Safety of probiotic *Escherichia coli* strain Nissle 1917 depends on intestinal microbiota and adaptive immunity of the host

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Short title: Safety aspects of *E. coli* Nissle

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ABSTRACT

Probiotics are viable microorganisms increasingly used in treatment of a variety of diseases. Occasionally, however, probiotics may cause clinical adverse effects including septicaemia. Here we addressed the role of the intestinal microbiota and the adaptive immune system to prevent translocation of probiotics e. g. *E. coli* Nissle. We challenged germfree (GF)-raised C57BL/6J, GF- and specific pathogen free (SPF)-raised *Rag1*–/– mice with probiotic *E. coli* Nissle 1917 (EcN), EcN mutant strains, commensal *E. coli* mpk or *Bacteroides vulgatus* mpk. Additionally, we reconstituted *Rag1*–/– mice with CD4+ T cells. *E. coli* translocation, dissemination, and mortality of mice were assessed. In GF-raised *Rag1*–/–, but not in SPF-raised *Rag1*–/– or in GF-raised C57BL/6J mice, oral challenge with EcN, but not with *E. coli* mpk, caused translocation and dissemination. Mortality was significantly higher in GF-raised EcN-challenged *Rag1*–/– mice (100% p<0.001) compared to SPF-raised *Rag1*–/– mice (0%) and GF-raised C57BL/6J mice (0%). Translocation and mortality promoted by EcN in GF-raised *Rag1*–/– mice was prevented when mice were T cell-reconstituted prior, but not after, EcN challenge. Co-colonization experiments revealed that *E. coli* mpk could not prevent translocation of EcN. Moreover, we demonstrate that neither lipopolysaccharide structure, nor the flagella, play a role in EcN translocation and dissemination. Our results suggest that if both microbiota and adaptive immunity are defective, translocation across the intestinal epithelium and dissemination of probiotic EcN may occur with potentially severe adverse effects. Future work needs to define the possibly related molecular principles of factors promoting probiotic functions, fitness and facultative pathogenicity.
Introduction

The human gastrointestinal tract accommodates a complex symbiotic microbiota, estimated to comprise more than 40,000 species and exceeding in some regions $10^{11}$ organisms (14), that provide help to maintain immune homeostasis within the gut-associated lymphoid tissues (20), (29), optimize nutritional uptake (13) and support the 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).

How important the cross-talk between the microbiota, intestinal epithelial cells and the innate and adaptive arms of the immune system is, becomes unmasked in a variety of intestinal pathological entities that include Crohn’s disease, ulcerative colitis, pouchitis, irritable bowel syndrome and necrotising enterocolitis (NEC). Immature or genetically compromised immunity (25) cause exaggerated intestinal inflammation (26), disruption or altered composition of the intestinal mucosa, which in turn disturb the homeostasis between the human host and their intestinal symbionts. These pathological events change the relative balance of beneficial vs. aggressive enteric symbionts, turn once-beneficial bacteria into pathogens (36) or select for novel opportunistic pathogens (2), (28). The qualitatively and quantitatively changed gastrointestinal microbiota, often described as small bowel bacterial overgrowth (SIBO) (14), (33) or dysbiosis (26), may substantially contribute to local chronic inflammation in a vicious cycle and provoke bacterial translocation leading to fatal sepsis.

This concept provides the rationale for selective therapeutic manipulation of the abnormal microbiota by probiotics in the described intestinal diseases, defined as viable microorganisms with beneficial physiological or therapeutical activities (36). Various in vitro and animal studies with probiotics, including the *E. coli* strain Nissle 1917 (EcN), have demonstrated the capacity of probiotics to reduce intestinal inflammation (29), (42), to strengthen the intestinal barrier against pathogens (46), (15), to increase the host innate...
immune functions (37), or to prevent adherent–invasive E. coli strains from adhering to
and invading human intestinal epithelial cells (9). Indeed, limited clinical trials using EcN or
other microorganisms suggest such therapeutic strategies to be efficacious in patients with
chronic idiopathic inflammatory bowel diseases (IBD) (36), (34), (35), (23), irritable bowel
syndrome (32) and NEC (25).

However, probiotic bacterial species are not equally beneficial and each may have
individual mechanisms of action due to specific metabolic activities and cellular structure
(10). Some case reports even seem to indicate that probiotics, including EcN, 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 concerning the existing microbiota and the intestinal
immune status are often not considered when probiotics are applied as therapeutics,
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 dilemma that living bacteria, which may turn out to be opportunistic pathogens, are
therapeutically administered to patients with impaired immune functions and altered
intestinal microbiota raises two important basic questions: how important is a functional
adaptive immune system to prevent clinical adverse effects of probiotics such as
translocation, bacteraemia and death? Lymphocytes of the gut-associated lymphoid tissue
play an essential role in controlling proliferation and differentiation of intestinal epithelial
cells (22) and the maintenance of the gut integrity (12). Secondly, how important is the
presence of the microbiota in this context? Symbionts have been shown to activate
epithelial innate immune signalling pathways, needed to fight microbial pathogens (20).

To resolve the aforementioned questions, we employed mice bearing targeted disruption
of the 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
germfree (GF) or specific pathogen free (SPF) conditions and were challenged with EcN.

We present evidence that a profound deficiency of the adaptive immune system in the presence of both functional innate immunity and intestinal microbiota does not allow EcN translocation and dissemination; however, in the absence of the intestinal microbiota, substantial translocation of EcN causes high mortality rates in \( \text{Rag1}^{-/-} \) mice. We further show that adoptive transfer of naïve CD4\(^+\) T cells to GF-raised \( \text{Rag1}^{-/-} \) mice after EcN challenge increases mortality rates significantly. Our data might indicate why immunocompromised patients with immature or disrupted intestinal microbiota and treated with probiotics show an enhanced risk for severe side effects due to bacterial translocation. Furthermore we demonstrate a T cell-mediated pathogenic mechanism involved in the fatal outcome of immunocompromised mice fed with probiotic EcN.
MATERIAL AND METHODS

Mice

C57BL/6J-Rag1tm1Mom (Rag1−/) (30) mice and C57BL/6J (B6) mice were used. Mice were bred either under SPF conditions in a barrier sustained facility, or under gnotobiotic conditions in isolators at the University of Ulm, Germany. Gnotobiotic mice were maintained in a germfree environment (GF) or were colonized exclusively with one bacterial strain, the *E. coli* strain Nissle 1917 (kindly provided by Dr. Sonnenborn, Ardeypharm, Germany), the mouse intestinal *E. coli* strain mpk or mouse intestinal *Bacteroides vulgatus* strain mpk (45). *E. coli* mpk (O not typable: H8) is assigned to the phylogenic group B1 (45). Initial colonization of GF mice was performed by feeding them a suspension of the respective bacterial strain(s). Successful colonization was controlled and monitored as detailed later. The gnotobiotic state was controlled weekly and at the time of necropsy, and this involved culturing for aerobic and anaerobic bacteria, gram stain examinations of feces and intestinal contents, as well as broad-range eubacterial 16S ribosomal DNA polymerase chain reaction (PCR) of stool samples from mice, as published previously (43). The absence of *Helicobacter spp.* was controlled by the routine veterinary monitory. Tumor necrosis factor-α deficient (TNF-α−/) mice, backcrossed on B6 over 10 generations (31) were kindly provided by R. Mocikat, Helmholtz Zentrum, Munich, Germany.

Bacterial challenge and determination of translocation by culture methods and PCR

We challenged groups of at least 4 GF-raised wild type mice and GF- or SPF-raised *Rag1−/−* mice at day 0 with the probiotic strain EcN, the non-pathogenic *E. coli* strain mpk or *B. vulgatus* mpk as described previously (45). In brief, mice were challenged with 1x10⁶ viable *E. coli* or *B. vulgatus* by oral gavage. EcN expresses flagella and exhibits a semi rough lipopolysaccharide (LPS) phenotype and does not produce known extracellular protein toxins (7), (17). EcN and the EcN mutant strains ΔfliC, deficient in the flagella
filament protein (37), ΔflgE deficient of the flagella hook gene (37), and an EcN strain, complemented with a plasmid containing a functional wzy copy of *E. coli* strain 536 (EcN (pBWBS36) (17)) (referred to as EcN wzy), which provides a smooth LPS phenotype to the bacterium, were used in mouse colonization experiments. All *E. coli* strains were grown in Luria-Bertani broth at 37°C over night, *B. vulgatus* was grown in Brain-Heart-Infusion (BHI) medium at 37° under anaerobic conditions. Where appropriate, ampicillin was added to the growth medium at a concentration of 100 µg/ml.

Bacterial CFUs in the mesenteric lymph nodes (MLN), liver, spleen and lungs and intestinal colonization (feces) 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 colony forming units (CFU) per plate were counted and expressed as log_{10}CFU/g organ (limit of detection: 50 CFU). For identification of EcN, PCR was performed using primers specific for the pMUT1 plasmid and pMUT2 plasmid as published previously (8).

**T cell transfer before and after bacterial challenge**

Lymphocytes were isolated by homogenising spleens of B6 or TNF-α−/− mice. Erythrocytes were eliminated by incubation of the homogenates in lysis buffer (160 mM NH_{4}CL, 170 mM Tris, pH 7.4). Splenic naïve CD4+ T cells were purified using MACS negative selection kit (Miltenyi, Bergisch Gladbach, Germany) following the manufacturer’s instructions. The purity of the obtained CD4+ T cell population was >90% with over 80% CD62L+CD4+ T cells. EcN-challenged GF- or SPF-raised Rag1−/− mice were reconstituted with 5x10^5 CD62L+ CD4+ naïve T cells intraperitoneally 3 d before bacterial challenge or at day 6 after bacterial challenge.
Histology and assessment of cytokine serum levels

Lung tissues were fixed in neutral buffered 4% formalin. Formalin-fixed tissues were embedded in paraffin and cut in 2 µm sections. Samples were stained with hematoxylin/eosin (H&E) (Merck, Darmstadt, Germany). Sections were analysed in a blinded fashion by one pathologist. Serum samples were stored at -80 °C. Levels of TNF-α were quantified by ELISA (BD Biosciences, Heidelberg, Germany), according to the manufacturer’s instructions.

Statistics

Statistical analysis was performed using Student’s t test, ANOVA or Kaplan Meyer log rank test, as indicated.
Results

The presence of a microbiota prevents translocation of EcN in immunodeficient
Rag1−/− mice and ensures survival after challenge with EcN

To assess the impact of the intestinal microbiota in protecting mice severely compromised
in adaptive immunity (30) from bacterial translocation, we challenged GF- or SPF-raised
Rag1−/− mice orally with EcN, 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−/− mice, raised under GF conditions were challenged with EcN or E. coli mpk and
bacterial translocation and dissemination was assessed by determination of bacterial
numbers in MLN, liver, spleen and lungs 7 days later. GF-raised Rag1−/− mice devoid of the
intestinal microbiota turned out to be highly susceptible to EcN challenge: high bacterial
CFU, identified by PCR as EcN, were detected in MLN, liver, spleen or lungs of the
animals (Fig. 1A I) and all GF-raised Rag1−/− mice succumbed the bacterial challenge
within 7 days (Fig. 1B). In contrast, SPF-raised Rag1−/− mice exhibiting a physiologic highly
diverse microbiota, did not reveal any translocation of EcN across the intestinal barrier,
independently of absence (Fig. 1A II) or presence of T cells (Fig. 1A III) and did all survive
the EcN challenge (Fig. 1B). To explore whether also other E. coli strains would
translocate in GF-raised Rag1−/− mice, we tested the commensal E. coli strain mpk in this
experimental setting. In contrast to EcN, translocation of E. coli mpk to MLN, liver, spleen
and lungs was not observed and none of the animals in this group died after bacterial
challenge up to 7 days (Fig. 1B). These results indicate that in absence of a microbiota
EcN but not E. coli mpk translocate and disseminate in GF-raised Rag1−/− hosts. The
presence of microbiota is required to prevent translocation of EcN in immunodeficient
mice, and the translocation of EcN across the intestinal barrier is a strain specific
phenomenon and therefore possibly related to EcN specific fitness or virulence factors.

T cells are required to prevent dissemination of EcN in GF mice
To assess the impact of the adaptive immune system in protecting mice devoid of an intestinal microbiota from bacterial translocation and dissemination, we challenged GF-raised $Rag1^{-/-}$ or B6 mice orally with EcN and determined bacterial translocation, dissemination and survival of mice. The data depicted in Figure 2A demonstrate that GF-raised $Rag1^{-/-}$ mice (Fig. 2A I), but not GF-raised B6 mice (Fig. 2A II), are susceptible to translocation and dissemination of EcN, indicating that T cells are required to prevent dissemination of EcN in mice devoid of a microbiota.

To test whether an adoptive transfer of T cells after EcN challenge would rescue $Rag1^{-/-}$ mice from EcN dissemination and mortality, we intraperitoneally reconstituted GF-raised $Rag1^{-/-}$ mice with $5 \times 10^5$ CD62L$^+$ CD4$^+$ naive T cells six days after EcN challenge (Fig. 2A III). Strikingly, T cell reconstitution after EcN challenge even led to an accelerated mortality of GF-raised $Rag1^{-/-}$ mice (Fig. 2B) than that observed in non T cell-reconstituted mice. In fact, all mice displayed high bacterial counts in MLN, liver, spleen and lungs of GF-raised $Rag1^{-/-}$ mice (Fig. 2A III), compared to non-T cell-reconstituted mice (Fig. 1A I and 2A I) and showed high mortality already 15 hours after T cell transfer (Fig. 2B). T-cell reconstitution of $Rag1^{-/-}$ mice 2 days after EcN challenge resulted in mild to severe exacerbation of disease, and the severity of disease correlated with mortality of mice (data not shown).

To investigate whether T cell reconstitution prior to EcN challenge would rescue $Rag1^{-/-}$ mice without intestinal microbiota from subsequent EcN translocation, we next reconstituted GF-raised $Rag1^{-/-}$ mice with T cells 3 days before EcN challenge. This treatment resulted in significantly less bacterial translocation and dissemination with a reduced bacterial burden in all investigated organs (Fig. 2A IV) and 100% of the mice survived during the whole period of observation (Fig. 2B). These results were corroborated in experiments with fully immunocompetent B6 mice without intestinal microbiota, which did not show EcN translocation and dissemination in any of the
investigated organs (Fig. 2A I). Consequently, all B6 mice survived the bacterial challenge (Fig. 2B).

Targeting of TNF-\(\alpha\) production in T cells does not reduce T cell-mediated mortality of GF-raised EcN challenged T cell-reconstituted \(\text{Rag}^1\) mice

In GF-raised mice challenged with EcN and reconstituted with T cell six days later the enhanced mortality was associated with significantly increased TNF-\(\alpha\) serum levels (Fig. 3A I), compared to non T cell-reconstituted mice (Fig. 3A II), suggesting systemic inflammatory events. Such increased TNF-\(\alpha\) levels were not observed in GF-raised non-challenged T cell-reconstituted \(\text{Rag}^1\) mice (Fig. 3A III), or in GF-raised EcN-challenged \(\text{Rag}^1\) mice which were T cell-reconstituted before bacterial challenge (Fig. 3A IV), as well as in GF-raised EcN-challenged B6 mice (Fig. 3A V). These results suggest that TNF-\(\alpha\) production was increased when translocated bacteria directly or indirectly stimulated T cells.

To address whether T cell derived TNF-\(\alpha\) may contribute to mortality in this model, GF-raised \(\text{Rag}^1\) mice were reconstituted with T cells from \(\text{Tnf}^{-}\) mice (31). The data depicted in Figure 3B demonstrated that the attempts to reduce mortality by adoptive transfer of \(\text{Tnf}^{-}\) T cells were unsuccessful although TNF-\(\alpha\) serum levels were reduced in these mice (Fig. 3A VI), compared to controls transferred with T cells from wild type mice (Fig. 3A I). These findings suggest that the enhanced mortality was largely mediated by other, TNF-\(\alpha\) independent, mechanisms.

To address possible mechanisms involved in mortality of GF-raised EcN-challenged \(\text{Rag}^1\) mice, histomorphological analyses were carried out (Fig. 3C). The substantially increased mortality of GF-raised EcN-challenged T cell-reconstituted \(\text{Rag}^1\) mice was particularly supported by the histology of the lungs: fibrin depositions, accumulations of alveolar macrophages, scattered neutrophils and signs of pleuritis were observed in these mice. In some aspects, these findings resembled diffuse alveolar damage as the morphological substrate of acute respiratory distress syndrome. GF-raised EcN-
challenged $\textit{Rag}^{1/-}$ mice without T cell reconstitution showed less histopathological changes with only moderate interstitial infiltrations, whereas GF-raised $\textit{Rag}^{1/-}$ mice displayed no significant pathology (\textbf{Fig. 3C}).

Neither semi-rough lipopolysaccharide nor flagella promote dissemination of EcN in and mortality of GF-raised EcN-challenged $\textit{Rag}^{1/-}$ mice

To explore the virulence factors of EcN which cause translocation and death in GF-raised EcN-challenged $\textit{Rag}^{1/-}$ mice, we next used EcN mutants in our animal model. First we addressed the role of flagella, which play an important role in cell adhesion and bacterial motility (39). As EcN is flagellated we hypothesized that the EcN flagella mutants $\Delta$\textit{fliC} and $\Delta$\textit{flgE} would not overcome the intestinal barrier and would not cause increased mortality in GF-raised $\textit{Rag}^{1/-}$ mice reconstituted with T cells after bacterial challenge.

EcN $\Delta$\textit{fliC} and EcN $\Delta$\textit{flgE} colonized the intestine of GF-raised $\textit{Rag}^{1/-}$ mice comparable to the EcN wild type strain (\textbf{Fig. 4A}). Determination of bacterial counts in MLN, liver, spleen and lungs revealed that the flagella mutants translocated and disseminated as effective as the EcN wild type strain. Thus, the mutant strains did not differ from the wild type strain concerning bacterial burden in the various organs of these mice 7 days after challenge (\textbf{Fig. 4B}), or with regard to mortality (\textbf{Fig. 4C}). Moreover, colonization with EcN wild type or mutants induced similarly enhanced serum TNF-$\alpha$ level (\textbf{Fig. 4D}). From these results we can conclude that flagella are not required for pathogenicity of EcN in GF-raised $\textit{Rag}^{1/-}$ mice.

Rough-type LPS which is present in the EcN wild type strain is known to be 100 times more effective in stimulation of epithelial cells compared with smooth-type LPS which is present in e.g. \textit{E. coli} mpk (1,4,19,45). Therefore, we tested whether a change in the LPS structure of EcN would yield different translocation, dissemination and mortality rates in $\textit{Rag}^{1/-}$ mice. To this end, EcN transfected with a plasmid containing a functional \textit{wzy} copy of \textit{E. coli} strain 536 (pBWB536, (17)) (EcN \textit{wzy}), which provides a smooth LPS phenotype to the bacterium was used for intestinal colonisation. Challenge of mice with EcN \textit{wzy} ...
resulted in equal colonization of the mouse intestine as compared to the EcN wildtype strain (Fig. 4A), and resulted in similar bacterial counts in MLN, liver, spleen and lungs (Fig. 4B). In line with this, a reduction of mortality (Fig. 4C) or TNF-α serum level (Fig. 4D) was not observed.

**E. coli** mpk is not sufficient to prevent translocation of EcN

In order to test whether competition of EcN with the intestinal mouse *E. coli* strain mpk would protect the mice from translocation and dissemination of EcN and increased mortality, GF-raised **Rag1**-/mice in a first step were challenged with *E. coli* mpk, after 3 days subsequently challenged with EcN and reconstituted with T cells on day 6 after EcN challenge.

As depicted in Fig. 5, *E. coli* mpk colonizes the intestine of GF-raised **Rag1**-/ mice (Fig. 5A I) as effective as EcN. Subsequent challenge with EcN did not augment the total intestinal CFU (Fig. 5A II). The ratio of *E. coli* mpk to EcN, determined by PCR based detection of the EcN specific plasmids pMUT1 and pMUT2, shows that the ratio of *E. coli* mpk to EcN was almost 50% each (Fig. 5B). This might indicate that EcN supersedes *E. coli* mpk by competition for similar biological niches. As shown in Fig. 5C challenge of **Rag1**-/ mice with *E. coli* mpk did not lead to translocation of *E. coli* mpk but did not protect from translocation and dissemination of EcN (Fig. 5C II) as confirmed by the EcN specific pMUT1 and pMUT2 PCR (data not shown). Additionally, mortality of mice was not reduced (Fig. 5D), but the increase of serum TNF-α level was obviated (Fig. 5E).

In contrast to EcN, *Bacteroides vulgatus* mpk colonization resulted in inhibition of EcN translocation as indicated by lower bacterial burden of EcN in the peripheral organs (Fig. 6A II). However, mortality of mice was unchanged (Fig. 6B).
DISCUSSION

In order to address safety aspects of probiotics we selected and used the probiotic bacterial strain EcN in GF- and SPF-raised wild type and Rag1\(^{−/−}\) mouse models. We analyzed the respective contribution of the intestinal microbiota and the adaptive immune system to prevent bacterial translocation across the intestinal epithelium, bacterial dissemination to various organs and death. The clinical relevance of these investigations derives from the fact that (i) probiotics have been administered to patients with therapy-related or disease-related immunosuppression and intestinal barrier dysfunction yielding in some cases severe side effects (3,25), and (ii) recent reports demonstrated an inflammatory potential of EcN (6), (44).

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

Herein we show that the intestinal microbiota is sufficient to prevent translocation of probiotic EcN to mouse organs. This 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 notion is based on our findings that Rag1\(^{−/−}\) mice raised under SPF conditions and exhibiting a physiologic, mature microbiota, did not reveal any bacterial numbers in MLN, liver, spleen or lungs and all survived the oral challenge with high CFUs of EcN. Results from other investigators reveal...
that SPF-raised C3H/HeJZtm mice, which carry a defective toll-like receptor (TLR)4-allele, and, hence, are defective in innate immunity, are also protected from EcN translocation (6).

Our findings also reveal 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 EcN challenge as were GF-raised Rag1−/− mice which were reconstituted with T cells prior to EcN challenge. This was an unexpected finding since GF-raised mice show various defects of the 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 lack of both the intestinal microbiota and the adaptive immune system allows the probiotic strain EcN to translocate and cause death in Rag1−/− mice: All GF-raised Rag1−/− mice succumbed the bacterial challenge within 7 days and high CFUs of EcN were found in MLN, liver, spleen or lungs of the animals. Interestingly, GF-raised C3H/HeJZtm mice are also susceptible to EcN translocation (6).

Thus, it seems that either two of the three components, i.e. the microbiota, the innate and the adaptive immune system, need to be in a competent state to form an effective barrier against microbial invasion and to prevent disease. These considerations may have important consequences for the administration of probiotics in general and EcN specifically.

This is in line with recent findings of Slack et al., showing that adaptive immunity is critical for successful mutualism in TLR signalling-deficient mice and that TLR signalling 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 signalling deficient mice (38). The authors suggest, that there is a flexible set point between innate and adaptive immunity, determined by the functional
performance of each system that acts to protect the host (38). However, in our animal
model of Rag1⁻/⁻ mice we can exclude a major role for B cells.

A major finding of the present study was that competition of EcN with the intestinal mouse
E. coli strain mpk did not prevent translocation and dissemination of EcN. This suggests
that the translocation of EcN might be an active process, depending on EcN specific
fitness or virulence factors that enable EcN to compete against E. coli mpk and to
overcome the intestinal barrier. However our data might provide a hint that anaerobic
intestinal bacteria like e. g. B. vulgatus might at least reduce translocation of EcN. Further
studies are necessary to clarify the molecular mechanisms underlying these effects. The
genome of EcN has been resolved and 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), microcins, and multiple-gene clusters coding for proteins
involved in iron acquisition (yersiniabactin, aerobactin, salmochelin, and Chu hemin
receptor) promoting bacterial fitness (16). Whether the probiotic feature of EcN is related
to its increased fitness or not is an important aspect 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 promoting probiotic functions, fitness, and
thus increased facultative pathogenicity in immunocompromised hosts (with e.g. defective
microbiota and T cell deficiency).

The reconstitution of GF-raised Rag1⁻/⁻ mice with naïve CD4⁺ T cells after EcN challenge
increased the mortality rate from 72% in non-reconstituted mice to 100%. Compared to
non-reconstituted mice, T cell reconstitution led to increased bacterial numbers in various
organs, more severe lung pathology and significantly increased serum TNF-α levels. Since
the transfer of Tnf-α⁻/⁻ T cells did not reduce the high mortality rate in GF-raised Rag1⁻/⁻
mice, other TNF-α independent effects might account for the increased mortality. The
concept suggested by Hotchkiss and Nicholson alludes, 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.
In a previous *in vitro* study, EcN revealed pro-inflammatory traits (44). To unravel the
virulence of EcN in GF-raised *Rag1*^-/-^ mice, on a molecular level, we therefore tested
isogenic flagella and LPS mutants of the EcN wild type strain. Neither deletion of the
flagella nor changes in the LPS structure of EcN did affect bacterial translocation,
dissemination and mortality.
We hypothesize, that the intestinal microbiota is essential to prevent the translocation of
EcN across the intestinal barrier. Once translocated, EcN seems to be able to evade host
innate immune defence like lysis by the complement protein complex or phagocytosis; T
cell mediated adaptive immune mechanism seem to be essential for the control and
clearance of translocated EcN. However, other studies report on serum sensitivity of EcN
in humans (17). Recent studies provide 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 *Escherichia coli* (ExPEC) strains but may comprise a separate group of *E. coli*
strains (27).
Our results suggest that administration of probiotic EcN preparations to
immunocompromised patients, presenting in addition 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 represent 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 reported on a severe sepsis in a
preterm infant due to treatment with probiotic EcN (18). Although experiments with
germfree, mono-colonized or gnotobiotic mice are not directly equivalent to the situation in patients, the data obtained with our model may provide an explanation why septic episodes have been observed in immunocompromised patients with immature or disrupted intestinal microbiota and treated with probiotics and demonstrate a T cell-mediated pathogenic mechanism causing fatal sepsis.
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Reference List


Figure legend

Figure 1: In Rag1<sup>-/-</sup> mice the microbiota prevents bacterial translocation across the intestinal epithelium and ensures survival after challenge with <i>E. coli</i> Nissle 1917 (EcN)

(A) Groups of at least 4 (I) GF- or (II) SPF-raised Rag1<sup>-/-</sup> mice were colonized with 1x10<sup>8</sup> CFU of EcN on day 0. Additionally, one group of (III) SPF-raised Rag1<sup>-/-</sup> mice was T cell reconstituted 6 days after EcN challenge, and (IV) GF-raised Rag1<sup>-/-</sup> mice were challenged with 1x10<sup>8</sup> CFU of commensal <i>E. coli</i> mpk. At day 7, bacterial CFUs were determined in MLN, liver spleen and lungs of the animals. Symbols represent data from a single animal. (*p < 0.05 compared with all other groups, one way ANOVA, Bonferroni multiple comparison post test).

(B) Kaplan Meyer survival curve of GF- or SPF-raised EcN Rag1<sup>-/-</sup> mice, SPF-raised EcN-challenged T cell-reconstituted Rag1<sup>-/-</sup>, and GF-raised Rag1<sup>-/-</sup> mice which were challenged with commensal <i>E. coli</i> mpk. (**p<0.01 Kaplan Meyer log rank test).

Figure 2: T cells are required to prevent translocation and dissemination of EcN in GF mice

(A) CFU in MLN, liver, spleen, lungs and feces of groups of at least 5 (I) GF-raised EcN-challenged Rag1<sup>-/-</sup> mice, (II) GF-raised EcN challenged B6 mice, (III) GF-raised EcN-challenged Rag1<sup>-/-</sup> mice which were reconstituted with naive T cells (IV), and GF-raised Rag1<sup>-/-</sup> mice which received naïve T cells 3 d before bacterial challenge with EcN. At day 7, bacterial CFUs were determined. Symbols represent data from a single animal. (**p < 0.01, ***p < 0.001 compared with all other groups, one way ANOVA, Bonferroni multiple comparison post test).

(B) Kaplan Meyer survival curve of GF-raised EcN-challenged B6 mice, GF-raised EcN challenged T cell-reconstituted Rag1<sup>-/-</sup> mice, and GF-raised Rag1<sup>-/-</sup> mice which received T cells 3 d before bacterial challenge. (**p<0.001 Kaplan Meyer log rank test).
Figure 3: Reconstitution of EcN-challenged Rag1<sup>−/−</sup> mice with Tnf-α<sup>−/−</sup> T cells does not reduce T cell-mediated mortality

(A) TNF-α cytokine concentration in serum of (I) GF-raised EcN-challenged T cell-reconstituted, (II) GF-raised EcN-challenged, (III) GF-raised T cell-reconstituted Rag1<sup>−/−</sup> mice, (IV) GF-raised Rag1<sup>−/−</sup> mice which received T cells of WT mice 3d before EcN-challenge, (V) GF-raised EcN-challenged B6 mice or (VI) GF-raised EcN-challenged Rag1<sup>−/−</sup> mice which received Tnf-α<sup>−/−</sup> naïve T cells was measured by ELISA. (*p<0.05, **p<0.01 paired student t test). (B) Kaplan-Meyer survival curve of GF-raised EcN-challenged Rag1<sup>−/−</sup> mice reconstituted with Tnf-α<sup>−/−</sup> or WT T cells. (C) Histology of lung tissues of GF-raised EcN-challenged T cell-reconstituted Rag1<sup>−/−</sup> mice, GF-raised EcN-challenged Rag1<sup>−/−</sup> mice, and GF-raised T cell-reconstituted uninfected control mice. All sections were stained with H & E.

Figure 4: Translocation in germfree Rag1<sup>−/−</sup> mice is independent of LPS or flagella mediated signals

Groups of at least 5 GF-raised Rag1<sup>−/−</sup> mice were challenged with 1x10<sup>6</sup> CFU ΔflIC, ΔflgE, EcN wzy or wild type EcN. Six days after challenge Rag1<sup>−/−</sup> mice were reconstituted with naïve T cells. (A) CFU in feces one day posttransfer. Each symbol represents one animal. (B) CFU in organs one day posttransfer. Each symbol represents one animal. (C) Kaplan-Meyer survival curve of Rag1<sup>−/−</sup> mice challenged either with EcN, ΔflIC, ΔflgE or EcN wzy after T cell reconstitution. (D) TNF-α concentration in sera of GF-raised T cell reconstituted EcN-, ΔflIC-, ΔflgE- or EcN wzy- challenged Rag1<sup>−/−</sup> mice. TNF-α concentration was measured by ELISA.

Figure 5: E. coli mpk is not sufficient to prevent translocation of EcN
Groups of at least 4 GF-raised \textit{Rag1}\textsuperscript{-/} mice were challenged with \num{1e8} CFU of commensal \textit{E. coli} mpk 3 days before challenge with EcN. Six days after EcN challenge \textit{Rag1}\textsuperscript{-/} mice were reconstituted with naïve T cells. (A) CFU of \textit{E. coli} mpk 3 days after challenge and total CFU (\textit{E. coli} mpk and EcN) 7 days after EcN challenge in feces. (B) Ratio of EcN to \textit{E. coli} mpk in feces 7 days after EcN challenge. EcN was identified by detection of the EcN specific cryptic plasmids pMUT1 and pMUT2. (C) CFU of EcN in MLN, liver, spleen and lungs one day posttransfer. (D) Kaplan-Meyer survival curve of GF-raised \textit{Rag1}\textsuperscript{-/} mice which were challenged with commensal \textit{E. coli} mpk 3 days before challenge with EcN and T cell-reconstituted six days after EcN challenge. (E) TNF-\alpha concentration in sera after T cell reconstitution. (*p<0.05 paired student t test).

\textbf{Figure 6:} \textit{B. vulgatus} mpk reduces translocation of EcN, but not the mortality of mice

Groups of at least 5 GF-raised \textit{Rag1}\textsuperscript{-/} mice were challenged with \num{1e8} CFU of commensal \textit{B. vulgatus} mpk 3 days before challenge with EcN. Six days after EcN challenge \textit{Rag1}\textsuperscript{-/} mice were reconstituted with naïve T cells. (A) CFU of EcN 7 days after challenge in peripheral organs and in feces (**p < 0.01, ***p < 0.001 compared to EcN-challenged T cell-reconstituted \textit{Rag1}\textsuperscript{-/} mice, one way ANOVA, Bonferroni multiple comparison post test). (B) Kaplan-Meyer survival curve of GF-raised \textit{Rag1}\textsuperscript{-/} mice which were challenged with commensal \textit{B. vulgatus} mpk 3 days before challenge with EcN and T cell-reconstituted six days after EcN challenge.
Figure 1

A

log_{10} CFU / g organ

mouse strain

Rag1-/-
Rag1-/-
Rag1-/-
Rag1-/-
colonization

GF
SPF
SPF
GF
challenge

EcN
EcN
EcN
Ec mpk
T cell transfer

- - + -

I

II

III

IV

B

survival (%)

days after bacterial challenge

GF Rag1-/- + Ec mpk
SPF Rag1-/- + EcN + T
SPF Rag1-/- + EcN

Figure 1
Figure 2
Figure 3

A

![Graph showing TNF-α levels](image)

- Mouse strain
  - Rag1-/-
  - Rag1-/-
  - Rag1-/-
  - Rag1-/-
  - B6
  - Rag1-/-

- Colonization
  - GF
  - GF
  - GF
  - GF
  - GF
  - GF

- EcN challenge
  - +
  - +
  - -
  - +
  - +
  - +

- T cell transfer
  - +
  - -
  - +
  - +
  - -

- Survival (%)
  - 0
  - 25
  - 50
  - 100
  - 300
  - 400
  - 600
  - 800

- Hours after T-cell transfer
  - I
  - II
  - III
  - IV
  - V

B

![Graph showing survival](image)

- GF Rag1-/- + EcN + WT T
- GF Rag1-/- + EcN + Tnfα-/- T

- Survival (%)
  - 0
  - 25
  - 50
  - 75
  - 100

- Hours after T-cell transfer
  - 0
  - 5
  - 10
  - 15
  - 20

C

![Histology images](image)

- GF Rag1-/- lung tissue
- + EcN + T
- + EcN
- + T

Figure 3
Figure 4

D

C

hours after T-cell transfer

survival (%)

Rag1-/- + EcN + T

Rag1-/- + fliC + T

Rag1-/- + flgE + ... flgE EcNwzy

EcN fliC flgE EcNwzy

log_{10} CFU/g feces

log_{10} CFU/g MLN

log_{10} CFU/g lung

log_{10} CFU/g spleen

log_{10} CFU/g liver

TNF-α (pg/ml)

0
100
200
400
500
700

0
5
10
0
5
10
0
5
10
0
5
10
EcN fliC flgE EcNwzy

Figure 4
Figure 5

A) 

mouse strain | $Rag^{1-/-}$ | $Rag^{1+}$
colonization | GF | GF
challenge | Ec mpk | 1. Ec mpk 2. EcN
T cell transfer | - | +

B) 

ratio EcN/Ec mpk (%)

C) 

C) 

mouse strain | $Rag^{1-/-}$ | $Rag^{1+}$
colonization | GF | GF
challenge | EcN | 1. Ec mpk 2. EcN
T cell transfer | + | +

D) 

survival (%)

E) 

mouse strain | $Rag^{1-/-}$ | $Rag^{1+}$
colonization | GF | GF
challenge | EcN | 1. Ec mpk 2. EcN
T cell transfer | - | +

Figure 5
Figure 6

A

mouse strain  
colonization  
challenge  
T cell transfer

Rag1-/-  
GF  
EcN  
+

Rag1-/-  
GF  
1. Bv 2. EcN

B

GF Rag1-/-  
Bv  
EcN  
T

survival (%)

hours after T-cell transfer