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Exploiting a host-commensal interaction to promote intestinal barrier function and enteric pathogen tolerance

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Abstract

Commensal intestinal bacteria can prevent pathogenic infection; however, limited knowledge of the mechanisms by which individual bacterial species contribute to pathogen resistance has restricted their potential for therapeutic application. Here, we examined how colonization of mice with a human commensal *Enterococcus faecium* protects against enteric infections. We show that *E. faecium* improves host intestinal epithelial defense programs to limit *Salmonella enterica* serotype Typhimurium pathogenesis *in vivo* in multiple models of susceptibility. *E. faecium* protection is mediated by a unique peptidoglycan hydrolase, SagA, and requires epithelial expression of pattern recognition receptor components and antimicrobial peptides. Ectopic expression of SagA in non-protective and probiotic bacteria is sufficient to enhance intestinal barrier function and confer resistance against *S. Typhimurium* and *Clostridium difficile* pathogenesis. These studies demonstrate that specific factors from commensal bacteria can be used to improve host barrier function and limit the pathogenesis of distinct enteric infections.

One Sentence Summary

SagA from *E. faecium* triggers protective intestinal epithelial cell programs that limit *S. Typhimurium* and *C. difficile* pathogenesis.

Introduction

The human intestine is populated by approximately 10^{13} bacteria, as well as eukaryotic viruses, bacteriophages and fungi (1). In addition to playing a crucial role in physiological processes such as digestion of food and detoxification of bile acids, these microorganisms have been implicated in conditions as diverse as metabolic syndrome (2, 3), cardiovascular

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diseases (4) and autism (5, 6). Gut microbes provide low grade stimulation of the intestinal immune system (7), and colonization by the microbiota activates host defense pathways in the gut epithelium and underlying immune cells that restrict invasion of both commensals and pathogens (8, 9). Accordingly, antibiotic-induced dysbiosis and changes in the relative abundance of several bacterial taxa correlate with increased susceptibility to inflammatory bowel diseases and enteric pathogens (10, 11). Recent studies have shown several beneficial effects of microbial metabolites on regulation of intestinal pathogen resistance and immune function. For example, microbiota regulation of bile acid metabolism has been shown to restrict *C. difficile* growth (12), and the production of short chain fatty acids by specific commensal bacteria modulates colonic anti-inflammatory T cell populations (13, 14) and attenuates enteric infections by enhancing intestinal epithelial cell integrity (15). Nonetheless, the precise mechanisms of protection elicited by many intestinal bacterial species remain to be determined. Notably, *E. faecium*, a commensal bacterium found in the intestines of insects, fish, birds, and mammals (16), can enhance resistance against enteric pathogens and has been utilized as a probiotic in livestock for decades (17). However, the protective mechanisms of *E. faecium* are unknown, and its use as a probiotic in humans is limited by its potential drug-resistance and pathogenesis in the form of vancomycin-resistant *Enterococcus* (VRE) (18). Therefore, dissecting the mechanisms of *E. faecium*-mediated pathogen resistance is required to reveal novel means to trigger these protective programs without introducing a potential pathobiont.

To investigate the protective mechanisms of *E. faecium*, we employed three different murine models of microbiota manipulation and evaluated key components of host immunity. We observed that *E. faecium* does not engage adaptive immune mechanisms, but instead enhances intestinal epithelial barrier effectors through innate immune pathways to restrict *Salmonella* pathogenesis *in vivo*. Additionally, we found that SagA, a secreted peptidoglycan hydrolase from *E. faecium*, is sufficient to increase the epithelial expression of barrier effectors and antimicrobial peptides *in vivo*. The ectopic expression of SagA in non-protective bacteria not only protects against the Gram-negative bacterial pathogen, *S. Typhimurium*, but also confers resistance against a major hospital-acquired Gram-positive enteric pathogen, *C. difficile*. These studies reveal a major protective mechanism of *E. faecium* in mammals and show that a specific secreted factor from a commensal bacterium, SagA, can be used to restrict the pathogenesis of distinct enteric infections.

Results

***E. faecium* limits *Salmonella* pathogenesis in multiple models of susceptibility**

To study the protective activity of *E. faecium* (human commensal isolate, strain Com15), we first compared it to another human commensal *Enterococcus* species, *E. faecalis* (strain OG1RF), in two non-typhoidal *Salmonella enterica* serotype Typhimurium infection models. Germ-free (GF) mice were mono-colonized with *E. faecium* or *E. faecalis* one week prior to oral infection with *S. Typhimurium*. Control GF mice with no pre-existing microbiota succumbed to infection within 6 days as did 50% of *E. faecalis* mono-colonized mice; however, *E. faecium* mono-colonized mice were significantly protected against early weight loss and exhibited decreased *S. Typhimurium* titers in the feces (Fig. 1, A and B and fig. S1).

This corresponded to 100% survival at day 6 post-infection and an overall survival of 42% for *E. faecium* mono-colonized mice (Fig. 1C). Because antibiotics deplete normal microbiota and increase susceptibility to several enteric infections, including *C. difficile* and *S. Typhimurium* (11), we also treated conventional specific pathogen-free (SPF) mice with a broad-spectrum antibiotic cocktail prior to colonization with *E. faecium* or *E. faecalis* and infection with *S. Typhimurium*. While antibiotic-treated *E. faecium*-colonized mice no longer exhibited significant decreases in early weight loss or average *S. Typhimurium* titers, they displayed an overall survival benefit (Fig. 1D–F). In fact, survival was significantly improved despite sub-optimal *E. faecium* colonization, as *E. faecium* was not as well maintained in mice after antibiotics were discontinued and the normal microbiota began to return (fig. S2).

These results suggested that protection was not dependent on secondary effects on the remaining microbiota, since *E. faecium* was protective in gnotobiotic mice and in antibiotic-treated mice from which no bacterial 16S rRNA could be amplified at the time of colonization (data not shown). Furthermore, although *E. faecium* treatment correlated in some cases with a delay in or clearance of infection (fig. S3), it did not eliminate *S. Typhimurium* colonization or influence expression of type III effectors involved in *S. Typhimurium* invasion of host cells (fig. S4). These results indicate that *E. faecium* does not act by direct bactericidal mechanisms, inhibiting *S. Typhimurium* virulence gene expression, or indirectly modulating the microbiota.

To investigate host mechanisms involved in *E. faecium*-mediated protection against *S. Typhimurium* pathogenesis *in vivo*, we analyzed intestinal immune cells after *E. faecium* colonization. *E. faecium* colonization did not induce significant changes in the relative frequency or cytokine production of intestinal myeloid cell and lymphocyte subsets (Fig. 1G and fig. S5). In addition, prolonged host survival in response to *E. faecium* colonization was preserved in *Rag1*^{-/-} mice, which lack B and T cells (Fig. 1H). These data suggest that although adaptive immunity contributes to overall *S. Typhimurium* resistance, it is not a major component of *E. faecium*-mediated protection.

E. faecium colonization rapidly enhances epithelial function and bacterial segregation

Mice treated with a single dose of streptomycin are rendered highly susceptible to *S. Typhimurium* infection (19). To characterize the timing of *E. faecium*-mediated protection, we next utilized the streptomycin model to rapidly induce *S. Typhimurium* susceptibility. As expected, streptomycin-treated control and *E. faecalis*-colonized mice showed severe *S. Typhimurium*-induced pathogenesis, as evidenced by rapid weight loss and mortality within 10 days of infection (Fig. 2A–C and fig. S6). In contrast, streptomycin-treated mice pre-colonized with *E. faecium* 4 hours before infection displayed significantly reduced early weight loss and prolonged survival, without affecting pathogen colonization (Fig. 2A–C and fig. S7). Of note, administration of *E. faecium* 24 hours after *S. Typhimurium* infection also conferred some protective effects, albeit greatly reduced compared to *E. faecium* administered before infection (Fig. 2, A and C), indicating that *E. faecium* colonization induces a protective response that must be triggered prior to infection.

S. Typhimurium produces several effector proteins that rapidly disrupt the intestinal epithelium and facilitate bacterial dissemination into the liver and other systemic tissues (20, 21). Accordingly, early (48h) after infection, control and *E. faecalis*-colonized mice exhibited severe edema, neutrophil infiltration, and destruction of intestinal epithelial integrity, while *E. faecium* pre-treated mice displayed markedly reduced *S. Typhimurium*-induced inflammation, pathology, and epithelial damage (Fig. 2, D and E). *E. faecium* colonization also attenuated *S. Typhimurium*-induced intestinal paracellular permeability, as measured by dissemination of FITC-dextran from the gut into the circulation (Fig. 2F). This corresponded to decreased *S. Typhimurium* invasion into peripheral organs (Fig. 2G). In addition, fluorescence in situ hybridization (FISH) using a universal bacterial probe showed improved bacterial segregation from the intestinal epithelium in *E. faecium*-colonized mice compared to controls (Fig. 2H), with bacterial contact much more apparent in untreated mice. These data show that *E. faecium* rapidly results in improved or maintained epithelial barrier function and *S. Typhimurium* resistance.

Intestinal epithelial cells upregulate barrier defenses upon *E. faecium* colonization

Given the beneficial effects of *E. faecium* on intestinal barrier integrity, we next examined *E. faecium*-induced changes in intestinal epithelial cell (IEC) gene expression. In the presence of microbial colonization, IECs produce numerous proteins that function to restrict bacterial contact and invasion of the epithelium, including antimicrobial peptides (AMPs), such as α -defensins (cryptdins in mice) and the C-type lectins RegIII β and RegIII γ , and mucous components like mucins (22, 23). Expression of many AMP and mucin genes is markedly reduced in response to antibiotic treatment (24, 25); however, we observed that *E. faecium* colonization after broad-spectrum antibiotic treatment restored expression of both cryptdin 2 and mucin 2 to pre-antibiotic levels and strongly increased expression of the C-type lectin *Reg3g* (RegIII γ) (Fig. 3A and fig. S8). The induction of *Reg3g* above steady-state levels suggests that *E. faecium*-derived signals are especially capable of prompting expression of certain AMPs. This increase was also present in streptomycin-treated, *E. faecium*-colonized mice as early as 4 hours post-colonization (Fig. 3B), consistent with the timing of protection observed against *S. Typhimurium* infection (Fig. 2, A and C). Using iDISCO+ tissue-clearing (26, 27) and light sheet microscopy to visualize three-dimensional mucin distribution in the intact intestine, we also observed enhanced MUC2 distribution along the epithelium in *E. faecium*-treated mice (Fig. 3C and Movies S1 and S2). This enhanced mucin distribution and the decreased bacterial contact with the epithelium observed by FISH (Fig. 2H) could be sufficient to limit *S. Typhimurium* invasion and dissemination to peripheral organs. Taken together, these data indicate that *E. faecium* stimulates protective programs in the intestinal epithelium that limit *S. Typhimurium* pathogenesis and result in prolonged host survival.

RegIII γ is required for *E. faecium*-induced bacterial segregation from the epithelium

The secretion of AMPs, including the RegIII-family, has been shown to play an important role in excluding bacteria from the epithelial surface both by direct bactericidal activity and by affecting bacterial spatial organization (28, 29). Although neither RegIII β nor RegIII γ are bactericidal toward *S. Typhimurium*, RegIII γ has also been shown to contribute to normal mucin distribution and bacterial segregation (28, 30). To determine the relevance of

the observed increase in expression of *Reg3g* to *E. faecium*-mediated protection, we colonized antibiotic-treated *Reg3g*-deficient (*Reg3g*^{-/-}) mice and infected them with *S. Typhimurium*. Absence of *Reg3g* significantly abrogated *E. faecium*-mediated protection against *S. Typhimurium* pathogenesis compared to *Reg3g*^{+/-} controls (Fig. 4A–C and fig. S9), while un-colonized *Reg3g*^{-/-} and heterozygous littermate controls were equally susceptible to infection-induced weight loss and mortality. These observations indicate that RegIIIγ is a required component of an *E. faecium*-induced protective program.

S. Typhimurium dissemination to peripheral organs like the liver can occur by invasion across IECs, disruption of tight junctions, or by unimpeded transport through microfold (M) cells. However, bacteria that are sampled through M cells are likely to be taken up by mononuclear phagocytes in the underlying Peyer's patches (PPs) and transported to the mesenteric lymph nodes (mLN) (31). In *Reg3g*^{+/-} mice, colonization with *E. faecium* prevented early *S. Typhimurium* invasion into the liver, whereas *Reg3g*-deficient mice suffered liver invasion comparable to untreated mice (fig. S10A). However, translocation of bacteria to the mLN was not significantly affected by colonization or genotype, implying that active transport of *S. Typhimurium* is intact in *E. faecium*-treated mice, regardless of *Reg3g* expression (fig. S10B). In addition, we did not observe significant *E. faecium*-specific changes in IEC transcription of tight-junction protein genes in wild-type (*Reg3g*-sufficient) mice (fig. S11). Therefore, to investigate how RegIIIγ might be contributing to *E. faecium*-mediated protection, we examined bacterial spatial organization and MUC2 distribution along the intestinal epithelium. In the absence of *Reg3g*, *E. faecium* colonization failed to decrease bacterial contact with the epithelium (Fig. 4, D and E) or increase MUC2 protein distribution and mRNA expression (Fig. 4F–H and Movies S3–6). Collectively, these data indicate that RegIIIγ is required for *E. faecium*-mediated bacterial segregation from the epithelium. Because many bacterial pathogens, including *S. Typhimurium*, require direct cell contact to induce pathology (32–35), by decreasing bacterial contact with the epithelium, RegIIIγ may afford enhanced pathogen resistance that is in addition to and distinct from its bactericidal effects. Along with mucins and other AMPs, this likely contributes to decreased *S. Typhimurium* invasion of peripheral organs and subsequent morbidity.

Epithelial pattern-recognition receptors are required for *E. faecium*-mediated protection

Expression of mucins and AMPs in the intestinal epithelium is upregulated in response to signaling through a number of pattern recognition receptors (PRRs) (36). Specifically, toll-like receptors (TLRs), and their signaling adapter, MyD88, have been shown to be required for IEC expression of *Muc2*, *Reg3b*, and *Reg3g* (37, 38). We therefore examined the requirement for MyD88 signaling in *E. faecium*-mediated protection by generating a tamoxifen-inducible, IEC-specific conditional *Myd88* knockout mouse (*Villin*^{Cre-ERT2}*xMyd88*^{f/f} (*i Vill Myd88*)). We administered tamoxifen to *i Vill Myd88* and Cre⁻ littermate controls one week prior to streptomycin treatment and followed this with *E. faecium* colonization and subsequent *S. Typhimurium* infection (fig. S12). We observed no significant difference in early weight loss or pathogen burden between *i Vill Myd88* and Cre⁻ mice (Fig. 5A and B and fig. S13). However, *E. faecium*-mediated prolonged host survival was abrogated in the absence of epithelial MyD88 expression (Fig. 5C). Furthermore, *E. faecium*-induced expression of AMPs, particularly *Defa2* (cryptdin 2) and

Reg3g, was impaired in *Vill Myd88* IECs (Fig. 5D). While macrophages are known to play a major role in *S. Typhimurium* pathogenesis and clearance (39), conditional deletion of *Myd88* in myeloid cells, in particular macrophages, by interbreeding *Myd88^{fl/f}* with *Lyz2^{Cre}* mice (*LysM Myd88*) still allowed for a significant survival benefit of *E. faecium* colonization prior to *S. Typhimurium* infection (fig. S14A and B). This benefit was evident despite a distinct increase in susceptibility to *S. Typhimurium* in mice harboring *Myd88*-deficient myeloid cells. These data indicate that although MyD88 expression in myeloid cells plays an important role in overall resistance to *S. Typhimurium*, *E. faecium*-mediated protection requires IEC, but not myeloid, MyD88 expression to improve barrier function and survival.

In addition to MyD88, previous studies have shown that mice with a mutation in the *Nod2* gene, another relevant PRR, also exhibit reduced intestinal production of cryptdins, MUC2, and RegIII γ (40–42). To investigate the contribution of NOD2 to *E. faecium*-induced protection and AMP expression, we compared the efficacy of *E. faecium* colonization in streptomycin-treated *Nod2*-deficient mice (*Nod2^{-/-}*) and heterozygous littermate controls. *E. faecium*-mediated protection was absent in *Nod2^{-/-}* animals in terms of early weight loss, pathogen burden, and survival (Fig. 5E–G and fig. S15). While induction of *Defa2* and *Muc2* expression was mostly maintained, *Reg3g* upregulation was present but significantly impaired in *Nod2^{-/-}* mice (Fig. 5H). This may indicate that other pathways, like MyD88, are able to contribute to upregulation of *Reg3g* in the absence of NOD2 signaling, and it implies that other NOD2-dependent, but RegIII γ -independent, responses to *E. faecium* may also be required for protection. Together, these data show that IEC innate signaling adapters and receptors known to contribute to *Reg3g* and AMP expression, namely MyD88 and NOD2, are required for *E. faecium*-mediated protection and improvement of barrier function.

E. faecium-derived SagA enhances barrier function and resistance to *S. Typhimurium*

In parallel studies performed in *C. elegans*, we identified that a *E. faecium*-derived secreted NlpC/p60 peptidoglycan hydrolase, secreted antigen A (SagA), is sufficient for protection against *S. Typhimurium* in worms (43). Deletion of *sagA* has been shown to render *E. faecium* non-viable, and it is encoded in the genomes of all sequenced *E. faecium* strains (44, 45); however, *sagA* is absent in *E. faecalis* and other known commensal species (46). Additionally, introduction of SagA expression into non-protective *E. faecalis* significantly improved early weight loss and survival compared to wild-type *E. faecalis* in gnotobiotic mice infected with *S. Typhimurium* (43). To determine what aspects of *E. faecium*-induced protection were mediated by SagA, we examined the intestinal epithelium in mice colonized with *E. faecalis*, *E. faecium* and *E. faecalis-sagA*. Both *E. faecium* and *E. faecalis-sagA*, but not wild-type *E. faecalis*, significantly induced expression of *Defa2*, *Muc2* and *Reg3g* in streptomycin-treated mice (Fig. 6A). Ectopic *sagA* expression in *E. faecalis* also abrogated *S. Typhimurium*-induced intestinal inflammation and pathology (Fig. 6, B and C), which was correlated with increased bacterial segregation from the epithelium and decreased liver invasion when compared to mice colonized with wild-type *E. faecalis* (Fig. 6, D and E). These results indicate that expression of *sagA* in *Enterococcus* is sufficient to increase epithelial barrier function and attenuate *S. Typhimurium* pathogenesis.

SagA-expressing bacteria improve barrier function and protect against *C. difficile* pathogenesis

Use of probiotic strains of bacteria has been proposed as an inexpensive strategy to decrease the incidence and severity of intestinal infection and dysbiosis without contributing to the increasing problem of antibiotic resistance (47). Although *Enterococcus* strains have been used as probiotics in livestock, their pathogenic potential makes them problematic for use in humans (18). We thus introduced *sagA* into a known non-pathogenic probiotic, *Lactobacillus plantarum* (48). Consistent with our findings with *E. faecalis-sagA* and *E. faecium* protection, *sagA*-expressing *L. plantarum* significantly prevented weight loss and improved survival upon *S. Typhimurium* infection compared to control *L. plantarum* (43). Bacterial segregation from the intestinal epithelium and MUC2 distribution were also maintained in *L. plantarum-sagA*-colonized mice relative to the prominent epithelial contact present in *L. plantarum*-vector controls (fig. S16 and Movies S7 and S8). These studies indicate that SagA protective activity is transferrable to other probiotic bacteria and can enhance epithelial barrier function to limit *S. Typhimurium* pathogenesis.

To determine if *E. faecium* and SagA protection was effective against other enteric pathogens, we investigated their activity against *C. difficile*. *C. difficile* is a leading cause of hospital-acquired infectious diarrhea that develops in antibiotic-treated patients. While existing antibiotics are effective in many patients, recurrent *C. difficile* infection is currently most effectively treated with fecal microbiota transplant (FMT) therapy (49). Following broad spectrum-antibiotic treatment, we found that mice colonized with *E. faecium*, *E. faecalis-sagA* or *L. plantarum-sagA* were protected against *C. difficile*-induced early weight loss when compared to non-*sagA*-expressing bacteria controls, despite similar colonization of each putative probiotic bacteria and similar early pathogen burden (Fig. 6, F and G and fig. S17). Further, the protection elicited by *sagA*-expressing bacteria led to significantly enhanced recovery and survival of mice (Fig. 6H and fig. S17). These data demonstrate that *E. faecium* and SagA provide effective protection against distinct enteric pathogens, suggesting broad efficacy of SagA-enhanced epithelial barrier function.

Discussion

The intestinal epithelium may be exquisitely responsive to modulation by metabolites and factors from commensal bacteria. In our studies, we characterize both epithelial and commensal microbial contributions to a protective mechanism in the mammalian intestine that reduces early pathogen invasion and tissue damage. Our results suggest that the commensal bacterium *E. faecium* triggers enhanced epithelial barrier function and pathogen resistance via its expression of a unique secreted peptidoglycan hydrolase, SagA. These observations complement our parallel studies that identified and biochemically characterized SagA as a protective factor in *C. elegans*, demonstrating extended worm survival following *S. Typhimurium* infection (43). Together, these studies depict a trans-phylum, microbiota-induced protective strategy that may be conserved among many species of animals and their cognate commensal bacteria (50, 51).

Our studies in mice indicate that *E. faecium* and SagA improve host pathogen resistance by increasing the expression of mucins and AMPs through MyD88 and NOD2 innate signaling

pathways, facilitating bacterial segregation from the epithelium. Although the molecular mechanisms involved in activation of MyD88 and NOD2 via SagA remain to be determined, our data suggest that these innate signaling pathways have unequal and partially redundant contributions to upregulation of AMPs in response to *E. faecium* colonization. In particular, RegIIIg upregulation was abrogated in *Myd88*-deficient epithelial cells but only partially hindered in *Nod2*-deficient animals. This is consistent with studies showing that the low levels of RegIIIg expression observed in *Nod2*^{-/-} mice can be boosted or recovered by colonization with certain cohorts of bacteria (42), and our observations suggest that this may be mediated by compensatory MyD88 signaling. Because *S. Typhimurium* is a Gram-negative pathogen whose cell wall lipid A and peptidoglycan are not readily accessible to RegIIIβ or RegIIIγ, respectively (52–54), these AMPs are not efficiently bactericidal toward *S. Typhimurium*, and this is evidenced by the ability of *S. Typhimurium* to colonize *E. faecium* and *E. faecalis*-SagA-treated mice despite robust RegIIIγ upregulation. However, in addition to its bactericidal activity, RegIIIγ has been shown to facilitate robust MUC2 distribution and proper bacterial spatial organization (28, 29), suggesting a mechanism by which SagA decreases both *S. Typhimurium* invasion and *C. difficile* toxin access to the epithelium (55, 56). In contrast to *Reg3g*, *Muc2* transcription was not significantly affected by either *Myd88*- or *Nod2*-deficiency. This indicates that increased *Muc2* expression is either redundantly induced by both MyD88 and NOD2 or via additional signaling pathways (57). Beyond transcriptional regulation, MUC2 proteins undergo disulfide-mediated polymerization to form complex net-like structures and are densely O-glycosylated, both of which contribute to the gel-forming properties of MUC2 upon secretion from goblet cells in the intestine (58). Additional pathways triggered by *E. faecium* and SagA may therefore contribute to host pathogen resistance by facilitating proper post-translational modification or secretion of mucins and other barrier effectors.

Beyond its benefit to the host and based on its conservation among all sequenced *E. faecium* strains (46), it is likely that SagA also contributes to *E. faecium*'s fitness and ability to colonize the intestines. Digestive tract microbiota are known to produce enzymes that facilitate bacterial access to nutrients (59, 60) and that can therefore influence bacterial survival in nutrient-poor conditions (61, 62). Determining whether and how SagA regulates the composition of gut microbes or influences niche competition in the intestines of mammals and other animals could provide important new insight into this complex host-commensal relationship.

The commensal intestinal microbiota have several potential beneficial modes of action that can be harnessed to fine-tune probiotics, including niche competition with pathogenic bacteria, activation of immune cells and augmentation of epithelial barrier function (63, 64). Our data demonstrate that SagA can be ectopically expressed in probiotic bacteria for potential prophylactic applications against diverse enteric infections, including clinically-challenging pathogens like *C. difficile*. There are nearly 500,000 new cases of *C. difficile* infection (CDI) each year in the U. S. alone, and approximately 20% of patients experience at least one recurrence (65). Use of non-CDI antimicrobials has been shown to be a significant risk factor for CDI recurrence, and the treatment strategy with the highest success rate for recurrent CDI is restoration of the healthy microbiota by FMT therapy (66). However, current probiotic formulations have shown only marginal or highly variable *in*

vivo efficacy against *C. difficile* in patients (67). Future studies are warranted to determine if low endogenous levels of commensal *E. faecium* can serve as a biomarker of CDI susceptibility and if administration of SagA-expressing probiotics can counteract this susceptibility or serve as a treatment alternative to FMT therapy. In conclusion, our findings uncover both host and microbial components of a protective program in the mammalian intestinal epithelium that could have important ramifications for the design of better probiotics.

Materials and Methods

Animals

C57BL/6J (000664), *Lyzz*^{Cre} (004781), *MyD88*^{fl} (008888), *Nod2*^{-/-} (005763), *Rag1*^{-/-} (002216) and *Reg3g*^{-/-} (017480) mice were purchased from Jackson Laboratories and maintained in our facilities. *Villin*^{CreERT2} mice were developed by Silvie Robine and generously provided by David Artis. Several of these lines were interbred in our facilities to obtain the final strains described in the text. Genotyping was performed according to the protocols established for the respective strains by Jackson Laboratories. Mice were maintained at the Rockefeller University animal facilities under specific pathogen-free (SPF) or germ-free (GF) conditions. GF C57BL/6J mice were obtained from Sarkis Mazmanian and bred and maintained in germ-free isolators in our facilities. GF status was confirmed by plating feces as well as by qPCR analysis (16S rRNA). Mice were used at 8–10 weeks of age for most experiments. Animal care and experimentation were consistent with the NIH guidelines and were approved by the Institutional Animal Care and Use Committee at the Rockefeller University.

Induction of MyD88 excision in Villin^{CreERT2}xMyD88^{fl} (iVill Myd88) mice

Mice were injected i.p. with 0.6mg tamoxifen/mouse for female mice and 0.8mg/mouse for males. Primers were designed to check floxed *MyD88* allele (primers 1&2) or tamoxifen-induced excision (primers 1&3) by PCR of genomic DNA, producing a ~357bp product for excised *MyD88* and a ~737bp product for unexcised. For mRNA expression, primers were designed for RQ-PCR (see below). *MyD88* primers used were:

PCR:	1	AGGCTGAGTGCAAACCTGGT
	2	GTTGTGTGTGTCGACCGTG
	3	ATACCGGAAGCAGATGGATG
RQ-PCR:	Forward	ACAGAAGCGACTGATTCCTATT
	Reverse	TGGTGCAAGGGTTGGTATAG

In pilot experiments, complete excision was detected at 5 days after a single dose of tamoxifen (fig. S10). For colonization and infection experiments, tamoxifen was injected once per day for two consecutive days, 7 days prior to streptomycin administration. In each experiment, intestinal tissue was collected post-mortem and genomic DNA and/or mRNA were isolated to check for correct *MyD88* excision.

Bacteria

E. faecium (strain Com15) was provided by Michael Gilmore, and *E. faecalis* (strain OG1RF) and *L. plantarum* (strain WCFS1) were generously provided by Balfour Sartor. *E. faecium* and *E. faecalis* were cultured in BHI broth (BD 237500), *S. Typhimurium* (strain 14028) was cultured in Luria Broth (LB) and *L. plantarum* was cultured in de Man, Rogosa and Sharpe (MRS) broth. *E. faecium*, *E. faecalis*, and *L. plantarum* were grown overnight at 37°C at 230 rpm shaking to stationary phase growth prior to colonization. *S. Typhimurium* was grown overnight followed by a 3.5 hour subculture at 37°C at 230 rpm to log phase growth prior to infection. *C. difficile* (strain VPI 10463) was provided by Eric Pamer and cultured in an anaerobic chamber on BHIS agar or broth with 16mg/L cefoxitin, 250mg/L cycloserine and 10mL/L 10% (w/v) taurocholic acid (henceforth denoted BHIS++) as described below and previously (68).

Colonization and *S. Typhimurium* infection of mice

To ensure effective colonization and induce infection susceptibility, specific pathogen-free (SPF) mice were gavaged with either a single dose of 20mg streptomycin 24 hours prior to colonization or a daily dose of AMNV antibiotic cocktail (4mg ampicillin, 2mg metronidazole, 4mg neomycin, and 2mg vancomycin) for 7–14 days as indicated in the figure legends. AMNV treatment was ceased 2 days prior to colonization. Germ-free (GF) mice were exported from isolators into autoclaved cages containing autoclaved food, water and bedding. Bacterial cultures were washed and resuspended in sterile PBS at 10^9 /mL for *E. faecium*, *E. faecalis*, and *L. plantarum* or at 10^7 /mL (SPF) or 10^3 /mL (GF) for *S. Typhimurium*. Mice were colonized by gavage with 100µL of the indicated bacterial suspension 4 hours before infection (for streptomycin-treated mice) or 2–7 days before infection (for AMNV-treated and GF gnotobiotic mono-colonized mice). Colonization was followed by infection with *S. Typhimurium* by oral gavage at the doses indicated above and in the figure legends. Weight loss was monitored from just before infection, and mice were euthanized when they reached 80% baseline weight or when they appeared hunched, moribund or exhibited a visibly-distended abdomen (indicative of peritoneal effusion), whichever occurred first. Death was not used as an end-point. Colony-forming units (CFU) in the feces were determined by plating 5 serial dilutions of feces suspended in sterile PBS on selective agars: Salmonella Shigella Agar (BD 211597) for *S. Typhimurium*, Enterococcosel Agar (BD 212205) for *E. faecium* and *E. faecalis*, and MRS Agar with 8ug/mL chloramphenicol for *L. plantarum*-vector and *-sagA*. Resulting quantities were normalized to feces weight. Experiments in which putative probiotics did not colonize or colonize equally well by day 2 post-colonization were terminated and not included in our analyses.

C. difficile infection of mice

Mice were gavaged with AMNV antibiotic cocktail daily for 7 days, and colonized as indicated above. In an anaerobic chamber, *C. difficile* spores were streaked onto BHIS++ plates and incubated overnight at 37°C. The following day, a single colony from the plate was inoculated into 5mL BHIS++ broth and incubated overnight at 37°C. 5mL sterile PBS was added, and broth culture tubes were then parafilmed and exported from the anaerobic

chamber for centrifugation at $2000 \times g$ for 5 minutes. Tubes were returned to the anaerobic chamber, supernatant was removed, and the bacterial pellet was resuspended in 3mL sterile PBS.

This suspension was loaded into 1-mL syringes fitted with gavage needles and exported from the anaerobic chamber in plastic bags. *C. difficile* suspensions were immediately transported to animal facilities for oral gavage of 100 μ L/mouse. Leftover inocula were serially-diluted and plated in the anaerobic chamber to confirm the number of CFU administered. Weight loss was monitored from just before infection, and mice were euthanized when they reached 80% baseline weight or when they appeared hunched or moribund, whichever occurred first. Death was not used as an end-point. CFU in the feces were determined in an anaerobic chamber by plating 5 serial dilutions of feces suspended in sterile PBS on BHIS++ agar plates. Resulting quantities were normalized to feces weight. Feces suspensions were then exported from the anaerobic chamber for quantification of aerobic colonizing bacteria as described above.

Pathological scoring

Representative portions of ileum, cecum and colon were fixed in Methacarn (methanol-Carnoy) solution and embedded in paraffin according to standard protocols (see 16S FISH below). 5- μ m sections were mounted on glass slides and stained with hematoxylin and eosin (H&E) for blinded histologic evaluation. Quantitative measures of inflammation were then recorded according to the methods of Barthel et al. (19). Briefly, each tissue section was microscopically assessed for the extent of submucosal edema, preservation of epithelial integrity and goblet cell retention (each category scored from 0–3), as well as for polymorphonuclear cell infiltration into the lamina propria (scored from 0–4). The sum of these scores represents the combined pathological score reported for each tissue. Scores >9 are indicative of severe inflammation, while scores of 5–8 are consistent with moderate inflammation. Scores <4 are associated with minimal to mild inflammation and frequently appear histologically normal.

Intestinal permeability and peripheral organ invasion

Epithelial barrier integrity was assessed by a FITC-dextran leakage assay and by measurement of *S. Typhimurium* invasion to the liver and mesenteric lymph nodes (mLN). At 48 hours post-infection, mice were gavaged with FITC-dextran 4 kDa (600 μ g/kg body weight, Sigma) dissolved in sterile PBS. After 4 hours, animals were anesthetized, and ~500 μ L blood were harvested by cardiac puncture. Serum was isolated using Z-Gel microtubes (Sarstedt), and FITC fluorescence (excitation 488nm, emission 518nm) was measured in triplicate on a FLUOstar Omega microplate reader (BMG Labtech). Concentration of FITC in serum (ng/mL) was determined using a standard curve. Background levels of FITC in serum from uninfected mice averaged ~450ng/mL, indicated by a dashed line in the figure. To measure Salmonella invasion, whole livers or mLN were homogenized in PBS with 0.1% Triton X-100 to release intracellular bacteria. 3 serial dilutions were plated on Salmonella Shigella Agar, and resulting quantities were normalized to organ weight prior to homogenization.

16S FISH

To visualize bacterial segregation from the intestinal epithelium, 16S rRNA FISH was performed with a universal probe (EUB388, Cy3-GCTGCCTCCCGTAGGAGT-Cy3) on sections fixed with Methacarn solution as described previously (69, 70). In brief, intestinal tissues (about 1 cm) including intestinal contents were fixed in Methacarn solution (60% methanol, 30% chloroform, 10% glacial acetic acid) for 6 hours at 4°C. After 3 washes with 70% ethanol tissues were embedded in paraffin. 5- μ m sections were cut, mounted on glass slides, and de-paraffinized with xylene and subsequently dehydrated. Probe was diluted (5 ng/ μ L) in hybridization buffer ((0.9 M NaCl, 20 mM Tris-HCl (pH 7.2), 0.1% SDS)) and hybridization was carried out at 50°C for 3 hours. Nuclei were stained with Hoechst and tissues were imaged on an inverted LSM 780 laser scanning confocal microscope (Zeiss). Two 0.4mm² images per tissue per mouse were acquired with four 2 μ m Z-stacks. Images were stacked, adjusted and analyzed in Image-J; bacterial contact was quantified as the number of 16S foci touching the epithelial brush border per mm of brush border length. All image analysis was performed blindly on randomized samples.

Tissue clearing - iDISCO

For 3D assessment of the colonic mucus layer, iDISCO tissue clearing and whole mount was performed as previously described ((26), updated at <https://idisco.info/>) with some modifications. Colonic tissues (about 1 cm) including contents were fixed in 4% PFA overnight at 4°C and then washed 3X in PBS. Samples were dehydrated in a series of methanol/PBS washes (30 minutes each: 20%, 40%, 60%, 80%, 100%; 1 hour: 100%) and then bleached in ice cold 5% H2O2/methanol overnight at 4°C. Samples were rehydrated in methanol/PBS (30 minutes each: 100%, 80%, 60%, 40%, 20%, 0%) and then washed 2X 1 hour in 0.2% Triton X-100/PBS. At this step, samples were opened longitudinally, and intestinal contents were gently washed out with PBS. Samples were then incubated in PTDG (1X PBS, 0.2% Triton X-100, 20% DMSO, 0.3 M glycine) for 2 days at 37°C, and then blocked in PTDD (1X PBS, 0.2% Triton X-100, 10% DMSO, 6% donkey serum) for 3 days at 37°C. After blocking, samples were washed 2X 1 hour in PTwH (1X PBS, 0.2% Tween-20, 10 μ g/mL heparin) and then stained with rabbit anti-MUC2 (1:100 in PTwH; Santa Cruz Biotechnology sc-15334) for 3 days at 37°C. Samples were washed every 2 hours (or as often as possible) in PTwH for 2 days, stained with donkey anti-rabbit AF568 (1:200 in PTwH, Life Technologies A10042) and EpCAM AF647 (1:200 in PTwH, Biolegend 118212) for 3 days at 37°C, and then washed every 2 hours (or as often as possible) in PTwH for 2 days. Tissues were mounted in 1.5% agarose in TAE and then dehydrated in a series of methanol/water washes (30 minutes each: 20%, 40%, 60%, 80%, 100%, 100%). Dehydrated samples were incubated in 66% dichloromethane/methanol overnight at room temperature, followed by 1 hour in 100% dichloromethane. Samples were then cleared in 100% dibenzyl ether and imaged on a light-sheet UltraMicroscope II (LaVision Biotec). 3D reconstruction was done in Imaris (Bitplane) and MUC2 and EpCAM volumes were quantified using the Surfaces masking function. All iDISCO analyses were performed blindly on randomized samples.

Intestinal cell isolation

Small intestines and large intestines were excised and freed of mesentery and fat. Feces were removed and Peyer's patches were excised from the small intestine. Both small and large intestines were opened longitudinally and washed in HBSS, followed by HBSS + 1mM DTT (Sigma-Aldrich). Tissue was cut into 1 cm pieces and incubated with 25 mL HBSS + 2% FBS + 5mM EDTA for 15 minutes at 37°C at 230 rpm. Supernatant containing intraepithelial lymphocytes was decanted and spun down (for RNA isolation from epithelial cells, see below). Tissue was washed with 25 mL HBSS + 2% FBS for 15 minutes at 37°C at 230 rpm. Supernatant containing additional intraepithelial lymphocytes was decanted and added to the previously-collected supernatant (IEL fraction). The gut tissues were then finely chopped and digested in HBSS + 2% FBS + 1X Sodium Pyruvate + 25mM HEPES + 50 µg/mL DNaseI + 0.05 mg/mL Collagenase VIII for 40 minutes at 37° C at 90 rpm followed by rigorous pipetting to dissociate *lamina propria* cells (LPL fraction). Cells were separated by discontinuous Percoll gradient (70%/35%) centrifugation. Hematopoietic cells were isolated from the interphase and stained for analysis by flow cytometry.

Lymph node and Peyer's patch cell isolation

Lymph nodes and Peyer's patches were mechanically disrupted and incubated with HBSS + 5% FBS + 0.5 mg/mL CollagenaseD (Roche) at 37 °C for 30 min. Cells were filtered through 100 µm cell strainers and washed. Erythrocytes were lysed with ACK Lysing buffer (Gibco). Resulting single-cell suspensions were then stained for analysis by flow cytometry.

Antibodies and flow cytometry analysis

Intestinal immune cell populations were characterized by flow cytometry. For analysis of cytokine-secreting cells, cells were incubated in the presence of 100ng/mL PMA (Sigma), 500ng/mL ionomycin (Sigma) and 10µg/mL brefeldin A (BFA) (Sigma) for 3 hours prior to staining. Cell populations were stained with antibodies against cell surface markers, followed by permeabilization in Fix/Perm buffer and intracellular staining in Perm/Wash buffer (BD Pharmingen). Fluorescent-dye-conjugated antibodies were purchased from BD-Pharmingen (anti-CD4, 550954; anti-CD45R, 557683) or eBioscience (anti-CD8α, 56-0081; anti-TCR-β, 47-5961; anti-IFN-γ, 25-7311; anti-TCRγδ, 46-5711; anti-CD8β, 46-0083; anti-TNF-α, 17-7321; B220 (RA3-6B2); F4/80 (BM8); Foxp3 (FJK-16s); Gr-1 (RB6-8C5); Ly6C (AL-21); Ly6G (1A8); MHCII (M5/114.15.2); NK1.1 (PK136); RORγt (B2D); Sca-1 (D7); Thy1.2 (53-2.1); CD3 (145-2C11); CD4 (RM4-5); CD8 (53-6.7); CD11b (M1/70); CD11c (N418); CD19 (1D3); CD44 (1M7); CD45 (30-F110); CD62L (MEL-14); CD64 (X54-5/7.1); CD103 (2E7); CD115 (AFS98). Flow cytometry data were acquired on an LSR-II flow cytometer (Becton Dickinson) and analyzed using FlowJo software (Tree Star).

Real-time quantitative PCR (RQ-PCR)

Gene expression programs in intestinal epithelial cells were assessed by RQ-PCR. Epithelial cells were harvested as described above and resuspended in Trizol (Invitrogen), RNA was isolated according to the manufacturer's protocol, and cDNA was generated using iScript cDNA Synthesis Kit (BioRad). RQ-PCR was performed using Power SYBR Green Master

Mix (Agilent) in an ABI 7900HT system. Relative gene expression was quantified by the Ct method and normalized to HPRT and GAPDH gene expression. Primers used were:

	Forward	Reverse
claudin-3	CCTGTGGATGAACTGCGTG	GTAGTCCTTGCGGTCGTAG
cryptdin 1	CTAGTCCTACTCTTTGCCCT	TTGCAGCCTCTTGATCTACA
cryptdin 2	CTATTGTAGAACAAAGAGGCT	TCTCCATGTTTCAGCGACAGC
cryptdin 3	GCGTTCTCTTCTTTTGCAGC	AAACTGAGGAGCAGCCAGG
cryptdin 4	GTCCAGGCTGATCCTATCCA	GGGGCAGCAGTACAAAAATC
cryptdin 5	GTCCAGGCTGATCCTATCCA	GATTTCTGCAGGTCCAAAAA
cryptdin 6	GTCCCATTCATGCGTTCTCT	AGGACCAGGCTGTGTCTGTG
Muc1	TGCTCTTACAAGTTGGCAGA	TACCAAGCGTAGCCCTATG
Muc2	ACAAAAACCCAGCAACAAG	GAGCAAGGGACTCTGGTCTG
Muc4	GGACATGGGTGTCTGTGTG	CTCACTGGAGAGTTCCTTG
Muc13	CTTTGTACTGTGGTGGGAGGA	TCAAGCAAGAGCAGCTACCA
Muc17	ATTCAGAGGCCCCAGGTAGT	TGGCTAAACACGCTTCTCCT
Muc20	CAGGAAGGACATGTGGACAA	TTCTCCTTGTACGGCTGAC
occludin	ATTCACCTTGCCATTGGAGG	ATTTATGATGAACAGCCCCC
Reg3g	TCCACCTCTGTTGGGTTTCAT	AAGCTTCCTTCTGTCTCTCC
ZO-1	TGCAATTCCAAATCCAAACC	AGAGACAAGATGTCCGCCAG

S. Typhimurium Pathogenicity Gene RQ-PCR

Expression of *Salmonella* pathogenicity genes during infection was assessed by RQ-PCR as previously described (71) with some modifications. Mouse fecal samples and cecal contents were suspended in 200 μ L PBS, vortex mixed extensively, and centrifuged at low speed (200 $\times g$, 3 minutes). This process was repeated twice more for each sample, collecting supernatant each time. Pooled supernatant from the three washes was centrifuged at high speed (10,000 $\times g$, 1 minute) and the resulting bacterial pellet was resuspended in 300 μ L Trizol Max Bacterial Enhancement Reagent (Life Technologies) preheated to 95°C. Following a 4 minute incubation at 95°C, 1 mL of Trizol (Invitrogen) was added and RNA was extracted according to the manufacturer's protocol. cDNA was generated using iScript cDNA Synthesis Kit (BioRad) and RQ-PCR was performed using Power SYBR Green Master Mix (Agilent) in an ABI 7900HT system. Primers for *invA* (72), *hilA*, *hilD*, *invC*, *invF*, *invJ*, *orgA*, *prgH*, *prgK*, *rtsA*, *sicA*, *sipA*, and *sipC* (73) have been previously described. Relative gene expression was quantified by the Ct method and normalized to *invA* gene expression.

Generation of *E. faecalis-sagA* and *L. plantarum-sagA*

E. faecalis-sagA and *L. plantarum-sagA* were generated as described in Rangan et al. (43).

Statistical Analyses

Comparisons and statistical tests were performed as indicated in each figure legend. Briefly, for comparisons of multiple groups over time or with 2 variables, a 2-way ANOVA was used

with an appropriate Bonferroni post-test comparing all groups to each other, all groups to a control, or selected groups to each other. Survival data were analyzed using a log-rank (Mantel-Cox) test with a Bonferroni correction for the degrees of freedom based on the number of comparisons made. To compare two groups with non-normal distribution or low sample-size, the medians of the two groups were compared using a Mann-Whitney test unless one data set contained only zero values. In these cases, a Mann-Whitney could not be performed because all values in the group were identical, and a Wilcoxon test was performed instead. For comparisons of multiple groups with only one variable, a 1-way ANOVA or Kruskal-Wallis test was performed for data with underlying normal or non-normal distribution, respectively, with Bonferroni, Dunnett's or Dunn's post-tests where appropriate. Statistical analyses were performed in GraphPad Prism software. A *P* value of less than 0.05 was considered significant, denoted as **p* 0.05, ***p* 0.01 and ****p* 0.001 for all analyses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. *E. faecium*-mediated *Salmonella* resistance does not require an adaptive immune response (A–C) Germ-free (GF) C57BL/6 mice were orally gavaged with 10^8 CFU *E. faecalis* or *E. faecium* 7d before oral infection with 10^2 CFU *S. Tm*. (A) Weight loss, (B) *S. Tm* bacterial burden in feces, and (C) survival are shown. Pooled data from 4 independent experiments, n=12 mice/group. (D–F) C57BL/6 mice were orally gavaged with a broad-spectrum antibiotic cocktail of ampicillin, metronidazole, neomycin, and vancomycin (AMNV) daily for 7d prior to gavage with 10^8 CFU *E. faecalis* or *E. faecium* or PBS, followed by infection with 10^6 *S. Tm*. (D) Weight loss, (E) *S. Tm* bacterial burden in feces, and (F) survival are shown. Pooled data from 4 independent experiments, n=14 mice/group. (G) Mice were

gavaged with AMNV daily for 7d prior to gavage with 10^8 CFU *E. faecium* (*E. fm*). 5d post-colonization, intraepithelial lymphocytes were isolated and analyzed by flow cytometry for relative frequency and cytokine expression of shown subpopulations. Pooled data from 2 independent experiments, n=5 mice/group. **(H)** *Rag1*^{-/-} mice were gavaged with AMNV antibiotic cocktail daily for 7d prior to gavage with PBS or 10^8 CFU *E. faecalis* or *E. faecium*, followed by infection with 10^6 *S. Tm*. Pooled data from 4 independent experiments, n=13–14 mice/group. (A, B, D and E) mean±SEM, 2-way ANOVA, p-value shown comparing *E. faecalis* to *E. faecium*. n.s.=not significant. (C, F and H) Log-rank analysis, p-value shown comparing *E. faecalis* to *E. faecium*. (G) mean±SEM, Mann-Whitney. *p 0.05, **p 0.01, ***p 0.001 for all analyses. Comparisons with no (*) or bar had p>0.05 and were not considered significant.

Fig. 2. *E. faecium* improves epithelial barrier function and bacterial segregation

(A–C) C57BL/6 mice were orally gavaged with streptomycin and given 10^8 colony-forming units (CFU) *E. faecalis* or *E. faecium* 4h before (b.i.) or 24h after (a.i.) oral infection with 10^6 CFU *S. Typhimurium* (*S. Tm*). (A) Weight loss, (B) *S. Typhimurium* (*S. Tm*) bacterial burden in feces, and (C) survival are shown. Pooled data from 3 independent experiments, $n=6-8$ mice/group. (D) Cecum tissue stained with H&E, harvested at 48h post-infection (p.i.) from mice given streptomycin and left uninfected or treated before infection as in (A–C). Representative images, 40X objective, from 1 of 3 independent experiments, $n=2-3$ mice/

group. Scale bar = 100 μ m. **(E)** Ileum, cecum, and colon tissues were harvested and prepared as in (D) and scored for 4 pathology parameters. Pooled combined pathology scores from 3 independent experiments, n=6–7 mice/group. **(F)** Intestinal permeability measured by FITC-dextran in the serum 48h p.i. from mice treated before infection as in (A–C). Dashed line = background level (uninfected). Pooled data from 3 independent experiments, n=7–9 mice/group. **(G)** *S. Tm* bacterial burden 48h p.i. in the livers of mice treated before infection as in (A–C). Pooled data from 4 independent experiments, n=11–12 mice/group. **(H)** FISH staining for all bacteria (universal 16S probe) and epithelial nuclei (Hoechst) from intestinal tissues 48h p.i. Representative images, 40X objective, from 1 of 3 independent experiments, n=4–6 mice/group. White arrows indicate bacteria in contact with or invading through the epithelium, and white dashed line designates zone of segregation from bacteria. (A and B) mean $\pm$ SEM, 2-way ANOVA, p-value shown comparing *E. faecalis* to *E. faecium* b.i., n.s.=not significant. (C) Log-rank analysis, p-value shown comparing *E. faecalis* to *E. faecium* b.i. (E) median $\pm$ range, Mann-Whitney comparing *E. faecalis* to *E. faecium* (Wilcoxon for colon, n.d.=none detected, score of zero). (F and G) bar=median, Mann-Whitney comparing *E. faecalis* to *E. faecium*. *p 0.05, **p 0.01 for all analyses. Comparisons with no (*) or bar had p>0.05 and were not considered significant.

Fig. 3. *E. faecium* colonization increases anti-microbial peptide and mucin expression

(A) C57BL/6 mice were orally gavaged with AMNV daily for 7d prior to gavage with 10^8 CFU *E. faecalis* or *E. faecium*. 4d post-colonization, intestinal epithelial cells (IECs) were isolated and analyzed by RQ-PCR for expression of shown genes vs unmanipulated specific pathogen-free (SPF) mice. Representative data from 1 of 3 independent experiments, $n=2-3$ mice/group. (B) Mice were given a single gavage of streptomycin followed by gavage with 10^8 CFU *E. faecium* 24h later. At indicated time-points post-colonization, IECs were isolated and analyzed by RQ-PCR. Representative data from 1 of 2 independent experiments, $n=2$ mice/group. (C) Captured images of tissue-cleared, MUC2- and Ep-CAM-stained colon from mice treated as in (J). Corresponding videos are Movies S1 and S2. Scale bar = 200 μ m. (A and B) mean $\pm$ SEM, 1-way ANOVA with Dunnett's post-test comparing each to antibiotic-only controls. * p 0.05, ** p 0.01, *** p 0.001 for all analyses. Comparisons with no (*) or bar had $p>0.05$ and were not considered significant.

Fig. 4. *E. faecium*-induced expression of *Reg3g* is required for protection against *S. Typhimurium*

(A–C) *Reg3g*^{-/-} mice or +/- littermate controls were given AMNV for 7d and colonized with 10⁸ *E. faecium* (*E. fm*) prior to oral infection with 10⁶ *S. Tm*. (A) Weight loss, (B) *S. Tm* bacterial burden in feces, and (C) survival are shown. Pooled data from 4 independent experiments, n=10–12 mice/group. (D–G) *Reg3g*^{-/-} mice or +/- controls were given streptomycin 24h before gavage with 10⁸ *E. fm* prior to oral infection with 10⁶ *S. Tm*. (D) FISH staining for all bacteria (universal 16S probe) and epithelial nuclei (Hoechst) from

intestinal tissues 48h p.i. Representative images, 40X objective, from 1 of 3 independent experiments, n=4–6 mice/group. White arrows indicate bacteria in contact with or invading through the epithelium, and white dashed line designates zone of segregation from bacteria. **(E)** Quantitation of bacterial contact from 4 images per mouse from **(F)**. **(F)** Captured images of tissue-cleared, MUC2- and Ep-CAM-stained colon from mice treated as in **(F)**. Corresponding videos are Movies S3–6. Scale bar = 200 μ m. **(G)** Quantitation of MUC2 volume as a ratio to volume of Ep-CAM from 4 sections per mouse from **(H)**. **(H)** *Reg3g*^{-/-} mice or +/+ controls were gavaged with streptomycin 24h before gavage with 10⁸ *E. fm*. IECs were harvested 4h later and analyzed by RQ-PCR for expression of shown genes. Pooled data from 2 independent experiments, n=3–6 mice/group. (A and B) mean $\pm$ SEM, 2-way ANOVA, p-value shown comparing *Reg3g*^{-/-} *E. fm* to *Reg3g*^{+/-} *E. fm*, n.s.=not significant. (C) Log-rank analysis, p-value shown comparing *Reg3g*^{-/-} *E. fm* to *Reg3g*^{+/-} *E. fm*. (E and G) Kruskal-Wallis with Dunn's post-test comparing all groups to *Reg3g*^{+/-} *E. fm*. (H) 1-way ANOVA with a Bonferroni post-test comparing all groups to each other. RegIII γ expression shown as a control, all groups significant compared to knock-out but not depicted on the graph. *p 0.05, **p 0.01, ***p 0.001 for all analyses. Comparisons with no (*) or bar had p>0.05 and were not considered significant.

Fig. 5. MyD88 and NOD2 are required for AMP induction and *E. faecium*-mediated protection (A–C) *iVill^{ΔMyd88}* or Cre⁻ (*MyD88^{f/f}*) littermate control mice were injected with tamoxifen 7 days prior to oral gavage with streptomycin. After 24h, mice were gavaged with 10^8 CFU *E. fm* followed 4h later by oral infection with 10^6 CFU *S. Tm*. (A) Weight loss, (B) *S. Tm* bacterial burden in feces, and (C) survival are shown. Pooled data from 3 independent experiments, n=7–11 mice/group. (D) Mice were treated and colonized as in (A–C). At 4 and 72h post-colonization (p.c.), IECs were isolated and analyzed by RQ-PCR for expression of shown genes. Pooled data from 2 independent experiments, n=2–4 mice/

group. **(E–G)** *Nod2*^{–/–} mice or +/- littermate controls were gavaged with streptomycin 24h prior to gavage with 10⁸ CFU *E. fm* followed 4h later by oral infection with 10⁶ CFU *S. Tm*. (E) Weight loss, (F) *S. Tm* bacterial burden in feces, and (G) survival are shown. Pooled data from 4 independent experiments, n=9–12 mice/group. **(H)** Mice were treated and colonized as in (E–G). At 4 and 72h post-colonization (p.c.), IECs were isolated and analyzed by RQ-PCR for expression of shown genes. Pooled data from 2 independent experiments, n=2–4 mice/group. (A, B, E, and F) mean±SEM, 2-way ANOVA, p-value shown comparing *E. fm*-colonized deficient to sufficient controls, n.s.=not significant. (C and G) Log-rank analysis, p-value shown comparing *E. fm*-colonized deficient to sufficient controls. (D and H) mean ±SEM, 1-way ANOVA with Dunnett's post-test comparing deficient to sufficient controls at the same time-point. *p 0.05 and ***p 0.001 for all analyses. Comparisons with no (*) or bar had p>0.05 and were not considered significant.

Fig. 6. The *E. faecium* protein SagaA is sufficient to improve host intestinal barrier function and resistance to *C. difficile*

Mice were given a single gavage of streptomycin followed 24h later by gavage with 10^8 CFU *E. fm*, *E. fs*, or *E. fs-sagA*. (A) At 72h postcolonization, IECs were isolated and analyzed by RQ-PCR. Pooled data from 2 independent experiments, n=2–4 mice/group. (B–E) Intestinal tissues 48h p.i. from mice colonized 4h prior to infection with 10^6 *S. Tm*. (B) Cecum tissue stained with H&E. Representative images, 40X objective, from 1 of 2 independent experiments, n=7 mice/group. (C) Ileum, cecum, and colon tissues were

prepared as in (B) and scored for 4 pathology parameters. Pooled combined pathology scores from 2 independent experiments, n=7 mice/group. (D) FISH staining for all bacteria (universal 16S probe) and epithelial nuclei (Hoechst) from Representative images, 40X objective, from 1 of 2 independent experiments, n=7 mice/group. White arrows indicate bacteria in contact with or invading through the epithelium, and white dashed line designates zone of segregation from bacteria. (E) *S. Tm* bacterial burden in the livers of mice 48h p.i. Pooled data from 3 independent experiments, n=9–11 mice/group. **(F–H)** Mice were given AMNV for 7d and colonized with 10^8 CFU of indicated bacteria 2d prior to oral infection with 10^6 *C. difficile*. (F) Weight loss, (G) *C. difficile* bacterial burden in feces at day 1 p.i., and (H) survival are shown. Pooled data from 3 independent experiments, n=6–9 mice/group. (A) mean±SEM, 1-way ANOVA with Dunnett's post-test comparing all to streptomycin-only controls. (C and E) Mann-Whitney. (F) mean±SEM, 2-way ANOVA, p-value shown comparing *E. fim* or *sagA*-expressing *E. fs* or *L. pl* to WT or vector controls, respectively. (G) 1-way ANOVA comparing all groups. n.s.=not significant. (H) Log-rank analysis, p-value shown comparing *E. fim* or *sagA*-expressing *E. fs* or *L. pl* to WT or vector controls, respectively. *p 0.05, **p 0.01, ***p 0.001 for all analyses. Comparisons with no (*) or bar had p>0.05 and were not considered significant.