Mucosal Lymphoid Infiltrate Dominates Colonic Pathological Changes in Murine Experimental Shigellosis

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Background. Shigella species are invasive human pathogens that cause acute rectocolitis by triggering a dysregulated inflammatory reaction in the colonic and rectal mucosa. Because mice are naturally resistant to shigellosis, there is no mouse model that mimics human disease. We explore the susceptibility of intestinal flora-depleted mice to shigellosis after intragastric infection with Shigella strains.

Methods. Mice given 5 g/L streptomycin as a beverage were infected intragastrically with cfu of either invasive or noninvasive Shigella strains.

Results. We found that invasive Shigella strains persist up to 30 days in feces, whereas the persistence of noninvasive Shigella strains was reduced. Colonization primarily involves the colon and the cecum and, to a lesser extent, the ileum. The hallmark of inflammation in the intestinal tissue is a dramatic expansion of the lymphoid follicles, in which a high apoptotic index is recorded.

Conclusions. We provide a murine model in which shigellae are able to reach their natural tissue target: the colon. Moreover, the absence of polymorphonuclear leukocyte recruitment and of epithelial cell lesions reveal some aspects of shigellosis that are usually hidden by the prevalence of this cell population. This novel model may contribute to the identification of new targets for vaccines and therapies.

Shigellosis is an acute rectocolitis [1] provoked by the ingestion of as few as 10–100 Shigella colony-forming units [2], and >150 million cases occur every year [3]. Shigellae invade the colonic mucosa by translocating through M cells of the follicle-associated epithelium that covers lymphoid nodules dispersed on the colorectal surface [4]. From there, shigellae penetrate intestinal epithelial cells (IECs), in which they inject effector proteins via a type III secretion apparatus [5]. These events initiate inflammation through the apoptotic killing of infected macrophages [6], due to the activation of caspase-1, resulting in the parallel release of mature interleukin (IL)–1β [7] and, possibly, a decreased expression of IL-1 receptor antagonist [8]. Invaded IECs produce proinflammatory cytokines and chemokines, particularly IL-8, a potent activator of polymorphonuclear leukocytes (PMNLs) [9, 10], after intracellular sensing of a peptidoglycan motif [11] via nucleotide-binding oligomerization domain protein 1 [12]; inflammation thus increases in intensity and extends according to the progression of shigellae in the epithelium. This is an essential feature, because mucosal inflammation disrupts the epithelial barrier and facilitates further invasion of the epithelium by shigellae [13–15]. PMNLs eventually eliminate invasive shigellae [16] by blocking their escape from the phagocytic vacuole. In summary, eradication of shigellae at the innate stage of the immune response occurs at the expense of massive inflammatory destruction of the epithelium. Although mice are the ideal animals to use in the experimental study of human infectious diseases, because of their natural resistance to intestinal infection by Shigella species, to date, alternative animal systems have
been used [17–20]. It is unclear why mice are resistant to developing shigellosis. Three major hypotheses have been put forth to account for this species specificity: (1) *Shigella* species cannot compete in the mouse colonic environment, which consists of commensal flora, mucins, and associated antimicrobial products; (2) mice lack a receptor that allows binding to and invasion of IECs; and (3) the murine epithelium is unable to relay and amplify the inflammatory reaction initiated by solitary nodules. In an attempt to understand the respective relevance of these hypotheses in the pathogenesis of the natural disease, we have developed a novel experimental mouse model. In this model, mice are intragastrically (ig) infected with *S. flexneri* 5a in the presence of the antibiotic streptomycin (Sm). Under these conditions, the murine colonic mucosa senses the presence of shigellae and mounts an inflammatory reaction characterized by a paucity of recruitment of PMNLs and destruction of the epithelial lining.

**MATERIALS AND METHODS**

**Bacteria, growth conditions, and genetic procedures.** The *S. flexneri* 5a strains used were the Sm-resistant (SmR) fully virulent wild-type strain M90T [21] and its avirulent Sm-susceptible (SmS) variant BS176, which lacks the virulence plasmid pWR100 [22, 23]. M90T SmR is a spontaneous SmR variant [21] of the original clinical isolate M90T [22]. BS176 is the original plasmidless variant of M90T [22]. To obtain BS176 SmR, BS176 was the receiver in an experiment of generalized P1-mediated transduction that was performed as described elsewhere [24] following Miller’s protocol [25] and using M90T SmR as the donor. M90T SmR and BS176 SmR were able to survive in media containing up to 5 mg/mL of Sm. *Shigella* strains were grown on trypticase soy broth (Becton Dickinson) or Hektoen enteric agar (HEA) (Oxoid). Sm (Sigma) was used at a concentration of 100 μg/mL.

**Infection of mice.** A minimum of 20 five-week-old female BALB/c mice (Charles River) were used per experimental group and received a 5 g/L Sm solution as a beverage for 48 h. After a 6-h starvation period, mice were infected ig by a polyethylene feeding tube with 1 × 10⁸ cfu of *S. flexneri* 5a strain M90T SmR or strain BS176 SmR in 100 μL of sodium bicarbonate (1.4%). The antibiotic treatment was maintained until the end of the experiment. A group of uninfected mice, which was otherwise treated as above, was used as a control.

**Recovery of shigellae from feces and organs of infected mice.** Feces were collected daily and examined for the presence of shigellae. Feces from 5 mice (0.2–0.3 g) were individually collected and homogenized in a sample tube containing 5 mL of saline solution (0.9% NaCl), serially diluted, and plated on plates with HEA and Sm. Bacterial counts were reported as colony-forming units per gram of feces. When this threshold was reached, the fecal pellets were collected, analyzed by individual mouse, and reported as colony-forming units per gram of feces as above. The minimum detectable value was 5 cfu/fecal pellet.

Relevant organs from mice euthanized at the desired time points were removed and prepared for histopathological examination, reverse-transcription polymerase chain reaction (RT-PCR) analysis, and counts of viable shigellae. For bacterial
Table 1. Histopathological scores for lesions in intestinal tissues from mice infected with *Shigella flexneri* 5a strain M90T or BS176 and from uninfected control mice.

<table>
<thead>
<tr>
<th>Group, hours after infection, organ</th>
<th>Score for interstitial areas</th>
<th>Score for epithelial damagea</th>
<th>Score for transcytosisb/c</th>
<th>Score for mononuclear cellsd</th>
<th>Score for lymphoid folliclesf</th>
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<tr>
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NOTE. A total of 6 mice were used in each group. P = .0022 for epithelial damage in colon from M90T-infected mice vs. BS176-infected mice at 72 and 120 h after infection and for lymphoid follicle activation of colon of M90T-infected mice vs. BS176-infected mice at 72 and 120 h after infection (Mann-Whitney U test).

a Scoring for transcytosis across the epithelium: 0, <10 cells; 1, 10–19 cells; 2, 20–49 cells; 3, 50–100 cells; 4, >100 cells.

b Scoring for epithelial damage: 0, absence of cellular damage; 1, presence of cytoplasmic vacuolization in some epithelial cells; 2, diffuse cellular vacuolization; 3, diffuse vacuolization and necrosis/detachment of singular/sporic epithelial cells; 4, diffuse and severe cellular vacuolization with areas of cellular detachment (groups of cells with microvesicular status).

c Scoring for neutrophils per high-power field: 0, <4 cells; 1, 5–19 cells; 2, 20–49 cells; 3, 50–100 cells; 4, >100 cells.

d Scoring for mononuclear cells per high-power field: 0, <49 cells; 1, 50–100 cells; 2, 101–200 cells; 3, 201–600 cells; 4, >600 cells.

e The mean areas of 10 different randomly selected follicles in gut-associated lymphoid tissues for each sample was measured. Scoring for mean area of activated lymphoid follicles: 0, none; 1, up to 0.17 mm²; 2, up to 0.32 mm²; 3, up to 0.27 mm².

Counts, tissue sections were placed in ice-cold saline solution (5 mL for liver, 4 mL for colon and ileum, and 2 mL for spleen and cecum) and homogenized, and serial dilutions were plated on plates with HEA and Sm. Bacterial counts were reported as colony-forming units per organ. The minimal detectable values were 50 cfu/liver, 40 cfu/ileum, 40 cfu/colon, 20 cfu/spleen, and 20 cfu/cecum.

**Histopathological analysis.** Samples for histopathological and immunohistochemical (IH) analysis were taken from the same areas of the organ of interest from 6 randomly chosen mice in each experimental group. For histopathological examination, 3-µm sections were deparaffinized, rehydrated, and stained with hematoxylin-eosin. The following monoclonal antibodies (MAbs) were used: mouse anti-*S. flexneri* 5a lipopolysaccharide (LPS; dimeric IgA; 6 mg/mL) [18], rat anti–murine CD3+ T lymphocytes, rat anti–murine CD8+ T lymphocytes (KT15), rat anti–murine macrophages (F4/80 antigen), rat antidendritic/interdigitating cells (Midc-8) (all from Serotec), rat anti–murine CD5+ B lymphocytes, mouse anti–murine CD21+ B lymphocytes, rat anti–murine CD32+CD16+ NK cells, and rabbit polyclonal IgG anti-mouse IL-2Rα (CD25) (all from Santa Cruz Biotechnology). For IH analysis, tissue sections were treated as described elsewhere [26]. Binding of antibodies was shown by use of avidin-biotin immunoperoxidase or avidin-biotin alkaline phosphatase (both from Vector Laboratories) with biotin-conjugated rabbit anti–rat IgG (Vector Laboratories) and biotinylated goat anti–mouse IgG (AO433; DAKO) (both at 1:200), respectively. The reactions were visualized with 3-1-diaminobenzidine (DAB; Sigma), Very Intense Purple, or Vector Blue and Vector Red (all from Vector Laboratories). For multiple stainings, the first chromogen used was DAB, with or without nickel addition, the second was VIP, the third was Vector Red, and the last was Vector Blue, with Meyer’s hematoxylin used as a nuclear counterstain.

The number of inflammatory cells and of lymphoid aggregates was assessed at ×400 and ×100 magnification, respectively, was scored as described by Dixon et al. [27], and was customized for murine samples as described by Happonen et al. [28]. The proapoptotic effect induced by *S. flexneri* was highlighted through TUNEL colorimetric staining (DeadEnd; Promega), in accordance with the manufacturer’s instructions.

**RNA extraction and RT-PCR.** Total RNA from homogenized ileum or colon of 3 mice per experimental group was extracted using Trizol solution (Invitrogen), in accordance with the manufacturer’s instructions. RT of total RNA (1 µg) and cDNA PCR was performed using the Super-Script One-Step RT-PCR with Platinum Taq (Invitrogen), in accordance with the manufacturer’s instructions. PCR conditions, primers, and densitometry are described elsewhere [24].

**ELISA.** Peripheral blood was obtained from the caudal vein at 24, 48, 72, and 96 h after infection. IL-6 levels in serum were determined by solid-phase ELISA (R&D Systems). The absorbance was measured at 450 nm, and concentrations were determined by interpolation of a standard calibration curve.

**Analysis of data.** The exact Mann-Whitney U test was used for statistical analysis of bacterial counts in feces and the cal-
culation of individual histopathological scores. \( P < 0.05 \) was considered to be statistically significant.

**RESULTS**

**Establishment of optimal conditions for infection.** The experimental conditions were determined on the basis of the results of several experiments aimed at evaluating the role that the resident flora play in establishing infection, the impact that different doses of Sm have on colonization of *S. flexneri*, and the distribution of microorganisms in the organs.

Mice were infected intragastrically with \( 1 \times 10^8 \) cfu of *S. flexneri* 5a SmR91 wild-type strain M90T. Shigellae persisted up to 3 days in feces. Bacterial counts in feces were \( 5 \times 10^6 \) cfu/g of feces at day 1, \( 7 \times 10^7 \) cfu/g of feces at day 2, and \( 1 \times 10^8 \) cfu/g of feces at day 3 after infection.

To assess how resident bacterial flora could affect colonization of *S. flexneri*, an antibiotic treatment was applied to eliminate local flora. We found that 5 g/L Sm given as a beverage 48 h before infection was sufficient to abolish all detectable aerobic flora from feces. M90T was administered at 3 doses (\( 1 \times 10^7 \), \( 1 \times 10^8 \), and \( 1 \times 10^9 \) cfu), and the inoculum of \( 1 \times 10^8 \) cfu was selected for use in the final experiments, because it allowed for an improved bacterial persistence in the feces, compared with that of the other inocula. The following protocol was applied in the final experiments: at 48 h before infection, a minimum of 20 mice per experimental group were given Sm (5 g/L) as a beverage, and the treatment was maintained until shigellae were cleared from the feces. After a 6-h starvation period, mice were infected with \( 1 \times 10^8 \) cfu of *S. flexneri* 5a strain M90T or BS176. Feces were collected daily. At different time points, depending on the experiment, at least 3 mice were euthanized, and the relevant organs were removed.

From day 1, most shigellae were present in the colon, cecum, ileum, and liver. This trend remained unvaried during the course of 7 days of infection, with a significant increase in the number of shigellae in the liver at day 7, compared with that
Figure 3. Histopathological characterization of colonic epithelium and lamina propria (LP). A–C, Hematoxylin-eosin (HE) staining of colonic sections from mice infected with Shigella flexneri 5a strain M90T (A) or BS176 (B) and from uninfected control mice (C) at 120 h after infection. In A, arrowheads point to transcytosis of polymorphonuclear leukocytes (PMNLs) throughout the epithelium. In B, the arrowhead indicates a PMNL under the LP. D–F, HE staining of chorion sections from mice infected with M90T (D) at 72 h after infection or BS176 (E) at 120 h after infection and from uninfected control mice (F). G–I, Immunohistochemical characterization of the interstitial monocyte infiltrate in sections of the LP from mice infected with M90T (G) or BS176 (H) and from uninfected control mice (I) at 120 h after infection. Avidin-biotin immunoperoxidase labeling of F4/80-positive macrophages immunostained with an IgA mouse anti–S. flexneri 5a LPS monoclonal antibody (brown and arrowheads) and CD3+ T lymphocytes (blue). A–C, Bars, 25 μm. D–I, Bars, 50 μm.
Figure 4. Immunohistochemical (IH) characterization of lymphoid follicles at 120 h after infection. A and G, Hematoxylin-eosin staining of the colonic mucosa (CM) showing the dramatic expansion of the lymphoid follicles in mice infected with *Shigella flexneri* 5a strain M90T (A) with respect to those in mice infected with strain BS176 (G). B and H, IH labeling showing CD3+ T lymphocytes (brown) in the follicles of mice infected with M90T. C and I, IH labeling showing a subpopulation of CD3+CD25+ lymphocytes in the follicles of mice infected with BS176. D and L, IH staining showing CD32+CD16+ NK cells (red and arrowheads) and CD8+ T lymphocytes (brown and arrows) around the follicles in the CM of mice infected with M90T (D) or BS176 (L). E and M, IH staining showing CD5+ B lymphocytes (purple-violet), interspersed dendritic cells (black), CD21+ B lymphocytes (brown), CD3+ T lymphocytes (blue), and CD32+CD16+ NK cells (red) in lymphoid follicles of mice infected with M90T (E) or BS176 (M). F and N, Schematic representation of E and M, respectively. Bars, 50 μm.
at day 1 (figure 1A). Histopathological analysis confirmed relevant morphological changes at early time points in the liver, colon, and, to a lesser extent, cecum (data not shown).

**Bacterial persistence.** To assess whether invasiveness plays a role in bacterial persistence and distribution, we compared Ig infection with M90T and infection with its noninvasive variant Sm" strain BS176. M90T persisted in feces for a mean ± SD of 30.2 ± 2.5 days, whereas BS176 persisted for a mean ± SD of 19.0 ± 2.2 days (figure 1B). After 7 days, the number of BS176 colony-forming units in feces rapidly decreased, whereas the number of M90T colony-forming units remained high. Similarly, during the first 7 days of infection, bacterial counts in the colon and cecum did not vary for either strain, with the number of colony-forming units of BS176 being ~1 log lower than the number of colony-forming units of M90T in these organs (data not shown).

**Histopathological features.** Histopathological analysis was performed on tissue sections removed from the colon and cecum of mice euthanized at 24, 72, and 120 h after infection. The severity of tissue inflammation was evaluated by recording qualitative and quantitative alterations of the intestinal mucosa as reported elsewhere [27, 28]. These alterations were quantified and recorded as the mean of scores calculated for each lesion, and these scores are reported in table 1.

In the intestinal tissue of M90T-infected mice, we observed a relative paucity of PMNLs, compared with the massive numbers of mononuclear cells and the dramatic expansion of the lymphoid follicles of gut-associated lymphoid tissues (GALT). In the intestinal tissue of BS176-infected mice, only a moderate number of mononuclear cells were found at 120 h after infection.

**Localization of the shigellae in the tissues.** The distribution of shigellae in the gastrointestinal tract was highlighted by immunostaining for *S. flexneri* LPS. The cecal and colonic contents of M90T-infected mice were strongly stained for *S. flexneri* LPS. However, although a scant positive result for *S. flexneri* LPS was found in the cecal mucosa, a stronger presence was detected in the colonic mucosa (CM). In the CM, at 24 h after infection, LPS was found in the cecal mucosa, a stronger presence was detected in the colonic mucosa (CM). In the CM, at 24 h after infection, LPS was mainly found in the cytoplasm of macrophages located in the central portion of villi beyond the epithelial layer (figure 2A). At 72 h after infection, LPS was detected in the deep chorion (figure 2C), whereas, at 120 h after infection, a large amount of LPS was found diffused within the CM and in scattered lymphoid cells of the activated follicles (figure 2E). In BS176-infected mice, small quantities of LPS

### Table 2. Apoptotic index in gut-associated lymphoid tissue (GALT) follicles and the lamina propria (LP).

<table>
<thead>
<tr>
<th>Group</th>
<th>In GALT follicles&lt;sup&gt;a&lt;/sup&gt;</th>
<th>In the LP&lt;sup&gt;b&lt;/sup&gt;</th>
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<tr>
<td></td>
<td>24 h</td>
<td>72 h</td>
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<tr>
<td>M90T</td>
<td>0.48 ± 0.12</td>
<td>2.20 ± 0.65</td>
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<tr>
<td>BS176</td>
<td>0.53 ± 0.21</td>
<td>0.72 ± 0.19</td>
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<tr>
<td>Control</td>
<td>0.56 ± 0.11</td>
<td>0.41 ± 10.09</td>
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<sup>a</sup> The apoptotic index was calculated as the percentage of TUNEL-positive cells (i.e., the number of TUNEL-positive mononuclear cells divided by the total number of lymphoid cells in GALT follicles, × 100) at 24, 72, and 120 hours after infection. A mean ± SE of 2203 ± 212 cells was counted for each sample.

<sup>b</sup> The apoptotic index was calculated as the percentage of TUNEL-positive cells (i.e., the number of TUNEL-positive mononuclear cells divided by the total number of lymphoid cells in diffuse infiltrate in the LP, × 100) at 24, 72, and 120 hours after infection. A mean ± SE of 586 ± 97 cells was counted for each sample.

<sup>c</sup> P<.0001 for values at 120 h vs. values at 24 h after infection.
Figure 6. Assessment of the expression of proinflammatory factors and production of interleukin (IL)–6. A, Agarose gel showing reverse-transcription polymerase chain reaction (RT-PCR) and densitometry of products of RNA extracted from colon and ileum. For each time point, 3 mice were euthanized, and mRNA was extracted from the intestinal tissue. The RT-PCR analysis was performed using primers for β-actin, IL-1β, IL-6, IL-12, interferon (IFN)–γ, tumor necrosis factor (TNF)–α, and inducible nitric oxide synthase (iNOS). In uninfected tissues, no expression of mRNA for these cytokines was observed (data not shown). Similar results were obtained in 3 additional identical experiments. Results obtained in 3 identical experiments had an SD within 18% of the values of the arbitrary units shown here. B, Serum IL-6 levels in mice infected with M90T or BS176 at 24, 48, 72, and 96 h after infection. Data are the geometric mean ± SE (error bars; n = 3 mice) for each time point.

were detected in colonic and cecal contents. At 24, 72, and 120 h after infection, LPS was immunostained in mucosal macrophages of the CM (figure 2B, 2D, and 2F).

Histopathological features of the epithelium and the structure and cell populations of the lamina propria (LP). In M90T-infected mice, at 72 h after infection, several mononuclear leukocytes and PMNLs were interspersed exclusively in the epithelial cell layer of the CM. At 120 h after infection, transcytosis of PMNLs across the vacuolated colonic epithelium could still be observed (figure 3A). However, in contrast to what was described for Ig infection of rabbits [29] and guinea pigs [30], no destruction of the epithelial lining was detected. In BS176-infected mice, at 120 h after infection, minor morphological changes characterized by vacuolization of a few ep-
Figure 7. Immunohistochemical (IH) analysis of tissue sections of liver. Hematoxylin-eosin staining of tissue sections of liver from mice infected with *Shigella flexneri* 5a strain M90T (A) or BS176 (B) and from uninfected control mice (C) at 72 h after infection are shown. IH staining shows *S. flexneri* lipopolysaccharide in the liver from mice infected with M90T (D) or BS176 (E) and from uninfected control mice (F) at 120 h after infection. In A, open arrows indicate a microgranuloma resulting in mononuclear cell aggregates with few neutrophils. In B, open arrows point to small aggregates of lymphocytes. Bars, 50 μm.

Epithelial cells in the absence of transcytosis of inflammatory cells were observed (figure 3B).

At 24 h after infection, the LP of M90T-infected mice showed an inflammatory infiltrate characterized by a few PMNLs and a significant number of mononuclear cells, consisting of CD3⁺ T lymphocytes, a few plasma cells, and macrophages (data not shown). At 72 h after infection, the mononuclear cell–infiltrating population increased (figure 3D), and a small number of PMNLs persisted. At 120 h after infection, the colonic villi were enlarged, and the LP appeared infiltrated by a large number of CD3⁺ T lymphocytes (histopathological score, 4) and scattered macrophages that were positively immunostained by mouse anti–*S. flexneri* 5a LPS (figure 3G). In BS176-infected mice, at 120 h after infection, the CM showed no significant infiltrate within the LP (figure 3E). Only a few CD3⁺ T lymphocyte infiltrates (histopathological score, 1) in association with a moderate presence (histopathological score, 2) of interspersed LPS-positive macrophages (figure 3H) were observed. No alteration in the epithelium (figure 3C), no cellular infiltrate (figure 3F), and a few or no CD3⁺ T lymphocytes were observed in the corresponding tissue from the uninfected control mice (figure 3I).

**Characterization of the expanded follicles in the CM.** The main sign of the presence of shigellae in the colonic tissue was the dramatic expansion of the lymphoid follicles. At 24 h after infection, we observed the presence of lymphoid aggregates in the CM of M90T-infected mice. The size of these well-structured follicles reached a peak at 120 h after infection (figure 4A). Their mantle area was covered by CD3⁺ T lymphocytes, primarily represented by CD3⁺CD25⁺ lymphocytes (figure 4B and 4C) and by many CD3⁺CD8⁺ lymphocytes (figure 4D) interspersed with a large number of CD32⁺CD16⁺ NK cells. The central area contained large arachnid-shaped dendritic cells (DCs) interspersed in a large population of CD5⁺ B lymphocytes and a few CD3⁺ T lymphocytes. An area of CD21⁺ B lymphocytes was immunostained around this central portion. These well-structured follicles, localized in the deep chorion, were consistently accompanied by CD32⁺CD16⁺ NK cells (figure 4E and 4F) and by a

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<td>24</td>
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<tr>
<td>72</td>
<td>1</td>
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<tr>
<td>120</td>
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**NOTE.** Liver from both BS176-infected mice and uninfected control mice had scores of 0 in both parameters analyzed at all time points.

ᵃ Microgranulomas were defined as well-circumscribed cell aggregates composed of >5 mononuclear phagocytes. Scoring for mean area of granuloma extension: 0, none; 1, up to 0.008 mm²; 2, up to 0.042 mm²; 3, up to 0.4 mm².

ᵇ Scoring for no. of hepatocytes showing degeneration (i.e., cloudy swelling and/or vacuolar degeneration) per high-power field: 0, none; 1–19 cells; 2, 20–49 cells; 3, 50–200 cells.
mixed population of CD3⁺ T lymphocytes that formed a diffuse, superficial, and subepithelial infiltrate, as described above. In the CM of BS176-infected mice, there were small to moderate lymphoid aggregates (figure 4G) not organized as a structured follicle. As was observed in M90T-infected mice, at 120 h after infection, a certain number of CD3⁺CD25⁺ lymphocytes covered the mantle area of the lymphoid aggregates (figure 4H and 4I). At this time point, CD3⁺CD8⁺ lymphocytes constituted the prevalent lymphocyte population, whereas few or no CD32⁺CD16⁺ NK cells were detected (figure 4L). The lymphoid aggregates mainly resulted in CD3⁺ T, CD21⁺ B, and CD5⁺ B lymphocytes (figure 4M and 4N).

**Apoptosis of infected cells in the lymphoid follicles of the LM.**

The amount and the localization of apoptotic cells in the CM was assessed by a TUNEL analysis (figure 5 and table 2). In the M90T-infected mice, from 24 to 120 h after infection, an increasing number of TUNEL-positive cells was present in the activated GALT (figure 5A). In contrast, in the BS176-infected
mice, a few TUNEL-positive lymphoid cells were detected (figure 5B). In uninfected control mice, a few TUNEL-positive cells were observed in the rare solitary lymphoid follicles of the CM (figure 5C), whereas a few apoptotic cells were counted in unstimulated lymphoid cells of the GALT (table 2).

Expression of proinflammatory cytokines in colonic tissue at early time points of infection and serum IL-6 production. We assessed the expression of IL-1β, IL-6, IL-12, interferon (IFN)–γ, tumor necrosis factor (TNF)–α, and inducible nitric oxide synthase (iNOS) mRNAs by RT-PCR in the colon and ileum of mice infected with M90T or BS176 and of uninfected control mice at 24, 48, and 72 h after infection (figure 6A). In the ileum, a considerable expression of mRNA for IFN–γ, TNF–α, and IL-12 was seen exclusively in M90T-infected mice only at 72 h after infection.

At 24 h after infection, in M90T-infected mice, a measurable expression of all these cytokines was observed in colonic tissues, whereas, in BS176-infected mice, only low levels of IL-12, TNF–α, and iNOS were detected. In M90T-infected mice, this trend remained unchanged, with a progressive increase in the expression of mRNA for these cytokines—except for mRNA for IL-6, whose low expression slightly decreased from that observed at 48 h after infection—until 72 h after infection. In BS176-infected mice, at 48 h after infection, weak expression of mRNA for IL-12, TNF–α, IFN–γ, and iNOS was present, whereas, at 72 h after infection, no or minimal expression of mRNA for these cytokines was observed (figure 6A). In M90T-infected mice, serum IL-6 production was high at 24 h after infection and then disappeared at 72 h after infection (figure 6B).

Damage by shigellae and localization in the liver. In M90T-infected mice, at 120 h after infection, the liver showed hepatic cell death and well-defined hepatic microgranulomas with mononuclear cell aggregates consisting of epithelioid and lymphoid cells (figure 7A). LPS was detected within the cytoplasm of many macrophages located in some central areas of these microgranulomatous lesions and in Kupffer cells (figure 7D). In BS176-infected mice, the liver did not show significant morphological changes, and little or no infiltration of mononuclear cells was observed (figure 7B). A positive response to LPS was detected only in Kupffer cells, in the absence of significant lesions (figure 7E). The lesions are scored in table 3.

DISCUSSION

In this study, we addressed 3 key hypotheses about the inability of mice to develop shigellosis. First, we examined whether the endogenous flora could protect the intestinal tissue from Shigella infection. We found that treatment of mice with Sm is a prerequisite for colonization of the intestinal tract by shigellae, which is in accordance with the role played by the intestinal flora in preventing oral infection with various enteropathogens, such as Salmonella species [31–33]. Then, we investigated the ability of Shigella species to invade the intestinal tissue. As in humans, the colon and eventually the cecum of the Sm-treated mice were the main targets for the invasion of a wild-type Shigella strain. However, in contrast to human shigellosis, no lesions or only a few abscesses were observed in the mice, despite the presence of a high number of shigellae. Finally, we considered the hypothesis that the murine epithelium may be unable to respond to intracellular shigellae by recruiting an inflammatory infiltrate. Interestingly, the presence of a mononuclear infiltrate and a dramatic expansion of lymphoid follicles dispersed along the CM and the cecal mucosa were the main features of the infected intestine, thus demonstrating that the intestinal epithelium is able to sense shigellae and to mount an inflammatory reaction. However, in contrast to the situation in human shigellosis, and in accordance with the paucity of lesions observed, a moderate number of PMNLs was observed in the mice.

In the rabbit ligated ileal loop model of Shigella infection, inhibition of IL-8 prevents the transmigration of PMNLs, thereby reducing the number of tissue lesions at the expense of bacterial dissemination in deeper layers of the CM [9]. In mice, the delivery of recombinant human IL-8 together with invasive Shigella strains through intraluminal instillation induces mucosal lesions similar to those seen in human disease [20]. In the present study, at 24 h after infection, shigellae were isolated from the liver, in which they induced microgranulomas, as is schematically shown in figure 8. These findings suggest that the absence of a murine equivalent of human IL-8 [34] might contribute to the prevention of the deleterious consequences of inflammation and favor the septic dissemination of shigellae into the liver. This confirms the results of our study showing that, in mice infected intravenously, shigellae reach the liver, where they induce microgranulomatous lesions [26]; again, the absence of PMNLs is a feature of these lesions.

In the CM of Sm-treated mice, several cell populations participate in the mononuclear infiltrate. CD32+CD16+ NK cells are localized superficially and subepithelially in the CM of M90T-infected mice. NK cells producing IFN–γ play an essential role in the resistance in mice after primary Shigella intranasal infection [35], whereas, in humans, high levels of mRNA for IFN–γ are found in the LP only during late-phase shigellosis [36].

In M90T-infected mice, only mature DCs migrating to secondary lymphoid organs, such as the expanded follicles, were observed. In M90T-infected mice, apoptosis of macrophages and the subsequent release of shigellae or their material in the expanded follicles might contribute to the maturation of DCs. Mature DCs can produce IL-12 and stimulate NK cells again [37], thus amplifying their activation and proliferation, the release of cytokines such as TNF–α and IFN–γ, and their bacteriolytic activity [38]. In accordance with this hypothesis, mRNA for both IL-12 and IFN–γ was detected only in colonic tissues.
of M90T-infected mice. In the rectal mucosa of humans with shigellosis [39], a large expansion of CD3+ T lymphocytes is observed. In mice, the large CD3+ T lymphocyte population primarily consists of a subset of CD3+CD25+ lymphocytes. CD4+CD25+ lymphocytes are a subset of naturally activated lymphocytes, CD45RBlow, which control inflammatory responses to commensal bacteria [40] by acting as a negative regulator of the immune response [41]. This cell population might play an important role in controlling the magnitude and the deleterious consequences of the murine intestinal tissue response to shigellae. Whether CD3+CD25+ lymphocytes play a role during natural shigellosis is still unknown. In the present study, a large number of CD3+CD8+ lymphocytes was present in the LP, particularly in the cortical areas of the expanded follicles of M90T-infected mice. This cell population is the T cell subset predominantly involved in the inflammation [39] of human rectal mucosa. Finally, in the present study, CD5+ B lymphocytes were diffusely distributed in the expanded central areas of follicles. In mice, CD5+ B lymphocytes predominate in the gut mucosa [42] and play a crucial role in the eradication of pathogens by stimulating the innate immune system via the classic pathway of complement activation [43].

In conclusion, it must be stressed that ig infection of Sm-treated mice is, to our knowledge, the first model of shigellosis in which the pathogens spontaneously interacted with the colonic tissue that represents the natural target of their infection. Furthermore, in accordance with the results of studies of intragastric infection of the macaque Macaca mulatta, the distribution of bacteria in this tissue is essentially concentrated around lymph nodes [4].

The study of infected murine colonic tissue in which PMNLs are poorly recruited helps us to understand the other cell populations involved in inflammation, their interactions, and their interplay with bacteria. This may facilitate the search for new vaccines and therapeutic approaches to shigellosis.

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References