



Sequestration of gut pathobionts in intraluminal casts, a mechanism to avoid dysregulated T cell activation by pathobionts

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T cells that express the transcription factor ROR γ , regulatory (Treg), or conventional (Th17) are strongly influenced by intestinal symbionts. In a genetic approach to identify mechanisms underlying this influence, we performed a screen for microbial genes implicated, in germfree mice monocolonized with *Escherichia coli* Nissle. The loss of capsule-synthesis genes impaired clonal expansion and differentiation of intestinal ROR γ ⁺ T cells. Mechanistic exploration revealed that the capsule-less mutants remained able to induce species-specific immunoglobulin A (IgA) and were highly IgA-coated. They could still trigger myeloid cells, and more effectively damaged epithelial cells in vitro. Unlike wild-type microbes, capsule-less mutants were mostly engulfed in intraluminal casts, large agglomerates composed of myeloid cells extravasated into the gut lumen. We speculate that sequestration in luminal casts of potentially harmful microbes, favored by IgA binding, reduces the immune system's actual exposure, preserving host–microbe equilibrium. The variable immunostimulation by microbes that has been charted in recent years may not solely be conditioned by triggering molecules or metabolites but also by physical limits to immune system exposure.

gut | CD4⁺ T cell | genetic screen | capsule | IgA

The relationship between mammals and microbes that populate their digestive tract is multifaceted. Several bacterial species can induce pathology but most are innocuous and even beneficial to the host. The immune system has evolved an array of innate or adaptive detectors for distinct conserved structures on pathogens, but microbes have also learned to utilize these sensors to manipulate the host to their own advantage (1, 2). Commensal bacteria that reside in the intestinal lumen possess surface structures quite similar to those of pathogens, but they achieve a harmless coexistence with the local immune system. These immunostimulatory molecules include structures indispensable to bacterial function, such as flagellins, peptidoglycans, and lipopolysaccharides, which act as ligands for Toll-like-receptor (TLR) sensors. Conversely, bacteria employ stealth strategies to limit recognition by the immune system, such as hiding their stimulatory components behind a polysaccharidic capsule (3). Importantly, these capacities to manipulate the immune system are widely shared between different phyla, and there is more variation between strains of a given species than there is between genera or phyla, a variability that is likely important to ensure flexibility and adaptability to bacterial species (4, 5).

Among the well-recognized lymphoid populations elicited by commensal bacteria are the T cells that express the ROR γ transcription factor. Earliest recognized were conventional CD4⁺ T helper (Th17) cells that express cytokines of the interleukin (IL)-17 family. These neutrophil-recruiting cytokines induce anti-microbial peptides and tight-junction proteins in intestinal epithelial cells, thereby buttressing gut barrier integrity and protecting against fungi and extracellular bacterial infections (6–10). On the other hand, Th17 cells can be detrimental to the host. They have been implicated in various inflammatory and autoimmune disorders (10, 11), and several studies have distinguished beneficial “homeostatic” types of Th17 cells needed for border control versus pathogenic types, typically marked by interferon gamma expression (12–14) although there is likely a continuum between these two entities (15, 16). The induction of Th17 cells by segmented filamentous bacteria (SFB) (17, 18) is the archetype of the control of T cell differentiation by microbes, but other commensals also have that ability (19, 20), including some used as probiotics (20). The other ROR γ ⁺ T cell population influenced by microbes is a subset of FoxP3⁺ T regulatory cells (Treg). Colonic Tregs are comprised of two major subsets, which can be distinguished operationally and phenotypically by the expression of specific transcription factors, ROR γ (or c-Maf) vs. Helios (or Gata3)

Significance

Intestinal symbionts regulate host physiology by influencing the accumulation and activation of various immunologic cell types via secreted metabolites or contact-triggering of host receptors. From this study, we propose a model in which interaction of the host with the commensal *Escherichia coli* Nissle and downstream T cells activation can be regulated by the presence of a bacterial capsule. Mutants lacking the capsule are sequestered in the gut lumen in large intraluminal casts, involving immunoglobulin A and host cell exudates, impeding recognition and activation of T cells. Because un-capsulated *E. coli* Nissle exacerbated colitis, this physical barrier could be a way for the host to avoid prolonged exposure to potentially harmful microbes.

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(21–27). The Helios⁺Gata3^{hi} population, not dissimilar to Treg populations found in other tissues, is homeostatically controlled by IL-33, and expresses several transcripts linked to tissue repair (e.g., Areg) (22). The RORγ⁺ Maf⁺ pool, a major component of colonic lamina propria (LP) Tregs, is far less frequent in other body locations (23, 24). Several functions have been attributed to colonic Tregs, in particular the RORγ⁺ population (2, 28). They influence the intensity of generic gut inflammation, control immunoglobulin A (IgA) production, dampen Th1/2/17-biased conventional T cell (Tconv) activation, and control food allergy (29). RORγ⁺ Tregs are strikingly responsive to fluctuations in bacterial load and composition: germ-free (GF) condition or treatment with broad-spectrum antibiotics result in reduced RORγ⁺ Tregs (23, 24, 30). Single strains of commensal bacteria can induce RORγ⁺ Tregs upon colonization of GF mice (23, 31, 32); this property is widespread (31, 33), and is shared by members of diverse genera, such as *Clostridiaceae*, *Bacteroides*, or *Staphylococcus*.

The mechanisms and molecules underlying microbial control of RORγ⁺ T cells remain uncertain. Induction of Th17 cell by SFB involves a multicellular cascade initiated by tight adherence of the bacteria to epithelial cells (2), but the pathways used by other commensals are unclear. For Tregs, several microbe-controlled metabolites have been reported to influence their induction, like short-chain fatty acids [somewhat controversially (23, 34–37)], or bile acids (37–40). In addition, the enteric nervous system seems to modulate the induction of RORγ⁺ Tregs by specific microbes (41, 42).

As a means to elucidate these mechanisms, we leveraged intraspecies variation. A first comparison involving a panel of *Bacteroides* strains did not succeed in identifying genes or operons correlated with RORγ⁺ Tregs, perhaps because of overly broad genetic variation (33). We then turned to *Escherichia coli* Nissle (EcN), a human commensal that can be readily manipulated and can induce moderate Th17 and RORγ⁺ Treg responses (33). Comparison with a genetically close but ineffective *E. coli* strain revealed several candidate operons, informing an ablation screen that identified a mutant unable to induce intestinal RORγ⁺ T cell responses. While addressing the mechanism of this phenotype, we found a surprising result. Instead of the fine molecular identification we had sought, we discovered physical containment of the microbe in luminal casts, large structures composed of dead extravasated immunocytes and IgA-coated microbes, and we propose that this physical barrier constrained microbe exposure and limited T cell responses.

Results

A Bacterial Screen Identifies a Capsule Mutant Unable to Induce Intestinal T Cells. Closely related strains within a bacterial species can vary widely in their immunostimulatory capacity, as exemplified by EcN versus *E. coli* BW25113 (EcBW). Monocolonization of GF mice with EcN resulted in the induction of both RORγ⁺ Tconvs and RORγ⁺ Tregs in the colonic lamina propria (LP), while EcBW was unable to elicit these populations (Fig. 1A). The two strains were found at similar densities in the feces, indicating that overall levels of colonization did not account for the difference (SI Appendix, Fig. S1A), implying that genetic variation between the two strains affected loci that influence the accumulation of colonic T cells. We compared EcN and EcBW genomes using the RAST annotation tool (43) and identified variation in ~1,000 genes (~600 genes unique to EcN and ~400 genes with low similarity, out of 4,832 total coding sequences in the *E. coli* genome) (Fig. 1B

and Dataset S1). We filtered out mobile elements, phage, and replication machinery or hypothetical proteins, leaving 200 coding sequences. Many of these sequences clustered in genomic islands, regions that allow rapid evolution and segregation of pathogenicity within the *E. coli* species (44, 45). Some of the differential loci related to mobile elements, phage specificity or growth (iron transporters), which are unlikely to be related to immune system crosstalk, but others encoded cell wall and outer membrane structures that could plausibly influence immunocytes. To assess the relevance of some of these candidate differences, we devised a screen that inactivated the corresponding operons in EcN, either by large deletion or by silencing genes known to be essential for the operon's function, as schematized in Fig. 1B. These mutants included Δ*kfiCD* for the K5 capsule, the semirough lipopolysaccharide (LPS) O-antigen (Δ*b1,3Glcuwaaw*), type 1 (Δ*fim*), F1C (Δ*foc*), and Curli (Δ*csgBA*) fimbriae and a recently described lipopeptide encoded in the colibactin cluster (Δ*clbA*) (listed in Dataset S2). Mutant bacteria were introduced into GF mice, the intestines of which they populated at similar levels, as judged by bacterial load in feces (SI Appendix, Fig. S1C); for some key mutants—see below—we also verified that the bacterial load in colonic or cecal content was unaffected (SI Appendix, Fig. S1D), as was the population of epithelial-adherent bacteria in the colon (SI Appendix, Fig. S1E). Detailed immunophenotyping on intestinal tissues from these mice (panels illustrated in SI Appendix, Fig. S2 A and B) revealed differences in lymphocyte populations (Fig. 1C). Mice monocolonized with the capsule mutant (Δ*kfiCD*) showed a clear reduction in EcN-induced RORγ⁺ T cells (both Tregs and Tconvs) in several tissues, almost reverting to GF levels (Fig. 1 C and D). The mutant lacking lipopeptide (Δ*clbA*) partially reproduced these effects in Tregs but not in Tconvs (46). Disruption of the LPS O-antigen or fimbriae did not affect immunocyte representation.

We elected to focus on the capsule deficiency because it gave most robust differences. Importantly, neither Δ*kfiCD*, nor EcN induced any inflammatory response in the gut or systemic dissemination in the mice. We first confirmed the results by constructing an independent mutant (Δ*kfiB*) that also affected capsule biosynthesis and yielded similar results (Fig. 1E). When comparing results across the entire screen, we observed very similar responses in the colon and cecum of monocolonized mice, with a strong correlation across mice (Fig. 1F). This relationship did not extend to the small intestine (Fig. 1G), indicating specificity for the terminal digestive tract in keeping with *E. coli*'s preferential localization. The same patterns were detected in IELs (SI Appendix, Fig. S2C). In addition, Tconvs secreted IL-17 in EcN-monocolonized mice, less so in Δ*kfiCD* and Δ*kfiB* (SI Appendix, Fig. S2D). Thus, induction of RORγ⁺ T cells by EcN was strongly dependent on the presence of the capsule.

EcN Δ*kfi* Capsule Mutants Are Highly Coated with Specific IgA. Binding of secretory IgA is an important means for mammalian organisms to constrain intestinal microbes (47–49), and there are strong relationships between the production and secretion of IgA by intestinal B cells and the microbes that affect RORγ⁺ T cells. SFB, the prototypic Th17-inducer, activates IgA production (50, 51), as did several Th17 inducers in our pan-phylum survey (33). Conversely, our recent work on maternal control of Treg setpoints demonstrated a negative correlation between RORγ⁺ Tregs and IgA levels (52). *E. coli* strains are known to be particularly targeted by secretory IgA, and antigen-specific IgAs target a wide range of their membrane-associated antigens (49, 53, 54).

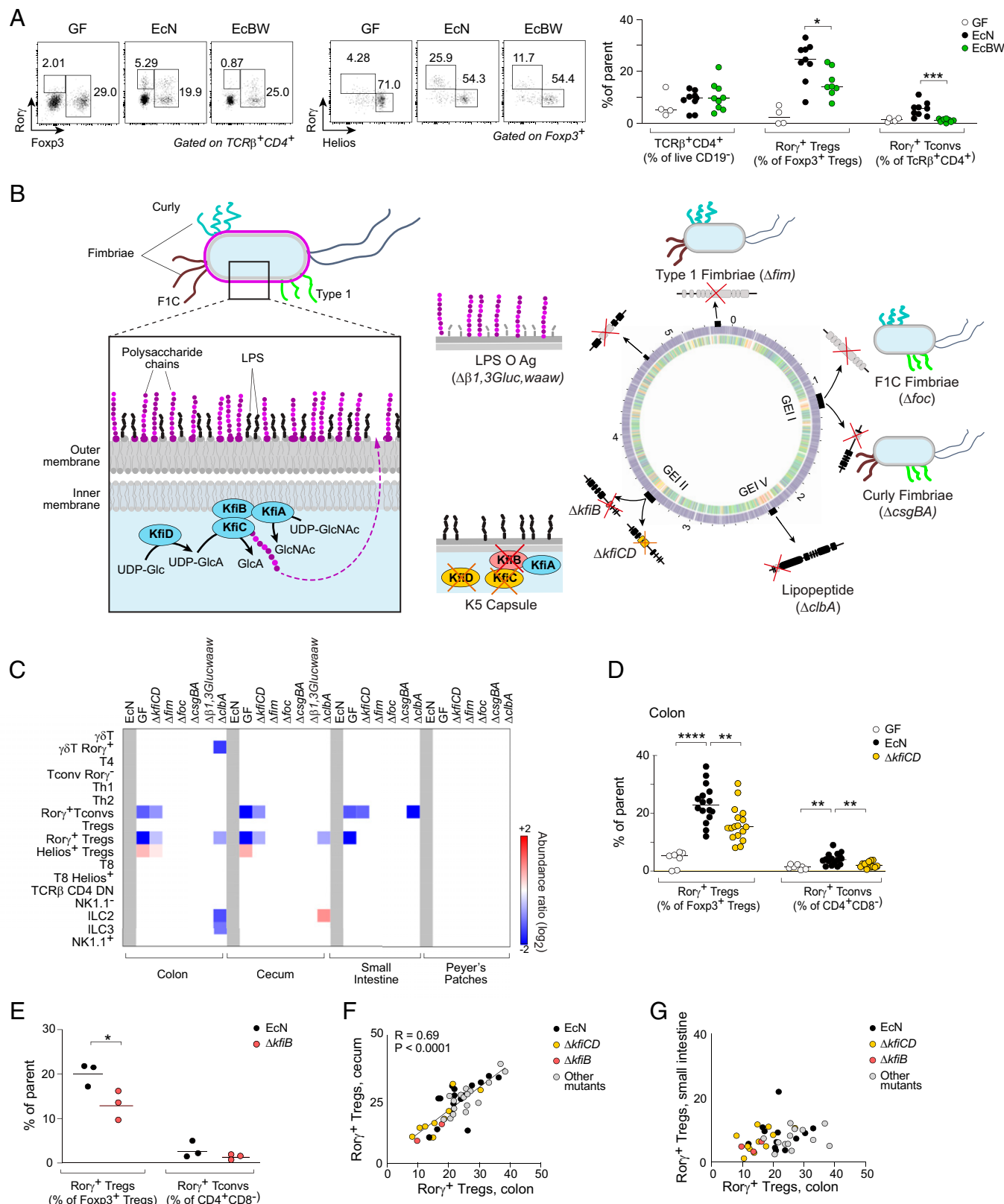


Fig. 1. Screening of EcN mutants' immunomodulatory activities. (A) Representative dot plots and proportions of CD4⁺ RORγ⁺ Tconv (Foxp3⁻ RORγ⁺) and RORγ⁺ Tregs from colonic LP in germfree and mice monocolonized with EcN and EcBW (4 independent experiments, *t* test **P* value < 0.05, ****P* value < 0.001). (B) *Left*: schematic illustration of EcN outer membrane structure with capsule polysaccharides (purple) and Kfi components of their biosynthetic pathway. *Right*: schematic of EcN and EcBW genome comparison (SI Appendix, Fig. S1B), with the positions of the mutants created here and their impact on EcN outer membrane. (C) Average fold changes (at *P* < 0.01) among immunocytes of different organs of mice monocolonized with the mutants, relative to EcN. (D) Frequencies of RORγ⁺ Treg and Tconv from colonic LP in GF and mice monocolonized with EcN and capsule mutant *ΔkfiCD* (five independent experiments, *t* test ***P* < 0.01, *****P* < 10⁻⁴). (E) As (D) for mutant *ΔkfiB* (**P* < 0.05). (F, G) Frequencies of RORγ⁺ Tregs in colon, cecum, and small intestine LP.

Thus, we asked whether the inability of the capsule mutants $\Delta kfiCD$ and $\Delta kfiB$ to induce ROR γ^+ T cells might affect their ability to induce IgA. Overall, the induction of IgA synthesis upon monocolonization of GF mice, assessed as total fecal IgA (Fig. 2A) or IgA in blood (Fig. 2B), was equivalent for EcN and the mutants. On the other hand, we noted a striking difference in coating by IgA of the microbes: $\Delta kfiCD$ and $\Delta kfiB$ cells were coated with significantly more IgA than were to wild-type (WT) cells and other mutants (Fig. 2C and D). This enhanced binding did not involve IgG (SI Appendix, Fig. S3A). Microbe-reactive IgA can bind specific surface molecules or can bind to structurally unrelated bacterial antigens (polyreactive IgA) (48, 49). Binding of serum IgA from EcN- or $\Delta kfiCD$ -monocolonized mice was highly specific: there was no binding to other commensal bacteria, not even the closely related EcBW (Fig. 2E). Conversely, monocolonization with several microbes did not elicit EcN-reactive serum IgA (SI Appendix, Fig. S3B). However, serum IgA from either EcN or $\Delta kfiCD$ -monocolonized mice bound more effectively to cultured $\Delta kfiCD$ than to WT EcN (Fig. 2F). The same heightened binding was observed with serum immunoglobulin G (IgG) (SI Appendix, Fig. S3C). Together, these results demonstrate that, despite their reduced ability to induce ROR γ^+ T cells, the capsule-deficient mutants

induced a strong IgA response and, in fact, exposed more epitopes for IgA binding, both in vivo and in vitro.

Single-Cell RNA and T Cell Receptor Sequencing Revealed a General Impairment in Activation of CD4 T Cells. Thus, the Δkfi capsule deficiency yielded an apparently paradoxical outcome, with reduced ability to induce ROR γ^+ T cells, both Tconvs and Tregs, but a heightened binding of IgA. The capsule is generally thought to be an essential shield for pathogens to evade host defenses and confer resistance to phages (3, 55, 56). Capsule components exert immunomodulatory activity by themselves (57). As an initial step of mechanistic exploration, we performed single-cell RNA sequencing (scRNA-seq) on CD4 $^+$ T cells from GF mice monocolonized by WT EcN or the $\Delta kfiCD$ mutant, using hash-tagging (58) to group cells from the three different types of mice into the same encapsulation for optimal comparison between groups. Three independent experiments were performed with sorted colonic CD4 $^+$ T cells from 17 mice, obtaining QC-passing data for 29,182 cells altogether. Dimensionality reduction and visualization on a Uniform Manifold Approximation and Projection (UMAP) (representative experiment in Fig. 3A) showed that cells from all mice resolved into the same general cell types, identified with signature genes and modules: naive

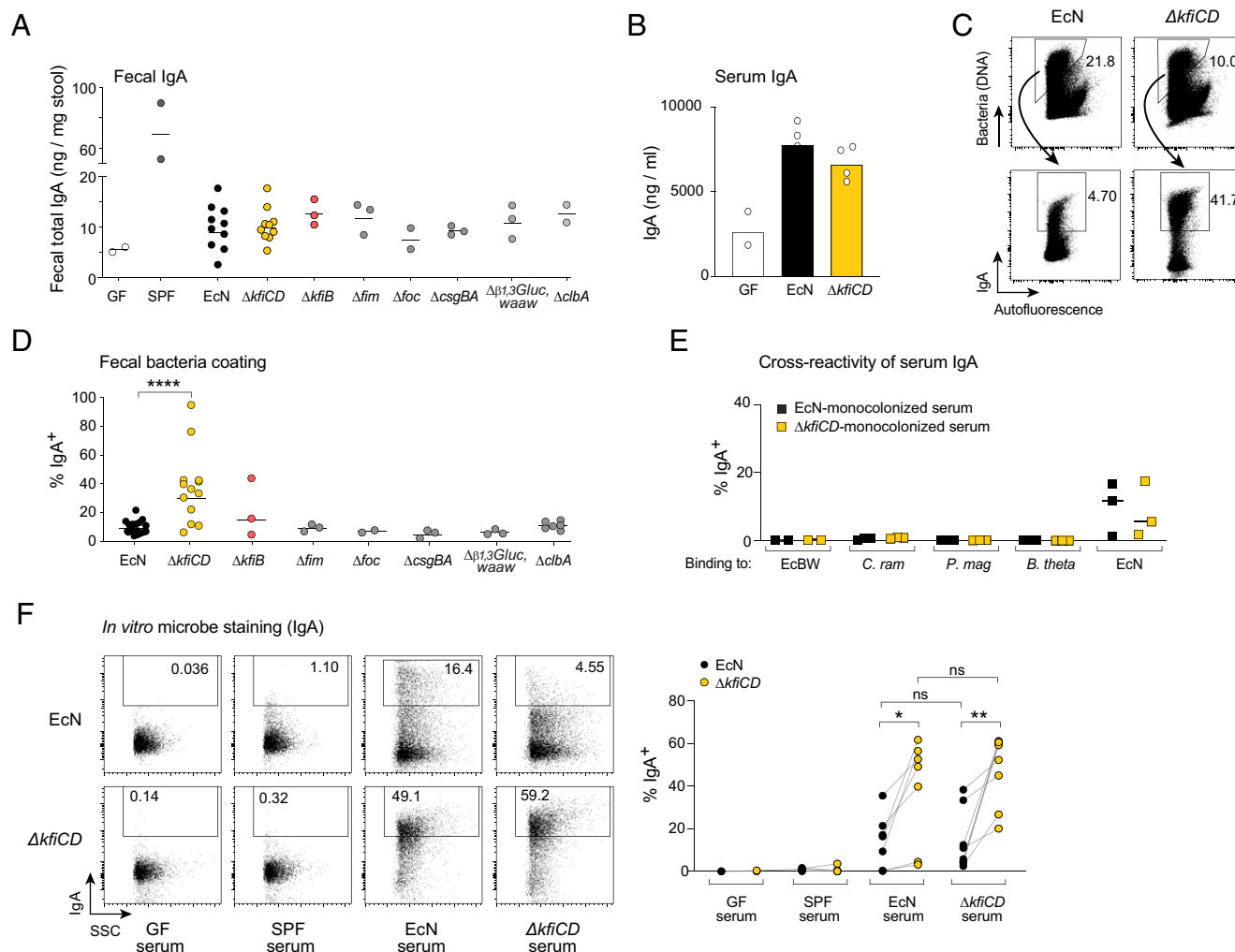


Fig. 2. EcN capsule-less Δkfi mutant is highly coated with specific IgA. (A) Total IgA (from ELISA) in stool of germ-free and monocolonized mice. (B) Total serum IgA (from ELISA) in germ-free and monocolonized mice (day 14). (C) Representative cytometry profiles and quantification (D) of IgA-coated bacteria in EcN or $\Delta kfiCD$ monocolonized mice (t test **** P < 0.0001). (E) Binding of serum IgA from EcN or $\Delta kfiCD$ monocolonized mice, assessed by flow cytometry, against EcBW, *Clostridium ramosum*, *Peptostreptococcus magnus*, and *Bacteroides thetaiotaomicron*. (F) Representative cytometry plots and quantification of binding to EcN or $\Delta kfiCD$ bacteria of serum IgA from EcN or $\Delta kfiCD$ monocolonized mice, GF or SPF serum for reference (paired t test * P < 0.05, ** P < 0.01).

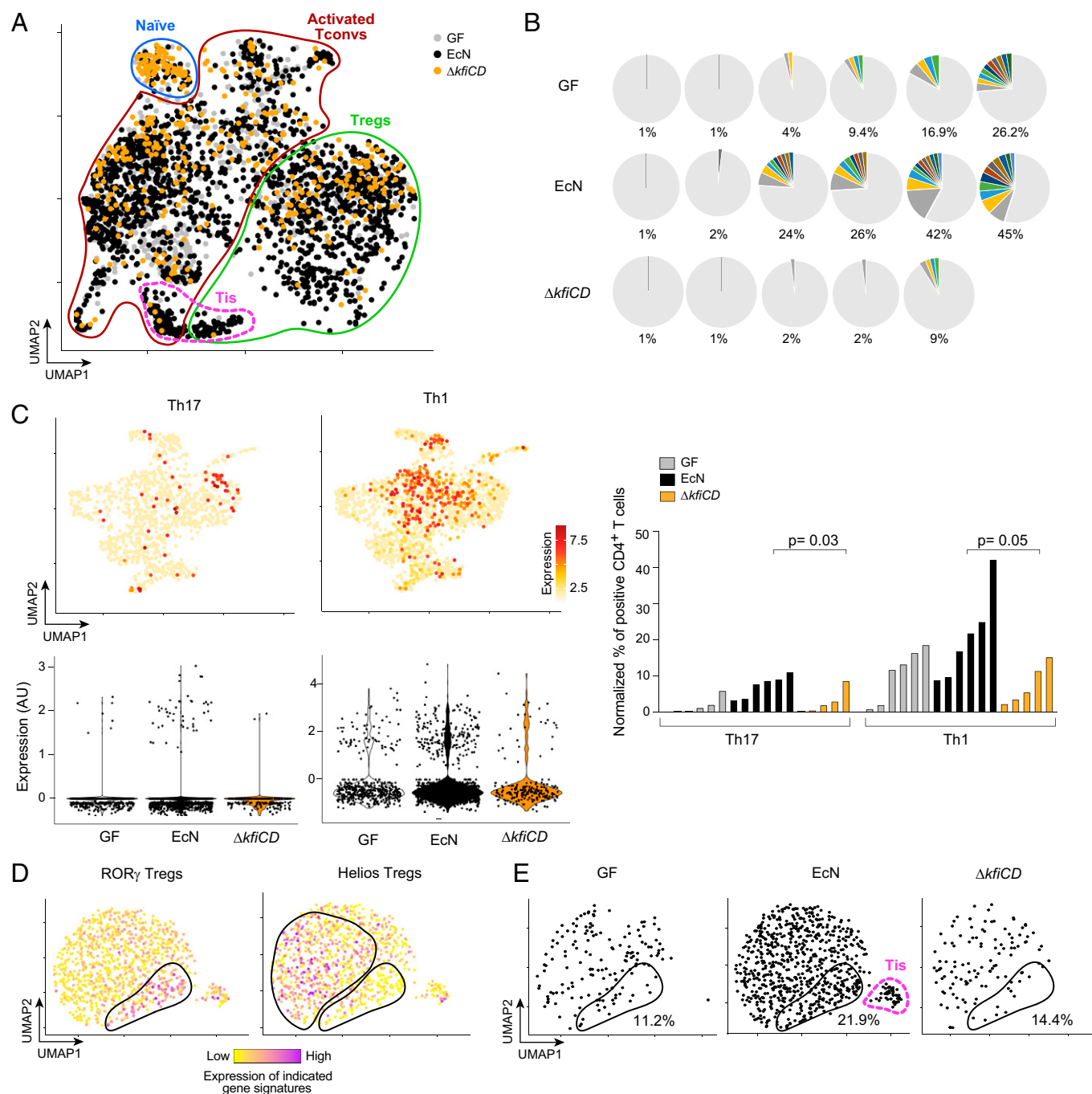


Fig. 3. T cell activation and clonal expansion are impaired in mice monoclonalized with the $\Delta kfiCD$ mutant. scRNA-seq and TCR-seq of GF or EcN and $\Delta kfiCD$ monoclonalized mice (day14). (A) UMAP projection of transcriptomes from CD4⁺ T cells; cell populations distinguished by expression of signature gene and modules (per S4A). One representative experiment shown. (B) Proportion of $\alpha\beta$ TCR clonotypes in individual mice, GF, or monoclonalized. Nonexpanded clones in gray, colored clonotypes present in two or more cells (Dataset S3 for details). (C) Projection on a UMAP dimensionality reduction (activated Tconv cells only) of averaged Th1 and Th17 signatures (listed in Materials and Methods), with corresponding violin plots below. Right: quantification of activated T conv cells positive for Th1 and Th17 gene signatures (normalized to total CD4⁺ T cells). Each bar corresponds to one mouse (Mann-Whitney *P* value). (D) UMAP projection (Treg cells from all mice in one experiment), color-coded for expression of ROR γ^+ and Helios⁺ Treg gene signatures (genes in Materials and Methods and Dataset S4). (E) As (D), but split between hash tagged cells from different mice; proportion of ROR γ^+ aTregs and the position of Tis Tregs are shown.

Tconvs, activated Tconv, and Tregs (SI Appendix, Fig. S4A). We also observed a unique cluster of cells expressing high levels of interferon signature genes, as previously reported [named “Tis” in (16)]. Here, Tis cells included both a Treg and a Tconv component (Fig. 3A). Cells from all mice populated the various clusters, with the expected preponderance of naive Tconvs from GF (also high with $\Delta kfiCD$). Tis cells were almost exclusively found in EcN-colonized mice, not in the GF or $\Delta kfiCD$ groups.

The scRNA-seq data were also processed to identify the $\alpha\beta$ T cell receptors (TCRs) expressed, revealing a range of clonality. Cells expressing the same $\alpha\beta$ TCR alpha and beta chain clonotype (identity at the level of CDR3 nucleotides), and thus resulting from clonal amplification, were observed at low frequencies in colons of GF- and $\Delta kfiCD$ -monoclonalized mice (9.6% and 3.0% on average, per 100 cells). Amplified clonotypes were more frequent in EcN-monoclonalized mice (23.6%

on average), some expressed by dozens of cells across the Tconv compartment (Fig. 3B and Dataset S3). These results indicate that EcN drove a stronger immune response than did the $\Delta kfiCD$ mutant.

Focusing the transcriptome analysis on the activated Tconv population (Fig. 3C), we asked whether the lesser clonal expansion seen in $\Delta kfiCD$ -colonized GF mice was also accompanied by lower Tconv differentiation toward effector phenotypes. We assessed the single-cell transcriptomes for signatures of Tconv differentiation. In accordance with Kiner et al. (16), no areas of the UMAP corresponded to discrete Th populations. Cells with high levels of the Th1 and Th17 signatures were observed after colonization with EcN, but markedly less frequent in $\Delta kfiCD$ -colonized mice (Fig. 3C). Accordingly, bulk RNA sequencing (RNA-seq) of sorted CD4⁺ T cells showed an over-representation of a curated gene signature of T cell activation (59) after WT vs. $\Delta kfiCD$ monocolonization (SI Appendix, Fig. S4B).

Thus, in terms of clonal expansion and effector differentiation, the capsule-deficient mutant was less potent at stimulating colonic Tconv cells, consistent with the flow cytometry data. We similarly interrogated the Treg compartment, parsing these cells into a specific UMAP and using curated gene signatures [(60) and Dataset S4] to visualize the main two subpopulations of colonic Tregs: ROR γ^+ and Helios⁺ (Fig. 3D). In this framework, colonization with neither WT nor mutant microbe brought forth a specific Treg subset not already present in GF hosts but only shifted their representation, with an increase in the ROR γ^+ component after EcN colonization (Fig. 3E). The exception was Treg Tis cells, which were only observed in EcN-colonized mice. Thus, these data further support the idea that when EcN lacks a capsule, it is unable to elicit a response promoting colonic CD4⁺ T conv activation and ROR γ^+ Treg accrual.

Cellular Relays? SFB activates Th17 cells through a multicellular cascade of events: epithelial cells are triggered by adhesion of SFB to release free radicals and serum amyloid A (SAA) proteins, which in turn activate myeloid cells (DCs) to produce IL-1b, IL-6 and IL-23, which then favor Tconv differentiation to ROR γ^+ Th17-producing cells (2). Our observations raised a similar question: since it seemed unlikely that capsule polysaccharides on EcN would directly activate T cells, what might be the relays that boost both ROR γ^+ Treg amplification and Th17 cell differentiation? Myeloid cells seemed a plausible candidate, since they can be directly activated by bacterial polysaccharides (32, 61), as demonstrated by experiments in vitro where treating DCs with *Bifidobacterium* polysaccharides enhanced iTreg generation in the same wells (32). The loss of capsule polysaccharides might be expected to unmask LPS. Immunophenotyping of myeloid cells in the colon of monocolonized mice (strategy in SI Appendix, Fig. S2 A and B) did not reveal changes in major myeloid populations, in their abundance (Fig. 4A) or in their activation state, as assessed by expression of CD86 and MHC-II molecules (Fig. 4B) (neither did other mutants in the panel) (SI Appendix, Fig. S5). In addition, gene-expression profiling of total colonic myeloid cells, performed 1 or 14 d after colonization with EcN or $\Delta kfiCD$, did not show any reproducible differences in independent experiments. Given the possible unmasking of LPS in $\Delta kfiCD$ cells, we assessed the impact of the mutation in vitro by challenging bone-marrow-derived macrophages (BMDM) with EcN or $\Delta kfiCD$. Both microbes were phagocytosed and then killed equally effectively by BMDM (Fig. 4C). They also induced similar endocytosis of the TLR4 receptor from the surface of

BMDM (reflected as loss of surface staining; Fig. 4D). This observation suggested that loss of the capsule may not have exposed more LPS to sensing by macrophages in this instance. The two microbes also activated BMDM similarly, judging from CD86 and MHC-II induction (Fig. 4E) or IL-6 secretion (Fig. 4F). Altogether, these data suggest that the presence or absence of the *E. coli* K5 capsule did not alter the bacteria's ability to trigger myeloid cells, and modified interaction with myeloid cells was not the relay between the $\Delta kfiCD$ mutation and its reduced ability to invoke T cell responses.

We next addressed the possibility of a differential interaction with other cell types. SFB and other Th17-inducing microbes are closely associated with intestinal epithelial cells (IECs), and this adhesion helps initiate the signaling cascade (17, 19). In addition, EcN's capsule modulates bacterial interaction with IECs in vitro (62, 63). To investigate the ability of EcN and $\Delta kfiCD$ to affect IEC barrier function, we prepared cultures of Caco-2 cells in Maestro Edge plates, exposed them to the microbes, and measured epithelial integrity over time by trans epithelial electrical resistance (TEER). Both bacteria induced a drop in TEER, sharp when initiated, but the transition occurred markedly faster with $\Delta kfiCD$ than with EcN (Fig. 5A). The timing of the TEER drop was dose-dependent, implying a cumulative signal delivered by the microbes over time, but the faster response to $\Delta kfiCD$ was significant at all doses (Fig. 5B). To test whether this was a unique trait induced by lack of capsule, we performed similar experiments with our other EcN mutants. Only the $\Delta kfiCD$ and $\Delta kfiB$ mutations affected IEC barrier function at an accelerated rate (Fig. 5C). Interestingly, the LPS O-antigen $\Delta b1,3Glucwaaw$ mutation slightly delayed the TEER drop, suggesting that in this context the semirough O-Ag had a detrimental effect on the integrity of IECs (Fig. 5C and SI Appendix, Fig. S6).

These results suggest that the loss of capsule actually increased the ability of *E. coli* to damage the IEC barrier. For an in vivo correlate of these findings, we analyzed extravasation from the gut into the circulation of fluorescein isothiocyanate (FITC)-dextran after oral gavage, as a test for intestinal barrier function in GF mice monocolonized with EcN or $\Delta kfiCD$. In contrast to the results obtained in vitro, no clear difference was noted between the two microbes at various times after colonization (Fig. 5D).

Luminal Casts Engulf the Capsule-Deficient Mutant. These results left us with a conundrum. The capsule-deficient mutants were able to perturb myeloid cells, induce IgA, and destabilize epithelial cells just as efficiently as the EcN parent strain (even more strongly for IECs): yet they seemed largely ignored by T cells. This paradox led us to hypothesize that, during monocolonization, $\Delta kfiCD$ and WT EcN might interact differentially with epithelial cells, engaging signaling pathways that lead to different T cell outcomes. To better understand the interaction of bacteria with the intestinal wall and immunocytes during GF monocolonization, we performed confocal imaging of colon tissue. Agglomerates of CD45-positive immunocytes were observed in the lumen of $\Delta kfiCD$ -monocolonized mice (Fig. 6A, higher magnification in Fig. 6B). These structures were absent in both GF and EcN-colonized mice (Fig. 6C). The intraluminal CD45⁺ structures were large (50–150 μ m equivalent to 4–15 cell diameters), with visible nuclei in some, but not all, cases, usually located alongside the epithelial border (Fig. 6B and SI Appendix, Fig. S7A). They included high densities of $\Delta kfiCD$ bacteria (Fig. 6B, Bottom) and also colocalized with IgA deposits (Fig. 6D and SI Appendix, Fig. S7B). These

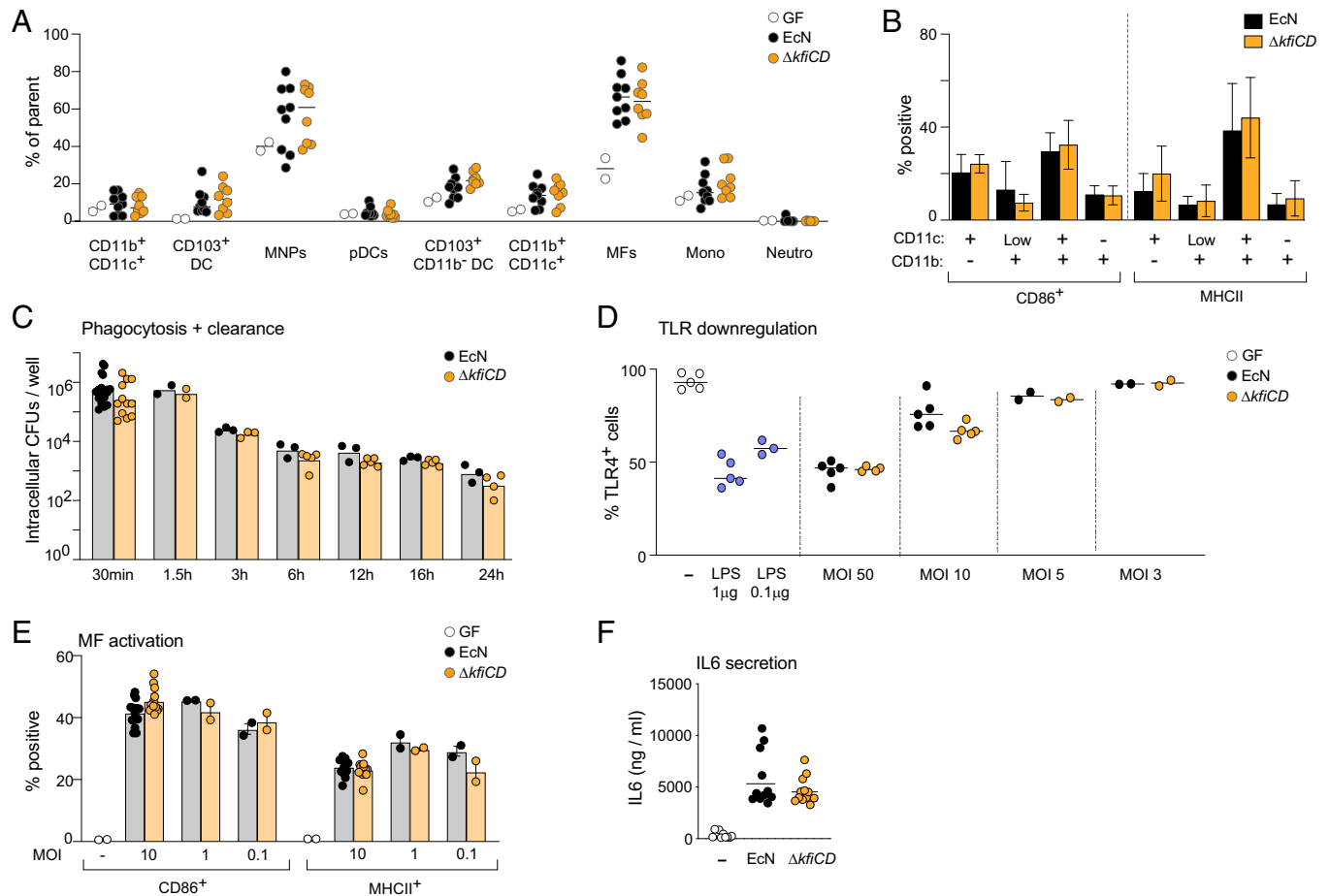


Fig. 4. Myeloid cells are similarly activated by both EcN and its capsule-less mutant. (A) Myeloid cell proportions, measured by flow cytometry, in colonic LP of GF, or EcN and $\Delta kfiCD$ monoclonized mice (day 14; gating strategy from (SI Appendix, Fig. S1E)). (B) Activation of colonic LP myeloid cells assessed by expression of CD86 and MHCII expression 24 h after monoclonization with EcN or $\Delta kfiCD$. (C) Phagocytosis and clearance by BMDM in culture at different times after infection with live EcN and $\Delta kfiCD$ at a multiplicity of infection (MOI) = 10, evaluated as cell-associated cfu. (D) TLR4 surface expression on immortalized BMDM after 20 min exposure to EcN or $\Delta kfiCD$ at different MOI (LPS used as a positive control). Different concentration of LPS was used as positive control. (E) BMDM activation 24 h after exposure to live EcN and $\Delta kfiCD$ in vitro, assessed as expression of CD86 and MHCII. (F) As (E), IL-6 concentration measured by ELISA from culture supernatants after 24 h.

structures were very reminiscent of the “intraluminal casts” described in a recent study, which appear during *Toxoplasma gondii* infection and accompanying overgrowth of *Proteobacteria* (64). These casts result from granulocyte extravasation into the lumen, where they contribute to the encapsulation of bacteria, and were proposed to be a mechanism by which the host contains pathobiont outgrowth during adventitious inflammation (65, 66).

We thus propose that the same phenomenon may operate here, as modeled in Fig. 7A: the capsule-deficient mutant, coated with high amounts of IgA, provokes myeloid cell extravasation and cast formation, encapsulating the microbes to exclude them and arrest an otherwise stronger insult to the epithelial border. We attempted to identify the extravasated cells by flow cytometric analysis of the luminal content, but very few live cells could be identified (with marker profiles suggestive of monocytes). We note that Molloy et al. (64) detected extravasated cells by flow cytometry only during a narrow period after *T. gondii* infection, a time window that may not apply after bacterial colonization of GF mice.

In this model, physical containment within solid structures of potentially aggressive bacteria (here $\Delta kfiCD$) is conditioned by the initial interactions by the epithelial layer, innocuous symbionts (here EcN) initiating a different response and remaining in the lumen. Encapsulation into casts may be an

unstable solution, however. Thus, we asked how EcN and $\Delta kfiCD$ microbes would behave in a context of adventitious damage to the IEC layer, for instance after damage induced by dextran sulfate (DSS). Mice housed under normal (SPF) conditions were fed DSS for 5 d, inoculated with either EcN or $\Delta kfiCD$ by oral gavage ($\sim 10^7$ colony-forming unit [cfu]/mouse), and more then maintained on a lower dose of DSS to maintain inflammation. Administration of $\Delta kfiCD$ had a detrimental impact on the mice, as shown by increased weight loss and sudden death in three of eight mice (Fig. 7B), while EcN had no such effect. In normal SPF mice, EcN colonized more effectively than did $\Delta kfiCD$, likely reflecting the protective role of the capsule, but the capsule-less mutant colonized more effectively under conditions of DSS-induced inflammation (Fig. 7C). Thus, we conclude that the containment offered by intraluminal casts can break down, revealing the more aggressive nature of the capsule-deficient mutant.

Discussion

Starting with a symbiotic strain of *E. coli*, one that amplifies both Treg and Th17 ROR γ^+ compartments, and one normally so innocuous that it is used as a probiotic, we initially aimed to identify molecules that induced ROR γ^+ T cells in the intestine by harnessing the power of microbial genetics. Instead, we

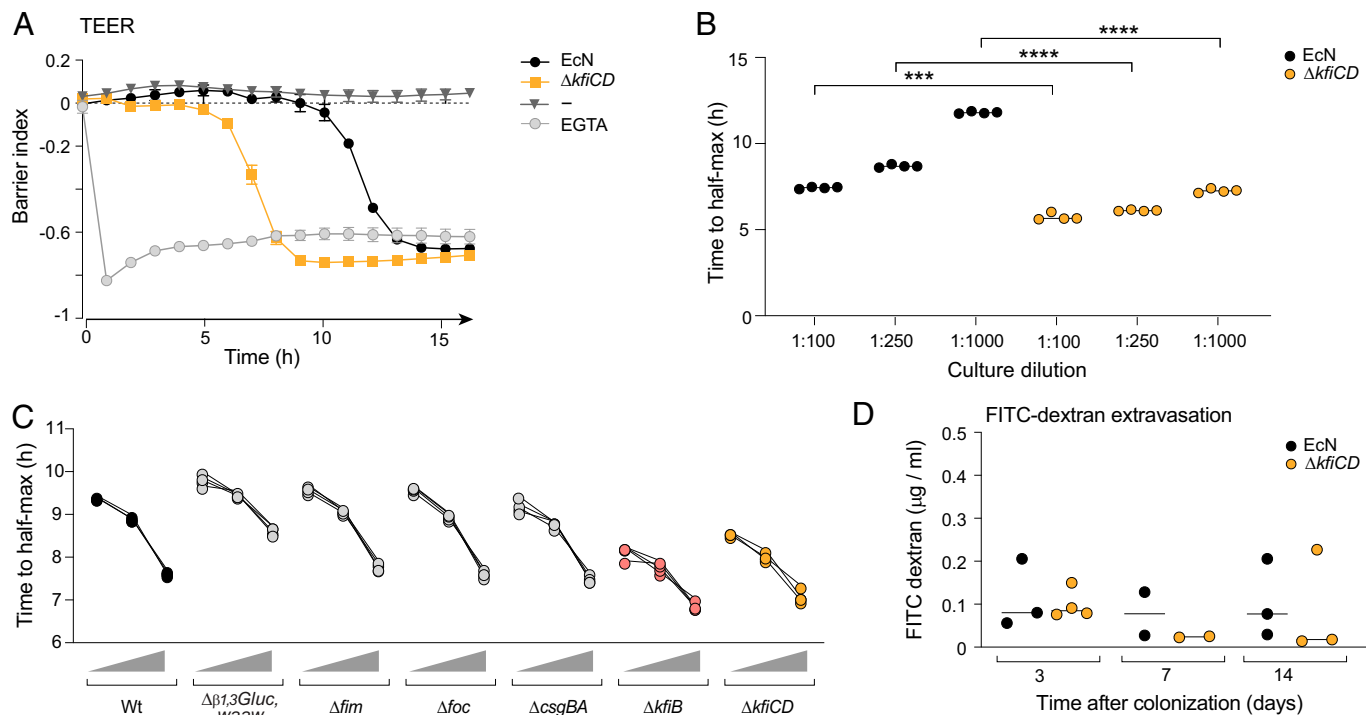


Fig. 5. Lack of capsule increases damaging effects on epithelial cells in vitro. (A) Caco-2 cells were cultured in CytoView-Z 96 plates, and TEER was recorded over time after challenge (1:1,000) with EcN or $\Delta kfiCD$ (EGTA as a positive control). The barrier index was calculated by “axion impedance module” as the ratio between cellular resistance at low frequency (1 Hz) vs. high frequency (41 Hz). (B) As (A), the time of transition (50% drop in barrier index) was measured in cultures supplemented with EcN or $\Delta kfiCD$ at different dilutions. (t test, *** $P < 0.001$, **** $P < 10^{-4}$); each point a quadruplicate within an experiment, representative of three experiments. (C) As (B), time to transition after exposure to various EcN mutants at three dilutions (1:1,000, 1:5,000, and 1:10⁴). (D) Assessment of in vivo gut permeability as leakage of FITC dextran (4 kDa) administered by gavage and detected in serum 4 h after oral gavage at different times after monoclonization with EcN or $\Delta kfiCD$ (each point a different mouse).

ended up rediscovering a strategy through which the host walls off a potentially inflammatory variant of normally tolerated symbionts, previously described in a different context.

In GF mice monoclonized with the capsule-deficient mutant, the colon lumen contained abundant agglomerates that included IgA-coated $\Delta kfiCD$ bacteria and CD45⁺ immunocyte. This disposition resembled the intraluminal casts that engulf *Proteobacteria* expansion during *T. gondii*-induced inflammation (64). In the model proposed above (Fig. 7A), we hypothesize that WT EcN induces ROR γ^+ Th17 and Treg cells by initiating a signaling cascade involving IECs that modulates T cell activation by DCs—this by analogy to mechanisms downstream of SFB triggering the epithelium (67–69). In contrast, loss of the capsule in the $\Delta kfiCD$ mutant, which would expose immunogenic components of the outer membrane, like LPS, leads to a more damaging interaction with IECs, as revealed by the faster loss of integrity in TEER assays. This heightened interaction with IECs would initiate a different response: instead of ROR γ^+ Th17 and Treg recruitment, enhanced extravasation of neutrophils and monocytes, leading to cast formation (plausibly involving DNA release and “netosis”). We also propose that encapsulation of microbes into the cast structures is conditioned by high amounts of bound IgA, as for $\Delta kfiCD$ here, as a sorting mechanism for selective inactivation. In this model, the $\Delta kfiCD$ mutant was not incapable of immunologic activation: it elicited a robust production of highly specific IgA, possibly through a T-independent route, although the apparently high specificity and affinity of the IgA induced by $\Delta kfiCD$ suggests that it was able to activate T follicular helper cells (70). On the other hand, as evidenced by the flow cytometry and scRNA-seq results, the $\Delta kfiCD$ strain was unable to activate the pathways connecting to ROR γ^+ T cells. The shielding of microbes within established casts would

prevent the adhesion and endocytosis events required for Th17 induction (19, 71). Prior to encapsulation, IEC activation by $\Delta kfiCD$ might be diverted in favor of myeloid cell extravasation instead of ROR γ^+ T cell inducing paths (e.g., not inducing IL-6), or elicit a dominant inhibitory response. Overall, the key distinction between a microbe that becomes physically excluded in casts vs. a commensal that elicits a mild “homeostatic” Th17 response (like EcN or *Bifidobacterium*) would be in the initial response of the IECs, working in tandem with the subsequent IgA response toward an effective exclusion of pathobionts.

How does this model integrate with other mechanisms proposed for IgA function? IgA is generally considered to discriminate between noninvasive and invasive microbes, hence protecting the host from bacterial colonization and disease (48, 49). Several mechanisms have been proposed: microbe killing (e.g., recruiting complement), inactivation of important functions (e.g., of adhesion or motility by binding to fimbriae or flagella), or modulation of bacterial gene expression (49). In other instances, some symbionts seem to have co-opted binding of IgA to actually enhance their productive colonization (72). Such effects cannot be excluded here, but the colocalization of IgA and bacteria in casts suggests that IgA binding to microbes might enhance their interaction with intraluminal phagocytes and inclusion in casts.

Encapsulation into robust cast structures is an effective way to physically segregate harmful pathobionts, but this isolation strategy might also be a dangerously unstable one should containment fail. Indeed, the harmful potential of $\Delta kfiCD$ was revealed in the context of DSS-induced inflammation, where it induced more severe inflammation (and death of half the animals). There may be correlates in human pathology, such as the *E. coli* blooms that accompany inflammatory bowel disease.

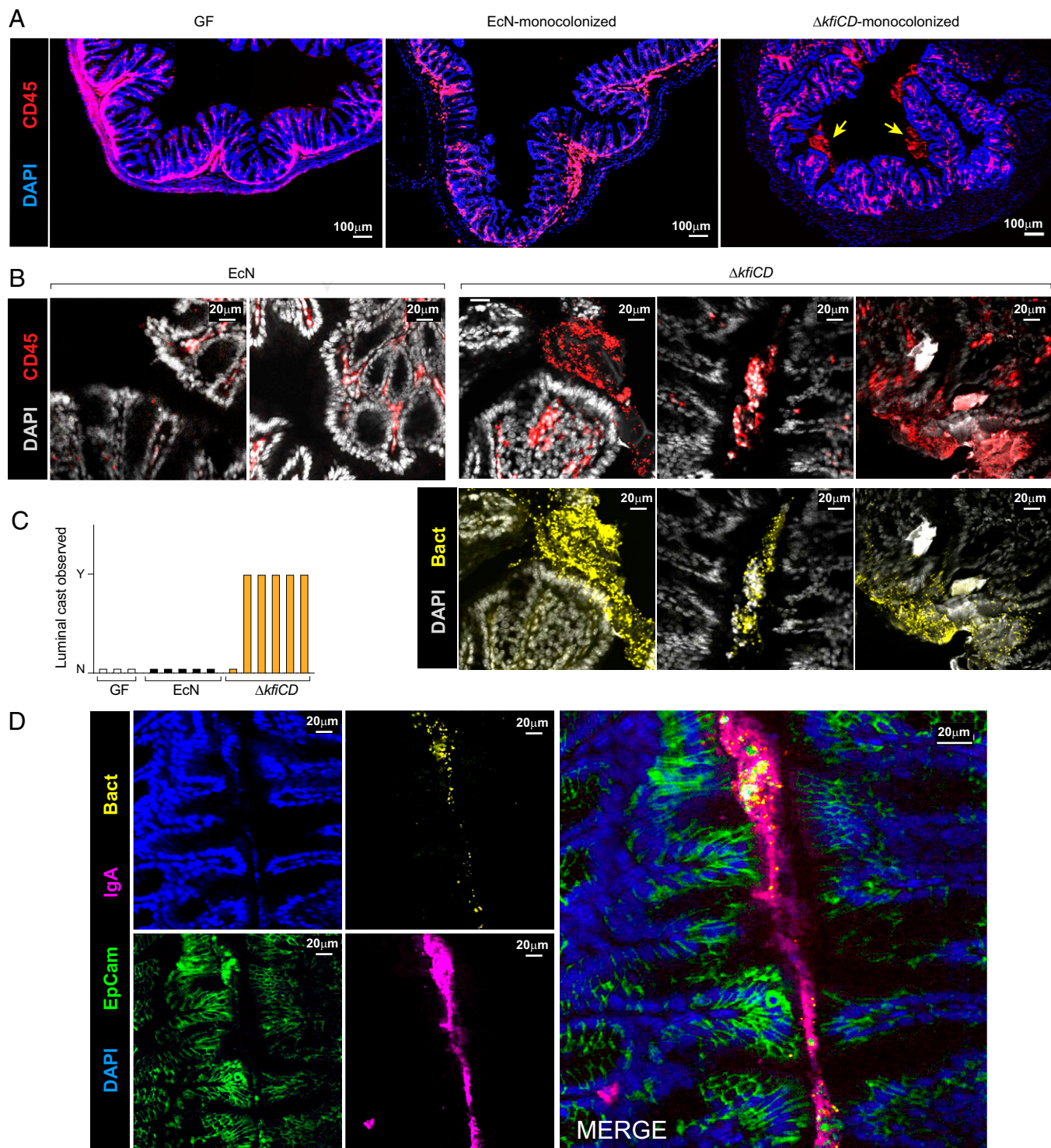


Fig. 6. Luminal IgA-immune cell casts engulf the capsule-less EcN mutant. Confocal imaging of colonic tissue was collected for imaging from GF, EcN, or $\Delta kfiCD$ monocolonized mice (day 14). (A) Confocal imaging of colonic tissue immunostained for pan-immunocytes (CD45) and cell nuclei (DAPI). Arrows point to intraluminal CD45⁺ structures. (B) As (A), with counterstaining for bacteria (anti-mCherry). (C) Quantification of intraluminal casts (scored as presence of discrete CD45⁺ structures, 100 μ m or more) in the colon of GF, EcN, and $\Delta kfiCD$ monocolonized mice. Each bar represents an individual mouse. (D) As (B), staining for IgA, Epcam (epithelial cells), and bacteria (anti-mCherry).

In fairness, and although this model does account for the different facets of the peculiar interaction of $\Delta kfiCD$ with the immune system, we have not formally demonstrated the causal connection between cast formation and the mutant's inability to trigger ROR γ ⁺ T cells. For instance, it is possible that the mutation perturbs the secretion of immunostimulatory molecules, or that it prevents components of the microbe to be routed into specific cellular compartments required to effectively trigger

ROR γ ⁺ cells. We also cannot rule out that the high degree of IgA binding to mutant microbes might be an epiphenomenon not related to cast formation.

In conclusion, this study highlights the importance of commensals microbe capsules as a means to minimize insult to the host and favor symbiotic relationships. More generally, it implies that the diversity of outcomes that microbial species induce by the immune system may result from variations in

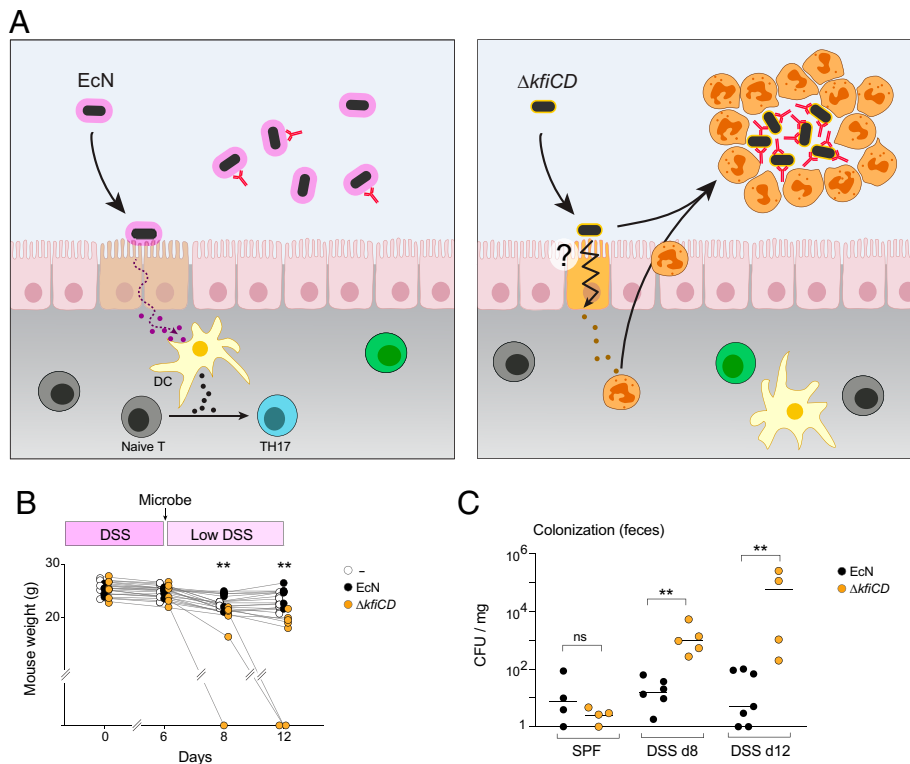


Fig. 7. Deleterious effects of the capsule-less mutant during DSS-induced colitis. (A) Schematic representation of proposed model, comparing the epithelial-initiated response to EcN or $\Delta kfiCD$ that leads either to $ROR\gamma^+$ cell activation or to myeloid cell extravasation. (B, C) Specific pathogen-free animals were fed 2.5% DSS for 6 d, then gavaged with 10^7 EcN or $\Delta kfiCD$ bacteria (DSS dose lowered to 1.5% for the remaining time). Weight was recorded and normalized to their initial weight. (C) As (B), measure of live bacteria (as cfu/mg of feces) at days 8 or 12 (t test, $**P < 0.01$).

physical barriers that they form or encounter in addition to the diversity of molecular agents they encode.

Limitations of Study. Much of this work, albeit not all, relied on monocolonization of GF mice. This reductionist strategy rendered more tractable the identification of differences in immunomodulatory properties between individual bacterial strains but there is no doubt that it does represent a simplified approximation of the far more complex network of interactions in the microbiota of normal mammals. Because the mutation shunted the $\Delta kfiCD$ microbe along an unanticipated path, and the project with it, we did not uncover the actual mechanism leading to homeostatic Th17 and Treg activation by the WT EcN symbiont. Nor do we know how IgA responses were triggered equally well by EcN and mutants (little Tfh induction was detected with either). Finally, the model proposed here attempts to integrate a number of threads in the data, but several aspects remain speculative in the in vivo context.

Materials and Methods

Bacterial Strains and Generation of EcN Mutants. Strains of the probiotic EcN 1917 wild-type and mutants (EcN; Mutaflor, DSM 6601) and EcBW utilized in this study are listed in [Dataset S2](#). Mutants of EcN were constructed using the lambda red recombinase system (73), as previously described (74).

Generation of Monocolonized Mice. For monocolonization, GF C57BL/6 mice at 4 wk of age were orally inoculated by gavage with LB-grown single bacteria ($\sim 1 \times 10^7$ cfu/mL). Each group of mice was housed in autoclaved cages, with autoclaved food and water and kept under sterile conditions for 2 wk. Fecal material, colon content, and cecum content were collected and plated at 2 wk after bacterial inoculation to ensure monocolonization by a single bacterial strain. Colonization were all preformed and processed at the same time of the day (10 AM) to reduce diurnal variability.

Intestinal Immunocyte Analysis. Intestinal tissues (colon, cecum, and small intestine) were collected at day 14 postmonocolonization in RPMI 2% fetal calf serum (FCS). Each intestinal section was then measured, cleaned, and treated with RPMI medium containing 1 mM DTT, 20 mM EDTA, 2% FCS at 37 °C for 15 min to remove epithelial cells, then dissociated in 1.5 mg/mL collagenase II (GIBCO), 0.5 mg/mL dispase, 1% FCS in RPMI with constant stirring at 37 °C for 40 min. Single-cell suspensions were then filtered and washed with 4% RPMI solution, stained with three constant panels of antibodies, and analyzed on a BD Symphony flow cytometer, with data analysis in FloJo. Fold-change values for population frequencies were calculated by dividing raw frequencies of the cytometry profiles of a given cell type for each mouse colonization by the average frequency obtained from control mice of the same experiment. For scRNA-seq and T cell receptor sequencing (three independent monocolonization experiments), $CD4^+$ T cells from distal colons of GF or monocolonized mice were sorted (DAPI⁻ $CD19^-$ $CD4^+$ $TCR\beta^+$), and encapsulated together for scRNA-seq on the 10x Genomics 5/v2 platform after hashtagging with Biolegend TotalSeq-C reagents (10 different hashtags per experiment). For immunofluorescence staining of colon, mice were monocolonized with bacteria expressing mCherry. Tissues were collected 14 d postmonocolonization from distal colon and fixed in 4% PFA overnight. The following day, tissues were place in 30% sucrose for 12–24 h and then embedded in OCT and frozen at -80°C .

Dynamics TEER Measurements. Single colony of EcN and its related mutant strains were grown overnight in Lennox Luria-Bertani (LB; BD Difco) at 37 °C to concentration of $\sim 1 \times 10^9$ cfu/mL and diluted in Caco2 growth media without antibiotics. The diluted bacteria cocultured with the cells (seeded at 3×10^5 cells/well 4 d prior to coculture with bacteria) in the CytoView-Z 96 plate and TEER was recorded over time (at 37 °C and 5% CO_2). The barrier index was calculated by Axio "Impedance" module (using AxIS Z software version 3.2.3/Axion BioSystems), as the ratio between cellular resistance at low frequency (1 Hz) vs. high frequency (41 Hz). The barrier index was measured at high temporal resolution of 1 min and was normalized to the reference time ($t = 0$). Additional correction was performed by normalizing to the barrier index of unstimulated cells.

Data, Materials, and Software Availability. The data reported in this paper have been deposited in the Gene Expression Omnibus database under accession numbers [GSE211049](#) (75) and [GSE213200](#) (76). Further details are available in [SI Appendix, SI Materials and Methods](#).

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