

## Safety of Probiotic *Escherichia coli* Strain Nissle 1917 Depends on Intestinal Microbiota and Adaptive Immunity of the Host<sup>∇</sup>

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**Probiotics are viable microorganisms that are increasingly used for treatment of a variety of diseases. Occasionally, however, probiotics may have adverse clinical effects, including septicemia. Here we examined the role of the intestinal microbiota and the adaptive immune system in preventing translocation of probiotics (e.g., *Escherichia coli* Nissle). We challenged C57BL/6J mice raised under germfree conditions (GF-raised C57BL/6J mice) and *Rag1*<sup>-/-</sup> mice raised under germfree conditions (GF-raised *Rag1*<sup>-/-</sup> mice) and under specific-pathogen-free conditions (SPF-raised *Rag1*<sup>-/-</sup> mice) with probiotic *E. coli* strain Nissle 1917, strain Nissle 1917 mutants, the commensal strain *E. coli* mpk, or *Bacteroides vulgatus* mpk. Additionally, we reconstituted *Rag1*<sup>-/-</sup> mice with CD4<sup>+</sup> T cells. *E. coli* translocation and dissemination and the mortality of mice were assessed. In GF-raised *Rag1*<sup>-/-</sup> mice, but not in SPF-raised *Rag1*<sup>-/-</sup> mice or GF-raised C57BL/6J mice, oral challenge with *E. coli* strain Nissle 1917, but not oral challenge with *E. coli* mpk, resulted in translocation and dissemination. The mortality rate was significantly higher for *E. coli* strain Nissle 1917-challenged GF-raised *Rag1*<sup>-/-</sup> mice (100%; *P* < 0.001) than for *E. coli* strain Nissle 1917-challenged SPF-raised *Rag1*<sup>-/-</sup> mice (0%) and GF-raised C57BL/6J mice (0%). Translocation of and mortality due to strain *E. coli* Nissle 1917 in GF-raised *Rag1*<sup>-/-</sup> mice were prevented when mice were reconstituted with T cells prior to strain *E. coli* Nissle 1917 challenge, but not when mice were reconstituted with T cells after *E. coli* strain Nissle 1917 challenge. Colonization experiments revealed that *E. coli* mpk could not prevent translocation of strain *E. coli* Nissle 1917. Moreover, we demonstrated that neither lipopolysaccharide structure nor flagella play a role in *E. coli* strain Nissle 1917 translocation and dissemination. Our results suggest that if both the microbiota and adaptive immunity are defective, translocation across the intestinal epithelium and dissemination of the probiotic *E. coli* strain Nissle 1917 may occur and have potentially severe adverse effects. Future work should define the possibly related molecular factors that promote probiotic functions, fitness, and facultative pathogenicity.**

The human gastrointestinal tract contains a complex symbiotic microbiota that is estimated to comprise more than 40,000 species and in some regions more than 10<sup>11</sup> organisms (14) and that helps maintain immune homeostasis in the gut-associated lymphoid tissues (20, 29), optimize nutritional uptake (13), and support development of the gut (40). The thick mucus layer that overlies the entire intestinal epithelium and an effective immune system keep this enormous bacterial load strictly sequestered on the luminal side of the gut, preventing penetration across the epithelial barrier (20).

The importance of the cross talk between the microbiota, intestinal epithelial cells, and the innate and adaptive portions of the immune system is indicated by a variety of intestinal pathological conditions, including Crohn's disease, ulcerative colitis, pouchitis, irritable bowel syndrome, and necrotizing enterocolitis (NEC). Immature or genetically compromised immunity (25) results in exaggerated intestinal inflammation (26) or disruption or altered compo-

sition of the intestinal mucosa, which in turn disturbs the homeostasis between the human host and its intestinal symbionts. Pathological events change the relative balance between beneficial and aggressive enteric symbionts, turn beneficial bacteria into pathogens (36), or select for novel opportunistic pathogens (2, 28). A qualitatively and quantitatively changed gastrointestinal microbiota, often described as small bowel bacterial overgrowth (SIBO) (14, 33) or dysbiosis (26), may contribute substantially to local chronic inflammation in a vicious cycle and provoke bacterial translocation that leads to fatal sepsis.

This concept provides the rationale for selective therapeutic manipulation of the abnormal microbiota by probiotics for the intestinal diseases that have been described; probiotics are defined as viable microorganisms with beneficial physiological or therapeutic activities (36). Various *in vitro* and animal studies with probiotics, including *Escherichia coli* strain Nissle 1917, have demonstrated the capacity of probiotics to reduce intestinal inflammation (29, 42), to strengthen the intestinal barrier against pathogens (15, 46), to increase the host innate immune functions (37), or to prevent adherent and invasive *E. coli* strains from adhering to and invading human intestinal epithelial cells (9). Indeed, limited clinical trials using *E. coli* strain Nissle 1917 or other microorganisms have suggested that this therapeutic strat-

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egy is efficacious in patients with chronic idiopathic inflammatory bowel diseases (IBD) (23, 34, 35, 36), irritable bowel syndrome (32), and NEC (25).

However, all probiotic bacterial species are not equally beneficial, and each species may have individual mechanisms of action due to specific metabolic activities and cellular structures (10). Some case reports even seem to indicate that probiotics, including *E. coli* strain Nissle 1917, might promote sepsis (6, 24), and severe adverse effects of probiotics have been observed in patients suffering from acute pancreatitis (3, 5).

Host characteristics, specifically characteristics of the existing microbiota and the intestinal immune status, are often not considered when probiotics are used as therapeutic agents, particularly in genetically or therapeutically immunocompromised patients, including very-low-birth-weight preterm infants (25) and severely ill IBD patients receiving anti-inflammatory therapy (11).

The fact that living bacteria that may turn out to be opportunistic pathogens are therapeutically administered to patients with impaired immune functions and an altered intestinal microbiota raises two important basic questions. First, how important is a functional adaptive immune system for preventing adverse clinical effects of probiotics, such as translocation, bacteremia, and death? Lymphocytes of the gut-associated lymphoid tissue play an essential role in controlling proliferation and differentiation of intestinal epithelial cells (22) and in the maintenance of gut integrity (12). Second, how important is the microbiota in this context? Symbionts have been shown to activate epithelial innate immune signaling pathways that are needed to fight microbial pathogens (20).

To answer the questions mentioned above, we employed mice having a targeted disruption of recombinase-activating gene 1 (*Rag1*<sup>-/-</sup>), in which T- and B-lymphocyte development is arrested at the CD4<sup>-</sup> CD8<sup>-</sup> double-negative thymocyte or B220<sup>+</sup> CD43<sup>+</sup> pro-B-cell stage (30). To study the role of the physiological microbiota, these mice were raised under germ-free (GF) or specific-pathogen-free (SPF) conditions and were challenged with *E. coli* strain Nissle 1917. Here we present evidence indicating that when there is a profound deficiency in the adaptive immune system in the presence of both functional innate immunity and the intestinal microbiota, translocation and dissemination of *E. coli* strain Nissle 1917 do not occur; however, in the absence of the intestinal microbiota, substantial translocation of *E. coli* strain Nissle 1917 causes high rates of mortality in *Rag1*<sup>-/-</sup> mice. We also show that adoptive transfer of naïve CD4<sup>+</sup> T cells to *Rag1*<sup>-/-</sup> mice raised under GF conditions (GF-raised *Rag1*<sup>-/-</sup> mice) after *E. coli* strain Nissle 1917 challenge increases the mortality rate significantly. Our data might explain why immunocompromised patients that have an immature or disrupted intestinal microbiota and are treated with probiotics have an enhanced risk for severe side effects due to bacterial translocation. Furthermore, we describe a T-cell-mediated pathogenic mechanism that is involved in a fatal outcome for immunocompromised mice fed the probiotic *E. coli* strain Nissle 1917.

#### MATERIALS AND METHODS

**Mice.** C57BL/6J-Rag1<sup>tm1Mom</sup> (*Rag1*<sup>-/-</sup>) (30) mice and C57BL/6J (B6) mice were used in this study. Mice were bred either under SPF conditions in a barrier-sustained facility or under gnotobiotic conditions in isolators at the University of Ulm, Ulm, Germany. Gnotobiotic mice were maintained in a germfree

(GF) environment or were colonized with only one bacterial strain, either *E. coli* strain Nissle 1917 (kindly provided by Sonnenborn, Ardeypharm, Germany), the mouse intestinal strain *E. coli* mpk, or the mouse intestinal strain *Bacteroides vulgatus* mpk (45). *E. coli* mpk (O not typeable:H8) has been assigned to phylogenetic group B1 (45). Initial colonization of GF mice was accomplished by feeding the animals a suspension containing the appropriate bacterial strain(s). Successful colonization was controlled and monitored as described below. The gnotobiotic state was controlled weekly and at the time of necropsy, and this involved culturing for aerobic and anaerobic bacteria, Gram stain examination of feces and intestinal contents, and performing broad-range eubacterial 16S rRNA gene PCR analyses of stool samples from mice, as described previously (45). The presence of *Helicobacter* spp. was controlled by routine veterinary monitoring. Tumor necrosis factor alpha (TNF- $\alpha$ )-deficient mice (*TNF- $\alpha$* <sup>-/-</sup> mice), backcrossed with B6 mice for 10 generations (31), were kindly provided by R. Mückat, Helmholtz Zentrum, Munich, Germany.

**Bacterial challenge and determination of translocation by culture methods and PCR.** We challenged groups of at least four GF-raised wild-type mice, GF-raised *Rag1*<sup>-/-</sup> mice, or *Rag1*<sup>-/-</sup> mice raised under SPF conditions (SPF-raised *Rag1*<sup>-/-</sup> mice) on day 0 with the probiotic *E. coli* strain Nissle 1917, the nonpathogenic strain *E. coli* mpk, or *B. vulgatus* mpk as described previously (45). In brief, mice were challenged with  $1 \times 10^8$  CFU of viable *E. coli* or *B. vulgatus* by oral gavage. *E. coli* strain Nissle 1917 expresses flagella, has a semirough lipopolysaccharide (LPS) phenotype, and does not produce known extracellular protein toxins (7, 17). *E. coli* strain Nissle 1917, an *E. coli* strain Nissle 1917  $\Delta$ flhC mutant deficient in the flagellum filament protein (37), an *E. coli* strain Nissle 1917  $\Delta$ flgE mutant deficient in the flagellum hook gene (37), and an *E. coli* strain Nissle 1917 strain complemented with a plasmid containing a functional copy of *wzy* from *E. coli* strain 536 [*E. coli* strain Nissle 1917(pBW536) (17)] (designated the *E. coli* Nissle 1917 *wzy* strain), which provides a smooth LPS phenotype, were used in mouse colonization experiments. All *E. coli* strains were grown in Luria-Bertani broth at 37°C overnight, and *B. vulgatus* was grown in brain heart infusion (BHI) medium at 37°C under anaerobic conditions. Where appropriate, ampicillin was added to the growth medium at a concentration of 100  $\mu$ g/ml.

The numbers of bacterial CFU in the mesenteric lymph nodes (MLN), liver, spleen, and lungs and in the feces (intestinal colonization) were determined by homogenization and plating of serial dilutions of the homogenates on blood and Endo agar (aerobic), as well as brain heart infusion (BHI) agar (anaerobic) at different time points after challenge. The numbers of CFU per plate were determined and expressed as log<sub>10</sub> CFU/g organ (limit of detection, 50 CFU). For identification of *E. coli* strain Nissle 1917, PCR was performed using primers specific for the pMUT1 plasmid and for the pMUT2 plasmid as described previously (8).

**T-cell transfer before and after bacterial challenge.** Lymphocytes were isolated by homogenizing spleens of B6 or *TNF- $\alpha$* <sup>-/-</sup> mice. Erythrocytes were eliminated by incubation of the homogenates in lysis buffer (160 mM NH<sub>4</sub>Cl, 170 mM Tris; pH 7.4). Splenic naïve CD4<sup>+</sup> T cells were purified with a MACS negative selection kit (Miltenyi, Bergisch Gladbach, Germany) by following the manufacturer's instructions. The purity of the CD4<sup>+</sup> T-cell population obtained was >90%, and over 80% of the cells were CD62L<sup>+</sup> CD4<sup>+</sup> T cells. *E. coli* strain Nissle 1917-challenged GF- or SPF-raised *Rag1*<sup>-/-</sup> mice were reconstituted with  $5 \times 10^5$  CD62L<sup>+</sup> CD4<sup>+</sup> naïve T cells intraperitoneally 3 days before bacterial challenge or at day 6 after bacterial challenge.

**Histology and assessment of cytokine levels in serum.** Lung tissues were fixed in neutral buffered 4% formalin. Formalin-fixed tissues were embedded in paraffin and cut into 2- $\mu$ m sections. Samples were stained with hematoxylin and eosin (H&E) (Merck, Darmstadt, Germany). Sections were analyzed in a blinded fashion by one pathologist. Serum samples were stored at -80°C. Levels of TNF- $\alpha$  were quantified by an enzyme-linked immunosorbent assay (ELISA) (BD Biosciences, Heidelberg, Germany) performed according to the manufacturer's instructions.

**Statistics.** Statistical analysis was performed using Student's *t* test, analysis of variance (ANOVA), or the Kaplan-Meier log rank test, as indicated below.

## RESULTS

**The presence of a microbiota prevents translocation of *E. coli* strain Nissle 1917 in immunodeficient *Rag1*<sup>-/-</sup> mice and ensures survival after challenge with *E. coli* strain Nissle 1917.**

To assess the impact of the intestinal microbiota on protection of mice with severely compromised adaptive immunity (30) from bacterial translocation, we challenged GF- or SPF-raised *Rag1*<sup>-/-</sup>

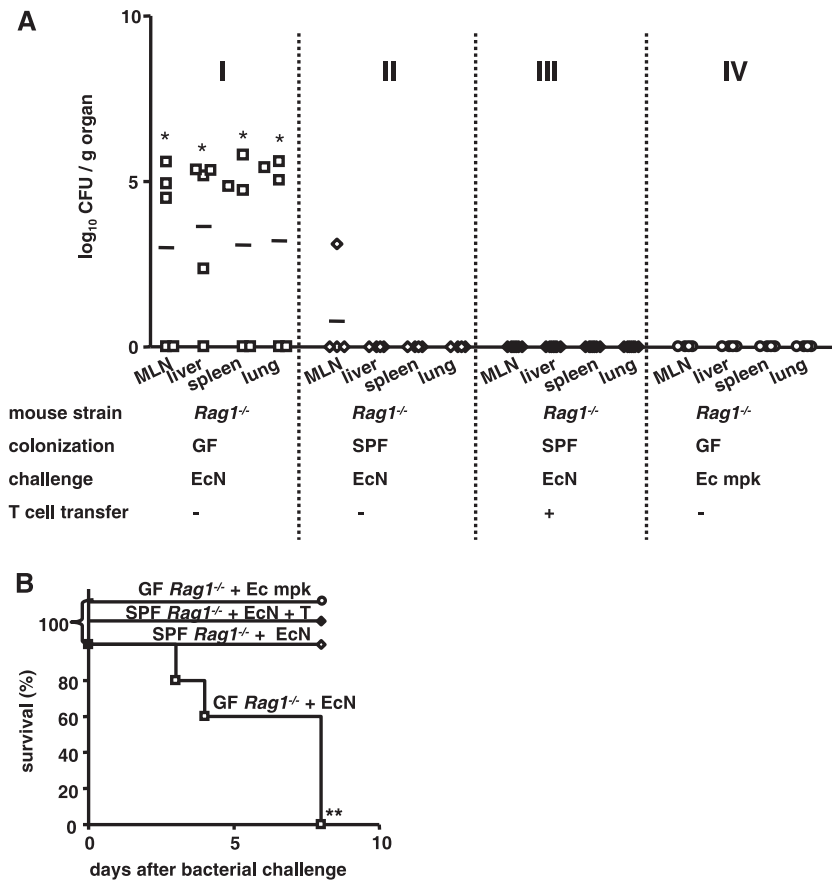


FIG. 1. In *Rag1*<sup>-/-</sup> mice the microbiota prevents bacterial translocation across the intestinal epithelium and ensures survival after challenge with *E. coli* Nissle 1917. (A) Groups of at least four GF-raised (panel I) or SPF-raised (panel II) *Rag1*<sup>-/-</sup> mice were colonized with  $1 \times 10^8$  CFU of *E. coli* Nissle 1917 (EcN) on day 0. Additionally, one group of SPF-raised *Rag1*<sup>-/-</sup> mice was reconstituted with T cells 6 days after *E. coli* strain Nissle 1917 challenge (panel III), and GF-raised *Rag1*<sup>-/-</sup> mice were also challenged with  $1 \times 10^8$  CFU of the commensal *E. coli* mpk (Ec mpk) (panel IV). At day 7, the numbers of bacterial CFU in the MLN, liver, spleen, and lungs of the animals were determined. Each symbol indicates the data for a single animal. \*,  $P < 0.05$  for a comparison with all other groups (one-way ANOVA, Bonferroni multiple-comparison post test). (B) Kaplan-Meier survival curves for *E. coli* strain Nissle 1917-challenged GF-raised ( $\square$ ) or SPF-raised ( $\diamond$ ) *Rag1*<sup>-/-</sup> mice, *E. coli* strain Nissle 1917-challenged T-cell-reconstituted SPF-raised *Rag1*<sup>-/-</sup> mice ( $\blacklozenge$ ), and GF-raised *Rag1*<sup>-/-</sup> mice which were challenged with the commensal *E. coli* mpk ( $\circ$ ). \*\*,  $P < 0.01$  (Kaplan-Meier log rank test).

mice orally with *E. coli* strain Nissle 1917, a probiotic *E. coli* strain used to treat IBD and other intestinal diseases (36), or with *E. coli* mpk, a commensal fecal mouse *E. coli* strain (45).

*Rag1*<sup>-/-</sup> mice raised under GF conditions were challenged with *E. coli* strain Nissle 1917 or *E. coli* mpk, and bacterial translocation and dissemination were assessed by determining the numbers of bacteria in the MLN, liver, spleen, and lungs 7 days later. GF-raised *Rag1*<sup>-/-</sup> mice devoid of the intestinal microbiota were found to be highly susceptible to *E. coli* strain Nissle 1917 challenge; high numbers of bacteria, identified by PCR as *E. coli* strain Nissle 1917, were detected in the MLN, liver, spleen, or lungs of the animals (Fig. 1A, panel I), and all GF-raised *Rag1*<sup>-/-</sup> mice succumbed to the bacteria within 7 days (Fig. 1B). In contrast, for SPF-raised *Rag1*<sup>-/-</sup> mice, which had a physiologically highly diverse microbiota, no translocation of *E. coli* strain Nissle 1917 across the intestinal barrier was observed, whether T cells were present (Fig. 1A, panel III) or not present (Fig. 1A, panel II), and all of these mice survived the *E. coli* strain Nissle 1917 challenge (Fig. 1B). To examine whether other *E. coli* strains translocated in GF-

raised *Rag1*<sup>-/-</sup> mice, we tested the commensal strain *E. coli* mpk under the same experimental conditions. In contrast to the results for *E. coli* strain Nissle 1917, translocation of *E. coli* mpk to the MLN, liver, spleen, and lungs was not observed, and none of the animals in this group died for 7 days after bacterial challenge (Fig. 1B). These results indicate that in the absence of a microbiota, *E. coli* strain Nissle 1917, but not *E. coli* mpk, translocated and disseminated in GF-raised *Rag1*<sup>-/-</sup> hosts. A microbiota is required to prevent translocation of *E. coli* strain Nissle 1917 in immunodeficient mice, and translocation of *E. coli* strain Nissle 1917 across the intestinal barrier is a strain-specific phenomenon and therefore may be related to *E. coli* strain Nissle 1917-specific fitness or virulence factors.

**T cells are required to prevent dissemination of *E. coli* strain Nissle 1917 in GF mice.** To assess the impact of the adaptive immune system on protection of mice devoid of an intestinal microbiota from bacterial translocation and dissemination, we challenged GF-raised *Rag1*<sup>-/-</sup> or B6 mice orally with *E. coli* strain Nissle 1917 and determined the bacterial translocation and dissemination and the survival of mice. Fig-

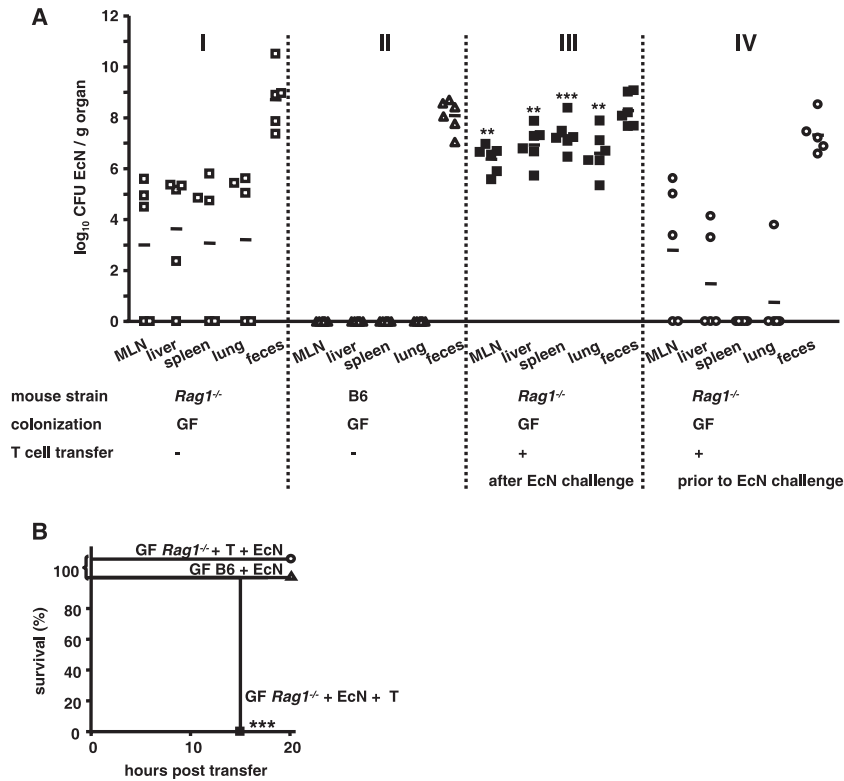


FIG. 2. T cells are required to prevent translocation and dissemination of *E. coli* strain Nissle 1917 in GF mice. (A) Numbers of CFU in the MLN, liver, spleen, lungs, and feces of groups of at least five *E. coli* strain Nissle 1917 (EcN)-challenged GF-raised *Rag1*<sup>-/-</sup> mice (panel I), *E. coli* strain Nissle 1917-challenged GF-raised B6 mice (panel II), *E. coli* strain Nissle 1917-challenged GF-raised *Rag1*<sup>-/-</sup> mice (panel III) which were reconstituted with naïve T cells (panel IV), and GF-raised *Rag1*<sup>-/-</sup> mice which received naïve T cells 3 days before challenge with *E. coli* strain Nissle 1917. At day 7, the numbers of bacterial CFU were determined. Each symbol indicates the data for a single animal. \*\*, *P* < 0.01 for a comparison with all other groups; \*\*\*, *P* < 0.001 for a comparison with all other groups (one-way ANOVA, Bonferroni multiple-comparison post test). (B) Kaplan-Meier survival curves for *E. coli* strain Nissle 1917-challenged GF-raised B6 mice (▲), *E. coli* strain Nissle 1917-challenged T-cell-reconstituted GF-raised *Rag1*<sup>-/-</sup> mice (■), and GF-raised *Rag1*<sup>-/-</sup> mice which received T cells 3 days before bacterial challenge (○). \*\*\*, *P* < 0.001 (Kaplan-Meier log rank test).

ure 2A shows that GF-raised *Rag1*<sup>-/-</sup> mice (Fig. 2A, panel I), but not GF-raised B6 mice (Fig. 2A, panel II), are susceptible to translocation and dissemination of *E. coli* strain Nissle 1917, which indicates that T cells are required to prevent dissemination of *E. coli* strain Nissle 1917 in mice devoid of a microbiota.

To test whether adoptive transfer of T cells after *E. coli* strain Nissle 1917 challenge rescued *Rag1*<sup>-/-</sup> mice from *E. coli* strain Nissle 1917 dissemination and mortality, we intraperitoneally reconstituted GF-raised *Rag1*<sup>-/-</sup> mice with 5 × 10<sup>5</sup> CD62L<sup>+</sup> CD4<sup>+</sup> naïve T cells 6 days after *E. coli* strain Nissle 1917 challenge (Fig. 2A, panel III). Strikingly, T-cell reconstitution after *E. coli* strain Nissle 1917 challenge led to accelerated mortality of GF-raised *Rag1*<sup>-/-</sup> mice (Fig. 2B) compared to the mortality observed for non-T-cell-reconstituted mice. In fact, high bacterial counts were obtained for the MLN, liver, spleen, and lungs of all GF-raised *Rag1*<sup>-/-</sup> mice (Fig. 2A, panel III) compared to the results for non-T-cell-reconstituted mice (Fig. 1A, panel I, and Fig. 2A, panel I), and the mortality rate was high 15 h after T-cell transfer (Fig. 2B). T-cell reconstitution of *Rag1*<sup>-/-</sup> mice 2 days after *E. coli* strain Nissle 1917 challenge resulted in mild to severe exacerbation of the disease, and the severity of the disease correlated with the mortality of mice (data not shown).

To investigate whether T-cell reconstitution prior to *E. coli* strain Nissle 1917 challenge rescued *Rag1*<sup>-/-</sup> mice without an intestinal microbiota from subsequent translocation of *E. coli* strain Nissle 1917, we next reconstituted GF-raised *Rag1*<sup>-/-</sup> mice with T cells 3 days before *E. coli* strain Nissle 1917 challenge. This treatment resulted in significantly less bacterial translocation and dissemination and a reduced bacterial burden in all organs investigated (Fig. 2A, panel IV), and all of the mice survived for the whole observation period (Fig. 2B). These results were corroborated by the results of experiments performed with fully immunocompetent B6 mice without an intestinal microbiota, in which *E. coli* strain Nissle 1917 translocation and dissemination were not observed in any of the organs investigated (Fig. 2A, panel I). Consequently, all B6 mice survived the bacterial challenge (Fig. 2B).

**Targeting of TNF-α production in T cells does not reduce T-cell-mediated mortality of *E. coli* strain Nissle 1917-challenged, T-cell-reconstituted GF-raised *Rag1*<sup>-/-</sup> mice.** In GF-raised mice challenged with *E. coli* strain Nissle 1917 and reconstituted with T cells 6 days later enhanced mortality was associated with significantly increased levels of TNF-α in the serum (Fig. 3A I) compared to the levels in non-T-cell-reconstituted mice (Fig. 3A, panel II), suggesting that there were

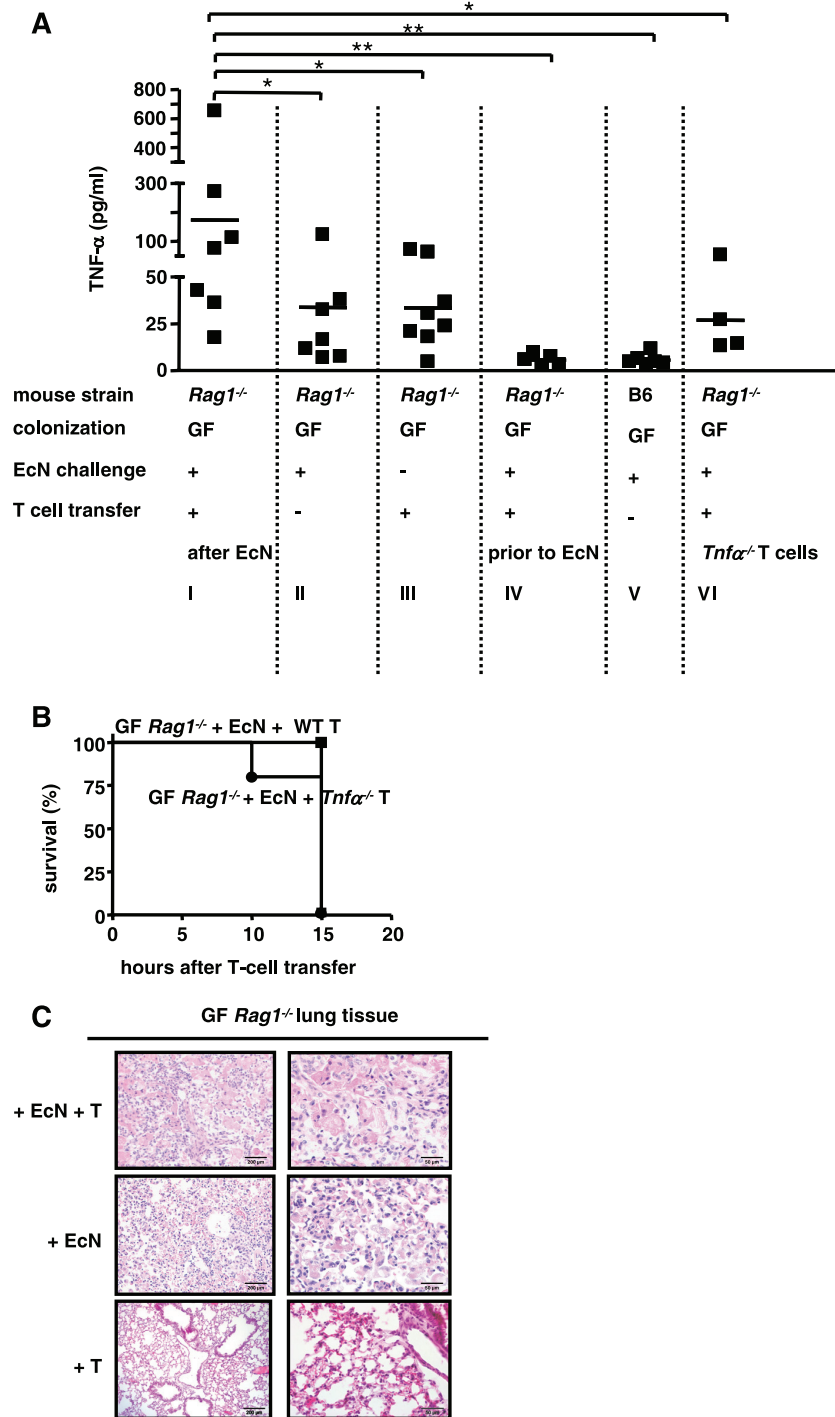


FIG. 3. Reconstitution of *E. coli* strain Nissle 1917-challenged *Rag1*<sup>-/-</sup> mice with *Tnf-α*<sup>-/-</sup> T cells does not reduce T-cell-mediated mortality. (A) TNF-α cytokine concentrations in serum of *E. coli* strain Nissle 1917 (EcN)-challenged T-cell-reconstituted GF-raised *Rag1*<sup>-/-</sup> mice (panel I), *E. coli* strain Nissle 1917-challenged GF-raised *Rag1*<sup>-/-</sup> mice (panel II), T-cell-reconstituted GF-raised *Rag1*<sup>-/-</sup> mice (panel III), GF-raised *Rag1*<sup>-/-</sup> mice which received T cells of wild-type mice 3 days before *E. coli* strain Nissle 1917 challenge (panel IV), *E. coli* strain Nissle 1917-challenged GF-raised B6 mice (panel V), and *E. coli* strain Nissle 1917-challenged GF-raised *Rag1*<sup>-/-</sup> mice which received *Tnf-α*<sup>-/-</sup> naive T cells (panel VI), as measured by ELISA. \*, *P* < 0.05; \*\*, *P* < 0.01 (paired Student *t* test). (B) Kaplan-Meier survival curves for *E. coli* strain Nissle 1917-challenged GF-raised *Rag1*<sup>-/-</sup> mice reconstituted with *Tnf-α*<sup>-/-</sup> cells (●) or wild-type T cells (WT) (■). (C) Histology of lung tissues of *E. coli* strain Nissle 1917-challenged T-cell-reconstituted GF-raised *Rag1*<sup>-/-</sup> mice, *E. coli* strain Nissle 1917-challenged GF-raised *Rag1*<sup>-/-</sup> mice, and T-cell-reconstituted GF-raised uninfected control mice. All sections were stained with H&E.

systemic inflammatory events. Increased levels of TNF- $\alpha$  were not observed in nonchallenged T-cell-reconstituted GF-raised *Rag1*<sup>-/-</sup> mice (Fig. 3A, panel III), in *E. coli* strain Nissle 1917-challenged GF-raised *Rag1*<sup>-/-</sup> mice that were T cell reconstituted before bacterial challenge (Fig. 3A, panel IV), or in *E. coli* strain Nissle 1917-challenged GF-raised B6 mice (Fig. 3A, panel V). These results suggest that TNF- $\alpha$  production was increased when translocated bacteria directly or indirectly stimulated T cells.

To examine whether T-cell-derived TNF- $\alpha$  may contribute to mortality in this model, GF-raised *Rag1*<sup>-/-</sup> mice were reconstituted with T cells from *Tnf- $\alpha$* <sup>-/-</sup> mice (31). Figure 3B shows that the attempts to reduce mortality by adoptive transfer of *Tnf- $\alpha$* <sup>-/-</sup> T cells were unsuccessful, although the levels of TNF- $\alpha$  in the serum were reduced in these mice (Fig. 3A, panel VI) compared to the levels in controls with T cells transferred from wild-type mice (Fig. 3A, panel I). These findings suggest that the enhanced mortality was mediated largely by other, TNF- $\alpha$ -independent mechanisms.

To examine the possible mechanisms involved in mortality of *E. coli* strain Nissle 1917-challenged GF-raised *Rag1*<sup>-/-</sup> mice, histomorphological analyses were carried out (Fig. 3C). The substantially increased mortality of *E. coli* strain Nissle 1917-challenged T-cell-reconstituted GF-raised *Rag1*<sup>-/-</sup> mice was particularly supported by the histology of the lungs; fibrin deposits, accumulation of alveolar macrophages, scattered neutrophils, and signs of pleuritis were observed in these mice. In some respects, these findings resembled the diffuse alveolar damage that is the morphological manifestation of acute respiratory distress syndrome. *E. coli* strain Nissle 1917-challenged GF-raised *Rag1*<sup>-/-</sup> mice without T-cell reconstitution had fewer histopathological changes and only moderate interstitial infiltration, whereas GF-raised *Rag1*<sup>-/-</sup> mice showed no significant pathology (Fig. 3C).

**Neither semirough lipopolysaccharide nor flagella promote dissemination of *E. coli* strain Nissle 1917 in and mortality of *E. coli* strain Nissle 1917-challenged GF-raised *Rag1*<sup>-/-</sup> mice.**

To examine the virulence factors of *E. coli* strain Nissle 1917 which cause translocation and death in *E. coli* strain Nissle 1917-challenged GF-raised *Rag1*<sup>-/-</sup> mice, we used *E. coli* strain Nissle 1917 mutants in our animal model. First, we examined the role of flagella, which play an important role in cell adhesion and bacterial motility (39). As *E. coli* strain Nissle 1917 is flagellated, we hypothesized that the *E. coli* strain Nissle 1917  $\Delta$ *flhC* and  $\Delta$ *flgE* flagellum mutants would not cross the intestinal barrier and would not cause increased mortality in GF-raised *Rag1*<sup>-/-</sup> mice reconstituted with T cells after bacterial challenge.

The *E. coli* strain Nissle 1917  $\Delta$ *flhC* and *E. coli* strain Nissle 1917  $\Delta$ *flgE* mutants colonized the intestine of GF-raised *Rag1*<sup>-/-</sup> mice like *E. coli* wild-type strain Nissle 1917 (Fig. 4A). Determination of the bacterial counts in the MLN, liver, spleen, and lungs revealed that the flagellum mutants translocated and disseminated as effectively as *E. coli* wild-type strain Nissle 1917. Thus, the mutant strains did not differ from the wild-type strain in terms of the bacterial burdens in the various organs of the mice 7 days after challenge (Fig. 4B) or in terms of the mortality that they caused (Fig. 4C). Moreover, colonization with *E. coli* wild-type strain Nissle 1917 and colonization with the mutants resulted in similarly enhanced levels of

TNF- $\alpha$  in the serum (Fig. 4D). From these results we concluded that flagella are not required for pathogenicity of *E. coli* strain Nissle 1917 in GF-raised *Rag1*<sup>-/-</sup> mice.

Rough-type LPS, which is present in *E. coli* wild-type strain Nissle 1917, is known to be 100 times more effective for stimulation of epithelial cells than smooth-type LPS, which is present in, e.g., *E. coli* mpk (1, 4, 19, 45). Therefore, we tested whether a change in the LPS structure of *E. coli* strain Nissle 1917 resulted in differences in translocation, dissemination, and mortality rates in *Rag1*<sup>-/-</sup> mice. To do this, *E. coli* strain Nissle 1917 transfected with a plasmid containing a functional copy of the *E. coli* strain 536 *wzy* gene (pWB536 [17]) (*E. coli* strain Nissle 1917 *wzy* mutant), which resulted in a smooth LPS phenotype for the bacterium, was used for intestinal colonization. Challenge of mice with the *E. coli* strain Nissle 1917 *wzy* mutant resulted in colonization of the mouse intestine that was the same as the colonization observed for *E. coli* wild-type strain Nissle 1917 (Fig. 4A) and in similar bacterial counts in the MLN, liver, spleen, and lungs (Fig. 4B). In line with this, a reduction in mortality (Fig. 4C) or in the TNF- $\alpha$  level in the serum (Fig. 4D) was not observed.

***E. coli* mpk cannot prevent translocation of *E. coli* strain Nissle 1917.** In order to test whether competition between *E. coli* strain Nissle 1917 and the intestinal mouse strain *E. coli* mpk protected the mice from translocation and dissemination of *E. coli* strain Nissle 1917 and increased mortality, GF-raised *Rag1*<sup>-/-</sup> mice were first challenged with *E. coli* mpk and then after 3 days were challenged with *E. coli* strain Nissle 1917 and reconstituted with T cells on day 6 after the *E. coli* strain Nissle 1917 challenge.

As shown in Fig. 5, *E. coli* mpk colonized the intestine of GF-raised *Rag1*<sup>-/-</sup> mice (Fig. 5A, panel I) as effectively as *E. coli* strain Nissle 1917. Subsequent challenge with *E. coli* strain Nissle 1917 did not increase the total number of intestinal CFU (Fig. 5A, panel II). The ratio of *E. coli* mpk to *E. coli* strain Nissle 1917, determined by PCR-based detection of the *E. coli* strain Nissle 1917-specific plasmids pMUT1 and pMUT2, showed that the ratio of *E. coli* mpk to *E. coli* strain Nissle 1917 was almost 1:1 (Fig. 5B). This might indicate that *E. coli* strain Nissle 1917 supersedes *E. coli* mpk during competition for similar biological niches. As shown in Fig. 5C, challenge of *Rag1*<sup>-/-</sup> mice with *E. coli* mpk did not lead to translocation of *E. coli* mpk but did not protect the mice from translocation and dissemination of *E. coli* strain Nissle 1917 (Fig. 5C, panel II), as confirmed by analysis of *E. coli* strain Nissle 1917-specific pMUT1 and pMUT2 PCR (data not shown). Additionally, the mortality of mice was not reduced (Fig. 5D), but the increase in the level TNF- $\alpha$  in the serum was eliminated (Fig. 5E).

In contrast to the results for *E. coli* mpk, *B. vulgatus* mpk colonization resulted in inhibition of *E. coli* strain Nissle 1917 translocation, as indicated by the lower *E. coli* strain Nissle 1917 burden in the peripheral organs (Fig. 6A, panel II). However, the mortality of mice was unchanged (Fig. 6B).

## DISCUSSION

In order to examine the safety of probiotics, we used the probiotic *E. coli* strain Nissle 1917 in GF- and SPF-raised wild-type and *Rag1*<sup>-/-</sup> mouse models. We analyzed the contributions of the intestinal microbiota and the adaptive im-

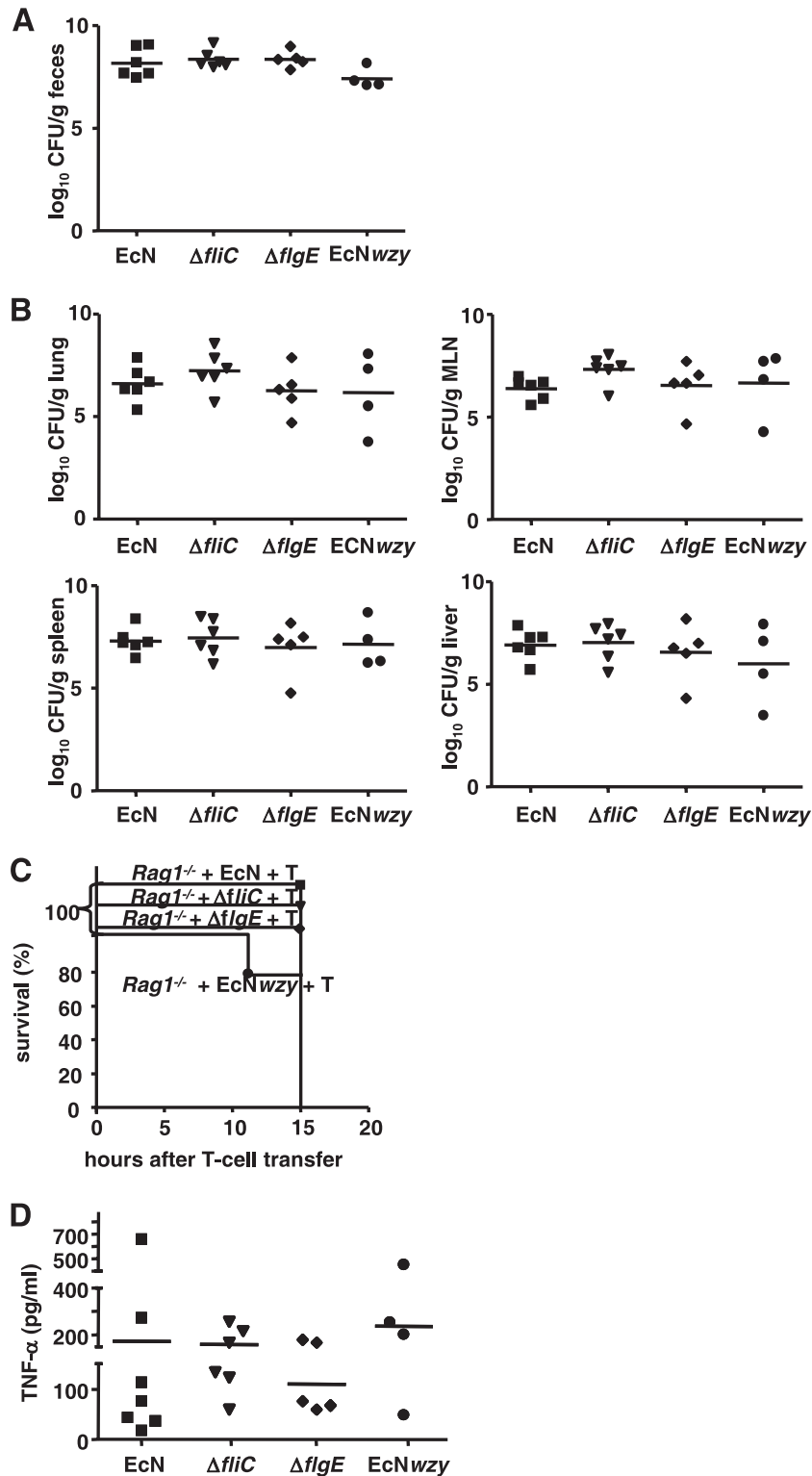


FIG. 4. Translocation in germfree  $Rag1^{-/-}$  mice is independent of LPS- or flagellum-mediated signals. Groups of at least five GF-raised  $Rag1^{-/-}$  mice were challenged with  $1 \times 10^8$  CFU of the  $\Delta fliC$  mutant, the  $\Delta flgE$  mutant, the *E. coli* strain Nissle 1917 wzy mutant (EcNwzy), or *E. coli* wild-type strain Nissle 1917 (EcN). Six days after challenge  $Rag1^{-/-}$  mice were reconstituted with naive T cells. (A) Numbers of CFU in feces at 1 day after transfer. Each symbol indicates the data for one animal. (B) Numbers of CFU in organs at 1 day after transfer. Each symbol indicates the data for one animal. (C) Kaplan-Meier survival curves for  $Rag1^{-/-}$  mice challenged with either *E. coli* strain Nissle 1917 (■), the  $\Delta fliC$  mutant (▼), the  $\Delta flgE$  mutant (◆), or the *E. coli* strain Nissle 1917 wzy mutant (●) after T cell reconstitution. (D) TNF- $\alpha$  concentrations in sera of T-cell-reconstituted GF-raised  $Rag1^{-/-}$  mice challenged with *E. coli* strain Nissle 1917, the  $\Delta fliC$  mutant, the  $\Delta flgE$  mutant, or the *E. coli* strain Nissle 1917 wzy mutant. TNF- $\alpha$  concentrations were determined by ELISA.

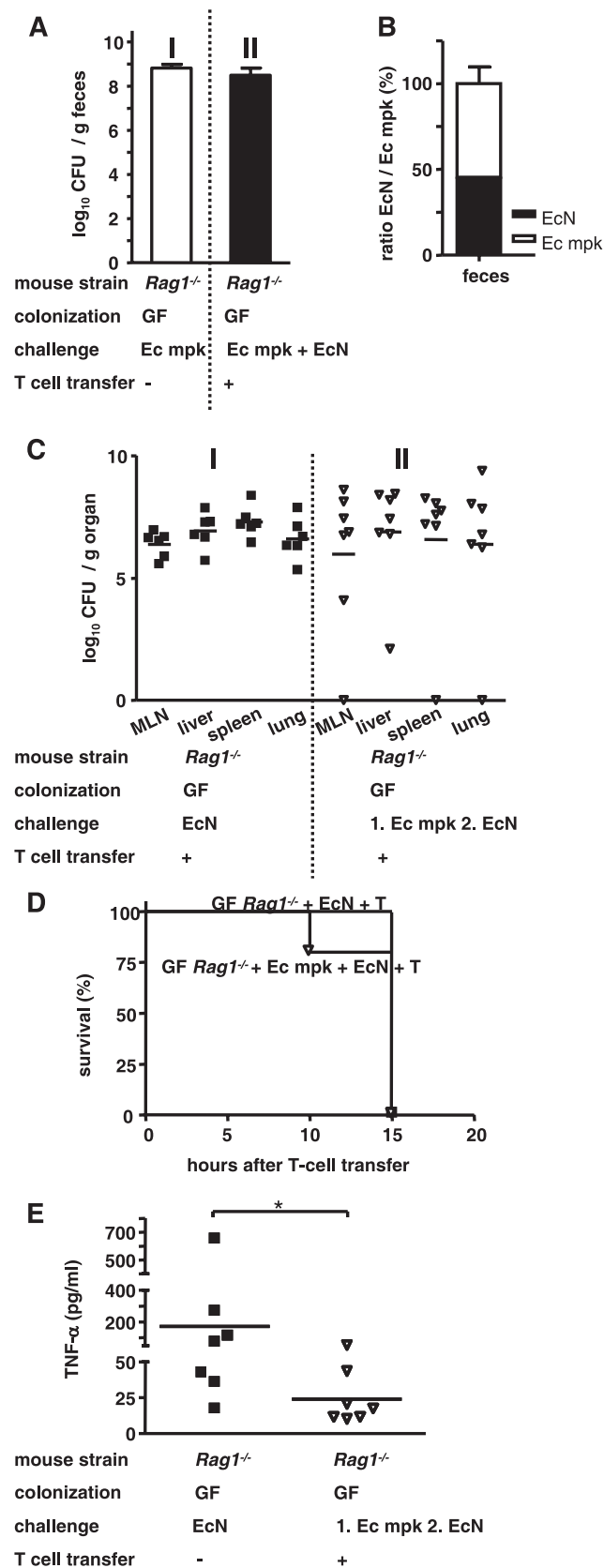


FIG. 5. *E. coli* mpk cannot prevent translocation of *E. coli* strain Nissle 1917. Groups of at least four GF-raised *Rag1*<sup>-/-</sup> mice were challenged with  $1 \times 10^8$  CFU of the commensal *E. coli* mpk (Ec mpk)

immune system to prevention of bacterial translocation across the intestinal epithelium, bacterial dissemination to various organs, and death. The clinical relevance of these investigations results (i) from the fact that probiotics have been administered to patients with therapy-related or disease-related immunosuppression and intestinal barrier dysfunction, in some cases resulting in severe side effects (3, 25), and (ii) from the fact that recent reports demonstrated the inflammatory potential of *E. coli* strain Nissle 1917 (6, 44).

Our results demonstrate that the intestinal microbiota prevents translocation of the probiotic *E. coli* strain Nissle 1917 even in the presence of a defective adaptive immune system. Conversely, in the absence of the intestinal microbiota, translocation of *E. coli* strain Nissle 1917 is prevented by a fully competent innate and adaptive immune system. However, when both the microbiota and adaptive immunity are defective, translocation of *E. coli* strain Nissle 1917 occurs. Once translocation of *E. coli* strain Nissle 1917 across the intestinal epithelium into internal organs has taken place, an attempt to rescue mice by adoptive transfer of immune cells (T cells) exacerbates the disease and accelerates mortality. Together, our results suggest that *E. coli* strain Nissle 1917 seems to overcome host innate immune defense mechanisms like lysis by the complement-protein complex or killing by phagocytes and that the defense against and clearance of translocated *E. coli* strain Nissle 1917 strongly depend on T-cell-mediated mechanisms.

Here we show that the intestinal microbiota is sufficient to prevent translocation of the probiotic *E. coli* strain Nissle 1917 to mouse organs. This finding suggests that the adaptive immunity is dispensable in this context, although lymphocytes have been shown to substantially impact the proliferation and differentiation of the intestinal epithelium (41). This suggestion is based on our findings that *Rag1*<sup>-/-</sup> mice raised under SPF conditions and exhibiting a physiologically mature microbiota did not have any bacteria in their MLN, liver, spleen, or lungs and that all of these mice survived oral challenge with high levels of *E. coli* strain Nissle 1917. Results of other investigators revealed that SPF-raised C3H/HeJZtm mice, which have a defective Toll-like receptor 4 (TLR4) allele and hence are defective for innate immunity, are also protected from translocation of *E. coli* strain Nissle 1917 (6).

3 days before challenge with *E. coli* strain Nissle 1917 (EcN). Six days after *E. coli* strain Nissle 1917 challenge *Rag1*<sup>-/-</sup> mice were reconstituted with naive T cells. (A) Numbers of CFU of *E. coli* mpk 3 days after challenge and total numbers of CFU (*E. coli* mpk and *E. coli* strain Nissle 1917) 7 days after *E. coli* strain Nissle 1917 challenge in feces. (B) Ratio of *E. coli* strain Nissle 1917 to *E. coli* mpk in feces 7 days after *E. coli* strain Nissle 1917 challenge. *E. coli* strain Nissle 1917 was identified by detection of the *E. coli* strain Nissle 1917-specific cryptic plasmids pMUT1 and pMUT2. (C) Numbers of CFU of *E. coli* strain Nissle 1917 in the MLN, liver, spleen, and lungs at 1 day after transfer. (D) Kaplan-Meier survival curve for GF-raised *Rag1*<sup>-/-</sup> mice which were challenged with the commensal *E. coli* mpk 3 days before challenge with *E. coli* strain Nissle 1917 and T cell reconstituted 6 days after *E. coli* strain Nissle 1917 challenge ( $\nabla$ ) and GF-raised, *E. coli* strain Nissle 1917-challenged *Rag1*<sup>-/-</sup> mice reconstituted with T cells ( $\blacksquare$ ). (E) TNF- $\alpha$  concentrations in sera after T-cell reconstitution. \*,  $P < 0.05$  (paired Student *t* test).



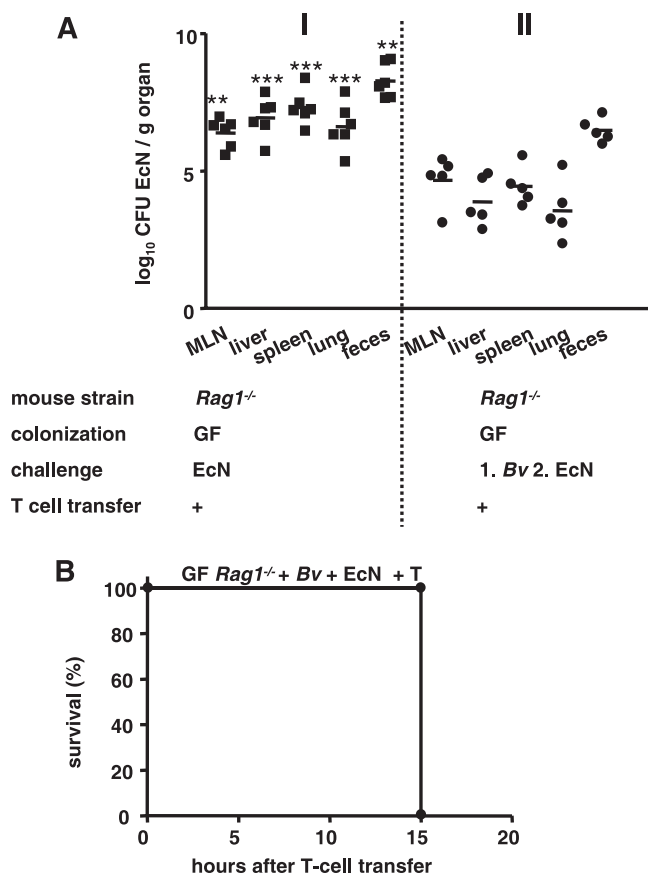


FIG. 6. *B. vulgatus* mpk reduces translocation of *E. coli* strain Nissle 1917, but not the mortality of mice. Groups of at least five GF-raised *Rag1*<sup>-/-</sup> mice were challenged with  $1 \times 10^8$  CFU of the commensal *B. vulgatus* mpk (*Bv*) 3 days before challenge with *E. coli* strain Nissle 1917 (Ecn). Six days after *E. coli* strain Nissle 1917 challenge *Rag1*<sup>-/-</sup> mice were reconstituted with naïve T cells. (A) Numbers of CFU of *E. coli* strain Nissle 1917 in peripheral organs and feces 7 days after challenge \*\*,  $P < 0.01$  for a comparison with *E. coli* strain Nissle 1917-challenged T-cell-reconstituted *Rag1*<sup>-/-</sup> mice; \*\*\*,  $P < 0.001$  for a comparison with *E. coli* strain Nissle 1917-challenged T-cell-reconstituted *Rag1*<sup>-/-</sup> mice (one-way ANOVA, Bonferroni multiple-comparison post test). (B) Kaplan-Meier survival curve for GF-raised *Rag1*<sup>-/-</sup> mice which were challenged with the commensal *B. vulgatus* mpk 3 days before challenge with *E. coli* strain Nissle 1917 and T cell reconstituted 6 days after the *E. coli* strain Nissle 1917 challenge.

Our findings also revealed that the microbiota is dispensable with regard to bacterial translocation in the presence of an intact adaptive immune system; GF-raised B6 mice were highly resistant to *E. coli* strain Nissle 1917 challenge, as were GF-raised *Rag1*<sup>-/-</sup> mice that were reconstituted with T cells prior to *E. coli* strain Nissle 1917 challenge. This finding was unexpected since GF-raised mice have various defects in their intestinal mucosa, including a smaller width, a sparse stroma in the lamina propria, wider microvillus brush borders, and small Peyer's patches (43).

An important finding of the present study is that a lack of both the intestinal microbiota and the adaptive immune system allows the probiotic *E. coli* strain Nissle 1917 to translocate and cause death in *Rag1*<sup>-/-</sup> mice. All GF-raised *Rag1*<sup>-/-</sup> mice succumbed the bacterial challenge within 7 days, and high

numbers of CFU of *E. coli* strain Nissle 1917 were found in the MLN, liver, spleen, or lungs of the animals. Interestingly, GF-raised C3H/HeJZtm mice are also susceptible to translocation of *E. coli* strain Nissle 1917 (6). Thus, it seems that two of the three components (i.e., the microbiota and the innate and adaptive immune systems) need to be in a competent state to form an effective barrier against microbial invasion and to prevent disease. These findings may have important consequences for administration of probiotics in general and *E. coli* strain Nissle 1917 specifically.

This is in line with recent findings of Slack et al., who showed that adaptive immunity is critical for successful mutualism in TLR signaling-deficient mice and that TLR signaling is required for the normal elimination of low numbers of bacteria that are translocated from the intestinal lumen into the mucosa, but that commensal-specific serum IgG responses, induced in response to translocated intestinal bacteria, can restore effective bacterial clearance in TLR signaling-deficient mice (38). Slack et al. suggested that there is a flexible set point between innate immunity and adaptive immunity, which is determined by the functional performance of each system that protects the host (38). However, we eliminated the possibility that B cells have a major role in our animal model of *Rag1*<sup>-/-</sup> mice.

A major finding of the present study was that competition between *E. coli* strain Nissle 1917 and the intestinal mouse strain *E. coli* mpk did not prevent translocation and dissemination of *E. coli* strain Nissle 1917. This suggests that translocation of *E. coli* strain Nissle 1917 might be an active process that depends on *E. coli* strain Nissle 1917-specific fitness or virulence factors that enable *E. coli* strain Nissle 1917 to compete with *E. coli* mpk and to cross the intestinal barrier. However, our data provide a hint that anaerobic intestinal bacteria like, e.g., *B. vulgatus* might at least reduce translocation of *E. coli* mpk. Further studies are necessary to clarify the molecular mechanisms underlying these effects. The genome of *E. coli* strain Nissle 1917 has been described, and the data revealed a number of so-called pathogenicity islands and genes encoding adhesins (type 1 and F1C fimbriae, Iha, curli, AIDA-I/Sap-like), proteases (Sat and Tsh), and microcins, as well as multiple-gene clusters coding for proteins involved in iron acquisition (yersiniabactin, aerobactin, salmochelin, and Chu hemin receptor) that increase bacterial fitness (16). Whether the probiotic characteristics of *E. coli* strain Nissle 1917 are related to its increased fitness is an important question and challenges the concept of probiotic bacteria in general. In fact, it is crucial to elucidate whether there is a direct or indirect molecular relationship between factors that promote probiotic functions, fitness, and thus increased facultative pathogenicity in immunocompromised hosts (hosts with, e.g., a defective microbiota and T-cell deficiency).

Reconstitution of GF-raised *Rag1*<sup>-/-</sup> mice with naïve CD4<sup>+</sup> T cells after *E. coli* strain Nissle 1917 challenge increased the mortality rate to 100% from the mortality rate of 72% observed for nonreconstituted mice. Compared to the results for nonreconstituted mice, T-cell reconstitution led to increased numbers of bacteria in various organs, more severe lung pathology, and significantly increased levels of TNF- $\alpha$  in the serum. Since transfer of *Tnf*- $\alpha$ <sup>-/-</sup> T cells did not reduce the high mortality rate in GF-raised *Rag1*<sup>-/-</sup> mice, other TNF- $\alpha$ -

independent effects might account for the increased mortality. The concept suggested by Hotchkiss and Nicholson indicates that death from sepsis might be the result of a substantially impaired immune response that is due to extensive death of immune effector cells (21). Our results suggest that once translocation of bacteria across the intestinal epithelium into internal organs has taken place, the response of the adaptive immune system exacerbates the disease and accelerates mortality.

A previous *in vitro* study of *E. coli* strain Nissle 1917 revealed proinflammatory traits (44). Therefore, to determine the virulence of *E. coli* strain Nissle 1917 in GF-raised *Rag1*<sup>-/-</sup> mice on a molecular level, we tested isogenic flagellum and LPS mutants of *E. coli* wild-type strain Nissle 1917. Neither deletion of the flagella nor changes in the LPS structure of *E. coli* strain Nissle 1917 affected bacterial translocation and dissemination or mortality.

We hypothesized that the intestinal microbiota is essential for prevention of the translocation of *E. coli* strain Nissle 1917 across the intestinal barrier. Once translocated, *E. coli* strain Nissle 1917 seems to be able to evade host innate immune defenses like lysis by the complement protein complex or phagocytosis; a T-cell-mediated adaptive immune mechanism seems to be essential for control and clearance of translocated *E. coli* strain Nissle 1917. However, other studies have reported serum sensitivity of *E. coli* strain Nissle 1917 in humans (17). Recent studies provided evidence that translocation of certain *E. coli* strains might occur via novel transcellular pathways activated in enterocytes by inflammatory and metabolic stress (27). The data of Macutkiewicz et al. suggest that translocating *E. coli* strains associated with infections are not opportunistic extraintestinal pathogenic *E. coli* (ExPEC) strains but may comprise a separate group of *E. coli* strains (27).

Our results suggest that administration of probiotic *E. coli* strain Nissle 1917 preparations to immunocompromised patients that also have a defective intestinal microbiota after, e.g., antibiotic therapy may lead to severe adverse effects and therefore should not be recommended. A typical target population may be very-low-birth-weight preterm infants or patients after organ transplantation. Although a recent study (25) concluded that probiotic bacteria, such as *Bifidobacterium* and *Lactobacillus*, fed enterally to very-low-birth-weight preterm infants for 6 weeks reduced the incidence of death or necrotizing enterocolitis, the authors mentioned that “occurrences of sepsis even seemed more frequent in the study group” (25). Recently, Guenther et al. described severe sepsis in a preterm infant due to treatment with the probiotic *E. coli* strain Nissle 1917 (18). Although experiments with germfree, monocolonized, or gnotobiotic mice are not directly equivalent to the situation in patients, the data obtained with our model may explain why septic episodes have been observed in immunocompromised patients that had an immature or disrupted intestinal microbiota, were treated with probiotics, and exhibited T-cell-mediated pathogenesis that resulted in fatal sepsis.

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