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# Chronic *Salmonella enterica* Serovar Typhimurium-Induced Colitis and Cholangitis in Streptomycin-Pretreated *Nramp1*$^{+/+}$ Mice

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***Salmonella enterica*** **subspecies 1 serovar Typhimurium is an enteric bacterial pathogen infecting a broad range of hosts. In susceptible *Nramp1*$^{-/-}$ (*Slc11α1*$^{-/-}$) mice, serovar Typhimurium cannot efficiently colonize the intestine but causes a systemic typhoid-like infection. However, after pretreatment with streptomycin, these susceptible (C57BL/6 and BALB/c) mice develop acute serovar Typhimurium-induced colitis (M. Barthel et al., Infect. Immun. 71:2839–2858, 2003). It was not clear whether resistant *Nramp1*$^{+/+}$ (*Slc11α1*$^{+/+}$) mouse strains would similarly develop colitis. Here we compared serovar Typhimurium infection in streptomycin-pretreated susceptible (C57BL/6) and resistant (DBA/2 and 129Sv/Ev) mouse strains: We found that acute colitis (days 1 and 3 postinfection) is strikingly similar in susceptible and resistant mice. In 129Sv/Ev mice we followed the serovar Typhimurium infection for as long as 6 weeks. After the initial phase of acute colitis, these animals developed chronic crypt-destructive colitis, including ulceration, crypt abscesses, pronounced mucosal and submucosal infiltrates, overshooting regeneration of the epithelium, and crypt branching. Moreover, we observed inflammation of the gall duct epithelium (cholangitis) in the 129Sv/Ev mice between days 14 and 43 of infection. Cholangitis was not attributable to side effects of the streptomycin treatment. Furthermore, chronic infection of 129Sv/Ev mice in a typhoid fever model did not lead to cholangitis. We propose that streptomycin-pretreated 129Sv/Ev mice provide a robust murine model for chronic enteric salmonellosis including complications such as cholangitis.**

Depending on the host organism, bacteria of the genus *Salmonella* can cause diseases such as enterocolitis, bacteremia, and enteric fever ("typhoid"). The infective dose and the host immune status dictate the extent of disease. In humans, *Salmonella enterica* subspecies 1 serovar Typhimurium causes a self-limiting enterocolitis (4), and bacteria usually do not disseminate beyond the lamina propria and gut-associated lymphoid tissue. In contrast, susceptible mice infected with serovar Typhimurium come down with and succumb to systemic disease but generally do not develop intestinal inflammation (6).

Oral administration of the antibiotic streptomycin renders mice more susceptible to oral serovar Typhimurium infection and intestinal colonization (8, 15, 24, 29). In pretreated mice, systemic infection is accompanied by acute intestinal inflammation resembling several features also observed in human *Salmonella* enterocolitis: the cecum and colon show polymorphonuclear neutrophilic infiltrates, epithelial destruction, and crypt abscesses (1, 4, 5, 9, 16, 35). Our initial studies were performed with susceptible (*Slc11α1*$^{-/-}$) C57BL/6 and BALB/c mouse strains. These studies had focused on the acute phase of inflammation (day 1 until day 4 postinfection [p.i.]) and were limited by the mice succumbing to the accompanying systemic disease.

Resistant (*Slc11α1*$^{+/+}$) mouse strains are able to control systemic serovar Typhimurium infection, and systemic bacterial loads remain low during the whole course of infection (25). It is still not known whether these resistant mice develop serovar Typhimurium colitis upon pretreatment with streptomycin.

Mouse strain-specific differences in susceptibility toward systemic serovar Typhimurium infection (19) are linked to multiple factors, including the major histocompatibility complex, Toll-like receptor 4 (41), and a "classical" locus on mouse chromosome 1, designated solute carrier family 11a member 1 (Slc11α1; formerly Nramp-1, *bcg*, *lsh*, *ity*). This locus encodes a divalent cation antiporter protein located in endosomes and lysosomes of professional phagocytes (40) and is believed to play a vital role in divalent cation homeostasis and antimicrobial activity (13). Slc11α1 controls intracellular parasites such as *Salmonella* spp., *Legionella* spp., *Mycobacterium* spp., and *Leishmania* spp. in reticuloendothelial organs (2, 3, 12, 18, 38, 39). Slc11α1 has been shown to interfere with the establishment of the *Salmonella*-containing vacuole in macrophages (7, 11, 16, 25, 32, 42). Significant numbers of bacteria have been observed in phagocytic cells of the lamina propria in the intestines of streptomycin-pretreated C57BL/6 mice (16). Thus, it was conceivable that the course of intestinal serovar Typhimurium infections might differ between resistant and sensitive mouse strains.

Here we studied oral serovar Typhimurium infection of the resistant mouse strains 129Sv/Ev and DBA/2 after pretreatment with streptomycin. We found that acute serovar Typhimurium colitis is similar in 129Sv/Ev, DBA/2, and C57BL/6

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mice. Long-term infections in the resistant mouse strains (i.e., 129Sv/Ev) were feasible and displayed characteristics of chronic, crypt-destructive colitis. Moreover, we observed the occurrence of inflammation of the gall duct (cholangitis) in 129Sv/Ev mice with chronic serovar Typhimurium-induced colitis. Thus, resistant (Slc11$\alpha$1$^{+/+}$) 129Sv/Ev mice do develop acute serovar Typhimurium colitis and may also provide an interesting model with which to study aspects of chronic intestinal inflammation and cholangitis.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** For infections, wild-type serovar Typhimurium SL1344 (streptomycin resistant) (17) was grown for 12 h at 37°C in Luria-Bertani broth containing 0.3 M NaCl, diluted 1:20 in fresh medium, and subcultured for 4 h under mild aeration. Bacteria were washed in ice-cold phosphate-buffered saline (PBS) and resuspended in cold PBS ($5 \times 10^7$ CFU/50 μl).

**Animal experiments.** Animal experiments were performed in individually ventilated cages (IVCs) at the Biologisches Zentrallabor (Universität Zürich) as described previously (1), using specific-pathogen-free (SPF) 6- to 9-week-old male or female C57BL/6 or DBA/2 mice (Harlan Horst, The Netherlands) or 129Sv/Ev mice bred at the research contract company (Füllinsdorf, Switzerland). Where indicated, mice were pretreated with 20 mg of streptomycin intragastrically 24 h prior to infection. The oral infection experiments ($5 \times 10^7$ CFU given intragastrically) were carried out as described previously (34). After 3 days of infection, mice were changed from IVCs with steel grid floors to IVCs with wooden litter. Experiments were performed with sex- and age-matched animals from different mouse strains. Animal experiments have been approved by the Swiss veterinary office and were performed as required by Swiss national and institutional regulations.

**Analysis of serovar Typhimurium loads in the intestine, mLN, spleen, and liver.** Spleen, liver, and mesenteric lymph nodes (mLN) were removed aseptically and homogenized in 4°C PBS (0.5% Tergitol, 0.5% bovine serum albumin) as described previously (34). The bacterial loads were determined by plating on MacConkey agar plates (streptomycin, 50 μg/ml). The minimal detectable values were 10 CFU/organ in the mLN, 20 CFU/organ in the spleen, and 100 CFU/organ in the liver. Cecal contents were collected at the indicated time points postinfection, and the bacterial loads were determined by plating. The minimal detectable value was 10 CFU per sample.

**Histological procedures.** Tissue samples were embedded in optimal cutting temperature compound (OCT; Sakura, Torrance, CA), snap-frozen in liquid nitrogen, and stored at −80°C. Cryosections (thickness, 5 μm) were mounted on glass slides, air dried for 2 h at room temperature, and stained with hematoxylin and eosin (HE) or used for immunohistochemistry.

Cecal pathology was independently evaluated by two pathologists in a blinded manner using thin HE-stained sections (thickness, 5 μm) and a histopathological scoring scheme as previously described (34).

**(i) Submucosal edema.** Submucosal edema was deduced from the extension of the submucosa and scored by morphometric analysis according to the formula % se = $(b - a)/c$, where se is submucosal edema, $a$ is the area enclosed by the mucosa (i.e., mucosa and intestinal lumen), $b$ is the area enclosed by the borderline between the submucosa and the tunica muscularis (i.e., submucosa, mucosa, and intestinal lumen), and $c$ is the area enclosed by the outer edge of the tunica muscularis (i.e., tunica muscularis, submucosa, mucosa, and lumen—the area of the whole cecal cross section). Submucosal edema scores are as follows: 0, no pathological changes; 1, detectable edema (<10%); 2, moderate edema (10 to 40%); 3, profound edema (≥40%).

**(ii) PMN infiltration into the lamina propria.** Polymorphonuclear granulocytes (PMN) in the lamina propria were enumerated in 10 high-power fields (magnification, ×400; field diameter, 420 μm), and the average number of PMN per high-power field was calculated. Scores are as follows: 0, fewer than 5 PMN per high-power field; 1, 5 to 20 PMN per high-power field; 2, 21 to 60 PMN per high-power field; 3, 61 to 100 PMN per high-power field; 4, more than 100 PMN per high-power field.

**(iii) Goblet cells.** The average number of goblet cells per high-power field (magnification, ×400) was calculated from 10 different regions of the cecal epithelium. Scores are as follows: 0, more than 28 goblet cells per high-power field; 1, 11 to 28 goblet cells per high-power field; 2, 1 to 10 goblet cells per high-power field; 3, less than 1 goblet cell per high-power field.

**(iv) Epithelial integrity.** Scores are as follows: 0, no pathological changes detectable in 10 high-power fields (magnification, ×400); 1, epithelial desquamation; 2, erosion of the epithelial surface (gaps of 1 to 10 epithelial cells per lesion); 3, epithelial ulceration (gaps of >10 epithelial cells per lesion; at this stage, there was generally granulation tissue below the epithelium).

The combined pathological score for each tissue sample was determined as the sum of these averaged scores. Thus, combined scores are as follows: 0, intestine intact without any signs of inflammation; 1 to 2, minimal signs of inflammation which are not signs of disease (these are frequently found in the ceca of SPF mice); 3 to 4, slight inflammation; 5 to 8, moderate inflammation; 9 to 13, profound inflammation.

Cholangitis was evaluated by inspecting 5-μm-thick HE-stained thin sections of the liver. An animal with cholangitis displayed at least 1 inflammatory lesion of a gall duct per 5-μm section.

**Immunohistochemistry.** Cryosections (thickness, 5 μm) were mounted on glass slides and air dried for 2 h at room temperature. Sections were fixed with acetone and stained with monoclonal rat anti-mouse B220/CD45R for B cells (RA3-6B2; Pharmingen 553084; 1:400), CD4 for T-helper cells (YTS 191; 1:200), CD8 for T cells (YTS 169; 1:50), F4/80 (1:50; Serotec) for macrophages, and CD11b (BMA clone 5CC; 1:3,000) and Ly6G (1:300; Jackson) for macrophages/PMNs. A goat anti-rat antibody (Caltag R 40000; 1:150) was used as the secondary antibody, and an alkaline phosphatase-conjugated donkey anti-goat antibody (1:80; Jackson) was used as the tertiary antibody. CD11c$^+$ cells were stained with Armenian hamster anti-mouse CD11c (HL3; Pharmingen 553799; 1:30). A goat anti-Armenian hamster antibody (Jackson 127-005-099; 1:200) was used as the secondary antibody, and an alkaline phosphatase-conjugated donkey anti-goat antibody (Jackson 795-055-147; 1:80) was used as the tertiary antibody. Alkaline phosphatase was visualized using naphthol AS-BI phosphate with New Fuchsin as the substrate, which yields a red precipitate (21).

**Statistical analysis.** Statistical analyses of the individual pathological scores for submucosal edema, PMN infiltration, loss of goblet cells, and epithelial integrity and of the combined pathological score were performed using the exact Mann-Whitney U test and SPSS, version 11.0, software, as described previously (34). $P$ values of <0.05 were considered statistically significant. Bacterial colonization was analyzed in a similar manner. To allow the statistical analysis of the bacterial loads, values for animals yielding "no CFU" were set to the minimal detectable value (10 CFU in the mLN, 20 CFU in the spleen, 100 CFU in the liver; intestinal content, between 67 and 400 CFU [see above]). Afterwards, the median values were calculated using Microsoft Excel 2003, and statistical analysis was performed using the exact Mann-Whitney U test and SPSS, version 11.0, software. $P$ values of <0.05 were considered statistically significant (NS, not statistically significant).

## RESULTS

**Infection of C57BL6, 129Sv/Ev, and DBA/2 mice with serovar Typhimurium for 3 days.** Streptomycin pretreatment renders susceptible (*Slc11α1*$^{-/-}$) SPF C57BL/6 mice highly susceptible to serovar Typhimurium colonization of the lower intestine and subsequent development of colitis within hours after infection. Investigations on long-term colitis were limited by the systemic disease emerging in parallel and leading to death of the susceptible animals at day 5 or 6 p.i. It was of interest to investigate whether resistant mice (23) would also develop colitis after streptomycin treatment and infection with serovar Typhimurium. For this study we have chosen the well-characterized mouse lines DBA/2 and 129Sv/Ev. The latter animals were of special interest because many transgenic and knockout mice are initially generated in this genotypic background.

Groups of five age- and sex-matched C57BL/6, 129Sv/Ev, and DBA/2 mice that were pretreated with either water or streptomycin (see Materials and Methods) were infected with wild-type serovar Typhimurium. As a control, groups of five age- and sex-matched C57BL/6, 129Sv/Ev, and DBA/2 mice were pretreated with streptomycin and mock infected with PBS. The mice were sacrificed at day 3 p.i. or at day 1 post-

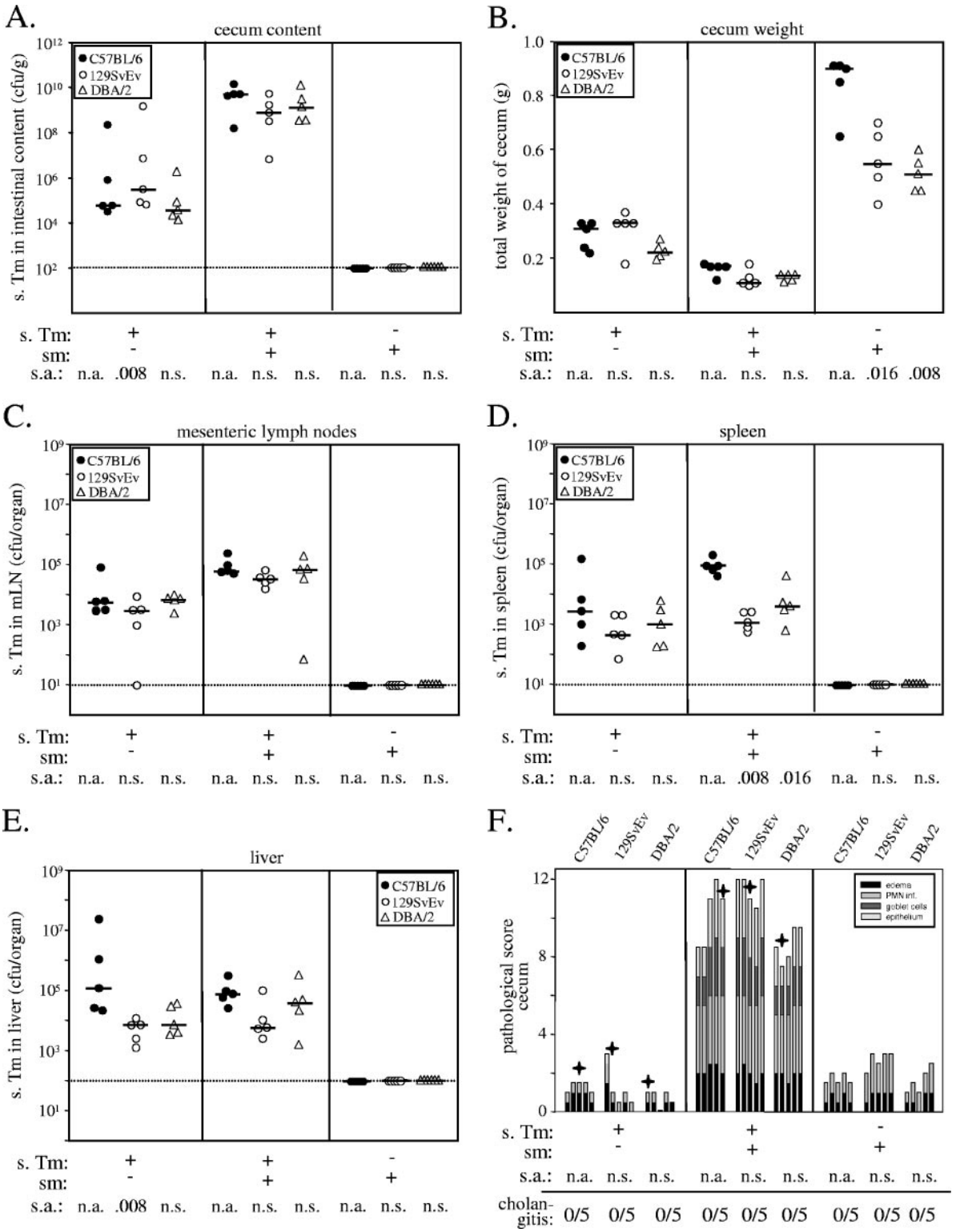



FIG. 1. Serovar Typhimurium infection of $H_2O$- or streptomycin-pretreated C57BL/6, 129Sv/Ev, and DBA/2 mice. Five C57BL/6, 129Sv/Ev, or DBA/2 mice per group were pretreated with either $H_2O$ or streptomycin and then infected intragastrically with $5 \times 10^7$ CFU wild-type serovar Typhimurium for 3 days. Five C57BL/6, 129Sv/Ev, or DBA/2 mice per group were pretreated with streptomycin and mock infected intragastrically with PBS for 1 day. (A) Bacterial loads in the cecal contents; (B) weight of cecum; (C) bacterial loads in the mLN; (D) bacterial loads in the spleen; (E) bacterial loads in the liver. Solid circles, C57BL/6 mice; open circles, 129Sv/Ev mice; triangles, DBA/2 mice. Dotted line, limit of detection. s. Tm, serovar Typhimurium; sm, streptomycin; s.a., statistical analysis versus C57BL/6; n.a., not applicable; n.s., not significant. (F) Histopathological analyses. HE-stained sections of cecal tissue were scored with respect to edema in the submucosa (black), PMN infiltration (medium gray), reduction in the number of goblet cells (dark gray), and desquamation/erosion/ulceration of the epithelial layer (light gray) (see Materials and Methods). Scores were plotted as stacked vertical bars. No cholangitis (indicated at the bottom as number of animals with cholangitis/total number of animals in the group) was observed in any animal from any group. Plus signs indicate animals for whom data area shown in Fig. 2.

mock infection, respectively, and analyzed as described in Materials and Methods.

As anticipated, serovar Typhimurium was not detected in the ceca or any of the organs of the mock-infected groups (Fig. 1A, C, D, and E, right panels). The weight of the cecum was considerably higher in the mock-infected groups than in any of the other groups (Fig. 1B), including the untreated control animals (data not shown). Enlarged cecal volumes are typically observed in germfree animals (37) and have been described earlier for streptomycin-pretreated mice (1). We also observed that the weights of the ceca of the streptomycin-pretreated, mock-infected C57BL/6 mice were significantly higher than those of 129Sv/Ev and DBA/2 mice (Fig. 1B, right panel) ($P = 0.016$ and $P = 0.008$, respectively). Without streptomycin pretreatment, cecum loads of serovar Typhimurium-infected C57BL/6, 129Sv/Ev, and DBA/2 mice were consistently low (median, $10^4$ to $10^6$ CFU/g) (Fig. 1A, left panel) and no differences could be found between the three mouse strains ($P \geq 0.05$). In contrast, the ceca of all streptomycin-pretreated, serovar Typhimurium-infected mice were highly colonized (medians, $5 \times 10^9$, $8 \times 10^8$, and $2 \times 10^9$ CFU/g for C57BL/6, 129Sv/Ev, and DBA/2 mice, respectively) (Fig. 1A, middle panel), and cecum weights were significantly lower than in the mock-infected animals, suggesting inflammation-induced changes ($P < 0.05$) (Fig. 1B, middle panel).

In line with earlier data (1), serovar Typhimurium loads in mLN were significantly higher in streptomycin-pretreated than in water-pretreated groups ($P < 0.05$; medians, $6 \times 10^4$, $3 \times 10^4$, and $7 \times 10^4$ versus $6 \times 10^3$, $3 \times 10^3$, and $7 \times 10^3$ CFU/organ for C57BL/6, 129Sv/Ev, and DBA/2 mice, respectively) (Fig. 1C) but did not differ significantly between susceptible C57BL/6 and resistant DBA/2 and 129Sv/Ev mice ($P \geq 0.05$).

When comparing resistant and susceptible mouse-strains, we found significantly higher serovar Typhimurium colonization of the spleens of streptomycin-pretreated C57BL/6 mice (median, $9 \times 10^4$ CFU/organ versus $1 \times 10^3$ and $3 \times 10^3$ CFU/organ in 129Sv/Ev and DBA/2 mice, respectively; $P = 0.008$ and $P = 0.016$, respectively).

The histopathology of serovar Typhimurium colitis was similar among streptomycin-pretreated C57BL/6, DBA/2, and 129Sv/Ev mice (Fig. 1F and 2G to L). Cecal and colon tissues were all characterized by submucosal edema, severe PMN infiltration into the lamina propria, PMN transmigration into the intestinal lumen (Fig. 2J, K, and L) (data not shown), ulceration of the epithelium, and severely reduced numbers of goblet cells. In the DBA/2 mice, signs of inflammation were significantly lower than in the 129Sv/Ev animals (Fig. 1F, middle panel) ($P < 0.05$). Crypt abscesses were frequently observed in all three mouse strains (data not shown).

None of the water-pretreated, serovar Typhimurium-infected animals showed inflammatory lesions in the cecum or colon (Fig. 2A to F) (data not shown). Interestingly, four of the water-pretreated, serovar Typhimurium-infected DBA/2 mice and four of the 129Sv/Ev mice showed mucosal inflammation restricted to the terminal ileum in the vicinity of a Peyer's patch (PMN infiltration, loss of goblet cells, and epithelial damage) (data not shown). These lesions were similar to the pathology observed in the large intestines of streptomycin-pretreated mice infected with serovar Typhimurium. Similar observations are only rarely made for C57BL/6 mice (B. Stecher, S. Hapfelmeier, and W.-D. Hardt, unpublished observation). This observation might be of interest for future studies of *Salmonella* enteritis but has not been analyzed further here.

From these data we conclude that the development of serovar Typhimurium colitis during the acute phase (up to 3 days p.i.) was similar in resistant (129Sv/Ev and DBA/2) and susceptible (C57BL/6) mice.

**Time course of serovar Typhimurium infection in C57BL6, 129Sv/Ev, and DBA/2 mice.** Next, we sought to exploit the natural resistance of 129Sv/Ev and DBA/2 mice to study the long-term development of serovar Typhimurium colitis in mice. For a time course experiment, we infected two groups (five mice per group) of streptomycin-pretreated C57BL/6 mice, six groups of streptomycin-pretreated 129Sv/Ev mice, and four groups of streptomycin-pretreated DBA/2 mice with wild-type serovar Typhimurium. At days 1, 3, 7, 14 (or 10 or 11 [see below]), 20, and 43 p.i., respectively, mice were sacrificed, and serovar Typhimurium loads in the cecal content, the mLN, and the liver were determined. Pathological changes in the cecum, colon, ileum, and liver were analyzed.

As found in previous studies using streptomycin-pretreated C57BL/6 mice (1, 34), serovar Typhimurium loads in the cecal contents of C57BL/6 mice ranged between $10^8$ and $10^{10}$ CFU/g at day 1 p.i. (Fig. 3A). Strong inflammation of the cecum was already observed at day 1 p.i.; PMN infiltration, epithelial damage, and the loss of goblet cells were more severe at day 3 p.i. (Fig. 3D).

Bacterial loads in the mLN and livers of C57BL/6 mice increased between day 1 and day 3 p.i. (medians, $1 \times 10^5$ CFU/organ and $3 \times 10^4$ CFU/organ, respectively) (Fig. 3B and C). This is typical for systemic typhoid-fever-like infections in susceptible C57BL/6 mice.

In streptomycin-pretreated DBA/2 mice, we generally observed $10^9$ to $10^{10}$ CFU serovar Typhimurium/g of cecal content toward the end of the experiment (11 days p.i.) (Fig. 3A). For unknown reasons, cecal colonization at day 3 p.i. was lower than at the other time points (compare also Fig. 3A and 1A).

At day 10 p.i., two of the DBA/2 mice showed signs of terminal systemic disease and were sacrificed. Only one of the sacrificed animals was analyzed further for colonization and inflammation (Fig. 3). The remaining three animals from this group became moribund at day 11 p.i. and were sacrificed. Thus, DBA/2 mice were indeed more resistant than C57BL/6 mice (Fig. 3) but were not entirely protected from fatal systemic infection at day 10 p.i. No experiments with longer infection times were carried out with the DBA/2 strain.

In sharp contrast to the DBA/2 mice, few external signs of systemic disease were noted with 129Sv/Ev mice throughout the course of the experiment (43 days). Only 2 out of 10 infected 129Sv/Ev mice became moribund between days 14 and 20 p.i. and had to be sacrificed. Those animals were not analyzed further. In an independent experiment, two streptomycin-pretreated 129Sv/Ev mice were infected with serovar Typhimurium for 20 days, analyzed as described above, and included in Fig. 3 (Fig. 3A, B, C, and D).

Cecal colonization of serovar Typhimurium in 129Sv/Ev mice at days 1 and 3 p.i. was similar to colonization in C57BL/6 mice ($10^9$ CFU/g). Cecal loads at days 7 and 14 p.i. were yet lower (medians, $3 \times 10^6$ and $6 \times 10^6$ CFU/g, respectively), but

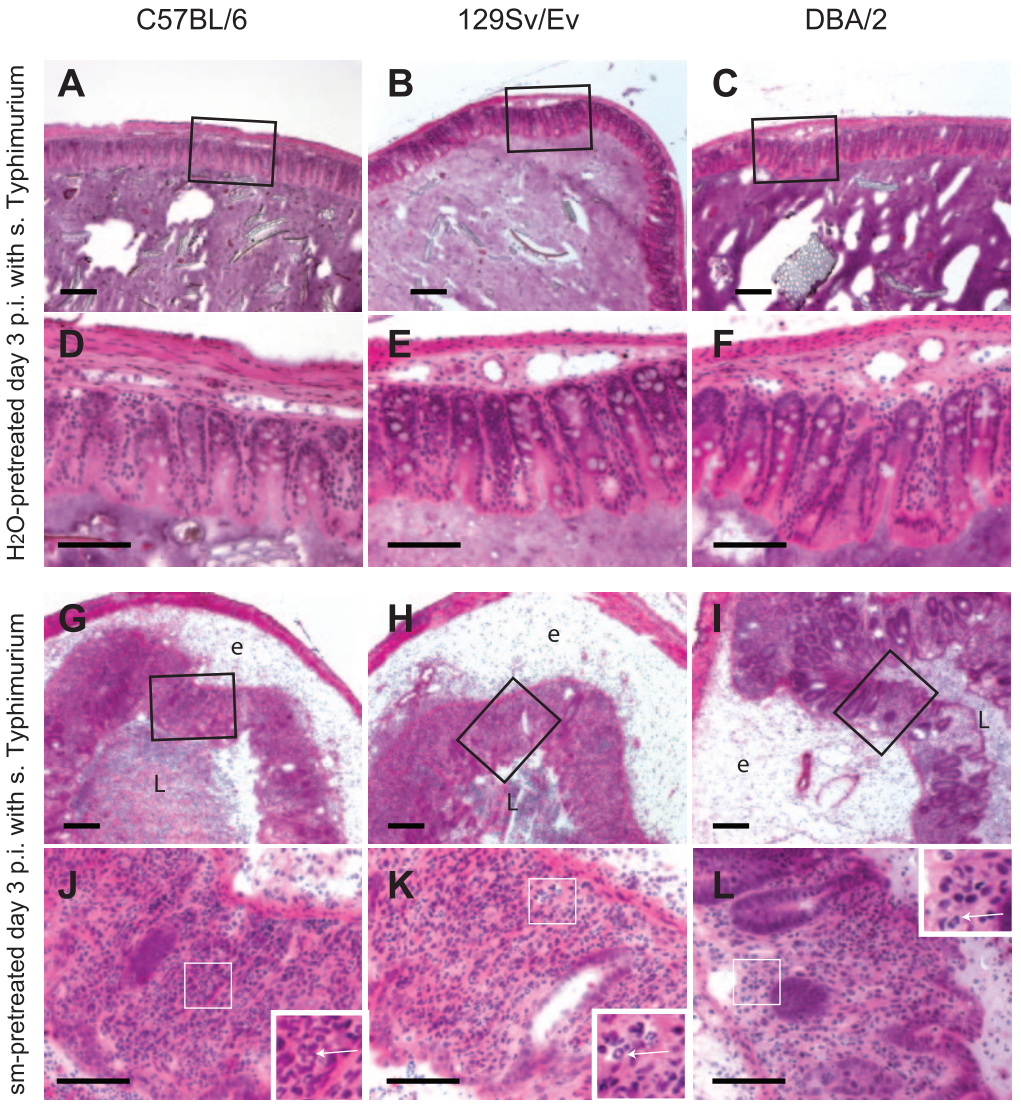



FIG. 2. Pathological changes in ceca of $H_2O$-pretreated or streptomycin-pretreated C57BL/6, 129Sv/Ev, and DBA/2 mice infected with wild-type serovar Typhimurium. Thin sections (thickness, 5 μm) of cecal tissues of mice from the experiment described in the legend to Fig. 1 were HE stained as described in Materials and Methods. (A to F) $H_2O$-pretreated serovar Typhimurium-infected C57BL/6, 129Sv/Ev, or DBA/2 mice; (G to L) streptomycin-pretreated serovar Typhimurium-infected C57BL/6, 129Sv/Ev, or DBA/2 mice. e, edema; L, cecal lumen. Panels D to F and J to L (bars, 100 μm) are enlargements of the boxed areas in panels A to C and G to I (bars, 200 μm), respectively.

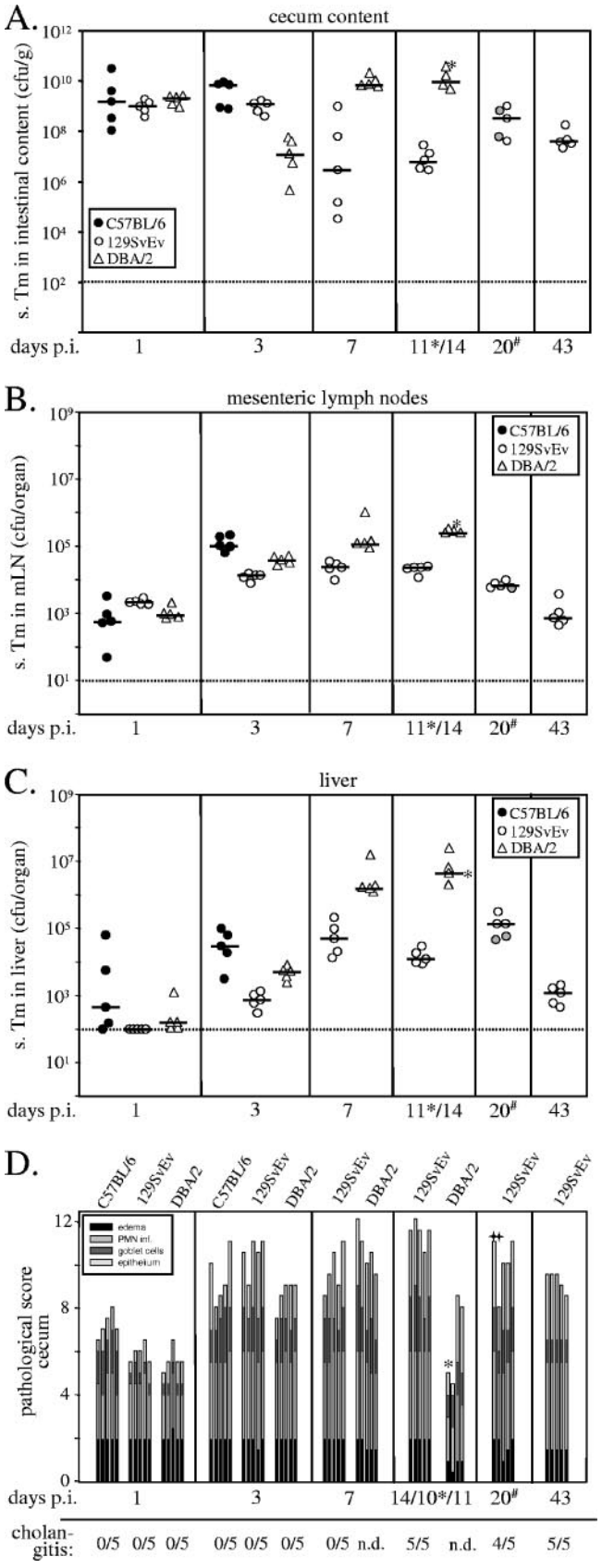
at days 20 and 43 p.i., cecal colonization levels increased again (medians, $3 \times 10^8$ and $4 \times 10^7$ CFU/g, respectively) (Fig. 3A).

At day 1 p.i., mLN loads in 129Sv/Ev mice were slightly but significantly higher than in both C57BL/6 and DBA/2 mice (median, $2 \times 10^3$ CFU/organ; $P < 0.05$). At all later time points, mLN colonization was consistently lower in 129Sv/Ev mice ($P < 0.05$) than in the other two strains, at a level of $10^4$ CFU/organ at days 3, 7, 14, and 20 p.i. and dropping to about $10^3$ CFU/organ at day 43 p.i. (Fig. 3B).

*Salmonella* loads in the livers of 129Sv/Ev mice increased to $10^2$ to $10^3$ CFU/organ at day 3 p.i. and to $10^4$ to $2 \times 10^5$ CFU/organ at days 7, 14, and 20 p.i. By day 43 p.i., liver loads had declined to $10^3$ CFU/liver (Fig. 3C). Thus, bacterial numbers in the livers of 129Sv/Ev mice never exceeded $3 \times 10^5$ CFU/organ. This is in line with our observation that the vast majority of 129Sv/Ev animals did not show visible signs of severe systemic disease upon serovar Typhimurium infection.

**Histopathological changes of the lower intestine.** In line with the data presented above, at days 1 and 3 p.i. serovar Typhimurium similarly induced colitis in streptomycin-pretreated C57BL/6, DBA/2, and 129Sv/Ev mice (Fig. 3D).

At day 7 p.i., cecal pathology in DBA/2 mice included massive infiltrates of inflammatory leukocytes (mainly PMN), severe loss of goblet cells, and large areas of ulceration with loss of the entire epithelial layer and exposure of the underlying granulation tissue. Numerous crypt abscesses were observed, and inflammation was also present in the colons of all five DBA/2 mice (data not shown). Overall, the colon inflammation was somewhat less severe than the cecum inflammation, and we observed adjoining regions of




ulceration and overemphasized epithelial regeneration (formation of fingerlike villi).

At day 10 or 11 p.i., the DBA/2 mice began to show severe signs of terminal systemic illness, and cecal inflammation was less severe at day 10 or 11 p.i. than at day 7 p.i. This also held true for the animal sacrificed at day 10 p.i. (Fig. 3D). In conclusion, streptomycin-pretreated DBA/2 mice cannot be used for long-term serovar Typhimurium infection assays because of a fatal overwhelming systemic infection by day 10 or 11 p.i. This is in line with previous studies in the murine typhoid model for *Salmonella* infection (27, 28).

The 129Sv/Ev mice were more resistant to systemic infection, and intestinal inflammation could be studied for extended periods. At day 7 we observed first signs of a "frustrated" regeneration of the cecal epithelium, as indicated by areas of fast-regenerating intact epithelium with increased numbers of goblet cells. These areas were interspersed with regions of severe ulceration. The extent of the epithelial ulceration and PMN infiltration peaked at day 14 p.i. Nevertheless, a few areas of frustrated regeneration prevailed.

Pathology in the liver was also maximal at day 14 p.i., manifested in strong PMN infiltration in the whole parenchyma, inflammatory foci, and microgranulomas (data not shown). Probably as a result thereof, two 129Sv/Ev mice had to be sacrificed between days 14 and 20 p.i., indicating that the animals went through a critical phase around/after day 14 p.i. However, the large majority of animals survived this critical phase and went on to develop a chronic colitis (see below).

At day 20 p.i., 129Sv/Ev mice displayed first signs of recovery, including reappearance of the intestinal epithelium and sporadic goblet cells (Fig. 4A; compare with Fig. 2H and K). We observed characteristics typical for chronic intestinal inflammation, i.e., crypt branching and overshooting regeneration (polypoid hyperplasia) (Fig. 4B). Nevertheless, some regions with epithelial ulceration still remained (Fig. 4C).

At day 43 p.i., we observed generalized recovery of the cecal epithelium (often associated with polypoid regeneration and crypt branching). The epithelium covered most of the cecal surface (Fig. 4D and E). However, small patches of ulceration

FIG. 3. Time course of serovar Typhimurium infection in streptomycin-pretreated C57BL/6, 129Sv/Ev, and DBA/2 mice. Five streptomycin-pretreated C57BL/6 mice per group were infected intragastrically with $5 \times 10^7$ CFU wild-type serovar Typhimurium for 1 and 3 days. Five streptomycin-pretreated DBA/2 mice per group were infected intragastrically with $5 \times 10^7$ CFU wild-type serovar Typhimurium for 1, 3, 7, and 10 or 11 days. Five streptomycin-pretreated 129Sv/Ev mice per group were infected intragastrically with $5 \times 10^7$ CFU wild-type serovar Typhimurium for 1, 3, 7, 14, 20, and 43 days. (A) Bacterial loads in the cecal contents; (B) bacterial loads in the mLN; (C) bacterial loads in the liver. Solid circles, C57BL/6; open circles, 129Sv/Ev; triangles, DBA/2. Dotted line, limit of detection. (D) Histopathological analyses. HE-stained sections of cecal tissue were scored with respect to edema in the submucosa (black), PMN infiltration (medium gray), reduction in the number of goblet cells (dark gray), and desquamation/erosion/ulceration of the epithelial layer (light gray) (see Materials and Methods). Scores were plotted as stacked vertical bars. The incidence of cholangitis (expressed as number of animals with cholangitis/total number of animals in the group) is indicated at the bottom. Asterisks indicate the DBA/2 animal killed at day 10 p.i. The two 129Sv/Ev animals from the second independent experiment are marked with light gray circles (A to C) or plus signs (D). s. Tm, serovar Typhimurium.

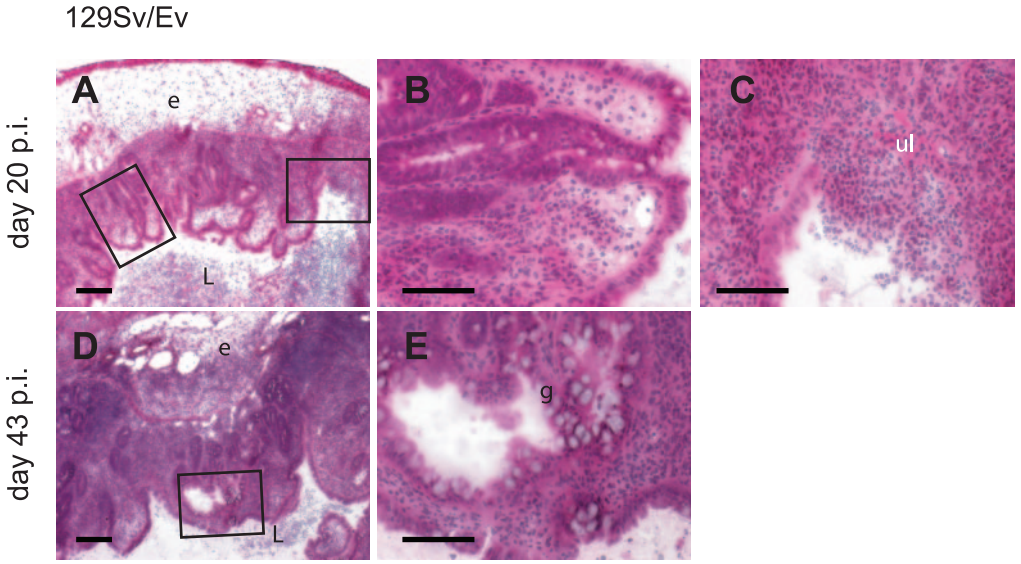



FIG. 4. Pathological changes in ceca of 129Sv/Ev mice chronically infected with wild-type serovar Typhimurium. Thin sections (thickness, 5 μm) of cecal tissues of mice from the experiment described in the legend to Fig. 3 were HE stained as described in Materials and Methods. Streptomycin-pretreated 129Sv/Ev mice were infected for 20 days (A to C) or 43 days (D and E). e, edema; L, cecal lumen; ul, ulceration. Bars, 200 μm (A and D) and 100 μm (B, C, and E). Boxed areas are enlarged in the panels on the right.

remained. Submucosal edema was less pronounced than at the earlier time points. In many regions of the cecum, the number of goblet cells was almost back to normal (Fig. 4E). Overall, a productive regeneration of the cecal epithelium had started by day 43 p.i.

Most notably, the large intestinal submucosae and laminae propriae of chronically infected mice showed extensive cellular infiltrates at day 43 p.i. (Fig. 4D and 5A). The appearance of the cecal mucosa was distinct from that of the acute inflammation manifested in both C57BL/6 and 129Sv/Ev mice at 3 days p.i. (Fig. 2G, H, J, and K and 5G). To illustrate the differences between acute and chronic serovar Typhimurium colitis, we performed a detailed immunohistochemical analysis of consecutive sections. Mucosal infiltrates in chronic colitis contained $B220^+$ cells (e.g., B cells), $CD4^+$ and $CD8^+$ cells (e.g., T cells, some dendritic cells), and high numbers of $CD11c^+$ and $F4/80^+$ cells (presumably dendritic cells and macrophages) (Fig. 5B to F). The number of cellular infiltrates into the submucosa and lamina propria in the chronic inflammation model was considerably higher than in the well-established acute serovar Typhimurium colitis model (Fig. 5G to L).

By day 43 p.i., systemic colonization declined and diffuse PMN infiltration of the liver was reduced, but there were still a small number of granulomatous lesions (Fig. 6A). These were analyzed in more detail by immunohistochemistry of consecutive sections. The lesions contained Ly6G (e.g., PMNs) (Fig. 6B)- and CD68 (e.g., tissue macrophages) (Fig. 6C)-positive cells in the center, and CD4-positive lymphocytic cells, presumably T cells, were also stained within these lesions (Fig. 6D). Such granulomas indicate the onset of an adaptive immune response against serovar Typhimurium.

**Cholangitis is observed in mice with chronic colitis symptoms.** In addition to inflammatory lesions of the liver parenchyma, we observed inflammation of the gall duct epithelium (cholangitis) and a mild portal inflammation in 129Sv/Ev mice chronically infected with serovar Typhimurium. Lesions were characterized by infiltration of Ly6G-positive cells (e.g., PMNs) in the vicinity of the gall duct (distinguished by columnar epithelial cells) (Fig. 7A and B). Reexamination of the livers from all experiments described above revealed that cholangitis occurred highly reproducibly in 129Sv/Ev mice at days 14, 20, and 43 p.i. Inflamed gall ducts were observed as early as day 14 p.i. and remained detectable at all later time points examined (Fig. 1F, 3D, and 7C to I).

To clarify, whether cholangitis is linked to chronic intestinal colonization and/or colitis, we compared the infection in 129Sv/Ev mice that were or were not pretreated with streptomycin (chronic typhoid fever model [25]). Streptomycin- or water-pretreated 129Sv/Ev mice (6 or 5 mice per group, respectively) were infected intragastrically with $5 \times 10^7$ CFU serovar Typhimurium. As a control, we included two mice that were treated with streptomycin and not infected. At day 20 p.i., mice were analyzed for serovar Typhimurium colonization of the cecum, mLN, liver, and spleen as well as for development of cecal inflammation and cholangitis.

Serovar Typhimurium efficiently colonized the ceca of streptomycin-pretreated mice (median count, $1.2 \times 10^8$ CFU/g) but was not detected in any of the other groups (Fig. 8A). Colonization levels of mLN, liver, and spleen were slightly but significantly higher in streptomycin- than in water-pretreated animals (Fig. 8B, C, and D) (median count in mLN, $1 \times 10^4$ versus $3 \times 10^3$ CFU/organ; median count in liver, $1 \times 10^4$ versus $1 \times 10^3$ CFU/organ; median count in spleen, $6 \times 10^3$ versus $5 \times 10^2$ CFU/organ). Colitis and cholangitis were detected exclusively in streptomycin-pretreated, serovar Typhimurium-infected mice (Fig. 8E). Thus, systemic colonization of serovar Typhimurium (i.e., liver) is not sufficient to lead to detectable cholangitis symptoms within 20 days p.i. Chronic intestinal inflammation and/or colonization seems to be required for the development of cholangitis.

Cholangitis has been observed in patients chronically infected with *Salmonella enterica* serovar Typhi and is thought to

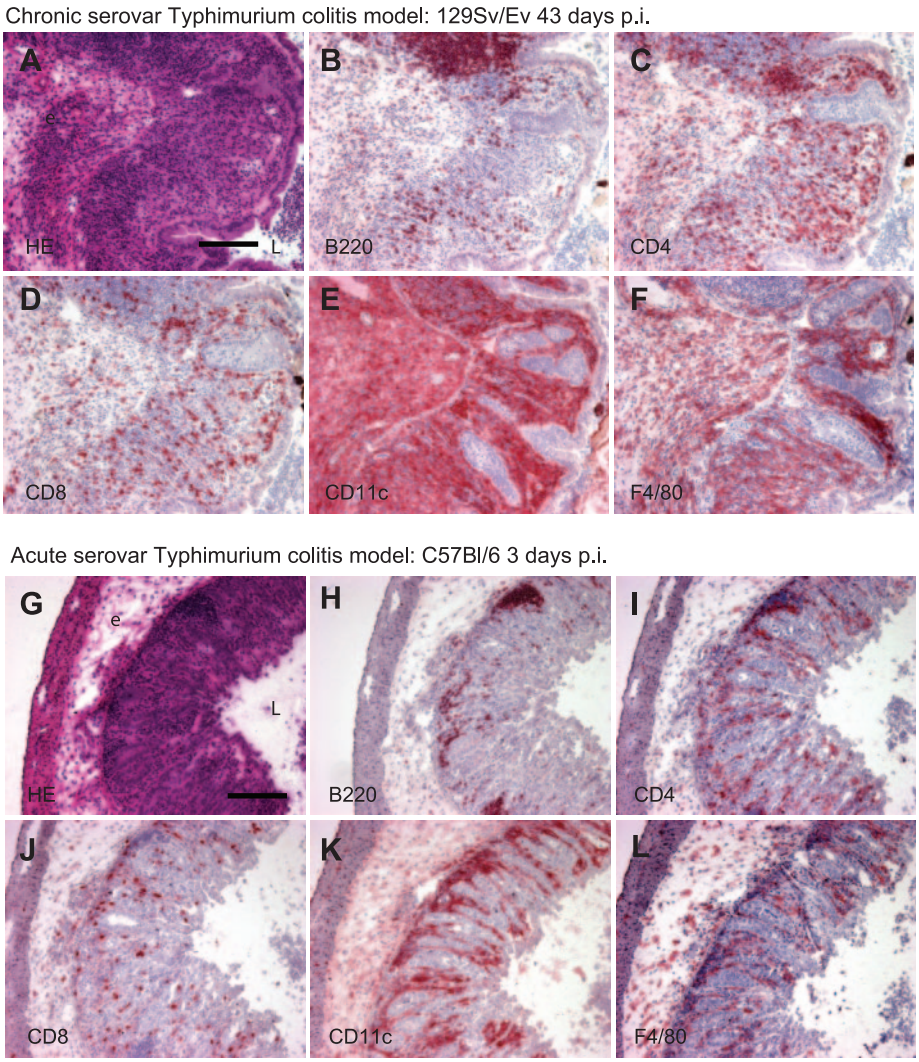



FIG. 5. Immunohistochemical analysis of chronic colitis in 129Sv/Ev mice. Serial thin sections (thickness, 5 μm) of cecal tissues of a streptomycin-pretreated 129Sv/Ev mouse infected for 43 days (A to F) from the experiment described in the legend to Fig. 3 were HE stained and immunohistochemically stained as described in Materials and Methods: Staining with HE (A and G), B220 (B and H), CD4 (C and I), CD8 (D and J), CD11c (E and K), and F4/80 (F and L) is shown. Bars, 200 μm. Tissues from a streptomycin-pretreated C57BL/6 mouse infected for 3 days (acute serovar Typhimurium colitis model) (G to L) with serovar Typhimurium are shown for comparison of the well established acute colitis model and the new chronic serovar Typhimurium colitis model.

be attributable to secondary responses primed by the massive granulomatous disease in the liver parenchyma (10, 30). Furthermore, cholangitis is observed in patients suffering from inflammatory bowel disease.

In conclusion, streptomycin-pretreated 129Sv/Ev mice develop chronic colitis and cholangitis after 2 weeks of infection in a highly reproducible fashion. These symptoms are clearly distinct from the acute streptomycin mouse colitis model described earlier. Long-term serovar Typhimurium infection of streptomycin-pretreated 129Sv/Ev mice may provide a valuable animal model for chronic intestinal inflammation and cholangitis.

## DISCUSSION

In earlier studies we have used a streptomycin-pretreated mouse model to analyze the molecular mechanisms leading to acute intestinal inflammation caused by serovar Typhimurium (1, 14, 33, 34). Since these studies had been performed exclusively with susceptible (*Slc11α1*$^{-/-}$) mice, it remained unclear whether resistant (*Slc11α1*$^{+/+}$) mice would show similar symptoms. Moreover, it was of interest whether resistant mouse strains would constitute an animal model for study of long-term infections. We found that acute serovar Typhimurium colitis was strikingly similar in Slc11α1$^{-/-}$ (C57BL/6) (1) and Slc11α1$^{+/+}$ (DBA/2 and 129Sv/Ev) mice with regard to severe PMN infiltration in the lamina propria, PMN transmigration into the intestinal lumen, submucosal edema, epithelial injury, and pronounced loss of goblet cells. In addition, streptomycin-pretreated 129Sv/Ev mice allowed study of long-term serovar Typhimurium colitis. These animals developed typical symptoms of chronic intestinal inflammation between days 7 and 43 p.i.

Two different Slc11α1$^{+/+}$ mouse strains were included in our study. 129Sv/Ev mice are known to be highly resistant to

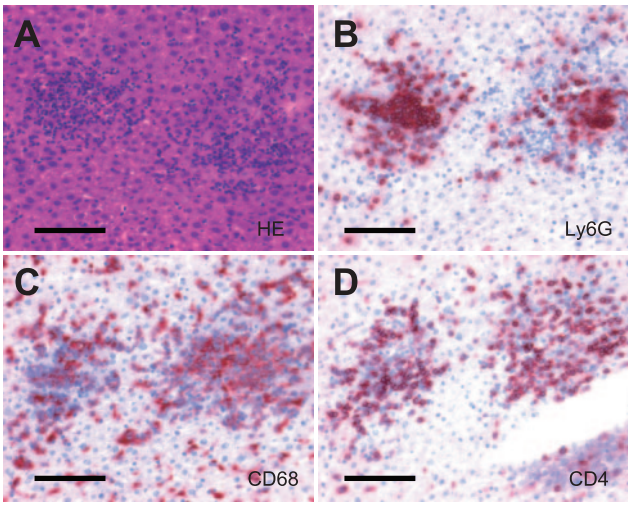



FIG. 6. Pathological changes in the liver of a 129Sv/Ev mouse infected with wild-type serovar Typhimurium for 43 days. Serial thin sections (thickness, 5 μm) of liver tissues of a streptomycin-pretreated 129Sv/Ev mouse infected for 43 days with serovar Typhimurium from the experiment described in the legend to Fig. 3 were HE stained and immunohistochemically stained as described in Materials and Methods. (A) HE; (B) Ly6G; (C) CD68; (D) CD4. Bars, 200 μm.

serovar Typhimurium infection for at least 360 days and have recently been employed for study of persistent infection (25). DBA/2 mice are less resistant to *Salmonella* (27, 28), resulting in a more pronounced systemic infection than that in 129Sv/Ev mice, with a fatal outcome of the disease by days 10 to 11 p.i.

In agreement with a detailed study in the mouse typhoid model (25), streptomycin-pretreated 129Sv/Ev mice were able to control systemic disease in spite of efficient intestinal colonization and severe colitis. In these mice, the acute inflammation progressed to a chronic form of disease between days 7 and 43 p.i. This chronic form of serovar Typhimurium colitis was characterized by reappearance of goblet cells, polypoid epithelial hyperplasia, and crypt branching. In addition, severe mucosal and submucosal infiltrates (T and B cells, dendritic cells, macrophages, and PMNs) were observed. Human cases of chronic diarrheal *Salmonella* infection have also been observed occasionally, and the chronic murine colitis symptoms resemble aspects of the human disease (20, 26, 31). Thus, serovar Typhimurium infection of streptomycin-pretreated 129Sv/Ev mice may provide a useful model for study of bacterial and host factors contributing to chronic colitis at a molecular level.

Unexpectedly, serovar Typhimurium infection of streptomycin-pretreated 129Sv/Ev mice caused inflammation of the gall duct epithelium (cholangitis) after 14 days of infection. This condition was observed neither in mice that received streptomycin only nor in mice orally infected with serovar Typhimurium (typhoid infection model). Therefore, we speculate that cholangitis occurs as a consequence of chronic colitis and that mere systemic colonization by serovar Typhimurium might not be sufficient. Cholangitis has also been observed in humans: 2 to 4% of all patients suffering from chronic colitis develop primary sclerosing cholangitis (10, 30, 36). This is of considerable interest, because cholangitis may represent a significant risk factor for cholangio- and colorectal carcinoma (22). Thus, serovar Typhimurium infection of streptomycin-pretreated 129Sv/Ev mice might provide a useful model for analyzing causal links between colonic inflammation, cholangitis, and cancer.

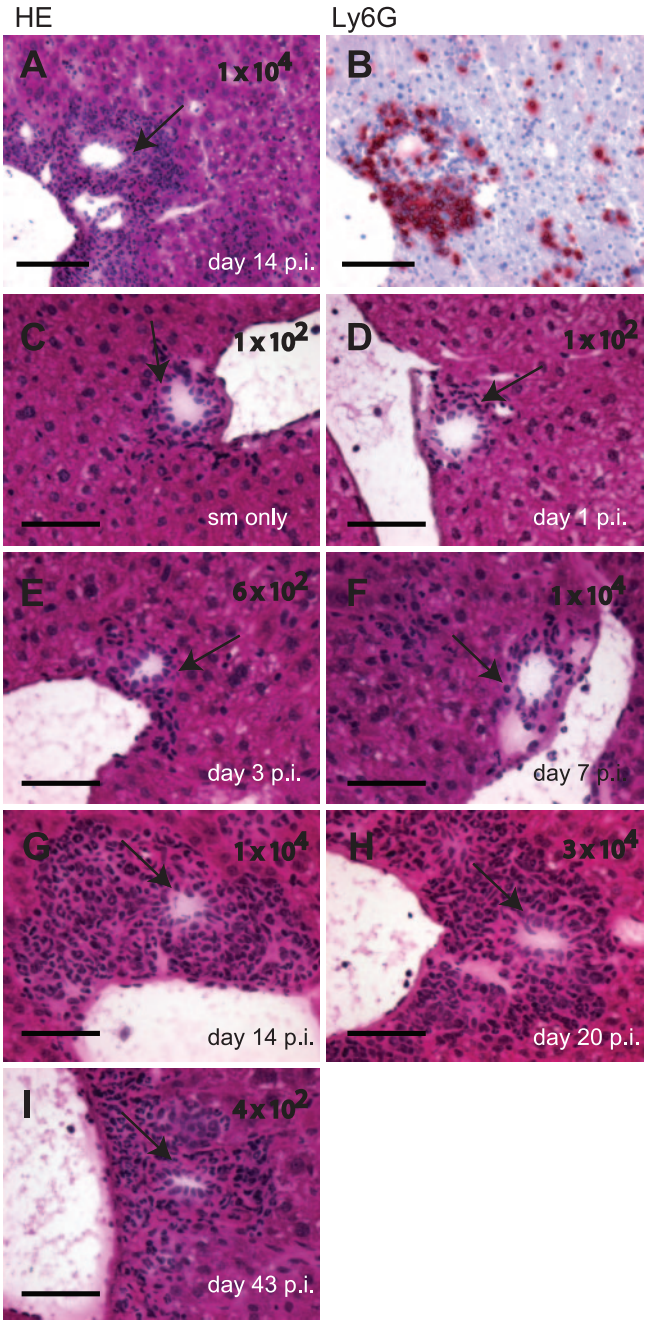



FIG. 7. (A and B) Cholangitis in streptomycin-pretreated, serovar Typhimurium-infected 129Sv/Ev mice. Serial thin sections (thickness, 5 μm) of liver tissues of a 129Sv/Ev mouse infected for 14 days with serovar Typhimurium from the experiment described in the legend to Fig. 3 were HE stained (A) and immunohistochemically stained for Ly6G (B) as described in Materials and Methods. (C to I) Time course of the development of cholangitis. Streptomycin-pretreated 129Sv/Ev mice were from the experiments described in the legends to Fig. 1 and 3. Animals either were not infected (C) or were infected with serovar Typhimurium for 1 day (D), 3 days (E), 7 days (F), 14 days (G), 20 days (H), or 43 days (I). Bars, 200 μm (A and B) and 50 μm (C to I). Arrows point at gall duct epithelium; numbers are CFU/liver in the respective animal.

In conclusion, streptomycin-pretreated 129Sv/Ev mice develop symptoms of acute serovar Typhimurium colitis early

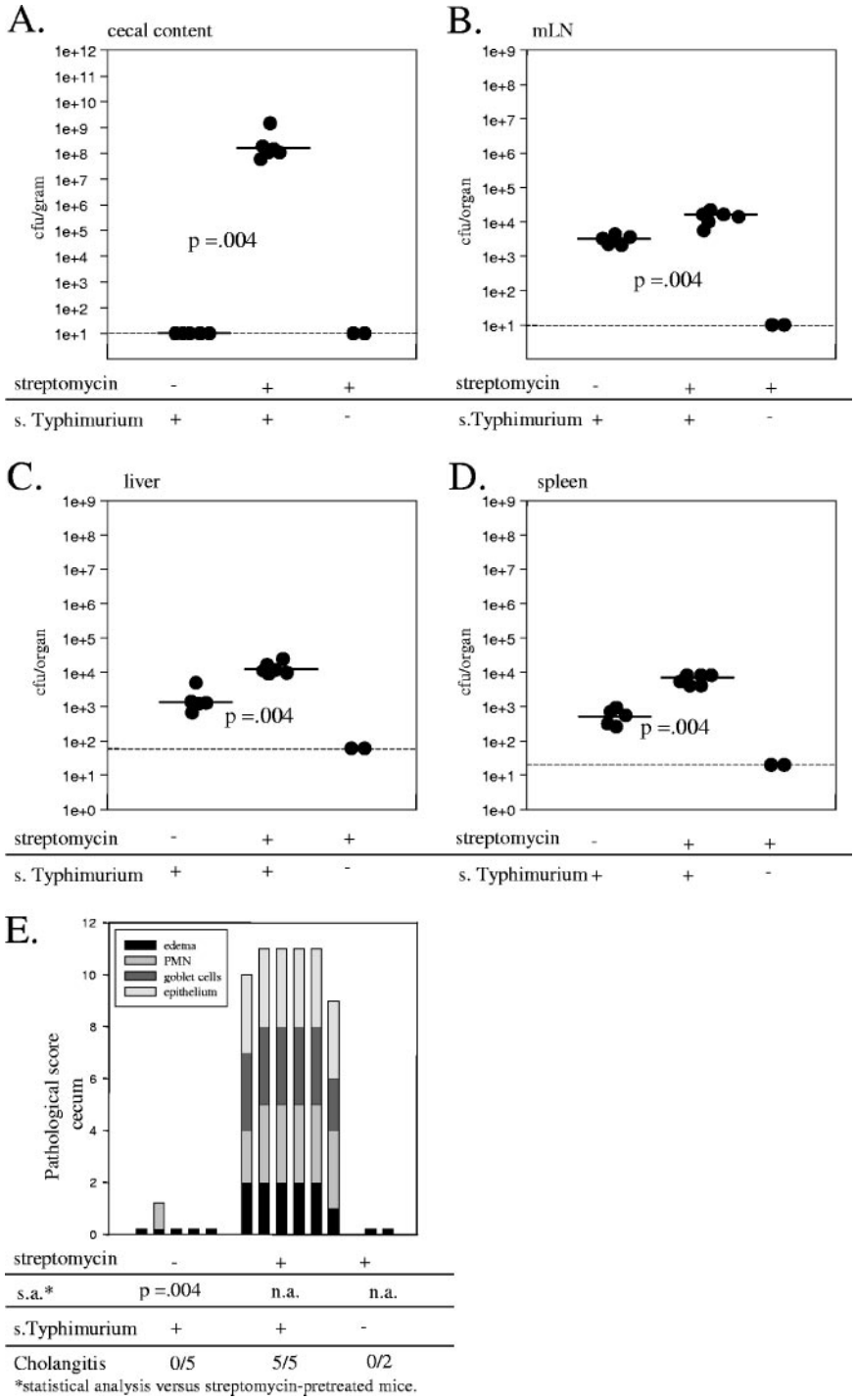



FIG. 8. Chronic infection in models for typhoid fever and colitis. 129Sv/Ev mice were $H_2O$ pretreated (five mice) or streptomycin pretreated (six mice) and then infected intragastrically with $5 \times 10^7$ CFU serovar Typhimurium for 20 days. Two mice were streptomycin pretreated and analyzed 20 days post-streptomycin treatment. (A to D) Bacterial loads in the cecal contents (A), mLN (B), liver (C), and spleen (D). Dotted line, limit of detection. (E) Histopathological analysis. HE-stained sections of cecal tissue were scored with respect to edema in the submucosa (black), PMN infiltration (medium gray), reduction in the number of goblet cells (dark gray), and desquamation/erosion/ulceration of the epithelial layer (light gray) (see Materials and Methods). The incidence of cholangitis (expressed as the number of animals with cholangitis/total number of animals per group) is indicated. Scores were plotted as stacked vertical bars.

after infection. In these mice, the intestinal colonization continues for at least 43 days and acute intestinal inflammation progresses to a chronic state that concomitantly leads to the complication of cholangitis. This may provide a useful model for study of the pathogenetic mechanisms of chronic colitis with the advantage that the host (i.e., mouse) and the causative agent (i.e., *Salmonella*) are easily accessible to genetic manipulations.

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