Containment of Tumor-Colonizing Bacteria by Host Neutrophils
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Abstract
Administration of facultative anaerobic bacteria like Salmonella typhimurium, Shigella flexneri, and Escherichia coli to tumor-bearing mice leads to a preferential accumulation and proliferation of the microorganisms within the solid tumor. Until now, all known tumor-targeting bacteria have shown poor dissemination inside the tumors. They accumulate almost exclusively in large necrotic areas and spare a rim of viable tumor cells. Interestingly, the bacteria-containing necrotic region is separated from viable tumor cells by a barrier of host neutrophils that have immigrated into the tumor. We here report that depletion of host neutrophils results in a noticeably higher total number of bacteria in the tumor. We now were also able to migrate into vital tumor tissue. Most remarkably, an increase in the size of the necrosis was observed, and complete eradication of established tumors could be observed under these conditions. Thus, bacteria-mediated tumor therapy can be amplified by depletion of host neutrophils. [Cancer Res 2008;68(8):2952–60]

Introduction
Since the middle of the 19th century, bacteria and bacterial products have been used in cancer therapy to treat solid tumors (1–3). At that time, it was believed that the rapid tumor shrinkage was caused by immune reactions against the infectious material. Thus, infection would only indirectly lead to the destruction of tumor cells. Now, the use of bacteria for tumor therapy is back in focus (1–3). Several bacterial species were discovered that specifically accumulate and proliferate in solid tumors. Despite of the growing number of bacterial species that apparently exhibit intrinsic tumor-targeting properties, no bacterium that is able to inhibit tumor growth completely, solely by its presence in the tumor, is known so far (4, 5). On the other hand, such natural tumor-targeting bacteria might aid therapeutic cancer treatment (6), as they can be exploited as highly specific carriers for the delivery of therapeutic factors into a solid tumor (3, 7–9).

Bacteria accumulate and grow throughout enlarged necrotic areas within solid tumors while leaving a rim of viable cells at the periphery of the tumor (6). Therefore, after an initial bacteria-induced shrinkage, the tumor starts to regrow again.

Hypothetically, bacteria should evenly spread throughout the tumor for efficient therapy. They should not only reach the nondividing or necrotic cells in hypoxic regions, but also viable, proliferating cells at the rim of the tumor. This has never been observed in immunocompetent hosts thus far.

For obligate anaerobic bacteria like Clostridium or Bifidobacterium spp., the limited spreading of the bacteria is explained by the physiologic constrains of the bacteria. Being obligate anaerobic, they should solely germinate and grow in hypoxic and anoxic regions of a tumor (10, 11). For facultative anaerobic bacteria, this issue is slightly more complex. Zhao et al. (12–14) successfully treated tumors in immunocompromised nude mice using a monotherapy with amino acid auxotrophic Salmonella typhimurium. They were able to detect bacteria in the viable tumor regions. This phenomenon has not been reported for fully immunocompetent animal models yet. Even facultative anaerobic species like Salmonella enterica serovar typhimurium SL2207 (S. typhimurium) or Escherichia coli do not reside outside necrotic areas of tumors in immunocompetent hosts, although they should not be affected by high oxygen pressure (15–17). Thus, the question arises: why are these bacteria kept inside necrotic and hypoxic areas instead of spreading evenly within the tumor?

Here, we investigated the location of three different, facultative anaerobic bacterial strains (S. typhimurium, E. coli TOP10, and Shigella flexneri M90T) in ectopic CT26 colon carcinomas after tumor colonization. Interestingly, in concordance with previous reports (18, 19), bacterial infection leads to an increase in necrosis and to a strongly increased proliferation of neutrophils. Bacteria were almost exclusively found within the necrotic area or in close contact with the infiltrating neutrophils, which surrounded the necrosis.

Importantly, when avoiding tumor infiltration of neutrophils by neutrophil depletion at the time of infection, bacteria were also found in vital tumor tissue. This was accompanied by a higher total number of bacteria inside the tumor and a strongly increased formation of necrosis. These data show that constrains exist within solid tumors that oppose bacterial dissemination, which are not only physiologic but also immunologic. Our findings offer new roads to increase the therapeutic potential of tumor-targeting bacteria.

Materials and Methods
Bacterial strains and growth conditions. S. typhimurium strain SL2207 (hisG, ΔaroA) was kindly provided by Bruce Stocker (20), and E. coli TOP10 was purchased from Invitrogen. Both bacterial strains were grown in Luria-Bertani medium supplemented with 30 μg/mL streptomycin.

S. flexneri M90T (Serotype 5, ΔnapA) was kindly provided by P.J. Sansonetti (21). Shigella were grown in tryptic soy broth supplemented with 200 μM/L Congo red, 30 μg/mL kanamycin, and 100 μg/mL DAP.

Bacteria were grown at 37°C in liquid medium with vigorous shaking at 180 rpm.

Cell lines and animals. Six-week-old female BALB/c mice were purchased from Harlan. CT26 colon carcinoma cells (ATCCRL-2638) were grown as monolayers in Iscove’s modified Dulbecco’s medium (IMDM; Life Technologies Bethesda Research Laboratories) supplemented with 10% (v/v) heat-inactivated FCS (Integro), 250 μM/L β-mercaptoethanol (Serva), and 1% (v/v) penicillin/streptomycin (Sigma).

Infection of tumor-bearing mice and recovery of bacteria from tissues. Six-week-old female BALB/c mice were s.c. inoculated at the
abdomen with $5 \times 10^5$ CT26 cells. Mice bearing tumors of $4$ to $7$ mm diameter were i.v. injected with $5 \times 10^6$ colony-forming units (CFU) of S. typhimurium or E. coli suspended in PBS and i.t. with $1 \times 10^7$ S. flexneri suspended in PBS with 100 μg/mL DAP, respectively. At day 2 postinfection (p.i.), mice were sacrificed and their tumors and spleens were transferred into 1 mL of sterile ice-cold PBS containing 0.1% (v/v) Triton X-100; livers were transferred into 2 mL of this solution. Tissues were homogenized by using a Polytron PT3000 homogenizer (Kinematica). For determination of total CFUs per organ, bacteria homogenates were serially diluted in PBS (Shigella in PBS with 100 μg/mL DAP) plated with the required antibiotics.

**Neutrophil depletion.** To deplete neutrophils, mice received three doses of 25 μg monoclonal rat anti-Gr1 (RB6-8C5) antibody diluted in 100 μL PBS i.p. 1 d before, simultaneously, and 1 d after infection. Depletion was controlled by testing blood samples from treated mice by flow cytometry.

**Flow cytometry of blood samples.** Erythrocytes of 50 μL blood were lysed in 1.5 mL erythrocyte lysis buffer, vortexed, incubated for 5 min at room temperature, and centrifuged for 5 min. This procedure was repeated once. Then cells were washed once with PBS and stained with rat anti-Gr1 FITC (RB6-8C5) and rat anti-CD11b PE (eBioscience) for 20 min on ice. Subsequently, cells were washed once with PBS and analyzed on a FACSCalibur (Becton Dickinson).

**Histology.** Tumors were removed from sacrificed mice and snap-frozen in Tissue-Tek OCT Compound (Sakura Finetek). Cryosections of 10 μm were cut with a microtome-cryostat (Cryo-Star HM560V, Microm) and placed onto glass slides. Slides were air dried at room temperature overnight and fixed in acetone at $-20^\circ$C for 3 min. Slides were rehydrated in PBS, blocked with 50 μg/mL bovine serum albumin and 1 μg/mL FcR blocker (rat antimouse CD16/CD32), and stained with the following reagents: polyclonal
rabbit anti-\textit{S. typhimurium} (Sifin), polyclonal goat anti-rabbit Alexa 488 (Sigma-Aldrich), polyclonal rabbit anti-\textit{S. flexneri} (Biomol), polyclonal goat anti-\textit{E. coli} (Biomol), polyclonal rabbit anti-goat Alexa 488 (Invitrogen), rat anti-Gr1 biotin (RB6-8C5), Streptavidin-cy5 (Invitrogen), rat anti-CD11b PE (eBioscience), Phalloidin Alexa Fluor 594 (Invitrogen), and DRAQ5 (Biotest). After staining, the slides were washed, dried, mounted with mounting medium (Neomount, Merck), and analyzed using a laser scanning confocal microscope (LSM 510 META, Zeiss). Images were processed with LSM5 Image Browser (Zeiss) and Adobe Photoshop 7.0. For paraffin sections, tumors were fixed in 10% (v/v) paraformaldehyde and embedded in paraffin wax. Sections (5 \mu m) were mounted on slides and stained with H&E. The stained paraffin sections were analyzed with an Olympus BX51 microscope, and pictures were taken with an Olympus U-CMAD3 camera. Areas of necrosis were quantified using the cell^D software from Olympus, which allows the calculation of marked areas inside a histologic section.

**Flow cytometry of tumor tissue.** Nonnecrotic tumor tissue was cut into 1 to 2 mm\(^2\) pieces. The pieces were rinsed twice with PBS and digested using dispase/collagenase/DNase suspension in IMDM (0.2 mg/mL;0.2 mg/mL;100 mg/mL) for 45 min in 37\(^\circ\)C. Cell suspension and remaining tissues were meshed using 50 \mu m disposable filters (Cell Trics, Partec); subsequently, erythrocytes were removed using erythrocyte lysis buffer. Single-cell suspensions of tumors were stained with the following fluorescent antibodies: PE-anti-CD11b (eBioscience), APC-anti-CD11b (eBioscience), PE-Cy7-anti-Ly-6G (Gr-1, eBioscience), PE-anti-CD8a and FITC-anti-CD8a (Ly-2, S3-6.7, eBioscience), biotin-anti-CD117 (c-kit, ACK-4, self-made), FITC-anti-CD3 (500A2, self-made), APC-anti-CD4 (L354, BD PharMingen), APC-Cy7-anti-CD45R (B220, RA36B2, BD PharMingen). The two self-produced antibodies were isolated from culture supernatants and prepared according to standard procedures. Flow cytometry was performed using a FACS Canto (Becton Dickinson). The number of single viable tumor

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**Figure 2.** Infiltration of neutrophils into CT26 tumors after bacterial infection. Cryosections of CT26 tumors from uninfected and infected mice were prepared 2 d p.i. In all pictures, Gr1-positive cells are stained in red, CD11b-positive cells are stained in blue, and bacteria (if present) are stained in green. A, low magnification overviews of Gr1-positive and CD11b-positive cells in noninfected CT26 tumors. B, location of \textit{S. typhimurium} and neutrophils inside CT26 tumors. B, left, low magnification overview of an \textit{S. typhimurium}–infected CT26 tumor. Middle and right, higher magnifications of the neutrophil border between viable and necrotic tumor tissue. C, location of \textit{S. flexneri} and neutrophils inside CT26 tumors. Left, low magnification overview of an \textit{S. flexneri}–infected CT26 tumor. Middle and right, higher magnifications of the neutrophil border between viable and necrotic tumor tissue. White bars, correspond to 100 \mu m in A and B, left and C, left and 10 \mu m in all other pictures. The micrographs are representative of several tumors from at least three independent experiments. D, percentage of living neutrophils in the tumors of control mice and \textit{S. typhimurium}–infected mice at different times p.i. Tumor cells were analyzed via flow cytometry. Black columns, results for control mice; gray columns, results for \textit{S. typhimurium}–infected mice; bars, SDs.
Neutrophils Impair Bacterial Distribution in Tumors

Figure 3. Bacterial dissemination in different organs with or without neutrophil depletion. Neutrophils were depleted by triple i.p. injections of 25 or 100 μg anti-Gr1, respectively, 1 d before (–1), simultaneously (0), and 1 d after (1) infection. A, at 2 d p.i., blood samples were taken and analyzed by flow cytometry for the presence of neutrophils. The percentage of such cells in the blood of neutrophil-depleted mice was normalized to the percentage of nondepleted tumor-bearing control mice. B, tumor-bearing anti-Gr1–treated and nondepleted mice were injected i.v. with Salmonella typhimurium SL7207 (left), E. coli TOP10 (middle), or infected i.t. with Salmonella flexneri M90T (right). Tumor, spleen, liver, and cervix were homogenized and plated and the CFUs per tissue were determined. Black columns, CFUs in neutrophil-depleted mice; gray columns, CFUs in nondepleted, infected mice. Left, depleted versus nondepleted mice; *, P < 0.005. Results are representative of at least two independent experiments with three to five mice. C, length of neutrophil depletion in the blood after triple injection of anti-Gr1 at consecutive days. Arrows, anti-Gr1 injection; bars, SDs. D, percentage of living neutrophils in the tumors of control mice and anti-Gr1–treated mice at different times after treatment. Tumor cells were analyzed via flow cytometry. Black columns, results for control mice; gray bars, results for anti-Gr1–treated mice; bars, SDs. All graphs are representative of multiple experiments with multiple mice.

cells was analyzed by propidium iodide staining. Data were analyzed with BD FACSDiva software (Becton Dickinson).

Tumor studies. Six-week-old female BALB/c mice were sc inoculated at the abdomen with 5 × 10^6 CT26 cells. Ten days after injection, mice were divided into four groups of 10 mice each. One group remained untreated, one group received a triple dose of anti-Gr1 i.p., one group received an i.v. injection of 5 × 10^6 CFU of either S. typhimurium or E. coli, and the fourth group received a triple dose of anti-Gr1 i.p. and an i.v. injection of 5 × 10^5 CFU of either S. typhimurium or E. coli. Tumor sizes were measured every other day, and the tumor volume was determined by the following formula: 4/3 × π × (h × w^2) / 8, wherein h = height and w = width. The depth of the tumors was assumed to equal tumor width.

Statistical analysis. Statistical analysis was performed using the Student's t test, with P < 0.05 considered as significant.

Results

Different bacterial strains accumulate preferably in solid tumors. BALB/c mice bearing an ectopic CT26 tumor were i.v. infected with S. typhimurium SL7207 and E. coli TOP10, respectively. Two days p.i., tumor, spleen, and liver were homogenized and the CFUs per organ were determined. Both bacterial strains exhibited a strong accumulation in the solid tumor (Fig. 1A). Whereas the number of Salmonella in the tumor was 50 to 100 times higher than in other organs (S. typhimurium i.v.), the E. coli strain showed an even stronger preference for the tumor. Here, 50 to 100,000 times more bacteria were found in the tumor compared with other organs (E. coli i.v.). S. flexneri M90T did not exhibit a preferential accumulation in tumor tissue after a systemic infection, although colonization of tumors took place to some extent (data not shown). Therefore, Shigella were given directly into the tumors in all the following experiments. Again, 2 days p.i., roughly 2,000 times more bacteria were found in the tumor compared with spleen and liver (S. flexneri i.t.). Interestingly, although the Shigella were given directly into the tumor, they were able to spread to other organs. Because this strain is impaired in its ability to produce the cell wall component DAP, they should not be able to replicate. This might explain why the number of Shigella in the tumor was remarkably lower than the number of E. coli or S. typhimurium.

This was corroborated, after the time course of tumor colonization, by S. typhimurium as a representative bacterium. CFUs per organ were determined on day 1, 3, 6, and 8 p.i. (Supplementary Fig. S1). S. typhimurium reached its highest count in the tumor between days 1 and 3 p.i. with roughly 1 × 10^8 bacteria per tumor. Later, the bacterial load of the tumor slowly decreased to...
1 × $10^5$ at the termination of the experiment. In contrast, in spleen and liver, bacterial numbers increase from $1 \times 10^5$ per organ at day 1 p.i. to $5 \times 10^6$ at day 8 p.i. (Supplementary Fig. S1).

**Distribution of different bacterial strains inside a solid tumor.** To determine where the bacteria accumulate inside the CT26 tumor 2 days p.i., the tumors were snap-frozen, cut into 10-μm sections, and stained with antibodies that detect the bacteria Phalloidin that stains the cytoplasm and DRAQ5 that stains the DNA. Particular regions of the tumor were determined by comparing antibody-stained cryosections with H&E-stained paraffin sections of infected CT26 tumors prepared in parallel. Figure 1B shows a low magnification overview of an H&E-stained paraffin section of an S. typhimurium–infected CT26 tumor 2 days p.i. The vital parts (v) of the tumor appear bluish, whereas the necrotic areas (N) of the tumor are stained purple. Two days p.i., tumors exhibited huge necrotic regions and a small viable rim surrounding the necrosis.

Figure 1C shows the distribution of S. typhimurium SL7207, E. coli TOP10, and S. flexneri M90T inside CT26 tumors. The first column shows low magnification overviews of CT26 tumors of mice infected with the different bacteria. Obviously, the distribution of all bacterial strains inside the tumor was similar: A large, inner area of the tumors was populated by bacteria, whereas a smaller, mainly outer area of the tumor remained bacteria-free. When comparing the antibody-stained cryosections with the H&E-stained paraffin section in Fig. 1B, it became clear that the bacteria-colonized areas correlate perfectly with the huge necrosis, whereas no bacteria were found inside the vital parts of the tumors. Thus, all bacteria tested here were almost exclusively found inside necrotic areas, sparing a rim of viable tumor tissue. The accumulation of bacteria inside the necrosis seemed not to be dependent on the route of infection. I.t. given, S. flexneri were also predominantly found inside the necrotic region (Fig. 1C).

**Accumulation of neutrophils at the site of bacterial colonization.** All bacterial strains tested were only facultative anaerobic and should have the ability to colonize the complete tumor. Because the bacteria were mainly found in the necrotic tumor area, we hypothesized that the particular bacterial distribution might be influenced by host responses against the infections. Therefore, we examined the composition of immune cells inside the tumor before and 2 days after bacterial infection. Immunohistology using anti-CD11b (blue) and anti-Gr1 (red) revealed a tremendous influx of neutrophilic granulocytes expressing both markers (purple staining) to the site of infection (Fig. 2). In uninfected control tumors (Fig. 2A), neutrophils were scattered all over the solid tumor. In some cases, they accumulated in the proximity of small necrotic areas (Fig. 2A, right). Of note, the strong red staining occasionally observed at the edge of tumors does not represent staining of neutrophils but is due to the staining of biotin in hair follicles by streptavidin.

In contrast to the control tumors, 2 days p.i., neutrophils had invaded the tumors in large numbers. They resided at the border between “healthy” tumor tissue that contained apparently viable cells and necrotic tissue that was populated by bacteria (Fig. 2B and C). In case of S. typhimurium (Fig. 2B) and E. coli (Supplementary Fig. S2), bacteria were mainly restricted to the necrotic area or in close contact with the neutrophils (Fig. 2B and C). Interestingly, S. flexneri was similarly distributed (Fig. 2C). In rare cases, a few S. flexneri could also be found in healthy tumor tissue. This might be correlated with the route of infection. When infecting the mice intratumorally, the injection channel might influence bacterial distribution in the tumor to some extent. According to flow cytometry analysis of Salmonella-infected and noninfected CT26 tumors, neutrophil infiltration into the tumor peaked around day 3 p.i. (Fig. 2D). Subsequently, the number of neutrophils in the tumor began to decline (Fig. 2D).

To ensure that the neutrophil barrier is not only a peculiar feature of CT26, another solid tumor was used with S. typhimurium and S. flexneri. Similar results were obtained with the adenocarcinoma TS/A (data not shown).
Together, the positioning of neutrophils around the necrotic and bacteria-colonized region of the tumor suggested that such host phagocytes were responsible for the containment of the microorganisms to this area and kept them from spreading.

Depletion of neutrophils enhances bacterial growth and dissemination inside the tumor. To test the effect of neutrophils on bacterial tumor colonization, neutrophils were depleted from the mice by a triple i.p. injection of 25 and 100 µg anti-Gr1, respectively. All mice survived the antibody treatment and subsequent short-term infection without apparent deterioration in health status. Two days p.i., the efficiency of neutrophil depletion was controlled by flow cytometry of blood from treated or untreated mice. A triple treatment with 25 µg anti-Gr1 was sufficient to reduce the number of neutrophils in blood to 4% of the number found in untreated control mice (Fig. 3A). A higher dosage of the antibody only slightly enhanced depletion. Thus, all further experiments were performed with a triple dose of 25 µg anti-Gr1.

Tumor-bearing mice were either treated with anti-Gr1 or left untreated and infected with the different bacterial strains. Two days p.i., mice were sacrificed and the CFUs per organ were determined (Fig. 3B). Neutrophil depletion led to a colonization of S. typhimurium in all tissues that was approximately four to five times higher than in nondepleted, infected controls. Thus, neutrophil depletion resulted in higher bacterial numbers inside the tumor, but at the same time, the bacterial burden also increased in the other organs tested.

An even stronger effect was observed for E. coli TOP10. The number of bacteria in the tumor was roughly seven times higher in depleted mice compared with nondepleted mice, whereas the other organs were only slightly more affected in depleted mice, if at all.

S. flexneri basically showed the same pattern as S. typhimurium, although a significant increase in bacterial load in tumors from anti-Gr1–treated mice compared with nondepleted mice could not be observed. This might be attributed to the infection route. Fluctuations in the size of the inoculum due to the small volume injected into the tumor probably resulted in the higher SDs observed and might obscure the effect of the depletion. Similar data were obtained for the other organs.

Neutrophil depletion was only transient (Fig. 3C). One day after the last anti-Gr1 administration, circulating neutrophils reach their lowest count in blood (~4% of the initial number of neutrophils). Two days later, neutrophils begin to reoccur. After 3 days, 80% of the initial number of neutrophils can be detected in circulation, and 4 days after anti-Gr1 treatment, the neutrophil count in blood is back to normal.

Flow cytometry of cells from tumor tissue yielded similar results (Fig. 3D). At day 1 p.i., the number of neutrophils in neutrophil-depleted mice was strongly reduced in the tumors. Neutrophils started reinfiltating the tumor by day 3 p.i. By day 8 p.i., the percentage of neutrophils in the tumor of depleted mice was approaching normal levels. In mice that were neutrophil-depleted and neutrophil-infected, neutrophils reached normal levels in the blood by day 4 (data not shown). In tumors, however, they never reached the high neutrophil levels observed in tumors of infected control animals. Corresponding tumor micrographs are depicted in Supplementary Fig. S3.

Depletion of neutrophils allows partial spreading of bacteria to vital tumor tissue. Parallel to the platings, tumors from Gr1-depleted mice were analyzed by histology. Figure 4 shows that most of the neutrophils in the tumor were depleted as intended. Figure 4A shows the distribution of S. typhimurium inside the solid tumor, whereas Fig. 4B highlights the distribution of S. flexneri. In both cases, the majority of bacteria still remained within the necrosis. Similar results were obtained for E. coli (Supplementary Fig. S4). As can be clearly seen at higher magnification (Fig. 4, middle), S. typhimurium was able to migrate beyond the few neutrophils that were left and was able to settle at both sites of this small neutrophil border. Obviously, the few remaining neutrophils were not sufficient to detain the bacteria from vital tumor tissue. The data obtained with the two other bacterial strains used were similar. Figure 4B (middle) shows S. flexneri settling at the border between necrotic and viable tumor tissue, whereas Supplementary Fig. S4 (middle) depicts E. coli inside the large necrosis.

Although the majority of the microorganisms still remained in necrotic areas, bacteria in neutrophil-depleted tumors could now also be found in association with living cells in the vital tumor tissue as highlighted in Fig. 4A and B and Supplementary Fig. S4 (last pictures). Similar results were obtained with S. typhimurium and S. flexneri in TS/A tumors (data not shown).

Depletion of neutrophils leads to an increase in necrosis. Structural changes in the tumor tissue were induced by neutrophil depletion in bacteria-colonized tumors. The area of necrosis in tumors from mice neither depleted nor infected represented below 15%. In tumors colonized by the different bacterial strains, an increase of the necrotic area of up to 70% at day 2 p.i. was observed. This was further increased up to 90% in neutrophil-depleted mice (Table 1).

Figure 5A displays the findings that are summarized in Table 1. In uninfected CT26 tumors (top), the necrotic areas were rather small and most of the necrotic cells still had distinct nuclei (right). A strong increase in necrosis could be observed upon infection, as shown exemplarily for S. typhimurium (middle). Besides this enlargement of the necrotic area, the barrier of neutrophils that separates necrosis from vital tumor tissue is clearly visible. The right picture of the middle panel shows a magnification of these densely packed neutrophils. The bottom panel shows the infected tumors after neutrophil depletion. No border of neutrophils is visible, and the expansion of the necrosis has become very distinct. An enlarged picture of the borderline between necrosis and vital tumor tissue (right) verified neutrophil depletion. Similar data were obtained for S. flexneri and E. coli (Supplementary Fig. S5).

### Table 1. Size of necrosis in infected (2 d p.i.) and uninfected CT26 tumors with and without depletion of neutrophils

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Bacteria</th>
<th>Anti-Gr1</th>
<th>Percentage necrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>CT26</td>
<td>Not infected</td>
<td>−</td>
<td>5–15%</td>
</tr>
<tr>
<td>S. typhimurium SL7207</td>
<td>−</td>
<td>60–65%</td>
<td></td>
</tr>
<tr>
<td>S. typhimurium SL7207</td>
<td>+</td>
<td>75–85%</td>
<td></td>
</tr>
<tr>
<td>E. coli TOP10</td>
<td>−</td>
<td>60–65%</td>
<td></td>
</tr>
<tr>
<td>E. coli TOP10</td>
<td>+</td>
<td>80–85%</td>
<td></td>
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<tr>
<td>S. flexneri M90T</td>
<td>−</td>
<td>65–70%</td>
<td></td>
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<tr>
<td>S. flexneri M90T</td>
<td>+</td>
<td>85–90%</td>
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NOTE: Percentage of necrosis was determined on paraffin sections after H&E staining by using cell^D software (Olympus).
Influence of neutrophil depletion on the therapeutic effect of tumor-colonizing bacteria. Having found a significant increase of the size of necrosis in infected tumors of neutrophil-depleted mice, the influence of this treatment on therapeutic efficacy of tumor-colonizing bacteria was examined. First experiments were performed using *S. flexneri*, because in their case, necrosis was drastically enlarged after neutrophil depletion. Differences could be observed between infected tumors of neutrophil-depleted and nondepleted groups (data not shown). Two of eight mice of the neutrophil-depleted group became tumor-free, whereas no mice became tumor-free from the nondepleted group. All other mice showed strong tumor growth after initial retardation. We interpret these findings such that the lack of pathogenicity of *S. flexneri* in the mouse and their lack of...
proliferation inside the tumor due to an impaired cell wall synthesis do not allow the bacteria to consequently destroy the tumor.

Thus, tumor growth from S. typhimurium–infected, neutrophil-depleted, and nondepleted mice was followed. Interestingly, all mice in which neutrophils had been depleted became tumor-free (Fig. 5B, top). Five of such mice eventually succumbed to the infection. In contrast, only 6 of 10 mice from the nondepleted group remained tumor-free. The four remaining mice showed strong tumor growth after initial retardation. None of the mice died from the infection.

Administration of lower doses did not lead to a more differential result. Infected mice were not killed, but tumors were not eradicated either.

Thereupon, experiments were performed with E. coli (Fig. 5B, middle). Infection of tumor-bearing, neutrophil-depleted mice quickly reduced tumor volume. All mice survived the infection and four of six mice were tumor-free after the treatment, whereas two of the mice showed moderate tumor regrowth 15 days p.i. The reduction of tumor volume was less intensive in the nondepleted control group. Here, only one of five mice was tumor-free after the treatment, whereas four of five mice showed strong tumor regrowth by day 7 p.i. Neutrophil depletion itself had no effect on tumor growth (Fig. 5B, bottom).

To compare the development of necrosis in tumor-bearing control mice and in tumor-bearing mice treated in various ways, H&E-stained paraffin sections were prepared on days 1, 3, 6, and 8 p.i. A comparison of histologic sections of control mice and of neutrophil-depleted and infected mice from day 8 p.i. are displayed in Fig. 5C. The complete set of data is shown in Supplementary Fig. S6. It is obvious that, in control mice, the size of necrosis is not changing during the course of the experiment. Identical results were obtained for mice in which only the neutrophils were depleted. Mice that were infected showed a strong increase in necrosis until day 6. In nondepleted mice, the tumor then starts to regrow because an increase in viable regions can be observed, whereas in mice infected and neutrophil-depleted, the tumor becomes completely necrotic. No viable regions were observable in all sections examined. Thus, the partial therapeutic effect of tumor-colonizing bacteria can be amplified by the transient removal of neutrophilic granulocytes.

**Discussion**

The accumulation of various bacteria in solid tumors is well accepted by now. Nevertheless, this therapy still has to prove its therapeutic potential. Although different kinds of bacteria-mediated tumor therapies have been tested thus far and some of them have shown promising results in immunocompromised nude mice (12–14), a complete regression of established tumors after colonization by tumor-targeting bacteria in immunocompetent hosts has been described rarely thus far (6). This indicates that the host response in tumors after colonization by bacteria is highly complex. It is not only restricted to the myeloid cells but most likely includes effector T cells, including regulatory T cells. The absence of the latter T cells in nude mice might explain the higher efficacy of bacterial colonization of tumors. Hence, additional measures are required to render bacteria a successful tool for cancer treatment in immunocompetent mice.

Along this line, it is essential to understand the peculiar properties of bacteria that enable them to colonize tumors, as well as the host reactions they trigger. This should then reveal the obstacles that have thus far impeded a complete destruction of colonized tumors. Accordingly, it is essential to understand why bacteria induce necrosis in one part of the tumor and leave another part unaffected.

In the present work, we investigated why bacteria like S. typhimurium, E. coli, or S. flexneri remain restricted to the supposedly hypoxic areas of necrosis within a solid tumor despite their potential to grow under aerobic conditions. It had been shown before that the colonization of tumors by bacteria induces a strong influx of host neutrophils. Such neutrophils settle at areas that directly border the necrotic regions (19). They might be attracted by necrosis to clear the dead cells, as is suggested by their accumulation at the small necrotic regions in uncolonized tumors. On the other hand, neutrophils are considered the life-saving first line of defense against bacterial infections. Thus, the bacterial pathogens that reside in the tumor most likely provide very strong attraction signals.

Nevertheless, the neutrophils seem to be impaired in clearing the infection. The absence of oxygen and an acidic extracellular pH, both characteristic conditions of necrotic areas inside solid tumors, have been reported to negatively influence the activities of neutrophils (reviewed in ref. 22). This might explain the coresidence of bacteria and neutrophils at the rim of necrotic regions. However, neutrophils are obviously still able to function as a border that inhibits bacterial dissemination into vital tumor tissue.

The barrier function of neutrophils could be clearly shown after depletion of these cells. After depletion, all bacteria tested improved their dissemination throughout the tumor, which could be proved by immunohistology. Neutrophil depletion also resulted in higher total numbers of bacteria in the tumor, an increase of necrosis, and a decrease of isolatable viable cells from tumor tissue. Obviously, neutrophil depletion amplifies the therapeutic effect of tumor-colonizing bacteria very strongly. This even resulted in a complete elimination of the established tumor in the mice when S. typhimurium or E. coli were used. In the case of Shigella, no difference was found for the CFUs in the tumors of depleted or undepleted mice although necrosis was strongly enhanced after neutrophil depletion. The lack of pathogenicity of such bacteria in mice and their impaired proliferation in the tumor might account for this phenomenon. However, this strong effect of neutrophil-depletion on bacterial tumor colonization could only be observed when neutrophils had been depleted at the time of infection. Neutrophil depletion after infection did not amplify the therapeutic effect of tumor-colonizing bacteria (data not shown).

Neutrophil-depleted or control mice infected with S. typhimu-
rium or E. coli that had become tumor-free were rechallenged with CT26 or TS/A tumor cells. CT26 cells were rejected in all cases, whereas TS/A cells formed tumors (data not shown). This indicates that the observed complete tumor remission was not a purely bacteria-induced effect but rather a specific immune response was induced in addition. This promising effect was obviously independent of a previous neutrophil depletion and was initiated by bacterial colonization of the tumor. A more detailed analysis will be required to characterize the inductive modalities and the effector cells.

Neutrophil depletion in tumor patients might seem as a “cruel intention,” as it seems to deprive the patient of one of his most important natural defense mechanisms. In our opinion, this is not the case. The effect of the bacterial infection on the patient strongly depends on the bacterial strain used and on the infection dose. In our experiments, all mice that were infected with...
nonpathogenic *E. coli* survived the infection despite of neutrophil depletion. In addition, neutrophils are quickly replenished after termination of antibody administration.

Systemic neutrophil depletion seems unrealistic for humans. A possible alternative could be the local depletion of neutrophils, e.g., via engineered bacteria that secrete depleting antibodies. In combination with promoters that are active only in tumors, a local depletion seems feasible. Alternatives could be small, neutrophil toxic molecules that are tumor-specifically expressed by the bacteria. One also has to bear in mind that depletion of neutrophils is an unwanted side effect of many chemotherapy regimens. Therefore, a combination of chemotherapy and bacterial treatment could be tested as an obvious possibility.

Another safety aspect is the quick elimination of the bacteria from the host at any time. Besides the use of antibiotics, this can be achieved by the employment of inducible suicide systems, as have been described by us recently (23).

Although neutrophil depletion did not result in complete dissemination of the bacteria within the tumor, the therapeutic results were promising. The combination of several steps of improvements will be required to render tumor-targeting bacteria a powerful alternative for conventional tumor treatment. Tumor-specific neutrophil depletion could be one of them.

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