

ORIGINAL ARTICLE

Early colonizing *Escherichia coli* elicits remodeling of rat colonic epithelium shifting toward a new homeostatic state

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We investigated the effects of early colonizing bacteria on the colonic epithelium. We isolated dominant bacteria, *Escherichia coli*, *Enterococcus faecalis*, *Lactobacillus intestinalis*, *Clostridium innocuum* and a novel *Fusobacterium* spp., from the intestinal contents of conventional suckling rats and transferred them in different combinations into germfree (GF) adult rats. Animals were investigated after various times up to 21 days. Proliferative cell markers (Ki67, proliferating cell nuclear antigen, phospho-histone H3, cyclin A) were higher in rats monocolonized with *E. coli* than in GF at all time points, but not in rats monocolonized with *E. faecalis*. The mucin content of goblet cells declined shortly after *E. coli* administration whereas the mucus layer doubled in thickness. Fluorescence *in situ* hybridization analyses revealed that *E. coli* resides in this mucus layer. The epithelial mucin content progressively returned to baseline, following an increase in KLF4 and in the cell cycle arrest-related proteins p21^{CIP1} and p27^{KIP1}. Markers of colonic differentiated cells involved in electrolyte (carbonic anhydrase II and *slc26A3*) and water (aquaglyceroporin3 (*aqp3*)) transport, and secretory responses to carbachol were modulated after *E. coli* inoculation suggesting that ion transport dynamics were also affected. The colonic responses to simplified microbiotas differed substantially according to whether or not *E. coli* was combined with the other four bacteria. Thus, proliferation markers increased substantially when *E. coli* was in the mix, but very much less when it was absent. This work demonstrates that a pioneer strain of *E. coli* elicits sequential epithelial remodeling affecting the structure, mucus layer and ionic movements and suggests this can result in a microbiota-compliant state.

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Introduction

Throughout life, the mammalian gut is engaged in dynamic interactions with a vast microbial community in conditions that should allow homeostasis (Brown *et al.*, 2013). Associations with the gut microbiota develop following every birth, as the newborn passes from a protected site to a densely colonized environment. The microbiota that colonizes first depends on the mode of delivery (Dominguez-Bello *et al.*, 2010). The human gut microbiota

evolves substantially over the first years of life and is formed by the successive emergence of diverse bacterial groups: aerotolerant bacteria establish first and strict anaerobes later (Palmer *et al.*, 2007; Adlerberth and Wold, 2009). Following weaning, the gut microbial community then converges toward that found in the adult intestine (Yatsunenکو *et al.*, 2012). The microbiota in infants conforms to the particular characteristics of the postnatal period, and displays functions different from those found in adults (Koenig *et al.*, 2011; Yatsunenکو *et al.*, 2012). Experimental studies have documented the contributions of these first colonizing microbes to health. Indeed, in mice, the bacterial status, and the antibiotic exposure, early in life may have long-lasting consequences for susceptibility to immune-mediated diseases (Olszak *et al.*, 2012; Russell *et al.*, 2012).

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The first cells that are confronted by this microbial consortium are intestinal epithelial cells, mainly in the colon where the bacterial density is the highest. The colonic epithelium is composed of tightly attached cells including columnar, goblet and enteroendocrine cells as differentiated epithelial lineages. These cells are continuously renewed by proliferation at the crypt base and terminal differentiation at the luminal surface (Van der Flier and Clevers, 2009). The epithelium displays various properties that sustain the complex and dynamic microbial communities of the gut. They include mucus secretion by goblet cells and the production of antimicrobial peptides (Hooper and Macpherson, 2010). A recent modeling approach predicted that these epithelial secretions act as a selective force providing competitive advantages to commensal intestinal bacteria that would otherwise be lost (Schluter and Foster, 2012). There is also evidence that the transport of ions and fluid, the primary physiological function of intestinal epithelial cells, can also have a role in commensal bacteria–host cell interactions (Keely *et al.*, 2012).

We investigated the responses of the colonic epithelium to early colonizing bacteria. We isolated dominant bacteria (*Escherichia coli*, *Enterococcus faecalis*, *Lactobacillus intestinalis*, *Clostridium innocuum* and a novel Fusobacterium spp.) from 15-day-old conventional (CV) suckling rats and the effects of these bacteria were tested over 21 days in growing adult gnotobiotic rats. This model, and not neonatal rats, was used because of the delay in the gastrointestinal tract maturation in rodents relative to humans in which the intestinal epithelium is mature at birth (Puiman and Stoll, 2008; Drozdowski *et al.*, 2010). We performed a kinetic study as colonic responses are progressive after the transfer of a complex microbiota into germfree (GF) animals (Cherbuy *et al.*, 2010; El Aidy *et al.*, 2012; Tomas *et al.*, 2013). The time points used correspond to before the total renewal of the colonic epithelium (3 days) and after one (7 days) or several (21 days) renewals of the colonic epithelium.

We report the prominent role of *E. coli* relative to other early colonizers isolated and insights into the involvement of the epithelial responses in the dynamics of homeostatic host–microbe interactions. We show that administration of *E. coli* elicits sequential remodeling of the colonic epithelium affecting the dynamics of proliferation, of mucus production and of ionic movements. These effects may contribute to a favorable microenvironment for commensal colonization.

Materials and methods

Selection of bacterial species

Freshly collected intestinal contents from six 15-day-old CV suckling rats were pooled, plated on selective media (desoxycholate citrate agar, bile

esculin agar, MRS and brain heart infusion supplemented with 0.5 mg ml⁻¹ cysteine), and cultured overnight aerobically and anaerobically for the isolation of *Enterobacteriaceae*, *Enterococcus*, *Lactobacillus* and anaerobic bacteria (Supplementary Figure S1). One colony per plate was picked and grown in appropriate liquid media to obtain the starting stocks, which were kept at -80 °C. The representativeness of each isolated group for the intestinal contents of suckling rats was assessed by PCR-temporal temperature gradient gel electrophoresis (TTGE) (Supplementary Figure S2).

Identification of bacterial species

Bacterial species were identified by analyses of the DNA sequence of the 16S rRNA gene. DNA was extracted from each bacterial culture as in (Tomas *et al.*, 2013). Primers GCclamp-U968 (GCclamp-5'-GAACGCGAAGAACCTTAC-3') and L1401 (5'-GCGTGTGTACAAGACCC-3') were used to amplify the V6–V8 regions of bacterial 16S rRNA genes (Gerard *et al.*, 2004). PCR products were purified using the Promega Wizard SV PCR clean-up system (Promega, Lyon, France) and sequenced by Eurofins MGW Operon (Ebersberg, Germany). Results of tests performed for isolated bacterial characterization are given in Supplementary Table S1. *E. coli*, *E. faecalis*, *L. intestinalis*, *C. innocuum* and the novel Fusobacterium spp. were identified and the strains were named CEC15, CEF15, CLI15, CCI15 and CFV15, respectively.

Bacterial culture conditions

Bacterial cultures were prepared separately from aliquots stored at -80 °C and cultured overnight at 37 °C: *E. coli*, *E. faecalis* and *L. intestinalis* were grown in aerobic conditions in LB and MRS media, respectively; *C. innocuum* and the novel Fusobacterium spp. were cultured in anaerobic condition in LYHBHI with 0.5% yeast extract, 5 mg l⁻¹ of hemin and 0.5 mg ml⁻¹ cysteine.

Gnotobiotic rats

All procedures were carried out according to European guidelines for the care and the use of laboratory animals with permission A78–196 from the French Veterinary Services. Animal experiments were evaluated and approved by the local ethics committee. Rats (males, Fisher 344) were bred at INRA gnotobiotic facilities (Jouy-en-Josas, France) as described in (Cherbuy *et al.*, 2010). Six groups of gnotobiotic rats were studied (Supplementary Table S2): GF rats, two groups of monoxenic rats inoculated with either *E. coli* CEC15 or *E. faecalis* CEF15 (Monocoli and Monofaecalis groups, respectively), one group of dixenic rats inoculated with *E. coli* CEC15 and *E. faecalis* CEF15 (Dicolifaecalis group), and two more complex gnotobiotic groups, inoculated with *E. faecalis* CEF15, *L. intestinalis* CLI15,

C. innocuum CCI15 and the novel Fusobacterium spp. CFV15 with (EELCF group) or without (ELCF group) *E. coli* CEC15 (Supplementary Table S2). CV rats were also used as complex microbiota control.

Inocula were prepared as follows: fresh overnight cultures were centrifuged at 4 °C for 20 min at 12 000 r.p.m., resuspended in LB medium and immediately introduced into the isolator. For mixed inoculations, bacterial strains were cultured separately, 1 ml aliquots were pooled, centrifuged and resuspended in 1 ml of LB medium. In all cases, GF rats were inoculated once by oral gavage with 1 ml of bacterial suspension. The samples used for the inoculation contained 10^9 c.f.u.ml⁻¹. Monocoli, Monofaecalis and Dicolifaecalis groups were killed 3, 7 or 21 days post inoculation. EELCF and ELCF groups were killed 3 or 21 days post inoculation. Each experiment was carried out independently at least three times. The rats used for these experiments were aged between 9 weeks and 12 weeks such that all rats were killed at the age of 3 months. Fisher 344 rats are in the growth phase at this age (Turturro *et al.*, 1999). Rats were anesthetized with isoflurane, the colons recovered and immediately used for cell isolation, histological procedures or permeability measurement in an Ussing chamber (Easy Mount, Physiologic Instruments, San Diego, CA, USA). Cecal contents were immediately frozen at -80 °C for bacterial enumeration.

E. coli and *E. faecalis* quantification

Bacteria in cecal contents were quantified by real-time quantitative PCR analyses targeting bacterial group-specific 16S rRNA genes as in (Tomas *et al.*, 2013).

Colonic epithelial cell isolation, and protein and RNA extraction

Colonic epithelial cells were isolated from the whole colon according to (Cherbuy *et al.*, 1995). Protein and RNA were extracted from colonic epithelial cells as in (Rul *et al.*, 2011). RNA quality was checked by R6K ScreenTape analyses (Agilent Technologies, Santa Clara, CA, USA) with an average RNA integrity number of 8 on a scale of 0–10.

Western blot analyses

Western blot (WB) analyses were performed as in (Thomas *et al.*, 2011). Signals on autoradiographic films were quantified by scanning densitometry with Biovision 1000 and Bio-1D software (Vilber Lourmat, Marne-la-Vallée, France).

Real-time quantitative PCR analyses of colonic epithelial gene expression

Colonic epithelial RNAs were analyzed by real-time quantitative PCR. Assays designed by Applied Biosystems (respectively Rn00709709-m1 and Rn00581754-m1) were used for the detection of *slc26A3* and aquaglyceroporin3 (*aqp3*) as in (Tomas

et al., 2013). Differences were calculated using the comparative 2- $\Delta\Delta$ Ct method (Livak and Schmittgen, 2001). Results obtained with the 7000 system software version 1.2.3 (Applied Biosystems) were normalized to those for the 18S rRNA gene (Hs99999901-s1) and compared with the mean target gene expression in GF rats.

Histology, immunostaining and in situ hybridization

Flushed colons were fixed in 4% paraformaldehyde, dehydrated and embedded in paraffin according to a standard protocol. Histological features were analyzed by hematoxylin–eosin–safran staining. Periodic acid-Schiff (PAS) and Alcian blue (AB) staining were performed as in (Wrzosek *et al.*, 2013). The EnVision+ System-HRP (DakoCytomation, Trappes, France) was used for immunohistochemistry and nuclei counterstained with Mayer's hematoxylin. In immunofluorescence (IF) experiments, fluorescence-labeled secondary antibodies were used and nuclei stained with Hoechst. Antigen retrieval was performed in buffer pH 6.0 or pH 9.0 (EnVision FLEX Target Retrieval Solution, DakoCytomation) for 40 min at 97 °C. The RNAscope kit (ACD, Hayward, CA, USA) was used according to the supplier's instructions for *in situ* hybridization. Probes were targeted in the 458–1795 *Wnt5a* mRNA region of the *Rattus norvegicus* (NM_022631.1).

Antibodies

The following primary antibodies were used: anti-Ki67 (DakoCytomation); anti-proliferating cell nuclear antigen (PCNA) (GeneTex, Irving, CA, USA), anti-KLF4 and anti-phospho-histone H3 (PH3) (Abcam, Cambridge, MA, USA); anti-cyclin A, anti-p21^{CIP1}, anti-p27^{KIP1}, anti-MUC2, anti-Bax, anti-carbonic anhydrase II (CAII) (Santa Cruz Biotechnology, Dallas, TX, USA); anti-ZO-1, anti-claudin-1, anti-occludin (Invitrogen, Saint Aubin, France). Anti-SKP1 (BD Transduction, Rungis, France) was used as the loading control in WB experiments.

Mucus thickness analyses and fluorescence in situ hybridization assays

Unwashed colons were gently bound *in situ*, coated with optimum cutting temperature compound and frozen in liquid nitrogen. Frozen sections were cut and slides placed in water-free Carnoy's fixative before IF assay with the anti-MUC2 antibody as described above. DNA was stained with Hoechst revealing both eukaryotic and bacterial cells. To complete Hoechst observation of bacteria, fluorescence *in situ* hybridization (FISH) experiments were performed. FISH for detection of *E. coli* CEC15 was unsatisfactory with slides from unwashed colon fixed in Carnoy's fixative. Therefore, slides were fixed in 4% paraformaldehyde and then hybridized with 4 ng μ l⁻¹ of the general bacterial probe Eub338 as described in (Rochet *et al.*, 2004).

Image acquisition and analyses

Crypt depth, PAS-, AB-, Ki67-, PCNA- and PH3-positive cells were scored using Nanozoomer-DigitalPathology.view software (Hamamatsu Photonics, Hamamatsu, Japan) as described in (Cherbuy *et al.*, 2010). Structured illumination microscopy was used to observe fluorescent signals and for photography (Zeiss Apotome, Oberkochen, Germany). The intensity of MUC2 staining and the

thickness of the mucus layer were evaluated with MacBiophotonics ImageJ software.

Tissue conductance and permeability measurements

Pieces of colon (0.5 cm²) were mounted in Ussing chambers as previously described (Ducroc *et al.*, 1988). Electrogenic ion transport was monitored as short-circuit current (I_{sc}) using an

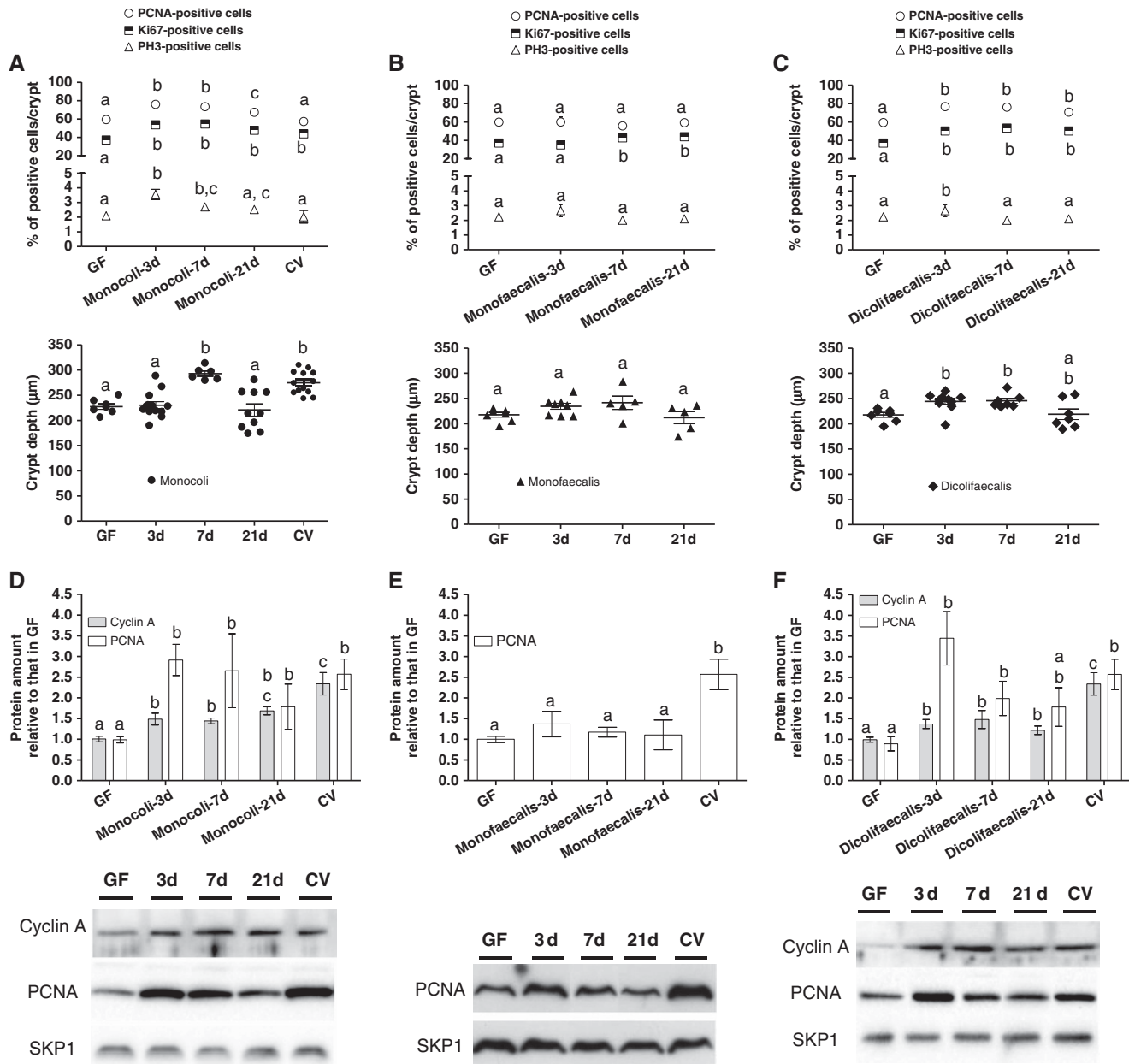


Figure 1 Histological and proliferation marker analyses of the colonic epithelium from gnotobiotic rats inoculated either with *Escherichia coli* CEC15 (Monocoli group) or with *Enterococcus faecalis* CEF15 (Mono/faecalis group) or both (Dicolifaecalis group). (A–C) Percentages of PCNA-, Ki67- and PH3-positive cells/crypt and colonic crypt depth measurement on colonic sections from (A) the Monocoli group ($n = 6–13$), (B) the Mono/faecalis group ($n = 7–8$) and (C) the Dicolifaecalis group ($n = 7–11$). Inoculated rats were killed 3, 7 and 21 days post inoculation. GF and CV rats were used as controls ($n = 7–13$). Representative photographs of Ki67 immunohistochemistry are shown in Supplementary Figure S3. (D–F) Western blot analyses of proliferative markers on isolated colonic epithelial cells in (D) Monocoli group ($n = 6–13$), (E) Mono/faecalis group ($n = 7$ to 8) and (F) Dicolifaecalis group ($n = 7–11$). Graphs show densitometric analyses of cyclin A and PCNA signals with a representative western blot image. SKP1 was used as a loading control. The mean values with letter designations are significantly different (P -value < 0.05).

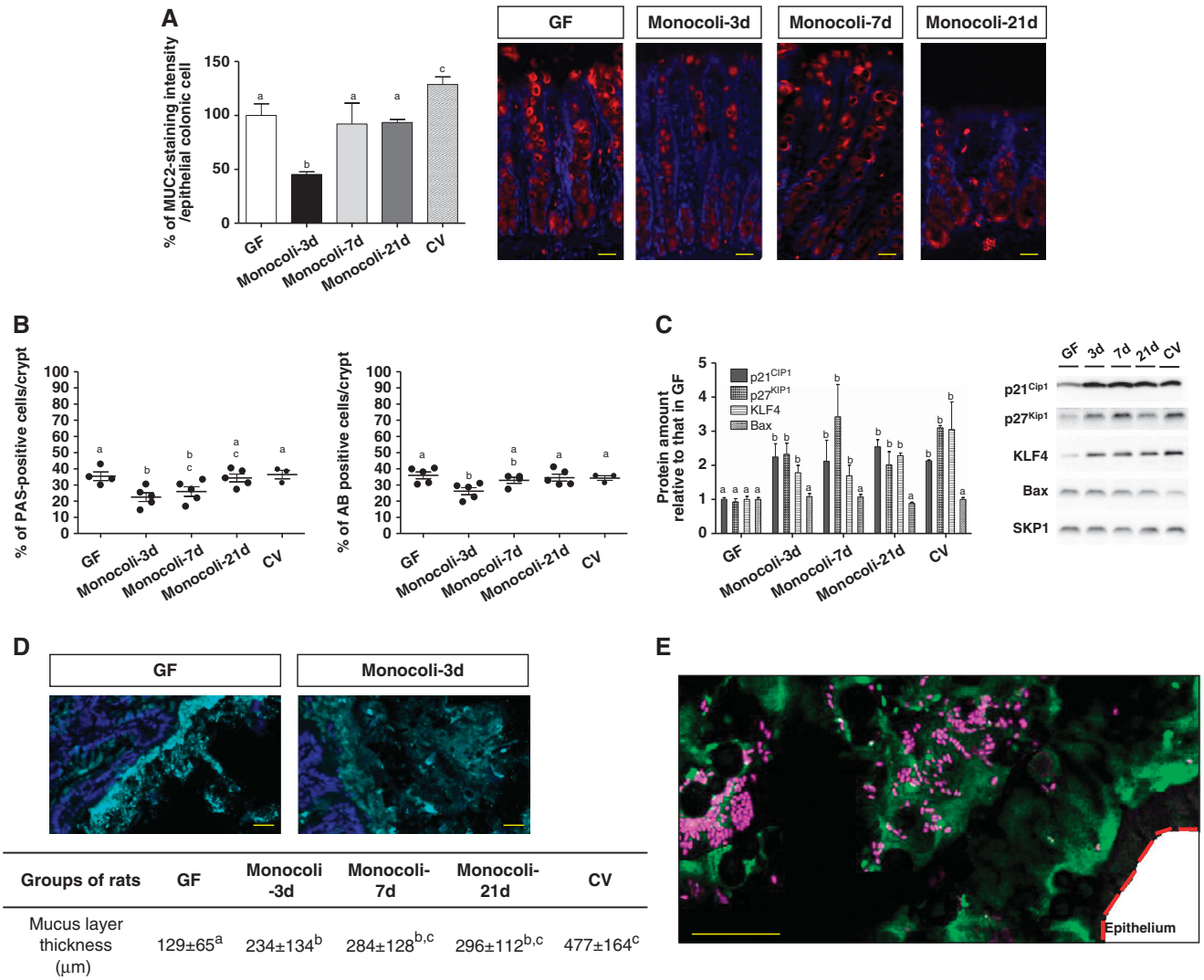


Figure 2 Analyses of the colonic goblet cell population and the mucus layer after inoculation with *Escherichia coli* CEC15. (A) MUC2-staining intensity/colonic epithelial cell of GF, Monocoli and CV groups ($n=4-6$ per group). Representative images of MUC2 staining with scale bar indicating $50\ \mu\text{m}$ for GF and Monocoli groups. See also Supplementary Figure S5A for MUC2 staining by immunohistochemistry. (B) Percentages of PAS- and AB-positive cells/crypt ($n=4-5$ per group). See Supplementary Figure S5B for representative photographs of PAS staining. (C) Western blot analyses of cell cycle arrest-related proteins (p21^{CIP1} and p27^{KIP1}), transcription factor KLF4 and the pro-apoptotic protein Bax in colonic epithelial cells isolated from GF, Monocoli and CV groups ($n=5-7$ per group). SKP1 was used as loading control. For each protein, a representative image and densitometric data are shown. (D) Immunofluorescence staining of MUC2 in unwashed colonic sections from GF, Monocoli and CV groups. Representative images of mucus layer stained with MUC2 in GF and Monocoli rats, after 3 days, with scale bar indicating $50\ \mu\text{m}$. Table reporting the thickness of the mucus layer in GF, Monocoli and CV groups ($n=5-6$ per group). Representative images obtained for Monocoli 7 days and 21 days are shown in Supplementary Figure S5D. (E) FISH analyses with the universal probe Eub338 to detect *E. coli* CEC15 in unwashed colonic sections of Monocoli 3 days. All values are means \pm s.e.m. Mean values with letter designations are significantly different (P -value < 0.05).

automated voltage clamp apparatus (DVC 1000; WPI, Hertfordshire, UK). Conductances at 30 min were calculated according to Ohm's law and paracellular permeability was further assessed by measuring mucosal-to-serosal flux of 4 kDa non-metabolizable fluorescein isothiocyanate-labeled dextran (FD4) over 90 min. At the end of the experiment, tissues were challenged with the cholinergic analog carbachol (CCh) on the serosal side ($100\ \mu\text{M}$) and ΔIsc was recorded.

Statistical analyses

Data are reported as means \pm s.e.m. Comparisons were performed using R 2.13.0 software (R Development Core Team, 2005). Kruskal-Wallis (Rcmdr package) and Tukey's range (nparcomp package) tests were used to identify significant differences between groups; if the Kruskal-Wallis test revealed differences, Tukey's range test was used for nonparametric pairwise comparisons to identify groups that were significantly different.

Mean values annotated with different letters (a, b, c, d) are significantly different ($P < 0.05$).

Results

Highlight on the prevailing proliferative effects of *E. coli* CEC15

E. coli is an early colonizer of the gut, reaching high densities within hours after birth (Adlerberth and Wold, 2009). We first monitored the effect of *E. coli* CEC15 after administration to GF (Monocoli group) using three proliferative markers: PCNA, involved in proliferation and DNA repair; Ki67, widely used as a proliferative marker; and PH3, a mitosis-specific marker. In the Monocoli group, the percentages of PCNA-, Ki67- and PH3-positive cells 3 days post inoculation were higher than in GF, indicating an expansion of the proliferative area in the crypt. These values remained high during the 21 following days, even though they were slightly lower after 21 than 3 days (Figure 1A and Supplementary Figure S3A). Twenty-one days post inoculation, the percentage of proliferative cells decreased toward the CV level. In parallel, we observed a substantial, transient, increase of colonic crypt depth 7 days post inoculation reaching the values obtained in CV (Figure 1A and Supplementary Figure S3A). Colonic crypt depth, however, was

not maintained 21 days post inoculation with *E. coli* CEC15. We similarly investigated the effect of one of the other pioneer bacteria, *E. faecalis*: the percentages of PCNA- and PH3-positive cells, and the crypt depth in Monofaecalis group remained similar to those in the GF (Figure 1B and Supplementary Figure S3B); only Ki67-positive cell count was slightly higher. In the Dicolifaecalis group, the effects of inoculation with the two strains together revealed the prominent effect of *E. coli* CEC15: Ki67-, PCNA- and PH3-positive cell counts were higher than in GF 3 days post inoculation (Figure 1C), and remained high after 21d for Ki67- and PCNA-positive cells; colonic crypt depth also increased transiently. WB analyses of colonic epithelial cells confirmed the proliferation-stimulating effect of *E. coli* CEC15. Cyclin A, a member of the cyclin family required for cell cycle progression, and PCNA were more abundant 3, 7 and 21 days post inoculation in Monocoli and Dicolifaecalis groups than in GF group (Figures 1D and F). In Monocoli samples, cyclin A and PCNA amounts tended toward those in CV. The amount of PCNA in the Monofaecalis group was similar to that in GF (Figure 1E).

We evaluated colonization in the three groups: there were 10^9 c.f.u. equivalent per g of cecal content in the Monocoli group and 10^8 in the Monofaecalis group after 21 days (Supplementary Figure S4). In Dicolifaecalis group, the levels of the

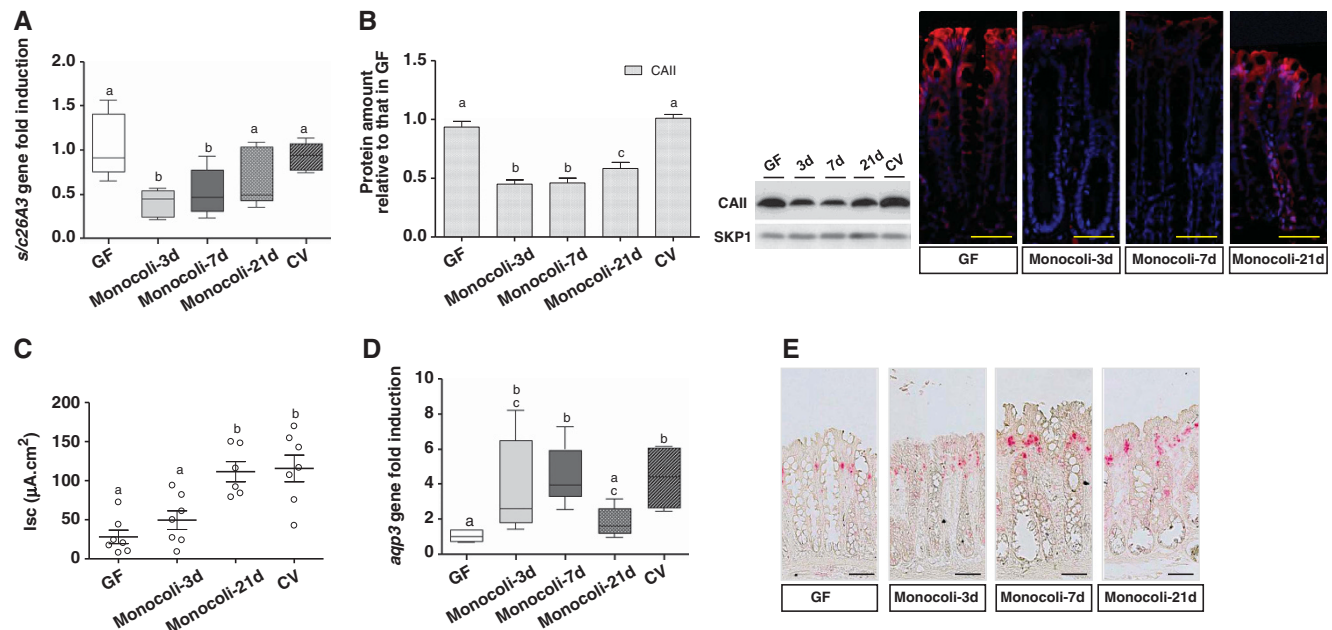


Figure 3 Analyses of colonic ion transporters after inoculation with *Escherichia coli* CEC15. **(A)** Relative abundance of *slc26A3* mRNA in colonic epithelial cells from GF, Monocoli and CV samples ($n = 5$ per group). Results are normalized to those for 18S rRNA gene expression and compared with values for GF rats. **(B)** Densitometric analyses and representative western blots for CAII protein performed on colonic epithelial cells isolated from GF, Monocoli and CV samples ($n = 5$ per group), and representative images of immunofluorescence. SKP1 was used as a loading control. **(C)** Measurement in an Ussing chamber of the short-circuit current (ΔIsc) induced after serosal stimulation of colonic tissue with the cholinergic agonist carbachol (CCh). **(D)** Relative amounts of *aqp3* mRNA in colonic epithelial cells of GF, Monocoli and CV groups ($n = 5$ per group). Results are normalized to values for 18S rRNA gene expression and compared with values for GF rats. **(E)** Representative *in situ* hybridization images of *wnt5a* in GF and Monocoli groups ($n = 4$ per group). All values are means \pm s.e.m. Mean values with letter designations are significantly different (P -value < 0.05). Scale bars indicate $50\ \mu\text{m}$.

two bacteria were similar 3 days post inoculation (10^9 c.f.u. equivalent per g of cecal content); the *E. coli* CEC15 count was stable after 21 days whereas that of *E. faecalis* CEF15 had declined (Supplementary Figure S4).

E. coli CEC15 elicits a sharp transient remodeling of colonic goblet cell populations and reinforces the mucus barrier.

In parallel to the increase in proliferative markers, we observed that the MUC2-staining intensity was

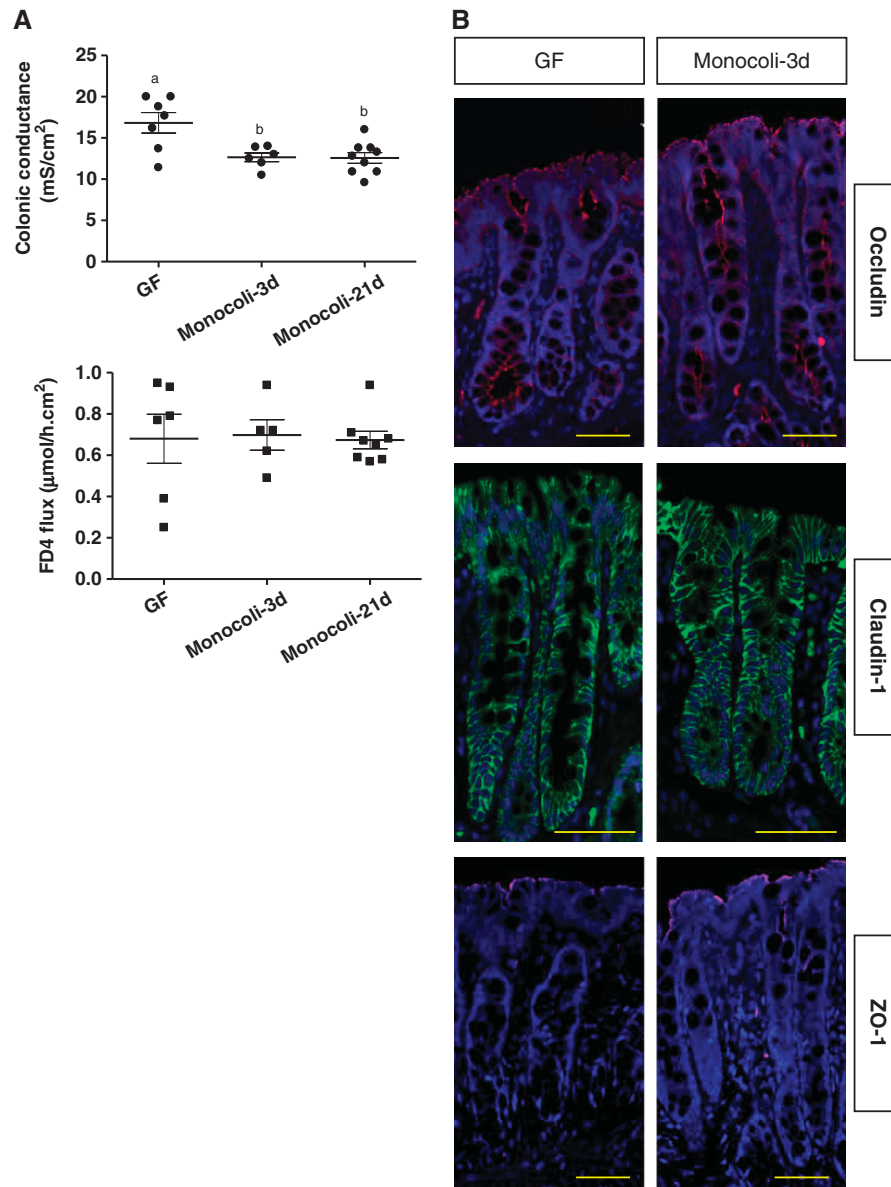
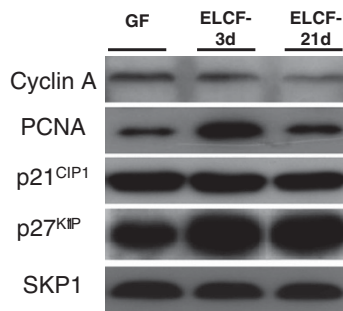
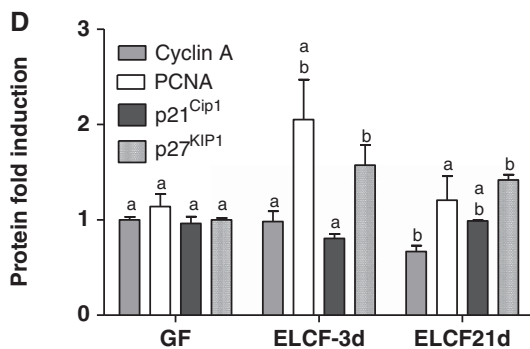
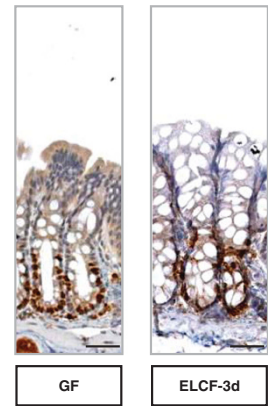
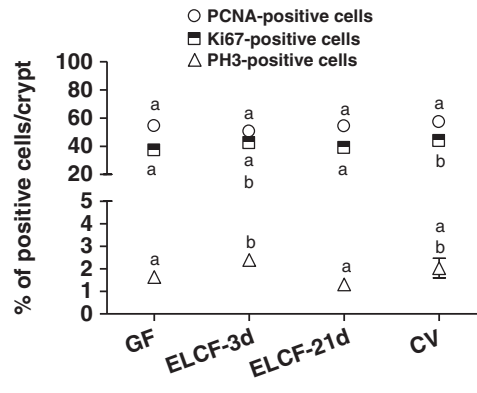
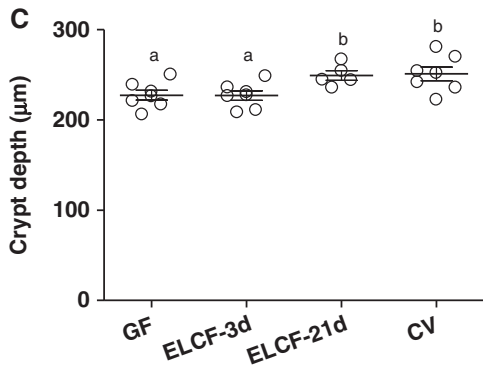
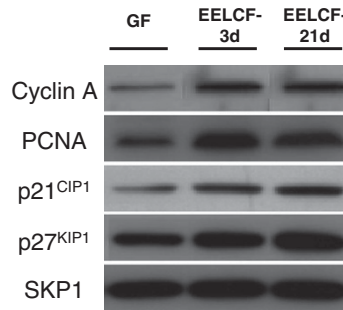
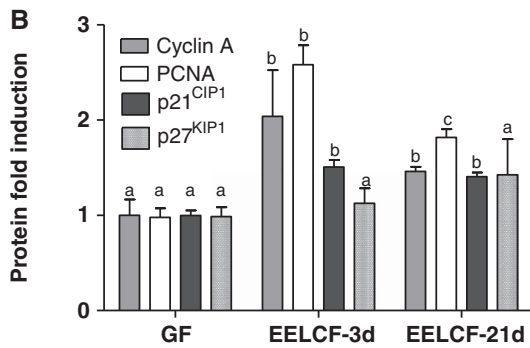
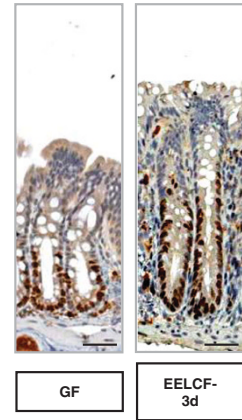
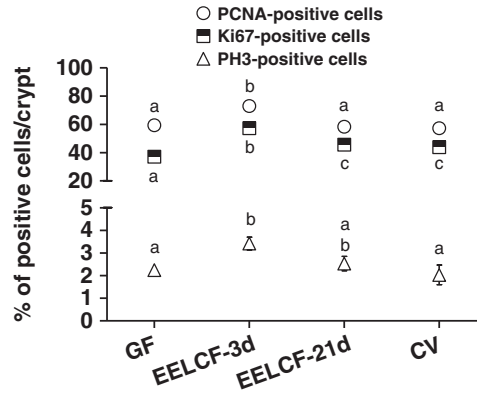
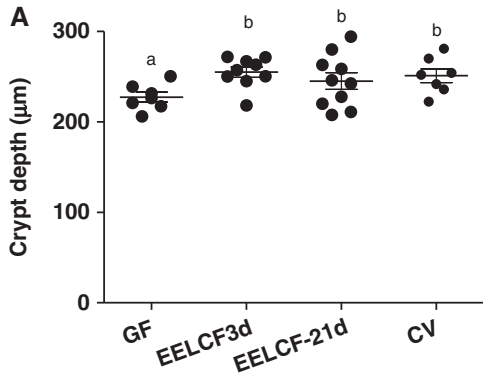


Figure 4 Analyses of the colonic permeability and tight junction proteins in Monocoli groups. (A) Tissue conductance and permeability measurements with 4 kDa FITC-labeled dextran (FD4). Colonic permeability was studied in an Ussing chamber after 30 min (colonic conductance, mS cm^{-2}); paracellular permeability was also assessed for 90 min by measuring mucosal-to-serosal flux of FD4 ($\mu\text{mol h}^{-1} \text{cm}^2$). (B) Representative immunofluorescence staining for occludin, claudin-1 and ZO-1. As no significant difference was observed between Monocoli-3, -7 and -21d, only one representative photograph of Monocoli-3d is shown. Scale bar indicates 50 μm .

Figure 5 Analyses of the colonic responses to two defined early colonizing microbiota containing and one not containing *Escherichia coli* CEC15. EELCF and ELCF groups were both inoculated with *E. faecalis* CEF15, *L. intestinalis* CLI15, *C. innocuum* CCI15 and a novel *Fusobacterium* spp. CFV15. The EELCF inoculum but not the ELCF inoculum, also contained *E. coli* CEC15. (A, C) Colonic crypt depth measurements and percentages of PCNA-, Ki67- and PH3-positive cells/crypt in colonic sections from (A) EELCF ($n = 6-9$) and (C) ELCF ($n = 5-7$) rats. Inoculated rats were killed 3 and 21 days post inoculation. GF and CV rats were used as controls ($n = 7-13$). The right part of the figure shows representative images of immunohistochemistry for Ki67 with the scale bar indicating 50 μm . (B, D) Western blot analyses of proliferative markers (cyclin A, PCNA) and cell cycle arrest-related proteins (p21^{CP1} and p27^{KIP1}) in isolated colonic epithelial cells from (B) EELCF and (D) ELCF groups ($n = 4-8$). Histograms show densitometric values and representative western blots are also presented. SKP1 was used as a loading control. All values are means \pm s.e.m. Mean values with letter designations are significantly different (P -value < 0.05).

lower in the Monocoli group than in GF 3 days post inoculation (Figure 2A and Supplementary Figure S5A); the staining intensity in the Monocoli

group increased thereafter and then remained stable over 21 days but without reaching CV values (Figure 2A and Supplementary Figure S5A).



Similarly, the percentages of PAS- and AB-positive cells 3 days post inoculation were below the GF values (Figure 2B); 7 days post inoculation, the number of AB-positive cells increased whereas PAS-positive cell counts remained low. By 21 days, the numbers of both PAS- and AB-positive cells were restored to GF and CV values (Figure 2B). Amounts of the two cell cycle arrest-related proteins, p21^{CIP1} and p27^{KIP1}, and the terminal goblet cell transcription factor KLF4, increased from 3 days post inoculation, that is, preceding the restoration of mucus storage in goblet cells and were similar to values for CV rats (Figure 2C). In double labeling experiments, Ki67 labeling and KLF4 labeling were partly superimposed in Monocoli-3d and to a lesser extent in Monocoli-21d whereas they were distinct in GF (Supplementary Figure S5C). The amount of the pro-apoptotic protein Bax was similar whatever the bacterial status of rats (Figure 2C). We performed MUC2 staining of unwashed colonic sections to study the mucus layer. It was twice as thick in the Monocoli group as in GF after 3 days but remained thinner than that in the CV group (Figure 2D). At later stages, 7 days and 21 days post inoculation, the mucus layer converged toward CV values (Figure 2D and Supplementary Figure S5D). FISH and Hoechst analyses indicated that *E. coli* CEC15 was embedded in the mucus layer from 3 days post inoculation and throughout the 21-day time course (Figure 2E). The numbers of CgA-positive cells also slightly varied in Monocoli group (Supplementary Figure S6). Crypt density was transiently increased: the number of crypts per mm of colonic section was 12.8 ± 0.2 for GF and reached a peak value 7 days post inoculation (15 ± 1.2).

E. coli CEC15 affects markers of colonic differentiated cells involved in electrolyte and water transport

We tested the effects of *E. coli* CEC15 on other primary colonic functions. We studied a major Cl⁻/HCO₃⁻ carrier, Slc26A3, that mediates Cl⁻ absorption and HCO₃⁻ secretion, and CAII that supplies protons and bicarbonate for Slc26A3 (Sterling *et al.*, 2002). Slc26A3 and CAII are localized in differentiated cells at the top of the colonic crypts (Schweinfest *et al.*, 2006). Both *slc26A3* gene expression and the amount of CAII protein were lower in the Monocoli group than in GF 3 days and 7 days post inoculation (Figures 3A and B). Values tended to increase toward CV values after 21 days (Figures 3A and B). We also analyzed ion secretion by the colonic epithelium by challenge with the cholinergic agonist CCh (Figure 3C). There was no difference in CCh-induced Δ Isc between GF and Monocoli rats at 3d. After 21d, Δ Isc was higher than in GF and in Monocoli 3 days, and reached CV values. Thus, the shift toward ion secretion under external stimuli is similar in Monocoli rats 3 days

post inoculation and GF rats, and is greater in Monocoli rats 21 days post inoculation and CV rats.

We next evaluated the expression of *aqp3*, a gene encoding a glycerol/water channel. Its pattern of expression was opposite to that of *slc26A3* and CAII protein amount. Expression of *aqp3* was stronger in Monocoli 3 days than in GF (Figure 3D). By 7 days, it was similar to that in CV rats, but then declined after 21 days (Figure 3D).

We also studied *Wnt5a* gene expression. The *wnt5a* gene is expressed in the mesenchyme just beneath the surface of the colonic epithelium; it causes the overlying epithelium to stop proliferating (Miyoshi *et al.*, 2011). In *in situ* hybridization, *Wnt5a* staining was stronger in Monocoli-7d and -21d than in Monocoli-3d and GF (Figure 3E) indicating that *E. coli* CEC15 colonization affected *Wnt5a* gene expression, which accompanied the related responses of the colonic epithelium to *E. coli* CEC15 colonization.

E. coli CEC15 preserves the epithelial barrier while remodeling the colonic epithelium

We tested whether *E. coli* CEC15 affected colonic epithelial permeability. We obtained contrasting data for conductance and FD4 flux. Colonic epithelial conductance was slightly lower in Monocoli-3d and -21d than in GF, suggesting that the epithelial barrier was reinforced by the presence of *E. coli* CEC15 (Figure 4A). FD4 flux did not differ between GF and Monocoli groups (Figure 4A). Also tight junction proteins (occludin, claudin-1 and ZO-1) did not differ between groups (Figure 4B). These observations indicate that epithelial barrier integrity is preserved after *E. coli* CEC15 administration, despite the strong remodeling effect. Data for colonic conductance and staining for tight junction were similar for GF and CV groups (data not shown).

Responses of the colonic epithelium to early colonizing simplified microbiotas differ according to the presence or absence of *E. coli* CEC15

We investigated the effects of *E. coli* CEC15 in a more diversified ecosystem. We used defined microbiotas composed of the early colonizing bacteria that we had isolated: *E. faecalis* CEF15, *L. intestinalis* CLI15, *C. innocuum* CCI15 and a novel Fusobacterium spp. CFV15 with (EELCF) and without (ELCF) *E. coli* CEC15. These two simplified microbiotas were used to inoculate GF rats and the presence of inoculated bacterial strains was studied in cecal contents by TTGE analyses and in unwashed colonic sections by FISH (Supplementary Figure S7). The TTGE profiles indicate that all the bacteria were present 3 days post inoculation for both groups and 21 days post inoculation for EELCF. In the ELCF group, Fusobacterium spp. CFV15 was

hardly detectable 21 days post inoculation (Supplementary Figure S7). In the EELCF group, the *E. coli* CEC15 count was 10^9 c.f.u. equivalent per g of cecal content both 3 days and 21 days post inoculation. Colonic crypt depth was greater in the EELCF group than in GF and remained similar to the CV values throughout the 21-day time course (Figure 5A). The percentages of PCNA-, Ki67- and PH3-positive cells were higher in EELCF than in GF 3 days post inoculation but had decreased toward GF and CV values by 21 days (Figure 5A). Similarly, WB analyses showed that the amounts of cyclin A and PCNA in the colon, like the amounts of p21^{CIP1}, were higher in EELCF than in GF (Figure 5B). The colonic responses to the mix without *E. coli* CEC15 differed in that the increase in colonic crypt depth was delayed. Indeed, an increase in colonic crypt depth similar to that of the EELCF and CV group was observed in the ELCF group not at 3 days but at 21 days post inoculation (Figure 5C). Moreover, the increase in colonic epithelial cell proliferation was strongly attenuated: PH3-positive cell numbers in ELCF-3d increased above GF values but were significantly lower than those observed for EELCF-3d rats (Figure 5C). Furthermore, the percentages of PCNA- and Ki67-positive cells, and cyclin A protein amounts were similar to GF and lower than EELCF-3d rats (Figures 5C and D). Also p21^{CIP1} protein amount remained low in ELCF rats relative to EELCF-3d rats (Figure 5D). However, in colonic epithelial cells isolated from ELCF-3d rats, the amount of PCNA protein increased, similar to that in EELCF and in p27^{KIP1}, slightly more than in EELCF (Figure 5D). All together, these data revealed that colonic epithelial cells responded differently to ELCF and EELCF.

Discussion

Regulation of the eukaryotic cell cycle by *E. coli* has been mostly studied in a context of pathogenesis. Some *E. coli* strains, including those residing in the human gut, produce bacterial toxins including colibactin, which modulate the eukaryotic cell cycle (Nougayrede *et al.*, 2006) and can trigger genomic instability in mammalian cells (Cuevas-Ramos *et al.*, 2010). A strong association is observed between colon tumor development and the colonization of the mucosa by *E. coli* strains harboring genes encoding colibactin (Arthur *et al.*, 2012). Interestingly, these bacterial effectors are not only associated with deleterious effects, as colibactin also supports the probiotic effects of *E. coli* Nissle 1917 (Olier *et al.*, 2012).

We identified an early colonizing strain of *E. coli* as a substantial player in remodeling of the colonic epithelium and suggest that this interaction could be a first step in the establishment of a

microbiota-compliant state. Monocolonization with *E. coli* CEC15 stimulated colonic epithelial proliferation whereas the other early colonizer, *E. faecalis* CEF15, did not. However, counts of colonizing *E. faecalis* CEF15 were 10-fold lower than those of *E. coli* CEC15 such that we cannot rule out the possibility that bacterial cell density could also be an important driver of host cell proliferation. In the presence of *E. coli*, the colonic crypts were poorly differentiated in the early phase post-colonization, but then differentiated cell markers (mucins, and ion and water transporters) converged progressively toward CV levels. Experiments with defined mixes composed of the other isolated bacteria revealed that the colonic epithelium responses differed according to whether or not *E. coli* CEC15 was present. As our experimental model involved adult rats, we showed this mechanism operates on epithelium that is mature, but naive in terms of exposure to microorganisms, which is similar to the situation in newborn humans.

Regulation of the eukaryotic cell cycle by commensal bacteria has frequently been described but its relevance to homeostatic relationships remains unclear. The stimulation of intestinal epithelial cell proliferation by commensal bacteria may be beneficial to the host. In mice, the recognition of commensal bacterial by Toll-like receptors initiates the colonic epithelial proliferation required for repair after epithelial injury (Pull *et al.*, 2005). We previously observed a transient expansion of the proliferative area after conventionalization with complex microbiotas, followed by a progressive return toward the homeostatic state of CV (Cherbuy *et al.*, 2010; Tomas *et al.*, 2013). This is accompanied by an increase in the colonic crypt depth. We report a similar pattern here, both when *E. coli* was inoculated alone or in combination with other bacteria. However, *E. coli* CEC15 inoculated alone did not fully mimic the conventionalization pattern: the increase in colonic crypt depth was not maintained suggesting that inoculation of *E. coli* alone is not sufficient to counterbalance the epithelial atrophy in GF rats.

Goblet cells were transiently altered in the Monocoli group. *E. coli* CEC15 administration was followed by a rapid decrease in epithelial MUC2 and mucopolysaccharide contents, and presumably these components are released to join the mucus layer, the thickness of which doubled 3d post inoculation. The epithelial cells then rapidly refilled with MUC2 and acidic mucopolysaccharides, whereas it took more time for neutral mucopolysaccharides to recover to the levels seen in GF and CV. *E. coli* CEC15 was found embedded in the mucus layer, consistent with intestinal colonization by commensal *E. coli* being dependent on its ability to grow in mucus. Studies *in vitro* and *in vivo* with mono-associated mouse models reveal that commensal *E. coli* can grow on monosaccharides derived from mucins (Chang *et al.*, 2004; Alpert *et al.*, 2009). In contrast, strains of *E. coli* unable to

survive in mucus fail to colonize the intestine (Moller *et al.*, 2003; Chang *et al.*, 2004). The occupation of nutritional niches by a mix of commensal *E. coli* strains prevents colonization by pathogenic *E. coli*, revealing the importance of pre-colonization by commensals for protection against pathogens (Maltby *et al.*, 2013). However, this effect does not seem to be restricted to *E. coli*, as two major representative bacteria of the human gut microbiota modify both goblet cells and the glycosylation profile (Wrzosek *et al.*, 2013).

The proteins Slc26A3 and CA are essential for Cl⁻ and HCO₃⁻ homeostasis (Höglund *et al.*, 1996). Both *slc26A3* expression and CAII protein abundance varied similarly after *E. coli* CEC15 administration and inversely to the expansion of the proliferative compartment. Thus, loss of differentiated cells and the associated increase in the number of proliferative cells may have consequences for ion fluxes. The effect of CCh was similar 3 days post inoculation as in GF, indicating that the capacity to shift toward ion secretion under external stimuli was unaffected during the early phase of colonization. Cl⁻ secretion and the resultant fluid transport are thought to be an innate defense mechanism in the intestine to flush out enteric pathogens. However, this mechanism can also target commensal bacteria as it has a selective effect on gut bacterial communities (Keely *et al.*, 2012). In our model, this mechanism only started to be established late post-inoculation concomitantly with the increase in *slc26A3* expression and CAII protein abundance. Possibly, the low ion secretion capacity early after *E. coli* administration may create a favorable environment for colonization. In contrast to *slc26A3* and CAII, the expression of the *aqp3* gene was enhanced from 3-day post-inoculation and thus paralleled proliferation. Deletion of *aqp3* leads to impaired colonic cell proliferation in a colitis model (Thiagarajah *et al.*, 2007), whereas deletion of *slc26A3* leads to enhanced colonic proliferative activity (Schweinfest *et al.*, 2006). Thus, the effects of *E. coli* CEC15 on ion transporters are coherent with their functions in cell proliferation. An increase in *Wnt5a* gene expression accompanied the responses of the colonic epithelium to *E. coli* CEC15. A recent study implicates *Wnt5a* in the bacterial epithelial cross talk contributing to the regulation of intestinal homeostasis: *Wnt5a* is weakly expressed in the colon of mice lacking commensal bacteria, whereas colonic *Wnt5a* expression is increased by conventionalization of GF mice (Neumann *et al.*, 2014).

Citrobacter rodentium infection, which mimics *E. coli* enteric infections in mice, leads to a highly proliferative and poorly differentiated colonic epithelium due to the downregulation of *slc26A3* and CAIV, (another member of the CA family) (Papapietro *et al.*, 2013). Thus, pathogenic and commensal bacteria modulate common targets; however, this process is worsened with pathogenic

bacteria, whereas it is balanced with commensal strains with the progressive induction of p21^{CIP1} and p27^{KIP1}, and *Wnt5a*. The remodeling effect of *E. coli* CEC15 also preserved epithelial barrier, unlike pathogenic strains of *E. coli* (Denizot *et al.*, 2012). Neither microscopic colonic damage, nor diarrhea, nor weight loss were observed in rats after *E. coli* CEC15 administration (data not shown). The values for *slc26A3* and *aqp3* gene expression, CAII protein amounts and ΔIsc after CCh stimulation were in the range of, or below, physiological values (those in CV rats).

E. coli is one of the first bacterial species to colonize the intestine and remains the predominant aerobic organism in the gastrointestinal tract in adulthood (Tenailon *et al.*, 2010). The pattern of colonization of the gut by *E. coli* has been changing substantially over recent decades (Nowrouzian *et al.*, 2003; Azad *et al.*, 2013), probably due to modern lifestyles (Adlerberth *et al.*, 2006; Blaser and Falkow, 2009). The prevalence of phylogenetic groups of *E. coli* is changing and this may have consequences for the host's health (Cuevas-Ramos *et al.*, 2010).

Our work shows that commensal early colonizing *E. coli* drives remodeling of the colonic epithelium affecting the structure of the epithelium, the mucus layer, and ion and water transport in rats without affecting the integrity of epithelium. Early colonizing *E. coli* is clearly highly significant, and may facilitate the establishment of favorable microenvironment for commensal colonization.

Conflict of Interest

The authors declare no conflict of interest.

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