COLONIZATION RESISTANCE AGAINST GENETICALLY MODIFIED *ESCHERICHIA COLI* K12 (W3110) STRAINS IS ABROGATED FOLLOWING BROAD-SPECTRUM ANTIBIOTIC TREATMENT AND ACUTE ILEITIS

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Escherichia coli K12 (EcK12) is commonly used for gene technology purposes and regarded as a security strain due to its inability to adhere to epithelial cells. The conventional intestinal microbiota composition is critical for physiological colonization resistance against most bacterial species including pathogens. We were therefore interested whether intestinal colonization by a genetically modified EcK12 (W3110) strain carrying a chloramphenicol resistance cassette was facilitated following broad-spectrum antibiotic treatment eradicating the intestinal microbiota or induction of small intestinal inflammation accompanied by distinct microbiota shifts. Whereas conventional C57BL/6 and BALB/c mice had virtually expelled the EcK12 (W3110) strain within the first 3 days upon peroral infection, EcK12 (W3110) could establish within the small and large intestines of gnotobiotic mice generated by quintuple antibiotic treatment. Gnotobiotic mice perorally infected with EcK12 (W3110) plus fecal transplant from conventional donors harbored lower intestinal EcK12 (W3110) loads compared to animals challenged with EcK12 (W3110) alone. Furthermore, EcK12 (W3110) infection of conventional mice after but not before induction of ileitis resulted in stable colonization of ileum and colon by EcK12 (W3110). Taken together, broad-spectrum antibiotic treatment and intestinal inflammation compromise colonization resistance and thus facilitate colonization of the intestinal tract with genetically modified EcK12 security strains.

Keywords: colonization resistance, intestinal microbiota, broad-spectrum antibiotic treatment, gnotobiotic mice, *Escherichia coli*, *E. coli* K12 (W3110), genetically modified security strains, acute small intestinal inflammation, *Toxoplasma gondii*, fecal transplantation

Introduction

After its first identification in 1922 and subsequent characterization, *E. coli* K12 (EcK12) has been extensively used for genetic cloning purposes [1–3]. Due to genetic defects, EcK12 is regarded apathogenic and is incapable of adhering to intestinal epithelial cells and thus of colonizing vertebrates [4, 5]. EcK12 (W3110) which differs from the K12 parental strain by a large chromosomal inversion, and genetically modified derivatives were not able to colonize the vertebrate intestinal tract at all [4, 5], and guinea pigs harboring a conventional intestinal microbiota

displayed colonization resistance upon peroral challenge [6].

We have recently shown that acute intestinal inflammation was accompanied by profound changes of the murine intestinal microbiota composition toward enterobacterial overgrowth [7–10]. We therefore hypothesized that changes within the intraluminal intestinal milieu with subsequent microbiota shifts and compromised epithelial barrier function during acute intestinal inflammation as well as broad-spectrum antibiotic treatment might compromise colonization resistance and subsequently facilitate colonization of genetically modified EcK12 strains.

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Materials and methods

Ethics statement

All animal experiments were conducted according to the European Guidelines for animal welfare (2010/63/EU) with approval of the commission for animal experiments headed by the “Landesamt für Gesundheit und Soziales” (LaGeSo, Berlin). Animal welfare was monitored twice daily by assessment of clinical conditions and weight loss of mice.

Conventional and gnotobiotic mice

Female C57BL/6 and BALB/c mice were bred and housed under specific pathogen-free (SPF) conditions in the Forschungsinstitut für Experimentelle Medizin (Charité, University Medicine Berlin, Germany). To eradicate the commensal gastrointestinal microbiota, conventionally colonized C57BL/6 mice were transferred to sterile cages and treated by adding ampicillin (1 g/l; Ratiopharm), vancomycin (500 mg/l; Cell Pharm), ciprofloxacin (200 mg/l; Bayer Vital), imipenem (250 mg/l; MSD), and metronidazole (1 g/l; Fresenius) to the drinking water ad libitum for 8–10 weeks as described earlier [7] starting at 8 weeks of age.

E. coli strains

The *E. coli* K12 (W3110) (Δ *rcnB::cat*) strain carries a chloramphenicol resistance cassette replacing the *rcnB* gene that has no known phenotype [11, 12]. We were thus able to unequivocally detect this *E. coli* K12 (W3110) derivative strain among the other commensal enterobacteria within the intestinal tract by culture on solid Luria–Bertani (LB) agar supplemented with chloramphenicol (see below). To generate another apathogenic *E. coli* control strain harboring the chloramphenicol resistance cassette, a commensal *E. coli* strain was isolated from a naive conventionally colonized C57BL/6 wildtype mouse [8, 13]. Subsequently, the chloramphenicol acetyltransferase (*cat*) resistance gene cassette was introduced via the plasmid pGEM-CAT which was constructed as described in detail for plasmid pHP-CAT [14].

Recolonization of gnotobiotic mice, fecal transplantation

Re-colonization experiments of gnotobiotic mice were performed as described earlier [7, 8]. In brief, 2 days prior to re-colonization experiments, antibiotic cocktail was withheld and substituted by sterilized tap water. The EcK12 (W3110) Δ *rcnB* strain was grown in supplemented brain heart infusion to a turbidity equivalent of 6 McFarland units (a bacterial load of approximately 10^9 to 10^{10} colony

forming units (CFU) per ml), harvested by centrifugation, washed once, resuspended in 5 ml phosphate-buffered saline (PBS), and administered by gavage in a final volume of 0.3 ml on three consecutive days. Bacterial loads of oral suspensions were determined by cultivation of serial dilutions on solid media (MacConkey and Müller-Hinton-Agar supplemented with 50 µg/ml chloramphenicol; Sigma-Aldrich, Steinheim, Germany). Gnotobiotic mice were perorally infected with 10^9 CFU EcK12 (W3110) Δ *rcnB* on three consecutive days. Another group of EcK12 (W3110) Δ *rcnB*-infected gnotobiotic animals was subjected to fecal transplantation the same day. Immediately before transplantation, fresh feces samples were collected from 10 healthy, conventionally colonized female and age-matched C57BL/6 donor mice, pooled, homogenized in 5 ml PBS and perorally transplanted by gavage in a final volume of 0.3 ml as described elsewhere [7, 15].

Induction of acute ileitis

For induction of acute ileitis, mice were infected perorally by gavage with 100 *Toxoplasma gondii* cysts (ME49 strain) from homogenized brains of intraperitoneally infected NMRI mice in a volume of 0.3 ml phosphate-buffered saline (PBS), as described previously [7, 16].

Sampling procedures

Mice were sacrificed by isoflurane inhalation (Abbott, Wiesbaden, Germany). Luminal samples from duodenum, terminal ileum, and colon of each mouse were removed under sterile conditions.

Cultural analysis of the intestinal microbiota

Cultural analyses of luminal intestinal contents were performed in serial dilutions on solid Columbia, MacConkey (Oxoid, Wesel, Germany) and Luria–Bertani (LB) agar supplemented with chloramphenicol (50 µg/ml; Sigma-Aldrich, Steinheim, Germany) under aerobic conditions (two days incubation at 37 °C). Bacterial species were morphologically and biochemically identified as described earlier [7]. The chloramphenicol resistance cassette in the orally administered EcK12 (W3110) strain enabled detection among the other enterobacterial commensal strains in the intestinal tract of conventionally colonized mice.

Statistical analysis

Medians and levels of significance were determined using Mann–Whitney *U* test. Two-sided probability (*p*) values ≤ 0.05 were considered significant. All experiments were repeated at least twice.

Results

Conventional intestinal microbiota prevents colonization of a genetically modified E. coli K12 (W3110) strain in mice

E. coli K12 (W3110) is considered a “security strain” [4–6, 17] and has been shown to be incapable of adhering to intestinal cells *in vitro*. We first investigated whether the genetically modified EcK12 (W3110) $\Delta rcnB$ strain (har-

boring a chloramphenicol resistance cassette) establishes within the intestinal tract of conventionally colonized, healthy mice following high-dose infection. C57BL/6 mice were perorally challenged with 10^9 CFU EcK12 (W3110) $\Delta rcnB$ on three consecutive days. Within 2 to 3 days following the latest infection, mice had virtually expelled the EcK12 strain from the small as well as the large intestinal tract (Fig. 1A). To exclude potential mouse strain-dependent effects on colonization resistance due to differences in abundance of commensal bacterial species

Fig. 1. Conventional mice display colonization resistance against *E. coli* K12 (W3110) strain. Conventionally colonized (A) C57BL/6 (black circles) and (B) BALB/c mice (white circles) were perorally challenged with 10^9 *E. coli* K12 (EcK12) (W3110) $\Delta rcnB::cat$ strain (containing a chloramphenicol resistance cassette) on day 0. Thereafter, kinetic analyses of EcK12 (W3110) $\Delta rcnB$ densities in luminal samples taken from duodenum, ileum, and colon at days 1, 2, 3, and 7 following infection were determined by culture (CFU, colony forming units). Numbers of mice harboring EcK12 (W3110) $\Delta rcnB$ out of the total number of analyzed animals are given in parentheses. Medians (black bars) and significance levels (p values) determined by Mann–Whitney U test are indicated. Data shown are representative for three independent experiments

Fig. 2. Colonization properties of traceable *E. coli* K12 (W3110) $\Delta rcnB::cat$ and commensal *E. coli* (pGEM-CAT) strain. A commensal *E. coli* strain was isolated from a naive conventionally colonized C57BL/6 wildtype mouse and a chloramphenicol resistance cassette introduced via the plasmid pGEM-CAT. Subsequently, conventional mice were infected either with 10^9 CFU of *E. coli* pGEM-CAT or *E. coli* K12 (EcK12) (W3110) $\Delta rcnB$ strain on three consecutive days. Thereafter, kinetic analyses of EcK12 (W3110) $\Delta rcnB$ (white circles) and *E. coli* pGEM-CAT (black circles) colonization densities in luminal samples taken from duodenum, ileum, and colon at (A) day 1, (B) day 3, and (C) day 7 following infection were performed by culture (CFU, colony forming units). Numbers of mice harboring the respective strain out of the total number of analyzed animals are given in parentheses. Medians (black bars) and significance levels (p values) determined by Mann–Whitney U test are indicated. Data shown are representative for three independent experiments

or defensin expression, the experiment was repeated in BALB/c mice with similar results (Fig. 1B). Thus, conventional colonized wildtype mice in our animal facilities display a strong colonization resistance against EcK12 (W3110), irrespective of the infected mouse strain.

Impact of genetic modification of E. coli K12 (W3110) security and commensal E. coli strain (pGEM-CAT) on intestinal colonization properties

We next asked whether lack of colonization by the EcK12 (W3110) Δ rcnB strain in conventional mice was due to genetic modification (i.e., introduced chloramphenicol resistance cassette) or a characteristic feature of this specific security strain. To address this question, a commensal *E. coli* strain was isolated from a naive conventionally colonized C57BL/6 wildtype mouse and the identical chloramphenicol resistance cassette was introduced via the plasmid pGEM-CAT. Subsequently, conventional C57BL/6 mice were infected either with 10^9 CFU of *E. coli* (pGEM-CAT) or EcK12 (W3110) Δ rcnB strain on three consecutive days. Irrespective of the administered strain, mice harbored comparably high *E. coli* loads in their colonic lumen 24 h following the last infection. From the ileum, the *E. coli* (pGEM-CAT) strain could be cultured at 2 orders of magnitude higher median numbers compared to EcK12 (W3110) Δ rcnB ($p < 0.001$; Fig. 2A). Thereafter, *E. coli* pGEM-CAT could be isolated from the colonic lumen in 100% of mice until day 7 post infection, whereas conventional animals had expelled the EcK12 (W3110) Δ rcnB strain by then (Fig. 2B and C). Thus, lack of EcK12 (W3110) Δ rcnB strain colonization in conventionally colonized mice cannot be attributed to introduction of the chloramphenicol resistance cassette alone, but is rather due to host colonization resistance and fitness characteristics exerted by the *E. coli* strain.

Colonization resistance against genetically modified E. coli K12 (W3110) is abrogated in mice following broad-spectrum antibiotic treatment

Next, we were interested whether eradication of the microbiota by antibiotic treatment leads to the abrogation of colonization resistance against security strain EcK12 (W3110) Δ rcnB. To accomplish this, we subjected C57BL/6 mice to 8 to 10 weeks of quintuple antibiotic treatment in order to completely remove the commensal intestinal microbiota. After antibiotic treatment, mice were replenished with 10^9 CFU EcK12 (W3110) Δ rcnB on three consecutive days. One additional group of mice received the genetically modified *E. coli* strain in combination with a complex intestinal microbiota by peroral transplantation of fresh fecal samples derived from naive, conventional colonized donor mice. Seven days following the last of three peroral challenges, all replenished gnotobiotic mice harbored EcK12 (W3110) Δ rcnB throughout their entire intestinal tract with

Fig. 3. Colonization resistance against *E. coli* K12 (W3110) strain is abrogated following antibiotic treatment. Conventionally colonized mice were subjected to quintuple antibiotic therapy for 8 to 10 weeks in order to eradicate the gastrointestinal microbiota. Generated gnotobiotic animals were perorally re-colonized either with *E. coli* K12 (EcK12) (W3110) Δ rcnB strain alone (white circles) or in combination with complex conventional, special pathogen free microbiota (+SPF) derived from feces of ten healthy naive mice (black circles) as described in methods. Seven days following recolonization, luminal EcK12 (W3110) loads were assessed in (A) duodenum, (B) ileum, and (C) colon by culture (CFU, colony forming units). Numbers of animals harboring the EcK12 (W3110) strain out of the total number of analyzed animals are given in parentheses. Medians (black bars) and significance levels (p values) determined by Mann–Whitney U test are indicated. Data shown are representative for three independent experiments

median loads as high as 10^8 CFU/g in the ileal and colonic lumen (Fig. 3). Fecal transplanted animals harbored comparable EcK12 W3110 loads in the intestinal tract 24 h postinfection (not shown). Interestingly, however, EcK12 (W3110) colonization densities declined over time. At day 7 p.i., recoverable EcK12 (W3110) numbers were approximately 4 and 6 orders of magnitude lower in the colonic and ileal lumen, respectively, compared to EcK12 (W3110) replenished mice without fecal transplantation (Fig. 3). Taken together, these data indicate that eradication of the entire intestinal microbiota through broad-spectrum antibiotic treatment abrogates colonization resistance and facilitates stable colonization of genetically modified strain at high levels. Furthermore, simultaneous application of EcK12 (W3110) and complex intestinal microbiota can partly restore colonization resistance.

Colonization resistance against genetically modified E. coli K12 (W3110) is abrogated in mice with acute intestinal inflammation

We next investigated whether EcK12 (W3110) Δ rcnB was capable of colonizing conventional mice under conditions

Fig. 4. Colonization resistance against *E. coli* K12 (W3110) strain is abrogated during acute small intestinal inflammation. To induce acute ileitis, conventionally colonized mice were perorally infected with *Toxoplasma* (*T.*) *gondii* (day 0). Mice were recolonized with 10^9 *E. coli* K12 (EcK12) (W3110) Δ *rcnB* strain either three, two, and one days prior to (PRE; white circles) or on days 3, 4, and 5 following *T. gondii* infection (POST; black circles). Seven days following ileitis induction, luminal EcK12 W3110 loads were assessed in (A) duodenum, (B) ileum, and (C) colon by culture (CFU, colony forming units). Numbers of animals harboring EcK12 (W3110) out of the total number of analyzed animals are given in parentheses. Medians (black bars) and significance levels (*p* values) determined by Mann–Whitney *U* test are indicated. Data shown are representative for three independent experiments

of compromised intestinal epithelial barrier function due to inflammation accompanied by distinct intestinal microbiota shifts. To address this question, susceptible C57BL/6 mice were perorally infected with 100 cysts of *T. gondii* strain ME49 in order to induce acute ileitis (day 0). Either 3 days before or after ileitis induction mice were perorally challenged with 10^9 CFU EcK12 (W3110) Δ *rcnB* on three consecutive days. Notably, mice with EcK12 (W3110) Δ *rcnB* challenge prior to *T. gondii* infection had expelled the bacteria by day 7 post ileitis induction. In contrast, all mice infected after ileitis induction harbored EcK12 (W3110) Δ *rcnB* with high median loads of between 10^7 and 10^8 CFU per g colonic and ileal content, respectively (Fig. 4). Overall, these results demonstrate that acute intestinal inflammation abrogates colonization resistance and thus facilitates stable colonization of a genetically traceable EcK12 (W3110) (security) strain in the intestinal tract of vertebrates.

Discussion

Findings of the study presented here clearly demonstrate that broad spectrum antibiotic treatment or acute small intestinal inflammation pose risk factors for colonization of the intestinal tract by *E. coli* security strains. The pronounced colonization resistance displayed by our conventional wildtype mice against the *E. coli* K12 (W3110)

strain was abrogated following antibiotic treatment or ileitis induction, leading to establishment of *E. coli* within the intestinal tract at high loads. We furthermore show that the intestinal microbiota plays a key role in colonization resistance. Stable colonization of EcK12 in the murine intestinal tract was only accomplished as long as the physiological colonization resistance exerted by the commensal bacterial microbiota composition was compromised. When colonization resistance was intact, however, the EcK12 (W3110) strain was indeed unable to establish within conventional C57BL/6 mice even at very high infection doses of 10^9 CFU live bacteria on three consecutive days (a rather “unnatural”, non-physiological infection mode). Under these conditions, virtually all bacteria had been expelled by the host after 48 h, regardless of its genetic background (i.e., C57BL/6, BALB/c). These results argue against a role of the mouse strain and, hence the specific intestinal microbiota composition as well as expression of antimicrobial host defense molecules, such as defensins, as a reason for the observed colonization resistance. We further excluded that the lack of intestinal colonization by the genetically modified EcK12 (W3110) strain was due to its inherent chloramphenicol resistance cassette by introducing the identical chloramphenicol acetyltransferase (*cat*) resistance gene into an apathogenic commensal *E. coli* strain we had isolated from a naive C57BL/6 mouse.

Our data are thus well in line with an earlier study demonstrating that later than 72 h post-infection, EcK12 (W3110) could no longer be cultured from the intestinal tract of guinea pigs any more [6]. Eradication of the entire intestinal microbiota following broad-spectrum antibiotic treatment with five different antimicrobials, however, facilitated stable EcK12 (W3110) colonization within the entire intestinal tract at high levels. This is somewhat surprising given that *E. coli* K12 is considered a security strain unable to adhere to intestinal epithelial cells [4, 5, 17]. Our data here provide evidence that vacant niches within the intestinal ecosystem can be easily and sufficiently taken over by non-residential species (i.e. the administered EcK12 strain). Simultaneous fecal transplantation of complex conventional microbiota with peroral EcK12 (W3110) challenge, however, resulted in significantly lower intestinal colonization densities of EcK12. Similarly, in a *Campylobacter* (*C.*) *jejuni* infection model, we showed earlier that replenishment of gnotobiotic mice with complex conventional flora 4 days prior to *C. jejuni* infection was sufficient to completely restore colonization resistance against this intestinal pathogen [13, 18]. Furthermore, Lawley and colleagues have recently demonstrated similar positive effects of microbiota restoration on severe infections with *Clostridium difficile* [19]. Collectively, all of these results suggest a potential clinical application for microbiota restoration through fecal transplantation.

Our data derived from infection experiments utilizing EcK12 (W3110) are also well in line with results from a very recent study of our group. Following broad-spectrum antibiotic treatment, a related EcK12 security strain, namely, MG1655, was not only able to stably colonize

the entire gastrointestinal tract at high loads upon oral infection, but also to induce intestinal inflammation in a murine ileitis model [15]. Within 7 to 9 days following peroral *T. gondii* infection, susceptible mice suffer from acute ileitis due to an underlying Th1-type immunopathogenesis mimicking acute Crohn's disease in humans [7, 10, 20]. During the first five days upon ileitis induction, mice not only exhibit a progressively compromised epithelial barrier function, but also distinct changes of the intestinal microbiota composition toward overgrowth of the inflamed ileal lumen with Gram-negative commensals, such as *E. coli* and *Bacteroides/Prevotella* spp., whereas bacterial diversity and Lactobacilli populations decreased [7, 8, 10]. Furthermore, lipopolysaccharide (from Gram-negative commensal *E. coli*) was able to induce and perpetuate immunopathology via TLR-4 signaling [8, 9]. Notably, following the application of EcK12 (W3110) before ileitis induction, colonization resistance was still preventing from intestinal EcK12 (W3110) colonization. Peroral challenge with EcK12 (W3110) between 3 and 5 days following *T. gondii* infection (a time window when first histopathological as well as microbiota changes within the ilea become overt [7]), however, led to high colonization densities in the inflamed small as well as un-inflamed large intestinal tract. This suggests that compromised barrier function plus microbiota changes during immunopathology abolish physiological colonization resistance and subsequently facilitate EcK12 establishment within the intestinal tract.

Taken together, our study indicates that eradication of the conventional intestinal microbiota and the induction of acute small intestinal inflammation, both facilitate stable colonization of the genetically modified EcK12 (W3110) security strain. One can only speculate whether this also holds true for the human host, but data from this work again underline that under conditions of compromised colonization resistance genetically modified presumably secure bacterial strains are capable of stably colonizing the vertebrate host even when lacking essential virulence properties. Hence, future investigations need to be undertaken to re-evaluate the potential risk profile of EcK12 security strains.

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Competing Interests

The authors have declared that no competing interests exist.

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