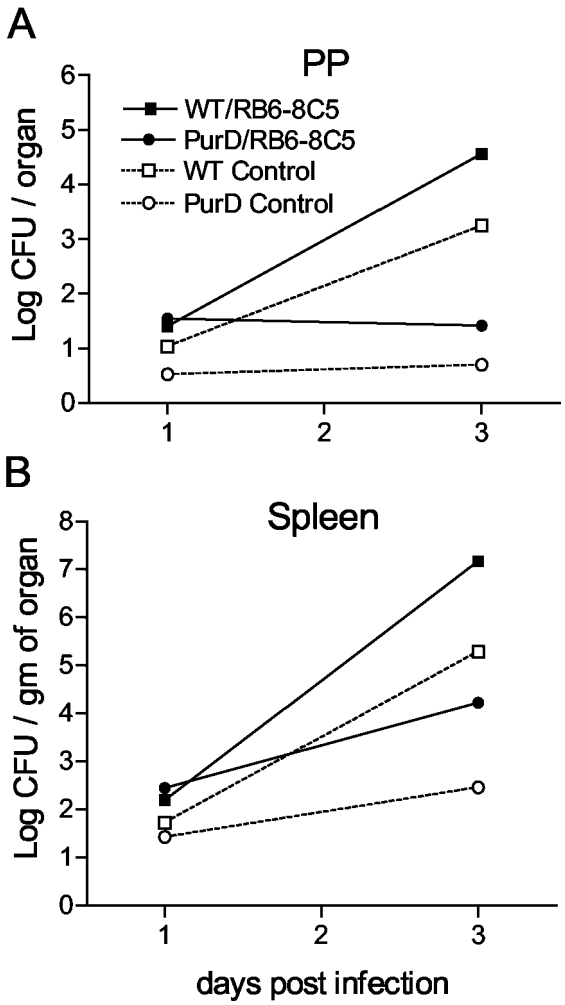

FIG. 7. Effect of neutrophil depletion on organ load with *Salmonella* WT and a *purD* mutant stain. Cohorts of neutropenic mice (solid symbols) and control mice (open symbols) were infected with *Salmonella* WT or an auxotrophic strain deficient in *purD*. On day 1, 2, or 3 after infection, the mice were sacrificed, and the mean numbers of CFU in the PP (A) or spleens (B) for *Salmonella* WT (squares) and the *purD* strain (circles) were determined ($n = 2$).

testinal epithelium. However, there is evidence that other mechanisms allow uptake of *Salmonella* (28). The role of *spv* genes of the *Salmonella* virulence plasmid is not fully understood. Mutants deficient in the SPV functions are also able to cause systemic infections after oral infection, but the rate of bacterial replication is greatly reduced. This reduced replication might allow sufficient control by the host innate immunity and an adaptive immune response at later stages of infection.

TABLE 2. Effects of neutrophil depletion on the course of infection with *Salmonella* WT and an auxotrophic mutant strain

Group	Mean CI (PurD mutant versus *Salmonella* WT)[a]			
	PP	MLN	Spleen	Liver
Control	0.00041	0.00182	0.00528	0.03
Neutropenia	0.00071	0.00213	0.00219	0.00087

[a] The CI was determined in various organs 3 days after oral infection ($n = 2$).

The observations on the role of neutrophils in immunity against salmonellosis reported here are in contrast to previous reports. A crucial role of neutrophils has been proposed based on intravenous infection studies with *S. enterica* serovar Dublin (27). In contrast, our studies based on the natural route of infection indicate that neutrophils have a supportive, but not essential function in controlling systemic murine salmonellosis. In a previous study the workers also investigated the roles of different populations of phagocytes after oral infection of mice with *S. enterica* serovar Typhimurium (14), and it was observed that the macrophage population had a pivotal role in controlling infection. The critical experimental parameter in the various studies appears to be the route of experimental infection with *Salmonella*. Intraperitoneal or intravenous infection results in a rapid confrontation between *Salmonella* and neutrophils present in the circulation. In contrast, infection of host organs after oral infection requires attraction of neutrophils to the site of infection, since the resident cell population is rather small in organs. We did not observe a significant influx of phagocytes on day 1 after oral infection, although *Salmonella* WT had already colonized various organs at this time. These observations indicate that the inflammatory response to *Salmonella* infection is not fast enough to prevent further colonization. In contrast, the massive influx of neutrophils to the sites of bacterial infection may contribute to pathogen-induced tissue damage and subsequent multiple organ failure.

The route of experimental infection with *Salmonella* is very important for interpretation of the host response. For example, different results have been obtained for the role of lipopolysaccharide binding protein in immunity to *Salmonella* depending on the route of infection (oral or intraperitoneal) (11, 31). The role of neutrophils reported here is also in contrast to previous observations based on an intraperitoneal infection model. As salmonelloses are food-borne diseases, we consider the oral route of infection the most suitable route for understanding the functions of various mechanisms of the immune system.

Marco et al. (20) reported that neutrophils play an important role in the initial resistance to *Listeria monocytogenes* after infection by the oral route. It was also observed that *Listeria* survives and multiplies within neutrophils. In another oral infection model for *L. monocytogenes*, mice treated with RB6-8C5 had a 5,000-fold-higher bacterial burden in the walls of the stomach, small intestine, cecum, or large intestine than control mice 3 days after infection (8). Systemic infection resulting from ingestion of *L. monocytogenes* was also greatly enhanced in neutropenic mice. Both of these studies supported the hypothesis that neutrophils play a critical role in the early defense against oral infection with *Listeria*, as well as in the control of subsequent systemic infections. In experiments with mycobacteria in which the aerosol infection route was used, CH3/HeJ mice (TLR4 deficient) had a reduced capacity to eliminate mycobacteria from the lungs and to control the spread of infection to the spleen and liver (1). CH3/HeJ mice had 10- to 100-fold-higher bacterial burdens than wild-type mice (CH3/HeN) and succumbed within 5 to 7 months, whereas most wild-type mice controlled the infection and survived for the duration of the experiment. The lungs of CH3/HEJ mice

TABLE 3. Effects of neutrophil depletion on the course of infection with *Salmonella* WT and an SPI2-deficient mutant strain

Group	CI (SPI2-deficient mutant versus *Salmonella* WT) (mean ± SD)[a]			
	PP	MLN	Spleen	Liver
Control	0.0168 ± 0.0311	0.0129 ± 0.0091	0.0178 ± 0.0128	0.0131 ± 0.0185
Neutropenia	0.0014 ± 0.0018	0.0096 ± 0.0132	0.0084 ± 0.0166	0.0043 ± 0.0038

[a] The CI was determined in various organs 3 days after oral infection ($n = 8$).

showed chronic pneumonia with increased neutrophil infiltration, reduced macrophage recruitment, and abundant acid-fast bacilli. In this model, high levels of infiltration of neutrophils did not allow control of mycobacterial infections.

Data reported here and in various previous studies highlight the different role of neutrophils in systemic and gastrointestinal infections with *Salmonella*. In pathogen-host models that do not result in systemic infections, infections are characterized by rapid attraction of neutrophils to mucosal sites. The pathogen appears to be unable to reach a safe intracellular location that could protect it from killing by neutrophils. In this situation, the invasion of human epithelial cells triggers the release of proinflammatory cytokines (17, 21). In contrast, systemic infections by *Salmonella* are characterized by invasion and destruction of M cells of PP. Infection by this route appears to bypass efficient induction of proinflammatory cytokines. We observed induction of proinflammatory cytokines only at later stages of infection when *Salmonella* had already reached systemic sites and proliferated so that the organ loads were high (unpublished observations). It should be noted that the observations described above resulted from the use of different host models, since a mouse model that resembles localized gastrointestinal infection is currently not available.

In conclusion, we investigated the role of neutrophils in the immune response against *S. enterica* serovar Typhimurium after oral infection of mice. Our observations demonstrate that neutrophils are not able to control systemic salmonellosis and support the hypothesis that *Salmonella* rapidly enters intracellular locations in host cells that allow replication of the pathogen, as well as systemic spread. Neutrophils make very different contributions during systemic salmonellosis and localized gastrointestinal infections. While rapid neutrophil attraction efficiently prevents the spread of the pathogen in localized infections, the neutrophil influx is delayed during systemic infections and cannot protect against lethal bacteremia. Further work is required to understand how these functions are modulated by chemokines and cytokines.

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