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Thomas Secher, Delphine Payros, Camille Brehin, Michèle Boury, Claude Watrin,  
Marion Gillet, Isabelle Bernard-Cadenat, Sandrine Ménard, Vassilia Theodorou,  
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# Oral Tolerance Failure upon Neonatal Gut Colonization with *Escherichia coli* Producing the Genotoxin Colibactin

Thomas Secher,<sup>a,b,c,d</sup> Delphine Payros,<sup>a,b,c,d</sup> Camille Brehin,<sup>a,b,c,d</sup> Michele Boury,<sup>a,b,c,d</sup> Claude Watrin,<sup>a,b,c,d</sup> Marion Gillet,<sup>e</sup> Isabelle Bernard-Cadenat,<sup>b,c,d</sup> Sandrine Menard,<sup>e</sup> Vassilia Theodorou,<sup>e</sup> Abdelhadi Saoudi,<sup>b,c,d</sup> Maiwenn Olier,<sup>a,b,c,d,e</sup> Eric Oswald<sup>a,b,c,d,f</sup>

INRA, USC 1360, Toulouse, France<sup>a</sup>; INSERM, UMR 1043, Toulouse, France<sup>b</sup>; CNRS, UMR 5282, Toulouse, France<sup>c</sup>; Université de Toulouse, UPS, Centre de Physiopathologie Toulouse Purpan, Toulouse, France<sup>d</sup>; INRA/INPT/UPS, UMR1331, Toulouse, France<sup>e</sup>; CHU Toulouse, Hôpital Purpan, Service de Bactériologie-Hygiène, Toulouse, France<sup>f</sup>

**The intestinal barrier controls the balance between tolerance and immunity to luminal antigens. When this finely tuned equilibrium is deregulated, inflammatory disorders can occur. There is a concomitant increase, in urban populations of developed countries, of immune-mediated diseases along with a shift in *Escherichia coli* population from the declining phylogenetic group A to the newly dominant group B2, including commensal strains producing a genotoxin called colibactin that massively colonized the gut of neonates. Here, we showed that mother-to-offspring early gut colonization by colibactin-producing *E. coli* impairs intestinal permeability and enhances the transepithelial passage of luminal antigen, leading to an increased immune activation. Functionally, this was accompanied by a dramatic increase in local and systemic immune responses against a fed antigen, decreased regulatory T cell population, tolerogenic dendritic cells, and enhanced mucosal delayed-type hypersensitivity response. Conversely, the abolition of colibactin expression by mutagenesis abrogates the alteration of oral tolerance induced by neonatal colonization by *E. coli*. In conclusion, the vertical colonization by *E. coli* producing the genotoxin colibactin enhances intestinal translocation and subsequently alters oral tolerance. Thus, early colonization by *E. coli* from the newly dominant phylogenetic group B2, which produces colibactin, may represent a risk factor for the development of immune-mediated diseases.**

The intestinal microbiota is the largest microbial community of the human microbiome, with an estimated  $10^{14}$  bacterial cells and more than a hundred times the number of genes of the human genome (1). The commensal intestinal microbiota favors the proper maturation and function of the host intestinal immune system, the maintenance of the barrier integrity, and the restriction of enteric microbes to the lumen (2, 3). Its composition is largely dependent on host and environmental factors encountered in the first year of life, such as mode of delivery, diet, and administration of drugs or antibiotics (4, 5). The commensal bacterium *Escherichia coli* belongs to the pioneer microflora colonizing the mammalian gut immediately after birth. Infants are stably colonized by *E. coli* within a few days after birth (6), and this bacterium become the predominant facultative anaerobic bacteria in the adult microbiota (7). The *E. coli* population is diverse, including life-threatening pathogenic strains, as well as harmless commensal and probiotic strains. *E. coli* can be differentiated into seven major phylogenetic groups (A, B1, B2, C, D, E, and F) (8). Recent studies showed that the prevalence of the B2 group is increasing among *E. coli* strains persisting in the microbiota of humans from developed countries (9, 10), including infants (11), while the A and B1 groups are predominant in developing countries (12). Up to 50% of commensal B2 group *E. coli* strains carried in their genome the *pks* gene cluster responsible for the synthesis of a peptide-polyketide hybrid genotoxin named colibactin (13). Mammalian cell intoxication with colibactin-producing *E. coli* induced transient DNA damage, senescence, and chromosomal abnormalities (14, 15). Recent evidences from our team confirmed and extended this result by showing that up to 15% of infants in a French maternity hospital (16) and 18% of Swedish infants carried commensal B2 *E. coli* expressing colibactin (17). There is a large body of evidence demonstrating that the incidence of inflammation-related diseases, including inflammatory bowel diseases, rheuma-

toid arthritis, or multiple sclerosis, is increasing in developed countries (18–20). Permanently exposed to a large load of potentially harmful antigens, the host intestinal mucosa has evolved and is matured after birth to recognize and control immune responses to microbe antigens, maintaining gut homeostasis (21). The intestinal immune system developed mechanisms that prevent untoward reactions against microbiota while providing efficient responses against pathogens. This physiological induction of immunological tolerance at the intestinal mucosa surface is referred to as oral tolerance. It has been suggested to operate, according to the dose of luminal antigen, via the induction of suppressive tolerogenic responses or via the induction of lymphocyte anergy or deletion (22, 23). This balance between effector and tolerogenic responses is tightly controlled, and disturbances in this host-commensal relationship may cause unrestrained activation of the intestinal immune system, leading to inflammation-related diseases (24, 25).

We report here that colibactin expression increased intestinal

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Address correspondence to Eric Oswald, eric.oswald@inserm.fr.

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permeability and enhanced translocation of B2 *E. coli* acquired by natural early gut colonization, during childhood, from the intestinal lumen to systemic compartments. In adults, we have observed an increase of the transepithelial passage through Peyer's patches (PP) correlated with enhanced gamma interferon (IFN- $\gamma$ ) levels. This alteration of the gut barrier homeostasis exacerbated the mucosal and systemic immune responses against a luminal antigen as observed in an experimental model of ovalbumin (OVA)-driven oral tolerance. Animals early colonized (bacterial colonization that occurs at birth) by colibactin-producing *E. coli* exhibited an enhanced OVA-specific response, which was correlated with a defect in T regulatory cells (Tregs) and tolerogenic dendritic cells (DCs). After subjection to a mucosal delayed-type hypersensitivity (DTH) reaction, colibactin-producing *E. coli*-colonized animals displayed exacerbated signs of inflammation that also correlated with a decreased in the Treg population. The invalidation of colibactin production reduced the propensity of *E. coli* to break down oral tolerance upon neonatal colonization, whereas the reintroduction of the genotoxicity ability restored it, demonstrating the implications of the genotoxin colibactin in altering oral tolerance in animals early colonized by commensal B2 *E. coli*.

## MATERIALS AND METHODS

### Experimental animal model of long-lasting gut bacterial colonization.

Wistar rats have been early colonized according to the model developed by Payros et al. (16). Briefly, primiparous timed-pregnant Wistar female rats were obtained from Janvier (Le Genest Saint-Isle, France) on gestational day 15 and housed separately. Pregnant females were housed under specific-pathogen-free conditions and had access to food and water supplemented with streptomycin (5 g/liter) *ad libitum*. Pregnant females were inoculated twice with  $10^9$  bacteria by intragastric gavage at postnatal day -1 (PND -1) and PND -5. The Toxalim animal facility (INRA, UMR 1331, Toulouse, France) is licensed by the French Ministry of Agriculture (agreement B31.555.13). All animal experiments complied with the European Union regulation, as reviewed by the regional ethics committee (CNREEA; MP/03/62/11/11). Bacterial colonization of offspring was monitored during childhood (PND8 to PND28) or at adulthood (PND56). To ensure a proper randomization, for each experimental group, three dams were used whose litters were pooled for each experimental readout.

### Bacterial strains, mutagenesis procedures, and growth conditions.

Bacterial strains used in the present study are listed in Table S1 in the supplemental material. The commensal *Escherichia coli* strain M1/5, kindly provided by Ulrich Dobrindt (Münster, Germany), was isolated from the stool of an asymptomatic human and maintained with minimal genetic manipulation. *pks* chromosomal isogenic mutants and complemented strains were generated in the laboratory (16). Before oral administrations, all *E. coli* strains were grown 6 h in Luria-Bertani (LB) broth supplemented with antibiotics at 37°C with shaking. These cultures were diluted 1:100 in LB broth without antibiotics and cultured overnight at 37°C with shaking. Bacterial pellets from these overnight cultures were resuspended in sterile phosphate-buffered saline to a concentration of  $10^9$  CFU/ml.

**Bacterial translocation.** Colon, mesenteric lymph node (mLN), spleen, and ileal PP homogenates were aseptically prepared in 0.6 ml of isotonic saline solution using a Precellys tissue homogenizer (Bertin Technologies). Tenfold serial dilutions of homogenates were plated on MacConkey agar plates (Biovalley) supplemented or not with appropriate antibiotics or on plate count agar (PCA) plates. Plates were incubated at 37°C and 5% CO<sub>2</sub>, and the numbers of CFU were enumerated after 24 h. Colonies found growing on MacConkey agar plates without antibiotic were considered *Enterobacteriaceae*. Colonies found growing on PCA agar

plates were considered total aerobic flora. Whole blood was directly assessed for bacterial translocation on MacConkey agar plates.

**Intestinal permeability.** *Ex vivo* paracellular and transcellular permeability were assessed in PP. Two PP biopsy specimens were mounted in Ussing chambers (Easy Mount; Physiologic Instruments), exposing a surface area measuring 0.1 cm<sup>2</sup>. The specimens were bathed on each side with 1 ml of oxygenated thermostated Krebs solution (Sigma). After equilibration, fluorescein isothiocyanate (FITC)-dextran (4 kDa) at a final concentration of 2.2 mg/ml or  $1 \times 10^7$  CFU of Alexa 488-conjugated *E. coli* K-12 BioParticles (Molecular Probes)/ml was added to the mucosal compartment. The epithelial permeability to FITC-dextran or Alexa 488-conjugated *E. coli* was determined by measuring the fluorescence intensity (FI) at 485/525 nm by using an automatic Infinite M200 microplate reader (Tecan). By measuring the specific activity of the markers expressed in FI/ $\mu$ g or FI/CFU found in the serosal compartment, the intestinal fluxes (in  $\mu$ g or CFU/2 h cm<sup>2</sup>) from mucosa to serosa were calculated. Permeability was calculated as the ratio of the flux to the initial concentration of the fluorescent marker. An *in vivo* intestinal permeability assay to assess barrier function was performed using FITC. Briefly, animals were treated orally with FITC (10 mg/kg [body weight] of FITC; molecular mass, 390 Da; Sigma-Aldrich). After 2 h, the animals were sacrificed, and serum was collected. The fluorescence intensity was measured at 485/525 nm using an Infinite M200 microplate reader. The FITC concentrations were determined from standard curves generated by serial dilution of stock FITC. FITC recovery was calculated by linear regression of sample fluorescence, taking account of rat body weight.

### Tolerance induction, immunization, and mucosal DTH response.

For oral tolerance induction, adult rats (PND56) received 20 mg of OVA (grade V; Sigma), prepared in bicarbonate buffer to avoid gastric degradation, by gavage on day 1. Control rats received bicarbonate buffer only. On days 7 and 14, all rats were immunized by subcutaneous injection of 100  $\mu$ g of OVA emulsified in complete Freund adjuvant (CFA; Sigma) at a 1:1 ratio (see Fig. S1 in the supplemental material). CFA was added to improve the potency of the immune response, as previously described (26). Rats were sacrificed at day 28. To assess the DTH, OVA- or control-fed rats were treated five times with 50 mg of OVA (grade V) by oral gavage every 2 days and again 7 days after the first immunization. Rats were sacrificed 2 h after the last gavage.

***Ex vivo* lymphocyte response and cytokine production.** Lymphocytes were isolated from mLN and cultured at  $2 \times 10^6$  cells/well in RPMI 1640 supplemented with 10% of inactivated fetal calf serum, penicillin (100 U/ml), streptomycin (100 U/ml), L-glutamine (4 mM), and 2-mercaptoethanol (50 mM) in 24-well plates at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>. Cell viability was assessed by trypan blue exclusion and was found to be similar in all groups. Cells were stimulated with 0, 10, or 100  $\mu$ g of OVA/ml for 72 h. Culture supernatants and jejunum or PP homogenates, prepared in radioimmunoprecipitation assay buffer, were tested for IFN- $\gamma$ , interleukin-17 (IL-17), IL-10, tumor necrosis factor alpha (TNF- $\alpha$ ), or IL-6 by enzyme-linked immunosorbent assay (ELISA; DuoSet kit; R&D Systems).

**Immunoglobulin assay.** OVA-specific IgG, IgG2a, IgG2b, and IgG1 serum levels were measured by sandwich ELISA. Briefly, 96-well plates were coated with 25  $\mu$ g of OVA/ml diluted in bicarbonate buffer. After the plates were washed and blocked, serum samples were incubated for 2 h. Subsequently, a 96-well plate was washed, and biotin-conjugated goat anti-rat IgG, IgG2a, IgG2b, or IgG1 antibodies (kindly provided by A. Saoudi) were added. After a washing step, peroxidase-conjugated streptavidin (R&D) was added. Tetramethylbenzidine was used as a substrate, and the optical density was measured at 450 nm using an Infinite M200 microplate reader. The titer was calculated by binary logarithm regression as the reciprocal dilution of the sample, where the extinction was 2-fold the background extinction.

**Flow cytometry.** Single cell suspensions were prepared from mLN and spleens by mechanical disruption. Before staining, Fc receptors were blocked with anti-CD16/CD32 antibody (Fc block; BD Biosciences). For

surface staining, the cells were incubated with anti-rat TCR $\alpha\beta$ -APC (clone R73), anti-rat CD4-Alexa Fluor 488 (clone Ox35), anti-rat CD8-Alexa Fluor 642 (clone Ox8), anti-rat CD25-PE (clone Ox39), anti-rat CD11c-PeCy7 (clone Ox42), anti-rat MHC-II-PerCP (clone Ox6), anti-rat CD103-Alexa Fluor 488 (clone Ox62), or anti-rat CD86-PE (clone 24F) for 30 min at 4°C. Intracellular Foxp3-PeCy7 staining was performed using fixation and permeabilization buffers according to the manufacturer's instructions (eBioscience). T-cell-associated antibodies were generated and kindly provided by A. Saoudi. DC-associated antibodies were purchased from Biolegend (San Diego, CA). Stained cells were analyzed on a MACSQuant cytometer (Miltenyi). The data files were analyzed using FlowJo (Tree Star, Inc.).

**MPO activity.** Myeloperoxidase (MPO), a marker of polymorphonuclear neutrophil primary granules, was determined in colonic tissues, according to a modified method of Bradley et al. (27). Segments of colon were homogenized in potassium phosphate buffer (KPB; 50 mM, pH 6.0). Endogenous catalases were inhibited by three cycles of freezing and thawing. Cell pellets were resuspended in the detergent hexadecyltrimethylammonium bromide buffer (0.5% in KPB; Sigma) to release MPO from the primary granules. After sonication on ice, suspensions were centrifuged at 13,000 rpm for 15 min at 4°C, and supernatants were assayed spectrophotometrically for MPO activity and protein content. Supernatants were diluted in KPB containing 0.167 mg of *o*-dianisidine dihydrochloride/ml and 0.0005% hydrogen peroxide. The absorbance at 450 nm was recorded using an Infinite M200 microplate reader at 30-s intervals over 5 min. MPO of human neutrophils (0.1 U/100  $\mu$ l) was used as a standard. The absorbance change at 450 nm for 1  $\mu$ mol of hydrogen peroxide/min at 25°C was calculated from the standard curve and equals 1 U of MPO activity. Protein concentrations were determined by the method of Lowry (Bio-Rad), and MPO activity was expressed as MPO units per g of protein.

**Histology.** Rat colonic tissues were fixed in 4% paraformaldehyde, embedded in paraffin, sectioned (5  $\mu$ m), and stained with hematoxylin and eosin. Colonic inflammation and damage were assessed in a blinded fashion according to the method of Cooper et al. (28). Each of three histological parameters was scored from 0 to 4, based on the severity of inflammation, the extent of inflammation, and epithelium damage, and correlated to the percentage of tissue involved. The histological score was the sum of these parameters.

**Statistical analyses.** Statistical evaluation of differences between the experimental groups was determined by using one-way analysis of variance, followed by a Bonferroni post test (which allows comparison of all pairs of groups). All tests were performed with GraphPad Prism 5.03 (GraphPad Software, Inc., San Diego, CA). All data are presented as means  $\pm$  the standard errors of the mean (SEM). A *P* value of <0.05 was considered significant.

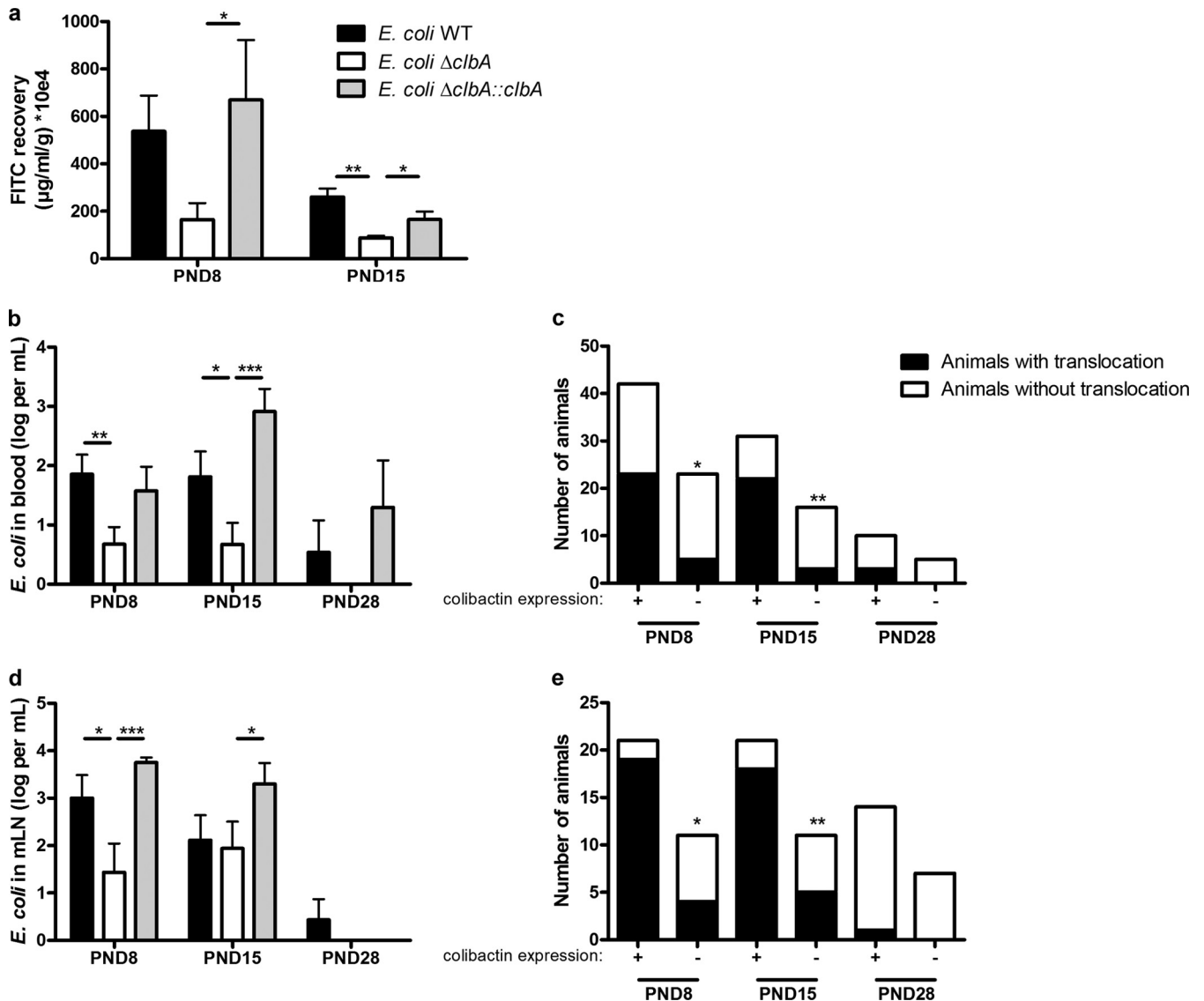
## RESULTS

**Colibactin impaired intestinal permeability and enhanced genotoxic *E. coli* translocation in neonates.** We found a significant increase in FITC passage at PND8 and PND15 in the blood of animals colonized since birth by colibactin-producing *E. coli* compared to animals early colonized by a non-colibactin-producing *E. coli* mutant strain (Fig. 1a). Increased intestinal permeability could expose the intestinal immune system to an increased load of luminal antigens and/or bacteria such as *E. coli*, which is predominant in the intestinal flora during the first weeks of life (11). Therefore, we decided to assess whether early colonization with a colibactin-producing *E. coli* strain could modulate bacterial translocation (BT) in extraintestinal compartments such as the mesenteric lymph nodes (mLN), spleen, and blood. Interestingly, we did observe an overall increase in the prevalence of total BT in the mLN (see Fig. S1c in the supplemental material) or spleen (see Fig. S1d in the supplemental material) at PND8 and PND15. Under

either condition, the expression of colibactin significantly enhanced specific B2 *E. coli* translocation from the intestinal lumen to the blood (Fig. 1b and c) and the mLN (Fig. 1d and e) at PND8 and PND15 and in the spleen at PND8 only (see Fig. S1e and S1f in the supplemental material). In addition, we monitored DNA damage in the intestinal compartment and observed that colibactin significantly increased the number of  $\gamma$ H2AX (a surrogate marker of DNA double-strand breaks)-positive cells in the intestinal laminae propriae of animals early colonized by *E. coli* (see Fig. S1b in the supplemental material). These phenomena were largely reduced at weaning (PND28) and were not detected in adult rats (data not shown). The increased levels of *E. coli* in the blood and mLN depend on colibactin expression by *E. coli* and are not due to enlarged gut colonization, since there was equivalent *E. coli* intestinal expansion regardless of the production of colibactin (see Fig. S1a in the supplemental material), but these increased levels are probably the result of increased intestinal permeability.

**Early gut colonization by colibactin-producing *E. coli* alters PP permeability.** The first element of the intestinal immune system that forms the interface between the host and the luminal environment is the PP. The PP are able to transport luminal antigens and bacteria toward the underlying component of the intestinal immune system, leading to either tolerance or systemic immune response. Thus, PP are considered important immune sensors of the intestine. We sought to determine whether the early gut colonization by colibactin-producing *E. coli* could specifically affect PP permeability. We showed in colibactin-producing *E. coli* colonized adult animals a significant augmentation of dextran-FITC permeability in PP (Fig. 2a). According to the PP ability to transport luminal antigens and bacteria, we next assessed the transmucosal passage of chemically killed *E. coli* in PP and observed that this translocation was facilitated when animals were colonized by colibactin-producing *E. coli* (Fig. 2b). This bacterial uptake was confirmed *in vivo* by the analysis of the bacterial load in PP, which revealed that even if the PP of all our animals were colonized by *E. coli*, colibactin expression significantly enhanced their colonization level (Fig. 2c). Consistent with these observations, we measured an increased production of IFN- $\gamma$  in colibactin-producing-*E. coli*-colonized animals compared to animals infected with non-colibactin-producing mutants (Fig. 2d), suggesting a dysregulated immune response.

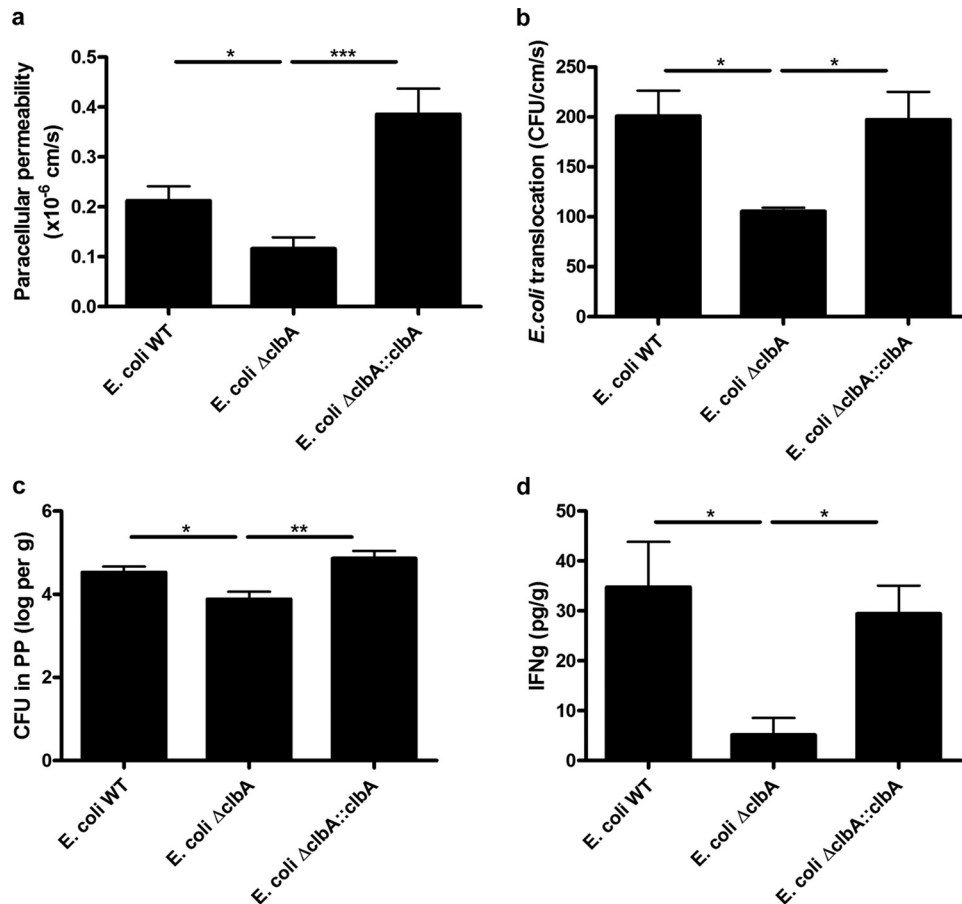
**Early gut colonization by colibactin-producing *E. coli* impaired tolerance against luminal antigen.** Increased permeability in the intestine (including PP) exposes the intestinal immune system to an increased load of luminal antigens, increasing immune stimulation. Thus, we hypothesized that early gut colonization by colibactin-producing *E. coli* may affect the gut immune responses when submitted to oral tolerance (see Fig. S2 in the supplemental material). It should be noted that this tolerization protocol did not modify *E. coli* colonization of the gut of these animals (see Fig. S3a in the supplemental material). Serum antigen-specific antibodies were quantified in all experimental groups. As expected, OVA-specific antibodies were largely produced in sensitized animals (without *per os* tolerization) in a similar manner regardless of the production of colibactin (see Fig. S3b in the supplemental material), whereas tolerized rats exhibited reduced titers. The titer of anti-OVA IgG and of the Th1-related IgG subclasses, IgG2a and IgG2b, were significantly enhanced in the serum of OVA-tolerized colibactin-producing-strain colonized animals compared to ani-



**FIG 1** Colibactin impaired intestinal permeability and enhanced *E. coli* translocation in neonates. (a) FITC recovery at PND8 and PND15 in the sera of rats early colonized with *E. coli* wild-type (WT; ■),  $\Delta clbA$  (□), or  $\Delta clbA::clbA$  (▨) strains. The means  $\pm$  the standard errors of the mean (SEM) for  $n = 6$  rats are shown (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ). (b) *E. coli* enumeration in blood at PND8, PND15, and PND28. The means for *E. coli* CFU (log) per ml  $\pm$  the SEM for  $n = 16$  to 26 (PND8 and PND15) or  $n = 6$  to 12 (PND28) rats are shown (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ). (c) Prevalence of blood *E. coli* translocation at PND8, PND15, and PND28. The absolute numbers of rats with (■) or without (□) blood translocation according to the expression of colibactin by *E. coli* for  $n = 16$  to 26 (PND8 and PND15) or  $n = 6$  to 12 (PND28) rats are shown (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ). (d) *E. coli* enumeration in mLN at PND8, PND15, and PND28. The means for *E. coli* CFU (log) per g  $\pm$  the SEM for  $n = 12$  (PND8 and 15) or  $n = 8$  (PND28) rats are shown (\*,  $P < 0.05$ ; \*\*\*,  $P < 0.001$ ). (e) Prevalence of mLN *E. coli* translocation at PND8, PND15, and PND28. The absolute numbers of rats with or without blood translocation according to the expression of colibactin by *E. coli* for  $n = 12$  (PND8 and 15) or  $n = 8$  (PND28) rats are shown (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ).

mals infected with non-colibactin-producing mutant *E. coli* (Fig. 3a, b, and c). In contrast, the levels of the Th2-related IgG1 subclass were not affected by *E. coli* colonization (Fig. 3d). At the mucosal levels, this correlated with an increased production of the prototypical Th1 cytokine, IFN- $\gamma$ , in the jejunum of animals early colonized by *E. coli* producing colibactin (Fig. 3e). In addition, analysis of the cytokine production upon OVA stimulation revealed that lymph nodes cells from animals that were sensitized by OVA produced large dose-dependent amounts of IFN- $\gamma$  and IL-17 (Fig. 3f and g, dotted bars). As expected, we observed that upon early gut colonization by non-colibactin-producing *E. coli*,

followed by oral tolerance with OVA, lymph node cells exhibited decreased production of IFN- $\gamma$  and IL-17 (Fig. 3f and g, white bars), which is a specific indication of induction or oral tolerance. Interestingly, upon early gut colonization by colibactin-producing *E. coli*, followed by oral tolerance with OVA, lymph node cells exhibited a sustained production of the proinflammatory cytokines IFN- $\gamma$  and IL-17 (Fig. 3f and g, gray bars). Altogether, these data demonstrated that oral tolerance tested here based on immunoglobulin, as well as IFN- $\gamma$  and IL-17, production is impaired by the neonatal gut colonization with commensal B2 *E. coli* strains, and this depends on the production of the genotoxin colibactin.

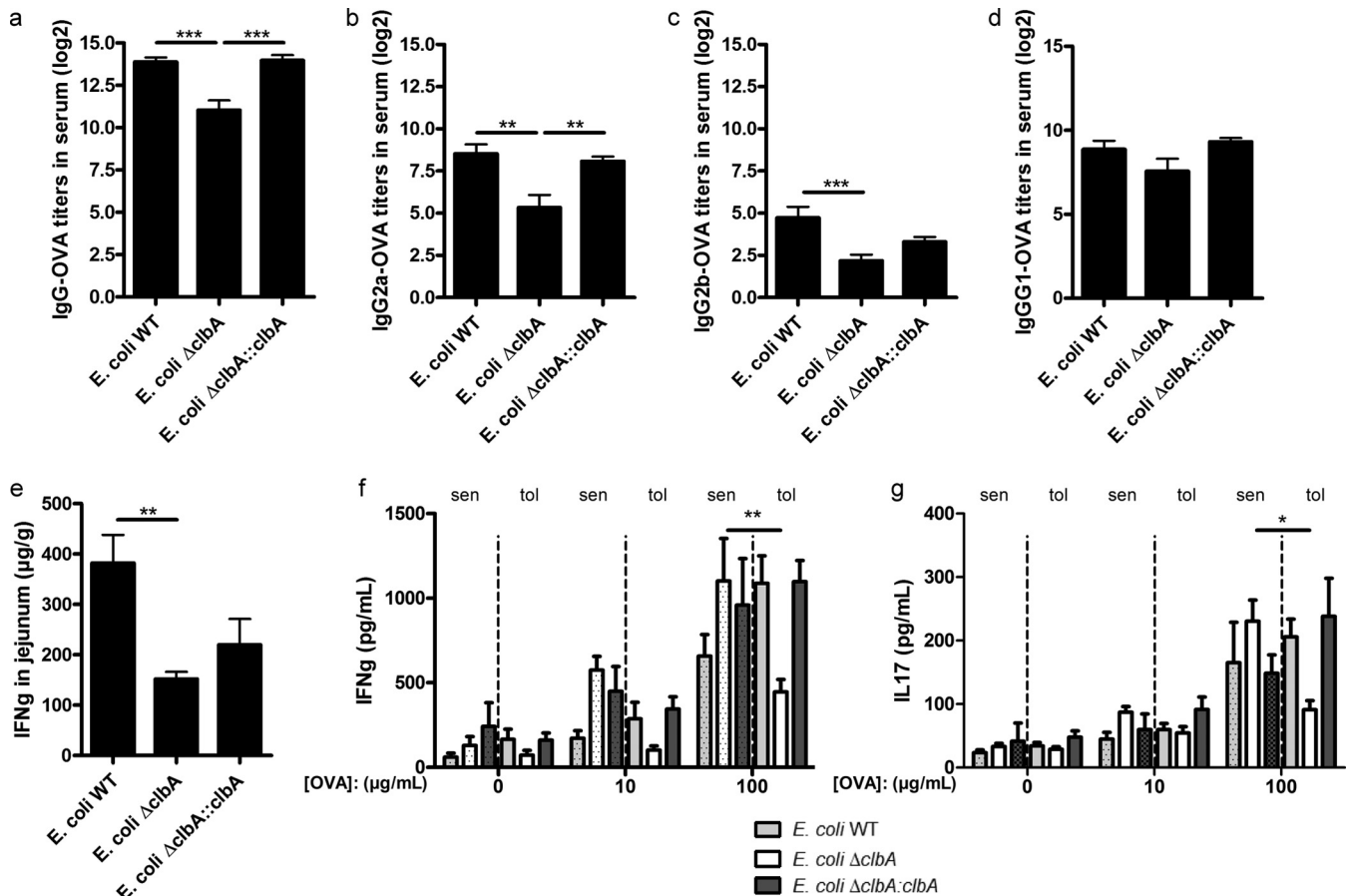


**FIG 2** Early gut colonization by colibactin-producing *E. coli* alters PP homeostasis. (a) Using chamber analysis, paracellular permeability of PP from rats colonized with *E. coli* WT,  $\Delta clbA$ , or  $\Delta clbA::clbA$  strains was assessed at PND28 by measuring mucosal to serosal flux to FITC-dextran. The means  $\pm$  the SEM for  $n = 10$  rats are shown (\*,  $P < 0.05$ ; \*\*\*,  $P < 0.001$ ). (b) Using chamber analysis of PP, the permeability to chemically killed K-12 Alexa 488-conjugated *E. coli* was assessed at PND28. The means  $\pm$  the SEM for  $n = 7$  (*E. coli* WT and  $\Delta clbA$ ) and  $n = 11$  (*E. coli*  $\Delta clbA::clbA$ ) rats are shown (\*,  $P < 0.05$ ). (c) *E. coli* enumeration in PP at PND28. The means of *E. coli* CFU (log) per g  $\pm$  the SEM for  $n = 20$  (*E. coli* WT and  $\Delta clbA$ ) or  $n = 12$  (*E. coli*  $\Delta clbA::clbA$ ) rats are shown (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ). (d) IFN- $\gamma$  production in PP was determined by ELISA at PND28. The mean levels of IFN- $\gamma$  per g  $\pm$  the SEM for  $n = 11$  (*E. coli*  $\Delta clbA::clbA$ ) or  $n = 7$  (*E. coli* WT and  $\Delta clbA$ ) rats are shown (\*,  $P < 0.05$ ).

**Diminished oral tolerance in animals early colonized by colibactin-producing *E. coli* is associated with reduced numbers of regulatory T cells and tolerogenic DCs.** Oral tolerance has been mechanistically related to T cell deletion or anergy or to active regulatory processes. Thus, we examined T cell and antigen-presenting cell populations in tolerized animals early colonized by *E. coli*. At 1 week after the second immunization, we observed a significant reduction in both CD4<sup>+</sup> CD25<sup>+</sup> FoxP3<sup>+</sup> regulatory T cells and CD103<sup>+</sup> CD11c<sup>+</sup> MHC-II tolerogenic DC frequency in the mLN of OVA-tolerized animals early colonized with colibactin-producing *E. coli* compared to non-colibactin-producing mutants (Fig. 4a and b). Interestingly, FoxP3<sup>+</sup> T cell and tolerogenic DC frequencies were also reduced in the spleen (see Fig. S3c and d in the supplemental material). This alteration in the mLN Treg population, followed by early gut colonization with *E. coli*, was not related to T cell deletion, since the total CD4<sup>+</sup> (Fig. 4c; see also Fig. S3e in the supplemental material) or CD8<sup>+</sup> (Fig. 4d; see also Fig. S3f in the supplemental material) T cell frequencies either were not or were only slightly modified regardless of the conditions.

**Early gut colonization with colibactin-producing *E. coli* enhanced intestinal DTH responses against luminal antigen.** The

consequence of feeding antigens to naive animals is the induction of systemic tolerance to this antigen. If this oral tolerance is impaired, active local DTH can be induced in the intestine by the readministration of the same antigen (29). We next determined whether the expression of colibactin by *E. coli* early colonization of the gut might modify the DTH response induced by the oral administration of OVA at high concentrations (see Fig. S4 in the supplemental material). Animals uniformly colonized by *E. coli* strains (see Fig. S5a in the supplemental material) were sacrificed 2 h after the last administration of OVA. Once again, we observed an increased production of OVA-specific IgG in the sera of animals early colonized by colibactin-producing *E. coli* (see Fig. S5b in the supplemental material), which characterized a defect in oral tolerance to OVA. We next assessed the morphology and inflammatory status of the gut mucosa. Jejunum histological analysis (Fig. 5a) revealed profound alterations with severe intestinal ulcerations and villus length reduction, marked edema, and inflammatory cell infiltration. These parameters were reduced in animals early colonized with non-colibactin-producing *E. coli*, which was confirmed by a semiquantitative assessment of microscopic damages (Fig. 5b). Mucosal inflammation macroscopically observed



**FIG 3** Early gut colonization by colibactin-producing *E. coli* impaired tolerance against luminal antigen. (a to d) OVA-tolerized adult rats early colonized by *E. coli* WT,  $\Delta clbA$ , or  $\Delta clbA::clbA$  strains were analyzed for serum levels of OVA-specific immunoglobulin. The mean IgG (a), IgG2a (b), IgG2b (c), and IgG1 (d) titers  $\pm$  the SEM for  $n = 18$  (*E. coli* WT) and  $n = 20$  ( $\Delta clbA$  and  $\Delta clbA::clbA$ ) rats are shown (\*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ). (e) IFN- $\gamma$  production in the jejunum was determined by ELISA. The mean levels of IFN- $\gamma$  per  $g \pm$  the SEM for  $n = 9$  rats are shown (\*\*,  $P < 0.01$ ). (f and g) OVA-tolerized (tol, full bars) or sensitized (sen, dotted bars) adult rats early colonized by *E. coli* WT,  $\Delta clbA$ , and  $\Delta clbA::clbA$  strains were analyzed for cytokine production analysis in OVA-restimulated mLN cells after 3 days of culture. The mean IFN- $\gamma$  (f) and IL-17 (g) production  $\pm$  the SEM for  $n = 8$  animals is shown (\*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ).

in the jejunum of rats early colonized by colibactin-producing *E. coli* was also correlated with increased production of two prototypal proinflammatory cytokines: TNF- $\alpha$  (Fig. 5c) and IL-6 (Fig. 5d).

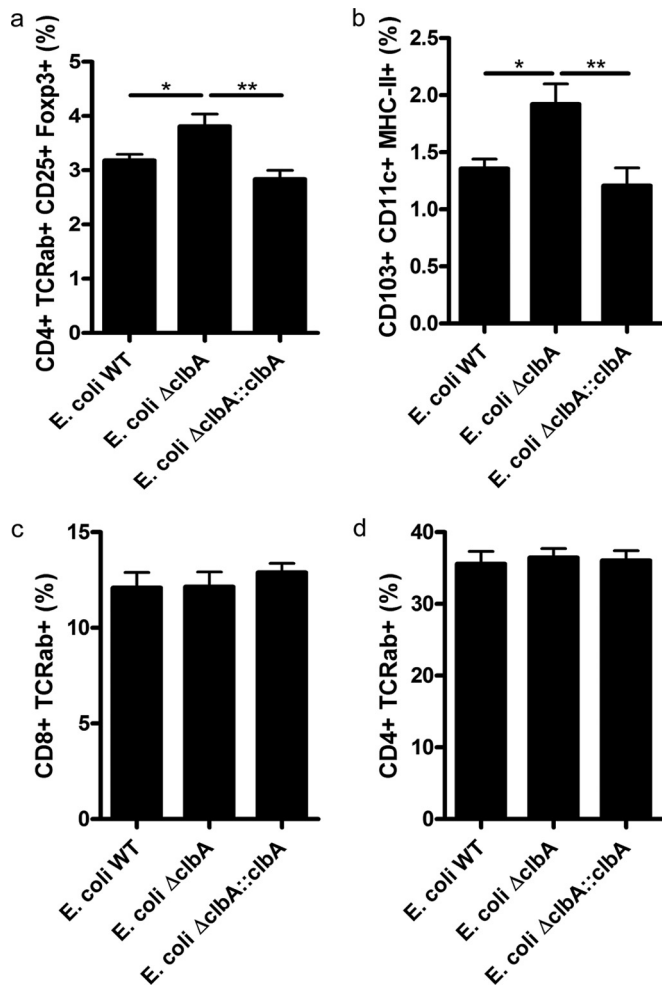
Interestingly, in animals early colonized by colibactin-producing *E. coli*, we observed a similar aggravation of intestinal DTH in the colon, which is the major site of *E. coli* settlement. This was characterized by enhanced mucosal crypt damage and inflammatory cell infiltrate (Fig. 5e and f). Colonic MPO activity, a marker of neutrophil infiltration, was significantly increased in animals early colonized by colibactin-producing *E. coli* (Fig. 5g). These intense alterations of intestinal mucosa in animals early colonized by colibactin-producing *E. coli* correlated with a significant decrease in mLN FoxP3<sup>+</sup> Treg frequency (see Fig. S5c in the supplemental material).

## DISCUSSION

Although the intestinal microbiota is essential for the development of functional immune system, certain types of microbes can affect the development and the maintenance of oral tolerance. Previous studies examining the role of commensal microbiota in the development of immune function and especially tolerance have used germfree (GF) or gnotobiotic animals. GF mice exhib-

ited decreased IgA production, reduced intraepithelial lymphocyte population, and PP or mLN maturation (30), whereas segmented filamentous bacteria or *Clostridium*-reconstituted animals displayed an increased prevalence in the Th17 (31) or Treg (32) population. In addition, tolerogenic antigen-presenting cells can be induced by *Bacteroides* or *Bifidobacterium* colonization (33, 34). However, the effects of these colonizing microbes in the artificial germfree model or in fully mature adults may not reflect the outcome of the intimate interaction that occurs between the pioneer microbiota, derived from the mother, and its host. As such, we sought here to investigate the impact of early postnatal interaction between a newly dominant phylogenetic group of *E. coli* and its host on the mucosal immune homeostasis at adulthood by using a model mimicking natural vertical transmission of intestinal microbes. The results obtained clearly demonstrated that neonatal vertical gut colonization by colibactin-producing *E. coli* impaired oral tolerance to luminal antigens.

We previously demonstrated that early gut colonization by genotoxic *E. coli* altered intestinal epithelial permeability in adults (16). We extended these results by showing that the production of colibactin impaired intestinal permeability and neonates colo-



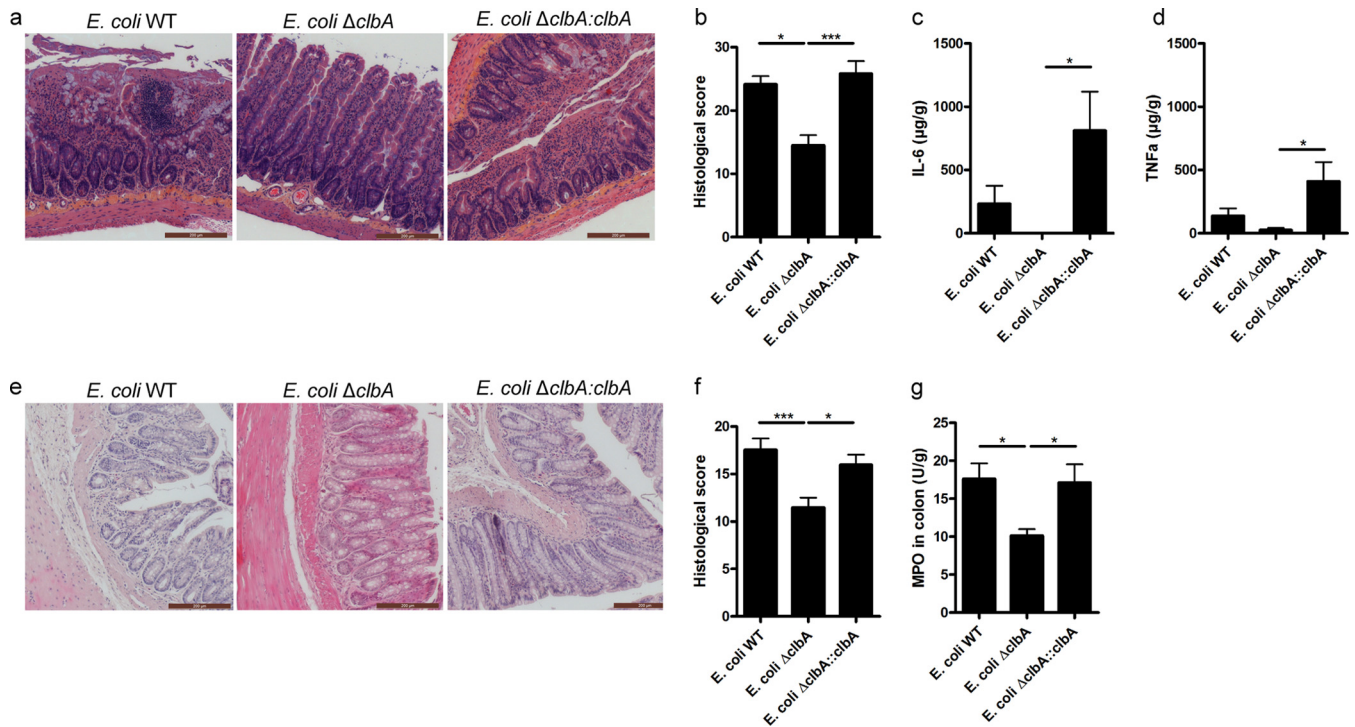
**FIG 4** Diminished oral tolerance in animals early colonized by colibactin-producing *E. coli* is correlated to a defect in regulatory populations. (a) Percentage of TCRab<sup>+</sup> CD4<sup>+</sup> CD25<sup>+</sup> FoxP3<sup>+</sup> cells in mLN of OVA-tolerized rats early colonized by *E. coli* WT,  $\Delta clbA$ , or  $\Delta clbA::clbA$  strains. The means  $\pm$  the SEM for 10 rats are shown (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ). (b) Percentage of CD103<sup>+</sup> CD11c<sup>+</sup> MHC-II<sup>+</sup> cells in mLN of OVA-tolerized rats early colonized by *E. coli* WT,  $\Delta clbA$ , or  $\Delta clbA::clbA$  strains. The means  $\pm$  the SEM for 8 rats are shown (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ). (c and d) Percentage of TCRab<sup>+</sup> CD4<sup>+</sup> (c) and CD8<sup>+</sup> (d) cells in mLN of OVA-tolerized rats early colonized by *E. coli* WT,  $\Delta clbA$ , or  $\Delta clbA::clbA$  strains. The means  $\pm$  the SEM for 10 rats are shown (\*\*,  $P < 0.01$ ).

nized by colibactin-producing *E. coli* exhibited impaired intestinal permeability, which was associated with an enhanced *E. coli* translocation. The historical definition for bacterial translocation (BT) as the passage of viable bacteria through the intestinal mucosa to the lamina propria (35) has become broader and now includes the passage of both nonviable and viable microbes and related products. BT is considered a hallmark of chronic infectious or inflammatory diseases, as well as trauma, and in healthy adult hosts numerous mechanisms are involved in attenuating BT and its harmful consequences. Nonetheless, the infancy period may be vulnerable to BT, since neonates displayed immature gut barrier, mucosal, and systemic immune systems and microbiota. Indeed, consistent with the findings of Steinwender and coworkers (36, 37), we detected the spontaneous transepithelial passage of luminal bacteria to the mLN of our mother-reared pups exclusively

before weaning. In addition, we demonstrated that the expression of colibactin enhances the dissemination of *E. coli* not only in the mLN but also in the blood and spleens of neonates, highlighting a defect in the anatomical containment of these commensals within the gut during childhood. Thus, this limited period of increased intestinal permeability, related in part to the immaturity of the gut barrier, allows the preferential passage of colibactin-producing *E. coli* which, along with the production of a large amount of bacterium-derived antigens, may influence the perinatal programming of the immune system that is critical for the induction of overall homeostasis (3). “Gut closure” occurs around weaning in rodents, limiting the transmucosal passage of luminal antigens (38). Nevertheless, enhanced transmucosal passage of bacteria was found to occur through human adult PP, suggesting that PP are important sites for bacterial sensing (39). In addition, PP are critical in the induction of immune tolerance between cells located in their lymphoid follicles and the intestinal microbiota (40). Based on these results, we chose to focus on the consequences of early gut colonization by *E. coli* on PP homeostasis in adults by investigating transepithelial permeability. Consistent with our previous findings (16), we observed an alteration in the permeability of PP from adult rats colonized since birth with genotoxic *E. coli*, characterized by a facilitated transepithelial passage of both bacteria and antigens, and the subsequent induction of local and systemic humoral and cellular immune responses after oral tolerance protocol, notably with an increased production of IFN- $\gamma$ , which is known to induce both para- and transcellular permeability. This observation is in accordance with a previous report showing a correlation between increased intestinal permeability to a 4-kDa protein and augmented translocation of lipopolysaccharide (41).

Having demonstrated that early gut colonization by colibactin-producing *E. coli* furthered intestinal permeability, especially in PP, thus enhancing exposition of the intestinal immune system to luminal antigens, we next extended these observations to functionally demonstrate that it may have an impact on the induction or oral tolerance to a fed antigen. It has already been suggested that a dysfunctional or damaged intestinal barrier may be involved in the suppression or oral tolerance (42, 43). Indeed, we observed that early gut colonization by colibactin-producing *E. coli* has a negative impact on the development of humoral systemic tolerance to OVA with an enhanced production of pro-Th1 IgG2 antibodies compared to rats colonized by non-colibactin-producing *E. coli*. This correlated with an increased production of IFN- $\gamma$  in the jejunum, as well as by lymphocytes restimulated by OVA, suggesting a stimulation of local and systemic immune response.

Other factors in addition to the enhanced access of luminal antigen to the intestinal immune system through a permeable intestinal barrier may also contribute to the downregulation of oral tolerance. Among them, the induction and maintenance of Tregs are mandatory for the induction of oral tolerance (44). In tolerized rats early colonized by colibactin-producing *E. coli*, flow cytometric analysis revealed a significant reduction in the proportion of mLN CD4<sup>+</sup> CD25<sup>+</sup> FoxP3<sup>+</sup> regulatory T cells, whereas other populations of T cells were not modified. Considering the distinct immunomodulatory potency of commensal or probiotic bacteria, which is related to specific molecular pattern expression, including specific enzymes, metabolites, or cellular structure (45, 46), it was of particular interest to investigate the mechanisms by which genotoxic *E. coli* could influence Treg population. Among these mechanisms, we observed that early gut colonization by



**FIG 5** Early gut colonization with colibactin-producing *E. coli* enhanced intestinal DTH responses against luminal antigen. (a) Representative microphotographs of jejunum H&E-stained sections from OVA-challenged adult rats early colonized with *E. coli* WT,  $\Delta clbA$ , or  $\Delta clbA::clbA$  strains. Scale bar, 200  $\mu$ m. Continuous arrows indicate cell infiltrates, and dotted arrows indicate mucosal ulcerations. (b) Histological evaluation of jejunum inflammation and damage in blinded fashion. The means  $\pm$  the SEM for  $n = 7$  (*E. coli* WT and  $\Delta clbA$ ) and  $n = 11$  (*E. coli*  $\Delta clbA::clbA$ ) rats are shown (\*,  $P < 0.05$ ; \*\*\*,  $P < 0.001$ ). (c and d) Levels of proinflammatory cytokines IL-6 (c) and TNF- $\alpha$  (d) production in jejunum homogenates as determined by ELISA. The mean levels of IL-6 or TNF per  $\mu$ g of tissue  $\pm$  the SEM for  $n = 7$  (*E. coli* WT and  $\Delta clbA$ ) and  $n = 11$  (*E. coli*  $\Delta clbA::clbA$ ) rats are shown (\*,  $P < 0.05$ ). (e) Representative microphotographs of colon H&E-stained sections from OVA-challenged rats. Scale bars, 200  $\mu$ m. Continuous arrows indicate cell infiltrates, and dotted arrows indicate mucosal ulcerations. (f) Histological evaluation of colon inflammation and damage in a blinded fashion. The means  $\pm$  the SEM for  $n = 7$  (*E. coli* WT and  $\Delta clbA$ ) and  $n = 11$  (*E. coli*  $\Delta clbA::clbA$ ) rats are shown (\*,  $P < 0.05$ ). (g) MPO quantification in the colon of OVA-tolerized rats. The means  $\pm$  the SEM for  $n = 7$  (*E. coli* WT and  $\Delta clbA$ ) and  $n = 11$  (*E. coli*  $\Delta clbA::clbA$ ) rats are shown (\*,  $P < 0.05$ ).

genotoxic *E. coli* reduced the number of tolerogenic DCs that preferentially induced Treg population (47). These data suggested that the overall impairment of oral tolerance occurring upon neonatal colonization by commensal *E. coli* depends on the production of colibactin and may be mediated by a diminished tolerogenic cell compartment.

Finally, we demonstrated that the breakdown of oral tolerance, occurring in rats colonized since birth with colibactin-producing *E. coli*, had dramatic consequences on mucosal immunopathology. The intestinal DTH response was exacerbated in these animals, showing profound tissue damages and increased inflammation. This may account for important clinical implication in food-sensitive enteropathic disorders, which originate from a defect in oral tolerance for DTH responses (29).

The paradigm of autoimmune diseases involving a specific genetic background and the exposure to environmental elements is now challenged by the addition of a third partner: the alteration of the intestinal barrier. It is now commonly accepted that its constitutive impairment leading to increased antigen delivery from the intestinal lumen to the gut submucosa is one of the processes preceding the induction of autoimmune diseases targeting extraintestinal organs (48, 49) whose prevalence is increasing in prosperous urban populations (19, 20, 50) and is related to microbial encounters (33). Interestingly, favorable socioeconomic fac-

tors, such as enriched dietary habits or increased levels of hygiene are presumably the main factors driving the *E. coli* population shift from phylogenetic group A to phylogenetic group B2, including colibactin-producing strains (51). In the present study, we demonstrated that the function of a single gene in the genomes of commensal *E. coli* strains is critical for immune homeostasis in the intestinal mucosa. Collectively, these data indicate that early acquired B2 colibactin-producing *E. coli* strains contribute to defective oral tolerance and could facilitate the development of dysregulated immune-mediated diseases at adulthood. This allows the development of new therapeutic strategies focusing on the reestablishment on the intestinal barrier function impaired after early intestinal colonization by genotoxic *E. coli*.

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