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Impact of *Enterococcus faecalis* in the regulation of chronic intestinal inflammation: structure-function relationship in microbe-host interaction.

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"*Don't be trapped by dogma, which is living with the results of other people's thinking. Don't let the noise of others' opinions drown out your inner voice. And most important, have the courage to follow your heart and intuition. They somehow already know what you truly want to become. Everything else is secondary."*

(Steve Jobs)

PUBLICATIONS AND PRESENTATIONS

Peer-reviewed original research articles

Ocvirk S, Sava IG, Lengfelder I, Lagkouvardos I, Steck N, Roh JH, Tchaptchet S, Bao Y, Hansen JJ, Huebner J, Carroll IM, Murray BE, Sartor RB, Haller D. Surface-associated lipoproteins link *Enterococcus faecalis* virulence to colitogenic activity in IL-10-deficient mice independent of their expression levels, *PLOS Pathogens*, 2015 June 12, 11(6): e1004911. doi:10.1371/journal.ppat.1004911

Chang JS, **Ocvirk** S, Berger E, Kisling S, Binder U, Skerra A, Lee AS, Haller D. Endoplasmic reticulum stress response promotes cytotoxic phenotype of CD8αβ+ intraepithelial lymphocytes in a mouse model for Crohn's disease-like ileitis, *Journal of Immunology*, 2012 Aug 1;189(3):1510-20

Oral presentations

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Sava IG, **Ocvirk** S, Steck N, Bao Y, Huebner J, Murray BE, Sartor RB, Haller D. *Enterococcus faecalis* polysaccharide antigen and lipoproteins mediate virulence in chronic inflammation and in infection models. 6th Conference of German Society of Hygiene and Microbiology (DGHM) section "Microbiota, Probiota and Host" (Seeon, Germany) 28/-30/06/2013

Ocvirk S, Steck N, Sava IG, Roh JH, Geillinger KE, Spanier B, Murray BE, Haller D. Additive effects of EpaB and gelatinase on *Enterococcus faecalis* biofilm formation and virulence in *C. elegans* and *G. mellonella*. 64th Conference of German Society of Hygiene and Microbiology (DGHM) (Hamburg, Germany) 30/09/-03/10/2012

Chang JS, **Ocvirk** S, Berger E, Kisling S, Binder U, Skerra A, Lee AS, Haller D. Critical role of ER chaperone Grp78 for the maintenance of intestinal CD8αβ+ T cell homeostasis in chronic intestinal inflammation. 4th Conference of German Society of Hygiene and Microbiology (DGHM) section "Microbiota, Probiota and Host" (Seeon, Germany) 15/-17/05/2011

Poster presentations

Ocvirk S, Sava IG, Steck N, Roh JH, Tchaptchet S, Hansen JJ, Huebner J, Murray BE, Sartor RB, Haller D. Colitogenic activity of *Enterococcus faecalis* requires lipoprotein-mediated activation of innate immune effector functions in IL-10-/- mice. Digestive Disease Week (Chicago, USA) 03/-06/05/2014

Ocvirk S, Sava IG, Steck N, Murray BE, Sartor RB, Haller D. Enterococcal polysaccharide antigen (Epa)-mediated adhesion contributes to virulence of pathobionts in infection and chronic inflammation models. 21st United European Gastroenterology Week (Berlin, Germany) 12/-16/10/2013

Ocvirk S, Sava IG, Steck N, Murray BE, Haller D. Enterococcal polysaccharide antigen (Epa) contributes to virulence of pathobionts in infection and chronic inflammation models. 16th International Congress of Mucosal Immunology (Vancouver, Canada) 17/-20/07/2013

Ocvirk S, Steck N, Sava IG, Roh JH, Geillinger KE, Spanier B, Murray BE, Haller D. Additive effects of EpaB and gelatinase on *Enterococcus faecalis* biofilm formation and virulence in *C. elegans* and *G. mellonella*. 5th Conference of German Society of Hygiene and Microbiology (DGHM) section “Microbiota, Probiota and Host” (Seeon, Germany) 15/-17/05/2012

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Chang JS, **Ocvirk** S, Berger E, Kisling S, Binder U, Skerra A, Lee AS, Haller D. The endoplasmic reticulum chaperone Grp78 links ER stress in intestinal T cells to chronic intestinal inflammation. 49th Scientific Congress of German Nutrition Society (DGE) (Freising, Germany) 14/-16/03/2012

Chang JS, **Ocvirk** S, Berger E, Kisling S, Binder U, Skerra A, Lee AS, Haller D. Critical role of ER chaperone Grp78 for the maintenance of intestinal T cell homeostasis. 15th International Congress of Mucosal Immunology (Paris, France) 5/-9/07/2011

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ABSTRACT

The commensal *Enterococcus faecalis* is among the most common causes of nosocomial infections. Recent findings regarding increased abundance of enterococci in the intestinal microbiota of patients with inflammatory bowel diseases and induction of colitis in IL-10-deficient (IL-10-/-) mice put a new perspective on the contribution of *E. faecalis* to chronic intestinal inflammation. Based on the expression of virulence-related genes in the inflammatory milieu of IL-10-/- mice using RNA-sequencing analysis, we characterized the colitogenic role of two bacterial structures that substantially impact on *E. faecalis* virulence by different mechanisms: the enterococcal polysaccharide antigen (Epa) and cell surface-associated lipoproteins (Lgt).

Germ-free wild type and IL-10-/- mice were monoassociated with *E. faecalis* wild type OG1RF or the respective isogenic mutants for 16 weeks. Intestinal tissues and mesenteric lymph nodes (MLN) were collected to characterize tissue pathology, loss of intestinal barrier function, bacterial adhesion to intestinal epithelium and immune cell activation. Bone marrow-derived dendritic cells (BMDC) were stimulated with bacterial lysates and bacterial adhesion to monolayers of intestinal epithelial cells investigated. *E. faecalis* virulence was tested in three different invertebrate models.

E. faecalis mutants lacking *epaB* or *lgt* showed significantly impaired virulence in *Galleria mellonella* and *Caenorhabditis elegans*. Consistently, the colitogenic activity of wild type *E. faecalis* (OG1RF score: 7.2±1.2) in monoassociated IL-10-/- mice was partially impaired in *E. faecalis* lacking enterococcal polysaccharide antigen (***ΔepaB*** score: 4.7±2.3; $p<0.05$) and was almost completely abrogated in *E. faecalis* deficient for lipoproteins (***Δlgt*** score: 2.3±2.3; $p<0.0001$). Gelatinase E-mediated degradation of E-cadherin in the epithelium was shown for all bacterial strains *in vitro* and in inflamed IL-10-/- but not wild type mice. Inactivation of *epaB* in *E. faecalis* impaired penetration into the colonic mucus layer of IL-10-/- mice and colon-like mucus produced by LS174T cells *in vitro*, reduced microcolony and biofilm formation *in vitro* and altered bacterial adhesion to intestinal epithelium of germ-free *Manduca sexta* larvae. Lipoprotein-deficient *E. faecalis* exhibited an impaired TLR2-mediated activation of BMDCs *in vitro* despite their ability to fully reactivate MLN cells as well as MLN-derived colitogenic T cells *ex vivo*.

E. faecalis virulence factors accounting for bacterial adhesion to mucosal surfaces as well as intestinal barrier disruption partially contribute to colitogenic activity of *E. faecalis*. Beyond their well-known role in infections, cell surface-associated lipoproteins are essential structures for colitogenic activity of *E. faecalis* by mediating innate immune cell activation.

ZUSAMMENFASSUNG

Das kommensale Bakterium *Enterococcus faecalis* gehört als opportunistisches Pathogen zu den häufigsten Ursachen nosokomialer Infektionen. Neue Studien zeigen jedoch einen erhöhten Anteil von Enterokokken in der Darmmikrobiota von Patienten mit chronisch-entzündlichen Darmerkrankungen (CED) und eine wichtige Rolle bei der Entzündungsentwicklung im IL-10-defizienten (IL-10-/-) Mausmodel. Bei einer Expressionsanalyse von Virulenz-assoziierten Genen bei *E. faecalis* aus monoassoziierten IL-10-/- Mäusen fanden wir zwei bakterielle Strukturen, die durch unterschiedliche Mechanismen erheblichen Einfluss auf die Virulenz von *E. faecalis* nehmen: Das Enterokokken Polysaccharid Antigen und Zelloberflächen-assoziierte Lipoproteine.

Keimfreie Wildtyp und IL-10-/- Mäuse wurden mit dem *E. faecalis* Wildtyp OG1RF oder isogenen *E. faecalis* Mutanten für 16 Wochen monoassoziiert. Darmgewebe und mesenteriale Lymphknoten (MLN) wurden gesammelt und auf Gewebspathologie, intestinale Barrierefunktion, bakterielle Adhäsion an das intestinale Epithel und die Aktivierung von unterschiedlichen Immunzellen untersucht. Zudem wurden dendritische Zellen hinsichtlich ihrer Stimulierbarkeit mit *E. faecalis*-Lysaten getestet und die Virulenz von *E. faecalis* in invertebraten Infektionsmodellen charakterisiert.

Beide *E. faecalis* Mutanten zeigten eine verminderte Virulenz in den Infektionsmodellen *Galleria mellonella* und *Caenorhabditis elegans*. Die kolitogene Aktivität von Wildtyp *E. faecalis* (OG1RF Score: 7.2±1.2) in monoassoziierten IL-10-/- Mäusen war bei *E. faecalis* ohne Enterokokken Polysaccharid Antigen teilweise verringert (*ΔepaB* Score: 4.7±2.3; $p<0.05$) und nahezu komplett abwesend wenn Lipoproteine fehlten (*Δlgt* Score: 2.3±2.3; $p<0.0001$). Alle *E. faecalis* Stämme waren in der Lage via Gelatinase E E-cadherin in intestinalen Epithelzellen *in vitro* und im Epithel von IL-10-/- Mäusen abzubauen. Die Deletion von *epaB* führte bei *E. faecalis* zu einer verringerten Mukus-Penetration im Colon von IL-10-/- Mäusen und bei Colon-artigem Mukus von LS174T Zellen *in vitro*, zu einer reduzierten Bildung von Mikrokolonien und Biofilmen *in vitro* und zu einer veränderten Adhäsion an das intestinale Epithel von monoassoziierten *Manduca sexta* Raupen. *E. faecalis* mit einer Defizienz für Lipoproteine, zeigten eine verminderte Aktivierung von dendritischen Zellen über TLR2 *in vitro*, jedoch waren sie in der Lage MLN-Zellen und aus MLN stammende kolitogene T-Zellen vollständig zu (re-)aktivieren.

Virulenz-assoziierte Strukturen tragen über die Vermittlung von Adhäsion an mukosale Oberflächen und Abbau der intestinalen Barriere teilweise zur kolitogenen Aktivität von *E. faecalis* bei. Dagegen stellen Zelloberflächen-assoziierte Lipoproteine, die ursprünglich nur mit bakterieller Virulenz in Verbindung gebracht wurden, durch die Aktivierung innater Immunzellen essentielle kolitogene Strukturen von *E. faecalis* dar.

1. INTRODUCTION

1.1 The intestinal microbiota: Microbe-host interactions in health & disease

Humans have co-evolved with a myriad of microbes including bacteria, eukaryotes, archaea and viruses that colonize their mucosal surfaces, but the significant importance of this microbial community living together with the human host has emerged only recently. In humans the microbiota comprises of over 100 trillions of bacteria, outnumbering the total number of host cells by the factor 10, and an bacterial gene pool (defined as the **microbiome**) that outnumbers host genes by approximately 450-fold [1,2]. During the last years, advances in next generation sequencing techniques allowed culture-independent analyses of microbial communities and shed light on a "virtual organ" in humans: the **intestinal microbiota** [3,4].

The diverse community of bacteria, residing in the lumen of the intestinal tract and colonizing mucosal surfaces, represent a functional equilibrium that maintains mucosal homeostasis by extensive **microbe-host interactions** resulting in a state of mutualistic relationship between the host and gut bacteria. The assembly of the intestinal microbiota is tightly regulated by the host's mucosal immune system to provide a stable ecosystem for commensal bacteria and protect from enteric pathogens [5]. The individual composition of the microbiota is determined by host genetics [6,7] and several other factors such as antibiotics [8] or diet [9,10]. Dietary perturbations seem to have a major impact on the microbiota composition [11] and it is tempting to speculate that particular dietary patterns may set the stage for alterations of the intestinal microbiota favoring disease-relevant bacterial species. Disturbances of the balanced microbe-host relationship resulting in an altered (functional) composition of the intestinal microbiota were linked to pathogenesis and susceptibility of disease settings like autoimmune diseases such as type 1 diabetes [12], metabolic diseases such as obesity [13,14] and type 2 diabetes [15], asthma [16], colon cancer [17] or inflammatory bowel diseases [18,19].

1.1.1 Organization of the intestinal tract: Interface functions & immune homeostasis

The human intestinal tract is colonized by different microbes with lowest density in the stomach and numbers continuously rising to the rectum. Being in permanent contact to microbes and related products, the intestinal mucosa is at the same time barrier and interface to the complex microbiota, absorbing nutrients and maintaining functional immune balance to preserve mucosal homeostasis. From the four main epithelial cell types in the gut, the intestinal epithelium is built of polarized **intestinal epithelial cells** (**IEC**) while goblet

cells produce mucus and enteroendocrine cells are responsible for gastrointestinal hormone production. Paneth cells occur only in the small intestine and are located in the crypt bottom, where they produce antimicrobial peptides and by this restrict access of bacteria to the epithelium. In the colon, the intestinal epithelium is covered by a mucus bilayer, which represents an impenetrable barrier to bacteria as well as a habitat for the commensal microbiota. In the small intestine the mucus layer is loosely connected to the epithelium facilitating the resorption of food constituents and allowing microbes to attach to the epithelial surface. In addition, the mucus contains host-derived reactive oxygen species that regulate bacterial access to the intestinal epithelium. Secretory Immunoglobulin A, which is released by IECs into the lumen, covers the intestinal epithelium and binds to commensal and pathogenic bacteria, maintaining a mutualistic relationship with the microbiota. The monolayer of IECs is connected by tight junctions that are composed of different transmembrane proteins and prevent the passage of luminal contents and microbes to the underlying lamina propria. The lamina propria is connective tissue containing different types of infiltrating immune cells such as dendritic cells, granulocytes or T-cells that build up the host defense against invading pathogens, but also confer tolerance against the normal commensal microbiota and luminal food constituents.

1.2 IBD as paradigm disease for microbe-host interactions

Considering the complex and dynamic relationships between the host, the intestinal microbiota and its metabolites, microbe-host interactions are a critical and tightly regulated prerequisite to induce and maintain mucosal (immune) homeostasis involving innate and adaptive immunity [20]. In addition, this interdependence is influenced by several environmental factors (diet, pathogenic bacteria, antibiotics, aging etc.) and genetic predispositions, making non-physiological alterations of this system an interesting chance to unravel the nature of microbe-host interactions. **Inflammatory bowel diseases** (**IBD**) are an exemplary model to study how the interplay of multigenic contributions to genetic susceptibility and environmental factors shape microbe-host interactions in a chronic intestinal inflammation scenario.

IBD are a heterogeneous group of chronic relapsing inflammatory conditions of the intestine comprising the two main manifestations Crohn’s disease (CD) and Ulcerative Colitis (UC). UC is characterized by inflammation that is restricted to the mucosal surface. UC starts in the rectum and by continuous proximal progression involves the whole colon leading to bloody diarrhea [21]. In contrast, CD is identified by focal lesions that expand to ulcerations extending through the bowel wall. While UC is limited to the colon, CD can occur in any part

of the gastrointestinal tract, but is often exclusively present in specific locations [22]. To date, the prevalence rates for IBD are by far highest in North America and Europe and stabilize in these regions on a high incidence level. During the last decades the incidence of IBD is also dramatically rising in low incidence areas such as developing countries [23], suggesting a strong impact of environmental factors ("westernization" of lifestyle and diet etc.) on disease etiology.

Several lines of evidence suggest that genetic [24] and environmental factors together contribute to IBD pathogenesis by triggering a loss of immune tolerance to the endogenous commensal microbiota [18,19]. In this multifaceted disease, genetic predisposition may set the stage for a defective innate immune response resulting in an altered microbe-host crosstalk that promotes intestinal dysbiosis evoking microbes that potentiate inflammatory processes. In this study, we focus on two main aspects of genetically shaped microbe-host interactions relevant in IBD pathogenesis**: Bacterial adhesion to mucosal surfaces** and **innate immune recognition of bacterial antigens**, especially of bacterial lipoproteins.

1.3 IBD susceptibility genes

To date, more than 160 single nucleotide polymorphisms (SNP) have been identified to be associated with increased risk for IBD [24]. Several of the loci associated with IBD appear in CD and UC, highlighting the shared basis of multigenic contributions to IBD risk. Many of these gene loci are involved in directing host responses to gut microbes including intestinal barrier integrity, innate immune defense and bacterial recognition as well as immune balance, autophagy and cellular stress responses [24]. This points to an important role for the intestinal microbiota in IBD pathogenesis, but the specific contribution of single risk factors to IBD-relevant microbe-host interactions remains unclear.

In the following chapters, selected key determinants that direct intestinal microbe-host interactions with focus on bacterial adhesion and innate immune recognition of bacterial antigens in genetically susceptible individuals are explained in more detail.

1.3.1 IBD susceptibility genes affecting the intestinal barrier

Playing a pivotal role in restricting access of the luminal milieu to the underlying host compartments, the **mucosal barrier** is build up by a tightly connected layer of IECs. The importance of this intact barrier is indicated by the aberrant intestinal permeability occurring in many IBD patients [25,26] and identified IBD-associated loci linked to barrier integrity [27]. For example, a polymorphism in the *CDH1* gene encoding for E-cadherin, which is an

adherens junction-regulating cell adhesion molecule expressed by IECs, is associated with CD resulting in mis-localized and impaired barrier integrity [28]. Consistently, a degradation of E-cadherin in colonic IECs by a bacterial protease exacerbates intestinal pathology in a mouse model of spontaneous colitis [29]. As another example, the expression of the protein tyrosine phosphatase N2 (PTPN2) is increased in biopsy specimens from CD patients and protects from IFN-γ-induced expression of claudin-2 [30], which is a pore-forming tight junction protein believed to contribute to increased intestinal permeability in IECs [31]. Mice deficient in *Ptpn2* elicit increased susceptibility to dextran sodium sulfate (DSS)-induced colitis [32]. As a subsequent result of an increased intestinal permeability in intestinal inflammation, increased levels of systemic antibodies directed against specific bacterial components can be detected in IBD patients [33–35].

Besides barrier function, the proper mediation of contact between IECs and microbes is critical to maintain mucosal homeostasis. The intestinal epithelium is covered by a **mucus bilayer** containing mucus glycoproteins, immunoglobulins and antimicrobial peptides. The mucin MUC2 is the main structural component of the colonic mucus and maintains the spatial organization of the impenetrable inner layer devoid of bacteria [36,37] and an outer layer that serves as habitat for the microbiota [37]. A loss of *Muc2* results in severe spontaneous colitis in mice [38]. An altered profile and impaired expression of Mucin-type O-linked oligosaccharides is observed in UC patients [39,40] and an IEC-specific deletion of this primary mucin component induces spontaneous colitis [40] and altered microbiota composition in mice [41]. Biopsies of inflamed mucosal tissue from IBD patients have thinner mucus layers compared to non-inflamed or healthy controls [42] and show increased numbers of bacteria penetrating the normally sterile inner mucus layer [43]. Importantly, the inner mucus layer is penetrable for bacteria in UC patients as well as in different mouse models of chronic colitis, for example in the IL-10-deficient (IL-10-/-) mouse [44]. MUC2 expression levels are significantly reduced in IL-10-/- mice [45] and a double-knockout of *Il-10* and *Muc2* exacerbates intestinal pathology [46]. It was shown that IL-10 directs mucus production of goblet cells [47] via prevention of protein mis-folding and endoplasmic reticulum (ER) stress. ER stress is induced by aberrant mucin assembly in mice resulting in spontaneous UC-like inflammation [48]. Importantly, the mucus-associated microbiota differs between IBD patients and healthy subjects regarding composition [49], with increased numbers of mucolytic bacteria in IBD patients [50], and the ability to penetrate the inner mucus layer [51], highlighting the importance of a functional mucus layer as interface to luminal factors.

The intestinal mucosal surfaces are covered by **Immunoglobulin A** (**IgA**), which is produced by plasma cells in the lamina propria and after transcytosis by IECs is secreted into the

lumen, where it binds commensal and pathogenic bacteria. This coating of bacteria is an integral part of the mucosal immune barrier and protects from enteric pathogens by neutralization and immune exclusion [52]. It is hypothesized that a detection of enteric pathogens by the intestinal immune system results in a production and secretion of high-affinity and pathogen-specific IgA when compared to the IgA production induced by commensal bacteria [53]. A recent study demonstrated that high IgA coating of commensal bacteria species isolated from IBD patients or a colitis mouse model correlated with their colitogenic activity and penetration capacity of the inner colon mucus layer in mice [51]. For example, IgA levels specific for the commensal bacterium *Enterococcus* (*E.*) *faecalis* are significantly increased in IBD patients [54]. The association of germ-free mice with members of the intestinal microbiota that induced strong IgA production in IBD patients exacerbated DSS-mediated colitis when compared to bacteria without strong IgA coating [51], suggesting that specific members of the commensal microbiota stimulate intestinal immunity and exert colitogenic traits in genetically susceptible individuals.

Being integral parts of the intestinal immune defense, host-derived **reactive oxygen species** and **antimicrobial peptides** (**AMP**) restrict bacterial proliferation and bacterial adhesion in close proximity to the intestinal epithelium. For example, hydrogen peroxide is released to the lumen by the membrane-associated NADPH oxidase, dual oxidase2 (DOUX2), which expression is highly conserved in the human gastrointestinal tract [55] and induced by commensal bacteria in mice [56]. *DOUX2* is among the risk loci for CD [57] and mice lacking DUOX in the gastric epithelium fail to restrict *Helicobacter felis*-colonization and related gastric inflammation [58], suggesting an important role of DOUX enzymes in mucosal immune defense. AMPs such as α-defensins, c-type lectins and lysozyme are secreted by Paneth cells and are important mediators of microbe-host interactions, which elicit antimicrobial effects and direct bacterial colonization of the mucosa [59] and microbiota composition in close proximity to the intestinal epithelium [60,61]. A defective AMP production by Paneth cells [62] as well as defects in Paneth cell functionality such as autophagy (*ATG16L1*) [63] were linked to increased IBD susceptibility.

1.3.2 IBD susceptibility genes affecting innate immune recognition

In order to maintain mucosal immune homeostasis, host cells need to differentiate between the commensal microbiota and pathogenic bacteria that pose a threat for the host. IECs and professional innate immune cells residing in the lamina propria exhibit a repertoire of different receptors to sense microbial structures from the lumen. These different **pattern recognition receptors** (**PRR**) are either cytoplasmic (NOD-like receptors, for example) or secreted

proteins (complement receptors, for example) or are expressed on the cell surface (C-type lectin receptors, Toll-like receptors, for example). PRRs play a crucial role in orchestrating pro-/anti-inflammatory innate immune responses directing underlying adaptive immunity. Genetically triggered dysfunctional PRR signaling was shown to promote altered mucosal homeostasis and aberrant microbe-host interactions – mechanisms that are also implicated in IBD pathogenesis.

Being the first identified IBD susceptibility gene, the **nucleotide oligomerization domain 2** (**NOD2**) of *CARD15* gene is a member of an intracellular receptor family that senses muramyl dipeptide (MDP), an integral part of peptidoglycan present in all Gram-negative and Gram-positive bacteria. Several sequence variants in the *NOD2* gene were described to be associated with increased susceptibility to CD in humans [64,65]. The predominant *NOD2* variants found in CD patients were shown to exert impaired MDP sensing [66–68]. In the small intestine of mice, NOD2 was reported to control the composition of the commensal microbiota [69] and *Nod2* deletion was associated with inability to control bacterial pathogens such as *Listeria monocytogenes* or *Helicobacter hepaticus* due to defective NOD2-dependent expression of α-defensins [62,70]. In the large intestine, NOD2-deficient (NOD2-/-) mice carrying a frameshift mutation are more susceptible to *Citrobacter rodentium*-induced colonic inflammation [71] and DSS treatment [72], while a complete deletion has no impact on DSS colitis development [70,73]. NOD2-/- mice are more susceptible to trinitrobenzene sulfonate (TNBS)-induced colitis [74] and induction of experimental antigen-specific colitis [75]. In addition, NOD2-/- mice are protected from inflammation by enhanced bacterial recognition through NOD2 in the virally pre-infected host [76] and *E. faecalis*-induced systemic infection [71]. Although mutations in *NOD2* as well as in several NOD2 signaling genes are associated with CD, the exact functional contribution of the NOD2 receptor to CD pathogenesis still needs to be mechanistically dissected.

Toll-like receptors (**TLR**) are important mediators of microbe-host interactions that maintain intestinal epithelial homeostasis and likely play an important role in IBD immunopathology [77]. TLRs are trans-membrane PRRs and are differently expressed by various cell types throughout the whole gastrointestinal tract including IECs, Paneth cells, where TLRs regulate the expression of AMPs [78], goblet cells and several immune cell subsets residing in the lamina propria, such as dendritic cells and macrophages [79]. The expression pattern of TLRs is controlled by several factors including endogenous commensal microbiota and exposition to ligands, cell type, activation and differentiation of cells [77,80]. This makes TLRs a key intermediator between innate and adaptive immunity and a balanced regulation of TLR responses to microbial stimuli in the lumen is critical to avoid aberrant immune activation. Due to the increased exposure of bacterial-derived TLR ligands in chronic

intestinal inflammation [81], stimulation of TLRs may contribute to propagation and/or modulation of pro-inflammatory responses directed against bacterial structures from the commensal microbiota.

For example, the **TLR5**-agonist bacterial flagellin was identified as a key antigen in CD stimulating intestinal pathology in mice [35,82] and promoting TLR5 expression in DSS-treated mice after oral infection with virulent *Escherichia* (*E.*) *coli* strains [83]. Mice lacking TLR5 show spontaneous colitis [84], but also attenuated intestinal inflammation after DSS-treatment [85]. SNPs in the *TLR5* gene were shown to be associated with human and canine IBD [86,87].

TLR4 is the pre-dominant receptor for bacterial lipopolysaccharide (LPS) [88–90] and constitutively expressed on IECs and lamina propria mononuclear cells in the intestine [91–93]. In IBD patients, TLR4 and -associated signaling molecules are upregulated [94–96] and several polymorphisms are associated with increased risk for IBD [97,98]. TLR4 deficiency or blocking leads to diminished tissue repair in DSS-induced colitis in mice [77,99,100], which may be caused by TLR4-induced anti-inflammatory cyclooxygenase-2 (Cox-2) expression in macrophages [101]. In addition, TLR4 expression is enhanced by pro-inflammatory cytokines IFN-γ and TNF [102,103] and an aberrant TLR4 response is triggered by TLR5-mediated altered microbiota composition [84], highlighting the interdependence of the host immune response and microbial factors in the context of intestinal inflammation.

TLR2 is constitutively expressed at basal level in IECs and mononuclear cells residing in the lamina propria [92,95,104,96]. TLR2 expression is also regulated in response to the endogenous microbiota [80] and bacterial lipoproteins were described as predominant immunobiologically active agonists of TLR2 [105,106]. TLR2 expression levels are significantly increased in the intestinal mucosa of IBD patients [95,107,108]. Lamina propria mononuclear cells and peripheral blood mononuclear cells isolated from CD or UC patients show increased TLR2 expression and secrete more TNF in response to TLR2 stimulation when compared to healthy controls [96,109]. Emerging evidence from genetic studies associate a TLR2-R753Q polymorphism with severe pancolitis in UC patients [110]. A loss of TLR2 results in impaired intestinal barrier integrity, defective protection from inflammation-induced apoptosis in IECs and impaired TLR2-dependent protective effects by trefoil factor 3 derived from goblet cells [111,112]. Consistently, TLR2-deficient (TLR2-/-) mice are more susceptible to DSS-induced colitis [77,111,112]. However, it was also reported that TLR2-/- mice are less susceptible to DSS-induced colitis [113], suggesting a heterogeneous functionality of TLR2 in the pathogenesis of IBD.

Taken together, these findings emphasize the importance of a balanced TLR signaling in the mucosal innate immune compartment directing subsequent mucosal immunopathology in chronic intestinal inflammation. The conflicting roles of TLRs demonstrated in mouse models of chronic intestinal inflammation may derive from the differential expression of TLRs by various cell types: TLR expression by IECs is implicated in protection of barrier functions, while TLRs expressed by underlying lamina propria immune cells trigger pro-inflammatory functions [114]. However, it should be mentioned that the differences in microbiota composition in TLR-deficient mice might be caused by breeding/housing conditions rather than TLR-deficiency [115].

The anti-inflammatory cytokine **interleukin-10** (**IL-10**) is an important mediator of mucosal homeostasis affecting also TLR-dependent microbe-host interactions. A polymorphism in the *IL-10* gene is associated with UC [116] and mutations in the IL-10 receptor are associated with intestinal hyper-inflammation resulting in early-onset colitis [117]. Mice deficient for IL-10 develop spontaneous colitis [118] and are a valuable model to mimic conditions of human chronic colitis [119]. In IL-10-/- mice the experimental colitis is mediated by an excessive aberrant immune response by Th1/Th17 cells that triggers the production of pro-inflammatory cytokines such as IFN-γ, IL-6 and IL-17 in the lamina propria [120–122].
Recent evidence suggests that TLR signaling is linked to intestinal inflammation observed in IL-10-/- mice: TLR4 deficiency increases inflammation in IL-10-/- mice [123,124]. However, inflammation in the IL-10-/- mouse model is abrogated when TLR-downstream adaptor protein MyD88 and dependent NF-kB signaling is missing [114,125], suggesting a differential role of TLRs in experimental colitis in IL-10-/- mice. Importantly, the development of spontaneous colitis in IL-10-/- mice is dependent on the presence of resident commensal bacteria [126] such as *E. coli* and *E. faecalis* [127] highlighting the essential contribution of microbiota-derived TLR signaling to colitis pathogenesis in IL-10-/- mice.

1.4 The impact of microbiota in IBD

While IBD susceptibility genes and pathways seem to have important functions in triggering the onset of disease, these risk loci are not likely to be sufficient for disease initiation in most cases. This is demonstrated in monozygotic twins concordant for UC or CD, where the concordance rate was higher than the average prevalence rates of the general population, but only ranging between 16% (UC) and 35% (CD) [128]. In addition, risk variants such as *NOD2* or the polymorphism T300A in *ATG16L1*, which is strongly associated with ileal CD [129,130] and implicated in clearance of IBD-related opportunistic pathogens [131], are also carried by healthy individuals [130]. Hence, the contributions of environmental factors to IBD

pathogenesis, such as diet or the intestinal microbiota, play a decisive role as disease trigger and/or modifier in the genetically predisposed host.

Several lines of evidence demonstrate that the intestinal microbiota is an essential factor in IBD pathogenesis: For example, IL-10-/- mice that are genetically susceptible for chronic colitis remain free of intestinal pathology under germ-free conditions [126]. Antibiotics represent a partially effective treatment of chronic intestinal inflammation altering the intestinal microbiota in IBD patients [132] and ameliorating CD-like ileitis in the $TNF^{\Delta ARE}$ mouse model [133]. The aberrant mucosal immune response in IBD is directed against the endogenous commensal microbiota [33,134] and diet-induced amelioration of DSS-induced colitis in mice is abrogated under germ-free conditions [135].

1.4.1 Dysbiosis in IBD

It is generally accepted that a disruption of the mutual relationship between the host and the endogenous intestinal microbiota, resulting in **intestinal dysbiosis**, is implicated in IBD etiology.

IBD-related intestinal dysbiosis is characterized by reduced microbial diversity including an expansion of potentially harmful bacteria (see chapter 1.5) and loss of beneficial bacteria [136]. Being the most consistent observation regarding microbial patterns associated with IBD, many studies demonstrated an overall reduced alpha-diversity of the intestinal microbiota in IBD with depletion of and reduced diversity of several taxa in the Firmicutes phylum and increased numbers of several members of Gammaproteobacteria [137–140]. Observations regarding shifts in the Bacteriodetes phylum are inconsistent in these studies. Beyond taxonomic profiling, alterations in metabolically important groups or specific members of the microbiota suggest a potential impact of dysfunctional bacterial metabolism by the disease-relevant microbiota [141]. For example, most studies report a depletion of butyrate-producing bacteria (butyrate is the primary energy source for colonic IECs) such as *Faecalibacterium prausnitzii* [142,143] or an increase of sulfate-reducing bacteria, such as *Desulfovibrio*, that show toxic effects on IECs via hydrogen sulfite production [144,145].

Observations from twins discordant for CD or UC confirmed the reduced bacterial diversity under disease conditions and demonstrated a correlation with an altered transcriptional profile of mucosal gene expression, suggesting impaired microbe-host interactions in the inflamed intestine [146,147]. Indicating a causal relationship of intestinal dysbiosis and chronic inflammation, the disease-associated microbiota isolated from $TNF^{\Delta ARE}$ mice, a mouse model of CD-like ileitis, but not from healthy mice induces experimental inflammation when transferred to germ-free $TNF^{\Delta ARE}$ mice [133]. But it remains unclear, if altered microbial compositions, observed in IBD patients and shaped by defective host genetics, in fact

promote intestinal inflammation or if the intestinal immunopathology leads to disease-associated but not -relevant alterations in microbiota assembly? In this context, several factors need to be considered when evaluating the fundamental impact of a perturbed microbiota in IBD pathogenesis: The microbiota community is unstable in IBD patients [148] and besides differences in sample type (biopsy or stool) and location of inflammation (ileal CD or colonic CD) [146], the microbiota differs between active and remission stages of disease [148,149]. In addition, antibiotic treatment amplifies a dysfunctional microbiota in CD patients [150] and inflammation itself impacts on the mucosa-associated microbiota resulting in altered composition compared to non-inflamed sites [151].

1.4.2 Dysbiosis - caused by genetic predisposition?

Since several genetic risk factors for IBD are involved in directing host responses to gut microbes, a disease-relevant microbiota can derive from genetically triggered immune responses. For example, IBD-associated risk alleles for *NOD2* and *ATG16L1* are associated with abnormal alterations in the human intestinal microbiota [152]. Again, the IBD susceptibility gene *NOD2* is an exemplary model to demonstrate how a genetically predisposed host immune response can be linked to complex disease-relevant alterations of the intestinal microbiota: Although showing no spontaneous disease phenotype, NOD2-/- mice exhibit an intestinal dysbiosis with increased loads of commensal bacteria in the terminal ileum and attenuated ability to prevent colonization with pathogenic bacteria [69]. This is followed by an altered microbiota composition, less diverse, but with increased numbers of Bacteroidetes and Firmicutes [153] and a depletion of Proteobacteria in the feces [154].

In summary, a genetic predisposition can contribute to the development of a disease-relevant microbiota, which *per se* is not sufficient to cause IBD and may only be a by-product of intestinal immunopathology. In addition to the cause-or-consequence relationship for dysbiosis in IBD it is unclear, if a disease-relevant microbiota depends on complex compositional perturbations or can be scaled down to few colitogenic species as ‘major driver’ of pathogenesis of intestinal inflammation?

1.5 The conceptual model of ‘pathobionts’ in IBD

Recent studies have emphasized the conceptual model of ‘symbionts’ and ‘**pathobionts**’ of the human commensal microbiota for disease conditions such as periodontitis [155] and chronic intestinal inflammation [156,157]. Both being members of the endogenous human microbiota, symbionts are beneficial microbes with mutualistic traits and pathobionts include opportunistic pathogens of the commensal microbiota that can elicit detrimental effects in

genetically susceptible individuals or under specific environmental conditions. One can argue about the simplistic approach of this concept classifying chronic intestinal inflammation as an equation of mutualistic, commensal and pathogenic microbial traits. But this concept raises legitimate questions regarding the multiple roles and wide-ranging contributions of commensal intestinal bacteria to IBD pathogenesis. Opportunistic pathogens are important triggers of infectious inflammation, but they might also contribute to pathogenesis of IBD targeting genetically susceptible populations. A close look at the 'dualistic character' of commensal bacteria that exert colitogenic traits under specific conditions may provide exemplary insight to the complex interdependence of the intestinal microbiota and the genetically predisposed host. In this context, the determination of novel mechanisms, by which opportunistic pathogens shape microbe-host interactions in chronic intestinal inflammation, and the identification of bacterial virulence-related structures with critical colitogenic function are key challenges.

1.5.1 Pathobionts implicated in IBD pathogenesis

Opportunistic pathogens shown to exert detrimental effects in susceptible mouse models include: *Helicobacter* (*H.*) *hepaticus* is a Gram-negative member of the intestinal microbiota with pathogenic potential that is able to regulate mucosal homeostasis [158] and is capable of triggering colitis in IL-10-/- mice in combination with specific microbiota composition [159,160]. Interestingly, the human symbiont *Bacteroides fragilis* is able to protect mice from *H. hepaticus*-induced experimental colitis via secretion of polysaccharide A that modulates host immune homeostasis [159]. One member of the microbiota that triggers *H. hepaticus*-induced colitis is *Bilophila wadsworthia* [161], which is resident in the gut lumen of healthy humans [162], but can also trigger colitis in IL-10-/- mice and may set the stage for intestinal dysbiosis after exposure to a diet rich in saturated fat [161]. Highlighting the dysregulated immune response to opportunistic pathogens of the commensal microbiota, CD patients homozygous for the IBD-associated *ATG16L1-T300A* risk allele show impaired clearance of pathobionts including Fusobacteria species [131].

Considering the spatial organization of the intestinal microbiota, mucosa-attached bacteria clearly play an essential role in initiation and perpetuation of inflammatory processes. Fusobacteria are Gram-negative bacteria that are known to reside in the oral cavity and in the gut of humans. Compared to healthy controls, increased numbers of different *Fusobacterium spp.* were detected in biopsies from either UC or CD patients, respectively [163,164]. Fusobacteria elicit invasive potential to colonic IECs [165], which is more prevalent in isolates from CD patients compared to healthy individuals [164].

Adherent-invasive *E. coli* (**AIEC**), an *E. coli* pathotype, are implicated in CD pathogenesis [166] occurring mainly in the ileal mucosa of CD patients [167]. While AIEC are also found in the intestinal mucosa of healthy individuals [166,167], the pattern is inconsistent for UC [167,168]. Demonstrating several pathogenic features, AIEC adhere to intestinal epithelial cells [169], exhibit invasive potential [170] and form biofilms on IECs [171,172]. Being an exemplary model to demonstrate the dynamic interplay of genetically driven host immunity, the commensal microbiota and pathobionts, AIEC manipulate the mucosal milieu at various levels: It was demonstrated that AIEC are able to impair intestinal barrier integrity in mice by triggering pore-forming claudin-2 expression in IECs [173] or by redistribution of E-cadherin and ZO-1 in an IEC monolayer *in vitro* [174,175]. Inflammation-related host factors such as the carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6), which is a glycoprotein highly expressed in ileal IECs of CD patients, act as receptors for AIEC facilitating the own adhesion capacity [176]. In addition, AIEC fuel chronic colitis by altering the intestinal microbiota in genetically susceptible mice lacking the flagellin receptor TLR5 [177] and take advantage of western diet-mediated dysbiosis promoting colonic inflammation in transgenic mice expressing human CEACAMs [178].

While the monoassociation of IL-10-/- mice with commensal *E. coli* induces spontaneous colitis in the proximal colon, the monoassociation with commensal ***E. faecalis*** results in severe distal colitis [127]. In a previous study, we were able to assign colitogenic activity to a known virulence factor of *E. faecalis* contributing to intestinal inflammation in IL-10-/- mice [29], suggesting that not only single bacteria, but specific bacterial structures are essential determinants and vital modulators of colitis pathogenesis shaping microbe-host interactions in genetically susceptible mouse models. Consequently, known putative virulence factors of these commensal bacteria need to be reconsidered in the context of IBD pathogenesis. Here, we use *E. faecalis* as a ‘pathobiont model organism’ to unravel the impact of microbe-host interactions in the regulation of chronic intestinal inflammation.

1.6 *Enterococcus faecalis* as model organism for pathobionts in IBD

The Gram-positive commensal *E. faecalis* is a member of the human intestinal core microbiota [1], but is also known for harboring several putative virulence genes mediating its pathogenicity [179]. The ability to acquire antibiotic resistance genes [180] and the emerging importance in nosocomial infections [181,182] highlight its role as an opportunistic pathogen. Several studies demonstrated the ‘dualistic character’ of *E. faecalis* under conditions of chronic intestinal inflammation making this an exemplary model to analyze bacterial virulence-related structures with potential colitogenic function in microbe-host interactions.

Fecal samples from CD patients show higher numbers of enterococci [183,184], especially of *Enterococcus faecium* [185], and UC patients have increased mucosal growth of *E. faecalis* correlating with high titers of *E. faecalis*-specific IgG antibodies [186] and disease severity [187]. In contrast to healthy controls, levels of systemic IgA specific for *E. faecalis* were significantly increased in IBD patients, but not for *E. coli* and other commensal bacteria [54]. Consistently, *E. faecalis* isolates from IBD patients are more likely to harbor virulence-related genes and activity [188]. In IL-10-/- mice, which is a valuable model to mimic conditions of human chronic colitis [119], monoassociation with *E. faecalis* induces severe intestinal inflammation [189]. This *E. faecalis*-driven inflammation is limited to the distal colon [127] and characterized by defective resolution of pro-inflammatory gene expression in the intestinal epithelium of IL-10-/- mice [190].

1.6.1 Colitogenic activity of E. faecalis virulence factors

Steck et al. recently identified that the zinc-dependent metalloprotease **gelatinase E** (**GelE**), a known virulence factor of *E. faecalis*, partially impairs intestinal epithelial barrier integrity in IL-10-/- mice [29]. Since the loss of GelE did not result in total diminution of intestinal inflammation, we screened for additional virulence factors expressed by *E. faecalis* under conditions of chronic inflammation. To identify which *E. faecalis* virulence factors exert significant colitogenic activity in a predisposed host, we targeted two genes that have been linked to virulence in different infection models such as *Galleria* (*G*.) *mellonella* and *Caenorhabditis* (*C*.) *elegans* (Table 1) and are thought to be critical for *E. faecalis* interaction with the host during chronic colitis. These include *epaB* from the enterococcal polysaccharide antigen cluster and *lgt*, a prolipoprotein diacylglyceryl transferase, which both are known to substantially impact on *E. faecalis* virulence by different mechanisms.

1.6.2 Enterococcal polysaccharide antigen

First, the **enterococcal polysaccharide antigen** (**Epa**) locus consists of 18 genes (*epaA*-*epaR*) encoding for enzymes and transporters that are sequentially involved in bacterial cell wall polysaccharide metabolism [191]. The *epa* gene cluster is suggested to synthesize a rhamnopolysaccharide (Fig.1A), which together with wall teichoic acid forms the secondary wall polysaccharides [192]. Epa confers protection against several different stress factors in *E. faecalis* [193] and is important for intestinal colonization capability of *E. faecalis* in a mouse model of transient colonization [194]. The *epaB* gene encodes a putative glycosyl transferase that mediates transfer of rhamnose to cell wall polysaccharides and seems to be critical for enterococcal shape determination [195]. Disruption of *epaB* results in reduced

virulence in mouse peritonitis [196] and urinary tract infection models [197], attenuated translocation across polarized human enterocyte monolayers [198], impaired resistance to polymorphonuclear leukocyte killing [199] and to phagocytosis in a zebrafish larva infection model [200].

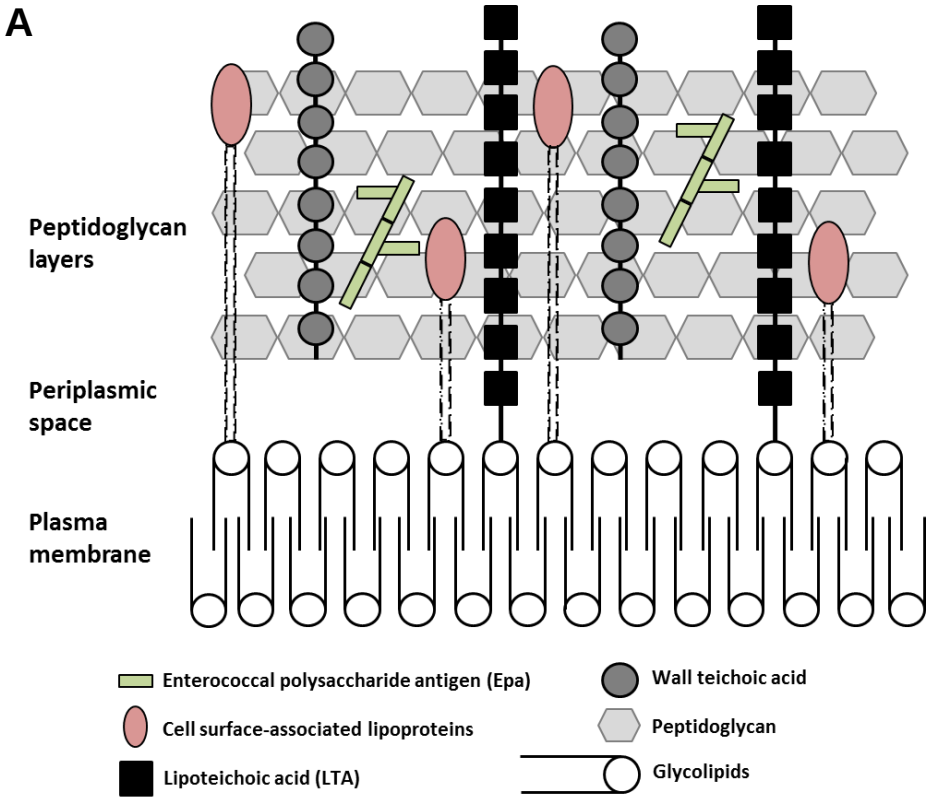

Figure 1: Schematic presentation of the cell wall of *E. faecalis*.

(**A**) Composition of cell wall constituents in Gram-positive *E. faecalis* OG1RF (adopted from [260]). The enterococcal polysaccharide antigen (**Epa**) is a rhamnopolysaccharide of the secondary wall polysaccharides of *E. faecalis*. The prolipoprotein diacylglyceryl transferase (**Lgt**) contributes to maturation of cell surface-associated lipoproteins.

1.6.3 Bacterial lipoproteins

Second, the **prolipoprotein diacylglyceryl transferase** (**Lgt**) contributes to maturation of bacterial lipoproteins (Fig.1A) by mediating the transfer of a diacylglyceryl moiety to conserved cysteine residues in Gram-positive bacteria [201]. Loss of Lgt results in enhanced growth of *E. faecalis* under oxidative stress or high Mn2+ concentrations *in vitro* and impaired virulence in an invertebrate infection model [202]. Although some lipoprotein-deficient Gram-positive pathogens show hypervirulent phenotypes [203,204], most of them have attenuated virulence [205] and impair TLR2-mediated activation of immune cells [203,206,207].

Table 1: Virulence factors identified to be relevant for colitogenic activity of *E. faecalis* in the IL-10-/- mouse model and their proposed cellular mechanisms

***E. faecalis* virulence factor (*locus*)**	**Identification of virulence activity**	**Identification of colitogenic activity**	**Cellular mechanisms of virulence relevant for colitogenic activity of *E. faecalis***
Secreted protease Gelatinase E (***gelE***)	*G. mellonella* [208] *C. elegans* [209] Zebrafish [200]	IL-10-/- mouse model [29]	**Degradation of E-cadherin at the intestinal barrier** [29]
Epa rhamno-polysaccharide (***epaB***)	Mouse infection models [196,197] *G. mellonella* [210] (confirmed by this study) *C. elegans* (this study) Zebrafish [200]	IL-10-/- mouse model (this study)	**Adhesion** to colonic epithelial cell surface *in vitro* and intestinal epithelium of *M. sexta* and colonic mucus penetration (all this study) **Biofilm formation** on abiotic surfaces ([211], this study), on colonic epithelial cell monolayer (this study) and biofilm-associated microcolony formation (this study) *Not investigated in this study:* **Phagocytosis resistance** [199,200] **Translocation** [198] *Not confirmed by this study:* **Intestinal colonization** [194]
Cell surface-associated lipoproteins (***lgt***)	*G. mellonella* (31) *C. elegans* (this study)	IL-10-/- mouse model (this study)	**Innate immune activation via TLR-2** (this study)

Enterococcus (*E.*) *faecalis*; Enterococcal polysaccharide antigen (Epa); *Galleria* (*G.*) *mellonella*; *Caenorhabditis* (*C.*) *elegans*; *Manduca* (*M.*) *sexta*.

1.7 Aim of the study

Although changes in composition, diversity and function of the intestinal microbiota were demonstrated in IBD patients, the specific contributions of individual bacteria and their virulence-relevant structures to chronic intestinal inflammation remain mainly unclear. Consequently, known putative virulence factors of commensal bacteria need to be reconsidered in the context of IBD pathogenesis. The investigation of colitogenic structure-function relationships in genetically susceptible mouse models is a helpful experimental system to understand the pathogenesis of this complex human disease.

In this study we aim at unraveling novel colitogenic activity of bacterial virulence factors in the ‘pathobiont model organism’ *E. faecalis* that are independent of GelE. For this reason, we monoassociate germ-free wild type and IL-10-/- mice with *E. faecalis* wild type OG1RF or isogenic mutants that lack either *epaB* or *lgt*. In addition, we use different invertebrate model systems and *in vitro* assays to investigate structure-function relationships. We are able to establish a novel correlation of intestinal inflammation and the presence of enterococcal polysaccharide antigen or cell surface-associated lipoproteins. We identify bacterial adhesion to mucosal surfaces as a factor contributing to *E. faecalis* colitogenic activity and bacterial cell surface-associated lipoproteins to be essential in mounting colitogenic activity of antigen-primed T cells in the colonic mucosa of IL-10-/- mice. Using these isogenic mutant strains we identify bacterial structures with critical colitogenic function and determine novel mechanisms by which opportunistic pathogens direct microbe-host interaction in the context of experimental colitis.

2. MATERIAL AND METHODS

2.1 Bacterial strains and growth conditions

2.1.1 Growth conditions

E. faecalis strains used in this study (Table 2) were cultivated in Brain Heart Infusion (BHI) broth (BD, Sparks, MD, USA) or on BHI agar (Roth, Karlsruhe, Germany) over night (ON) at 37°C under aerobic conditions with shaking at 160 rpm, unless otherwise indicated. For all assays, *E. faecalis* were taken from stationary phase ON culture. Growth rates of *E. faecalis* strains were assessed with a Bioscreen C (Oy Growth Curves, Helsinki, Finland) at standard conditions (37°C, aerobic conditions with shaking at 160 rpm) and automatically detected every 30 min by measuring the optical density (OD) at 600 nm.

The isogenic mutants for *epaB* and the reconstituted *epaB* strain were generated in *E. faecalis* OG1RF and provided by the Division of Infectious Diseases of the University of Texas (Houston, USA). The isogenic mutants for *lgt* and the reconstituted *lgt* strain were generated in *E. faecalis* OG1RF and provided by the Division of Infectious Diseases of the University Medical Center Freiburg, Germany. Please see reference [212] for details on how *E. faecalis* mutants were generated.

Table 2: *E. faecalis* strains used in this study

Strain	Characteristics (Abbreviation)	Reference
OG1RF	Wild type strain, isolated from the human oral cavity.	[213]
TX5264	OG1RF *gelE* in-frame deletion mutant (*ΔgelE*)	[209]
TX5692	OG1RF *epaB* deletion mutant (*ΔepaB*)	This study.
TX5706	Reconstituted OG1RF *epaB* deletion mutant (*ΔepaB rec.*)	This study.
Δlgt	OG1RF *lgt* deletion mutant (*Δlgt*)	This study.
rec. *Δlgt*	Reconstituted OG1RF *lgt* deletion mutant (*Δlgt rec.*)	This study.

2.1.2 Generation of bacterial lysates

E. faecalis from stationary phase ON cultures (n=2-3 cultures/*E. faecalis* strain) were washed once with phosphate-buffered saline (PBS) (Sigma-Aldrich, St. Louis, MO, USA) and resuspended in 500 µL PBS. The suspension was transferred to 2 mL micro tubes (Sarstedt, Nümbrecht, Germany) together with 250 mg zirconia glass beads (Roth, Karlsruhe, Germany). The lysates were prepared with four cycles for 45 sec each in a bead beater always followed by 45 sec cooling on ice, centrifuged to collect the supernatant free of glass beads and filter sterilized. Finally, the protein concentration was detected by Bradford assay

with a Roti-Nanoquant-kit (Roth, Karlsruhe, Germany) according to the manufacturer's instructions.

2.2 Animal experiments

2.2.1 Invertebrate models

Galleria mellonella infection model

G. mellonella larvae (HW-Terra KG, Wirtsgrund, Germany or Evergreen, Augsburg, Germany) were infected with the different *E. faecalis* wild type and mutant strains. *E. faecalis* strains were cultivated ON under routine conditions in 10 mL BHI broth and washed once with PBS. *G. mellonella* larvae were sorted by weight and each larva infected at the second to last proleg with 5 µL bacterial suspension containing $2x10^5$ colony forming units (CFU) or 5 µL PBS as control using a 20 µL syringe and 0.210 x 51 mm needles (both from Hamilton Bonaduz AG, Bonaduz, Switzerland). Per group, 30 larvae were incubated at 37°C in petri dishes containing litter. Insect mortality was monitored at 3 to 6 hours intervals for up to 3 days post infection.

Caenorhabditis elegans infection model

C. elegans (wild type strain Bristol N2, which was a generous gift from the Chair of Nutritionional Physiology of the Technical University of Munich) were maintained and cultivated as previously described [214]. For nematode infection, *E. faecalis* strains were cultivated ON under routine conditions in 10 mL BHI broth. After washing once with PBS, bacteria cultures were 10-fold concentrated, 150 µL spread in the middle of each nematode growth medium (NGM) agar plate and incubated at 37°C ON. NGM agar plates were prepared as follows: 3 g NaCl (VWR Chemicals, Leuven, Belgium), 2.5 g Pepton (AppliChem, Darmstadt, Germany), 17 g high-strength agar (Serva, Heidelberg, Germany) and 1 mL cholesterol (AppliChem, Darmstadt, Germany) (from 0.5 g dissolved in 100 mL ethanol stock) were dissolved in 1000 mL dH2O, autoclaved and 0.5 mL 1M CaCl2 (Sigma-Aldrich, St. Louis, MO, USA), 1 mL 1M MgSO4 (Roth, Karlsruhe, Germany), 25 mL 1M KH2PO4 (AppliChem, Darmstadt, Germany) (pH=6) and 10 mL Nystatin solution (AppliChem, Darmstadt, Germany) added. The NGM agar was filled in 6 cm petri dishes (Sarstedt, Nümbrecht, Germany) and stored at 4°C. Plates were equilibrated at room temperature before *C. elegans* L4 larvae (n=3 x 30/group) were transferred individually onto the bacterial lawn of the respective *E. faecalis* strain or *E. coli* OP50 strain as food control. Nematodes were transferred to fresh NGM plates with the corresponding *E. faecalis* or *E.*

coli OP50 strain every second day. Worms were considered dead, if they failed to respond to touch. The number of viable and killed worms was determined daily for about 25-30 days.

Manduca sexta monoassociation model

The caterpillar *M. sexta* was used to investigate commensal-host interactions *in vivo*. *M. sexta* eggs, which were a generous gift from the Chair for Neurobiology of the Philipps-University in Marburg, were surface sterilized with 80% ethanol and in petri dishes transferred to a germ-free small isolator (at 26°C, controlled humidity and regular light/dark cycle). After hatching the larvae were maintained in groups in small containers and reared on a wheat germ-based artificial diet, which was autoclaved and regularly checked for contaminations. The wheat germ-based diet was prepared as follows: 3 L H2O were boiled and 120 g agar-agar (Roth, Karlsruhe, Germany) added. While stirring, 700 g of a wheat mixture (2 g CaCl2 (Sigma-Aldrich, St. Louis, MO, USA), 2 g NaCl (VWR Chemicals, Leuven, Belgium), 6.7 g sorbic acid (AppliChem, Darmstadt, Germany), 20.4 g ascorbic acid (AppliChem, Darmstadt, Germany), 54 g casein (Alfa Aesar, Karlsruhe, Germany), 219 g soy flour, 405 g organic whole-grain flour, 20 mg Nystatin (AppliChem, Darmstadt, Germany)) and 6 mL rapeseed oil (Sigma-Aldrich, St. Louis, MO, USA) were added subsequently. After cooling (45 to 48°C) 45 mL of a vitamin mix (45 mg nicotinic acid, 22.5 mg riboflavin, 10.5 mg thiamine, 10.5 mg pyridoxine, 10.5 mg folic acid, 0.9 mg biotin (all AppliChem, Darmstadt, Germany)) were added, mixed and 10 mL of 10% phosphate-buffered formaldehyde (PFA) added to the mash. To examine the effects of dextran sodium sulfate (DSS) on *M. sexta* growth and midgut morphology, DSS (AppliChem, Darmstadt, Germany) was dissolved in sterile filtered dH2O and added (at a total concentration of 3% in the diet) to the mash when PFA was added. The mash was stored in small containers at 4°C.

Newly molted 3rd instar larvae were distributed to containers and received diet soaked with different *E. faecalis* strains or PBS as control or diet containing DSS (always n=5-6/group). Successful and similar monocolonization by all strains was tested and monitored regularly by plating serial dilutions of midgut content (n=3-4 larvae/group) at BHI agar. All larvae were sacrificed during the 5th instar larva stadium and approximately 1 cm of the midgut was fixed in 4% PFA or Carnoy's solution for histological analysis. Sections of 5 µm were prepared for immunofluorescence staining and pictures taken (n=3-4 larvae/group) as described in section 2.5.2. For immunofluorescence staining of *E. faecalis* an antibody raised in rabbit against purified enterococcal lipoteichoic acid (LTA) was used [215]. *In vivo* imaging of *M. sexta* was performed with an IVIS Lumina system (Caliper Life Sciences, Mainz, Germany).

2.2.2 Mouse experiments

Germ-free 129S6/SvEv wild type and IL-10-/- mice were maintained at the gnotobiotic facility of the Division of Gastroenterology and Hepatology, University of North Carolina, Chapel Hill, USA. Animal protocols used in this study were approved by the Institutional Animal Care and Use Committee of the University of North Carolina, Chapel Hill, NC (IACUC-ID: 12-300.0, accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care) and performed according to the guidelines in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

Germ-free mice (n=8-10/group both females and males) were colonized at the age of 8-9 weeks with either *E. faecalis* wild type OG1RF or *ΔepaB* or *Δlgt* mutant strains by rectal swab and sacrificed after 16 weeks of monocolonization.

2.2.3 Isolation of luminal and mucosa-associated bacteria

To test colonization of mice (n=8-10/group) luminal content was sampled from ileum, cecum and colon, serial dilutions (till 10^{-8}) were plated at BHI agar and CFU were counted after ON incubation at standard conditions.

In addition, gut tissue sections from ileum and colon (approximately 0.5 cm long) were collected, cut longitudinally, weighted and stored in 1.5 mL tubes (Eppendorf, Hamburg, Germany) with 500 µL PBS under sterile conditions. Tissue samples were washed four times with PBS. After removal of PBS from last washing step, 500 µL PBS containing 0.1% Dithiothreitol (DTT) (AppliChem, Darmstadt, Germany) were added to each sample, vortexed for 15 sec at maximum level and incubated at RT for 2-3 min. The PBS/DTT solution was discarded, 500 µL PBS added and tubes shaken by hand for 15 sec. After two additional washing steps with PBS, the amount of PBS according to the 10^{-1} dilution step of the corresponding initial weight was added to each tube and samples vortexed at 1000 rpm, 26°C for 30 min. Finally, serial dilutions (till 10^{-4}) were plated at BHI agar and CFU were counted after ON incubation at standard conditions.

2.2.4 Organ culture

To detect spontaneous secretion of cytokines by colonic tissue of wild type and IL-10-/- mice monoassociated with the different *E. faecalis* strains, approximately 0.5 cm tissue sections were taken from the middle colon of all mice (n=8-10/group), weighted and incubated with 1 mL of RPMI-1640 (Sigma-Aldrich, St. Louis, MO, USA) containing 1% antibiotics/antimycotics (Sigma-Aldrich, St. Louis, MO, USA) for 24 hours in cell culture-

treated 12-well plates (Thermo Scientific, Roskilde, Denmark). Supernatants were taken, cytokines quantified by ELISA measurement and calculated according to the initial tissue section weight.

2.2.5 Microbial RNA-sequencing of virulence-related E. faecalis genes

Ribosomal RNA-depleted bacterial RNA was isolated from the colon content of wild type and IL-10-/- mice (n=8 mice/group) monoassociated with *E. faecalis* wild type OG1RF for 16 weeks by Jonathan Hansen and Sandrine Tchaptchet from the Division of Gastroenterology and Hepatology of the University of North Carolina in Chapel Hill as described previously with the following exceptions [14]. After the second round of DNase treatment, RNA was recovered using RNeasy mini columns (Qiagen, Venlo, Netherlands) and then depleted of rRNA using Ribo-Zero Magnetic kit (Epicentre, Madison, WI, USA) according to the manufacturer's instructions prior to library construction. Samples were submitted to the Washington University at St. Louis Genome Technology Access Center for library preparation and sequencing on the Illumina HiSeq2000 instrument (Illumina, San Diego, CA, USA) to generate 190 million unidirectional 50nt reads. Each read was aligned to the *E. faecalis* OG1RF annotated genome available on NCBI using the Bowtie 2 algorithm and the counts per million reads per kilobase of each ORF were calculated.

The selection of virulence-relevant genes (Table 3) was focused on known virulence factors in *E. faecalis* OG1RF [179] including the *epa* cluster [195] and *lgt*-dependent (predicted) lipoproteins involved in *E. faecalis* or Gram-positive bacteria virulence [216]. The graphical analysis was performed by Ilias Lagkouvardos from the Chair of Nutrition and Immunology of the Technical University of Munich as follows: The expression profiles of all virulent factors were first centered and scaled and then visualized as heatmaps using the package EMA (PMID: 21047405) in R. Samples and gene profiles were clustered using Ward's method based on their Spearman rank correlation distance and each gene's under- or over-expression per genotype was represented as the log2 of the groups mean expression fold changes.

Table 3: Virulence-related genes of *E. faecalis* OG1RF selected for RNA-sequencing

OG1RF ID	Gene	Definition
OG1RF_10051	*adcA*	metal ABC superfamily ATP binding cassette transporter, binding protein
OG1RF_10057	-	oligopeptide ABC superfamily ATP binding cassette transporter, binding protein
OG1RF_10136	-	iron (Fe3+) ABC superfamily ATP binding cassette transporter, binding protein
OG1RF_10139	*fhuG*	iron ABC superfamily ATP binding cassette transporter, membrane protein
OG1RF_10423	*prsA*	peptidyl-prolyl cis-trans isomerase
OG1RF_10537	*aatB*	amino acid ABC superfamily ATP binding cassette transporter, binding protein
OG1RF_10540	-	oligopeptide ABC superfamily ATP binding cassette transporter, binding protein

OG1RF_10550	-	family 8 polysaccharide lyase
OG1RF_10593	*opuCC*	ABC superfamily ATP binding cassette transporter, binding protein
OG1RF_10634	*oppA*	oligopeptide ABC superfamily ATP binding cassette transporter, binding protein
OG1RF_10680	*bopD*	LacI family transcriptional regulator
OG1RF_10841	*traC*	oligopeptide ABC superfamily ATP binding cassette transporter, binding protein
OG1RF_10878	*ace*	collagen adhesin protein
OG1RF_10897	-	glutamine ABC superfamily ATP binding cassette transporter, binding protein
OG1RF_10993	-	spermidine/putrescine ABC superfamily ATP binding cassette transporter
OG1RF_11130	-	pheromone cAM373 lipoprotein
OG1RF_11186	*modA*	molybdenum ABC superfamily ATP binding cassette transporter, binding protein
OG1RF_11229	-	oligopeptide ABC superfamily ATP binding cassette transporter, binding protein
OG1RF_11253	*tig2*	peptidyl-prolyl isomerase
OG1RF_11354	-	iron (Fe) ABC superfamily ATP binding cassette transporter, binding protein
OG1RF_11459	*lgt*	prolipoprotein diacylglyceryl transferase
OG1RF_11502	*oppA2*	oligopeptide ABC superfamily ATP binding cassette transporter, binding protein
OG1RF_11525	*sprE*	SprE protein
OG1RF_11526	*gelE*	Gelatinase
OG1RF_11527	*fsrC*	sensor histidine kinase FsrC
OG1RF_11528	*fsrB*	FsrB protein
OG1RF_11529	*fsrA*	FsrA response regulator
OG1RF_11679	*efaA*	BC superfamily ATP binding cassette transporter, binding protein
OG1RF_11721	*epaR*	sugar transferase
OG1RF_11722	*epaQ*	hypothetical protein
OG1RF_11723	*epaP*	brp/Blh family beta-carotene 15,15'-monooxygenase
OG1RF_11724	*epaO*	group 2 glycosyl transferase
OG1RF_11725	*epaN*	group 2 glycosyl transferase
OG1RF_11726	*epaM*	ABC superfamily ATP binding cassette transporter, ABC protein
OG1RF_11727	*epaL*	ABC superfamily ATP binding cassette transporter, membrane protein
OG1RF_11728	*epaK*	hypothetical protein
OG1RF_11729	*epaJ*	hypothetical protein
OG1RF_11730	*epaI*	group 2 glycosyl transferase
OG1RF_11731	*epaH*	dTDP-4-dehydrorhamnose reductase
OG1RF_11732	*epaG*	dTDP glucose 4,6-dehydratase
OG1RF_11733	*epaF*	dTDP-4-dehydrorhamnose 3,5-epimerase
OG1RF_11734	*epaE*	glucose-1-phosphate thymidylyltransferase
OG1RF_11735	*epaD*	group 2 glycosyl transferase
OG1RF_11736	*epaC*	group 2 glycosyl transferase
OG1RF_11737	*epaB*	group 2 glycosyl transferase
OG1RF_11738	*epaA*	phospho-N-acetylmuramoyl-pentapeptide-transferase
OG1RF_11914	*cpsB*	phosphatidate cytidylyltransferase
OG1RF_11915	*cpsA*	di-trans,poly-cis-decaprenylcistransferase
OG1RF_11916	*metQ*	ABC superfamily ATP binding cassette transporter, binding protein
OG1RF_12089	*t0*	oligopeptide ABC superfamily ATP binding cassette transporter, binding protein
OG1RF_12303	-	family 8 polysaccharide lyase
OG1RF_12311	*traC2*	peptide ABC superfamily ATP binding cassette transporter, binding protein
OG1RF_12351	-	ferric (Fe+3) ABC superfamily ATP binding cassette transporter, binding protein
OG1RF_12366	-	peptide ABC superfamily ATP binding cassette transporter, binding protein
OG1RF_12449	-	M protein trans-acting positive regulator
OG1RF_12464	-	ABC superfamily ATP binding cassette transporter, binding protein

OG1RF_12472	-	ABC superfamily ATP binding cassette transporter, binding protein
OG1RF_12509	-	pheromone cAD1 lipoprotein
OG1RF_12576	*spoIIIJ*	stage III sporulation protein J

2.2.6 RNA isolation and gene expression analysis

Tissue sections taken for RNA isolation (n=8-10 mice/group) were stored in RNA stabilization reagent RNAlater (Qiagen, Venlo, Netherlands), stored at -80°C and RNA was isolated using the NucleoSpin RNA II Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. The RNA concentration was determined with a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). RNA was transcribed to cDNA using 500 ng of RNA and the Superscript III system (Life Technologies by Thermo Scientific (Carlsbad, CA, USA)). RNA expression profiles were investigated using a Light Cycler 480 system (Roche, Basel, Switzerland) with 1 µL of cDNA, 5 µL of Brilliant III Ultra-Fast QPCR 2x master mix (Agilent, Santa Clara, CA, USA), 400 nM primers and 200 nM probe. Primers specific for genes of interest and probes were generated using the Universal Probe Library (Roche, Basel, Switzerland) and are listed in table 4. Relative mRNA expression was calculated using the $2^{-\Delta\Delta Cp}$ method and normalized to *Gapdh* expression. Data were presented as fold change against wild type mice monoassociated with *E. faecalis* wild type OG1RF.

Table 4: Primers used for quantification of cytokine expression in murine tissues

Gene	Primer	Sequence
TNF	Forward	TGCCTATGTCTCAGCCTCTTC
	Reverse	GAGGCCATTTGGGAACTTCT
IL-6	Forward	GATGGATGCTACCAAACTGGAT
	Reverse	CCAGGTAGCTATGGTACTCCAGA
IL-12p40	Forward	ATCGTTTTGCTGGTGTCTCC
	Reverse	GGAGTCCAGTCCACCTCTACA
IL-22	Forward	TTTCCTGACCAAACTCAGCA
	Reverse	TCTGGATGTTCTGGTCGTCA
FOXP3	Forward	CCCACACCTCTTCTTCCTTG
	Reverse	CATGACTAGGGGCACTGTAGG
GATA-3	Forward	TTATCAAGCCCAAGCGAAG
	Reverse	TGGTGGTGGTCTGACAGTTC
RORγt	Forward	GCAGAACTGCCCCATTGA
	Reverse	GACATTCGGCCAAACTTGA

T-bet	Forward	TCAACCAGCACCAGACAGAG
	Reverse	AAACATCCTGTAATGGCTTGTG
KC	Forward	AGACTCCAGCCACACTCCAA
	Reverse	TGACAGCGCAGCTCATTG
MIP3α	Forward	CTTGCTTTGGCATGGGTACT
	Reverse	TCGTAGTTGCTTGCTGCTTC
GAPDH	Forward	TCCACTCATGGCAAATTCAA
	Reverse	TTTGATGTTAGTGGGGTCTCG

RNA isolated from *M. sexta* midgut tissue samples (n=3-4 larvae/group) was transcribed to cDNA using 500 ng of RNA and the Superscript III system (Life Technologies by Thermo Scientific (Carlsbad, CA, USA)). PCR was run with the following setup: 1 cycle preDenaturation (95°C, 1 min), 30 cycles with Denaturation (95°C, 15 sec), Annealing (54°C), Extension (72°C, 30 sec) using 12.5 µL of MyTaq Red Mix (Bioline, London, UK), 0.5 µL 20 µM primers and 200 ng template cDNA. Afterwards, 8 µL of the transcript were added to a 2% agarose-gel (Bioline, London, UK) including a 100 bp ladder (New England BioLabs, Ipswich, MA, USA) and ran for 90 min at 100V.

The RNA expression profile of PGRP in *M. sexta* midgut tissue was performed with the following setup: 1 cycle preDenaturation (95°C, 10 min), 30 cycles with Denaturation (95°C, 15 sec), Annealing (54°C, 15 sec), Extension (72°C, 30 sec) using 5 µL of Maxima SYBR 2x master mix (Life Technologies by Thermo Scientific, Carlsbad, CA, USA), 2 µL template cDNA and 0.5 µL 20 µM primers for PGRP (Table 5). Primers for specific genes of interest regarding *M. sexta* PRRs and AMPs were generated and used as described before [217] and are listed in table 5. Relative mRNA expression was calculated using the $2^{-\Delta\Delta Cp}$ method and normalized to *rpS3* expression. Data were presented as fold change against *M. sexta* monoassociated with *E. faecalis* wild type OG1RF.

Table 5: Primers used for quantification of expression of *M. sexta* PRRs and AMPs

Gene	Primer	Sequence	Product size (bp)	Function
PGRP	Forward	ACGGTATCACTTCCGTCCAC	516	Peptidoglycan binding
	Reverse	CATTCTGGCCATCTCCTGAT		
Hemolin	Forward	ACAGCAACAACACAGGTGAA	1273	LPS, LTA binding
	Reverse	TTAAGCAACAATCACGAGCG		
Immulectin-2	Forward	GACTCTTGCGAGTCGTGTGA	953	LPS binding
	Reverse	GACTGTTTGGGTCCTTTTCG		

Lebocin	Forward	ACGTGCGTAGTGTGAACGAG	170	Antibacterial
	Reverse	CGCAGATTATGAGTTACGACGA		
Cecropin	Forward	TTCTTCGTCTTCGCTTGCTT	161	Antibacterial
	Reverse	CCTTTGAAAATGGCGGTTG		
Lysozyme	Forward	TACAAACGCCACAAGTTCCA	309	Bacteriolytic
	Reverse	AGGCTTGTAACCAAGGACGA		
Attacin B	Forward	GGTCACGGCGCTACTCTTAC	341	Antibacterial
	Reverse	TTGGGCATCTCGAACTTCTT		
Moricin	Forward	TCGCGTTGTCGCTATTATTTT	327	Antibacterial
	Reverse	CAGAAGATTCCGAAGGGAGA		
rpS3	Forward	CTGGCTGAGGATGGCTACTC	186	
	Reverse	TTTCTCAGCGTACAGCTCCA		

Pattern recognition receptor (PRR); Antimicrobial peptide (AMP); Peptidoglycan recognizing protein (PGRP); Lipopolysaccharide (LPS); Lipoteichoic acid (LTA); primers adopted from [217], table adopted from [218].

2.3 Primary cell culture experiments

2.3.1 Isolation of cells from mesenteric lymph nodes

Mesenteric lymph nodes (MLN) were aseptically collected from wild type and IL-10-/- mice (n=3-4 mice/group). Non-fractionated cells were isolated by tissue homogenization and filtration through 70 µm cell strainers (Thermo Scientific, Waltham, MA, USA). MLN cells ($5x10^5$ cells/well) were then cultured in cell culture-treated 96-well plates (Thermo Scientific, Roskilde, Denmark) in 100 µL/well RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) containing 10% fetal calf serum (Biochrom, Berlin, Germany) and 1% antibiotics/antimycotics (Sigma-Aldrich, St. Louis, MO, USA). Cells were stimulated for 72 hours with corresponding bacterial lysates (15 µg/mL) from the *E. faecalis* strain that was used for monocolonization and supernatant collected and used for cytokines measurement, as indicated.

2.3.2 Generation and stimulation of bone marrow-derived dendritic cells

Dendritic cells were generated from bone-marrow (BMDC) of pooled wild type, IL-10-/- or TLR2-/- mice as described before [219] with minor modifications. Femur and tibia were collected from mice (n=2-4) and stored in PBS. After washing once briefly in 70% ethanol and twice in PBS, the end parts of the bones were cut off and flushed with 10 mL PBS through 70 µm cell strainers (Thermo Scientific, Waltham, MA, USA) into 50 mL Falcon tubes (Sarstedt, Nümbrecht, Germany) using a 0.55 x 25 mm syringe (Terumo, Leuven, Belgium).

Cells were centrifuged and the cell pellet resuspended in 1 mL red blood cell lysis buffer (BioLegend, San Diego, CA, USA) and incubated for 1-3 min at RT. Subsequently, 9 mL PBS were added, cells centrifuged, resuspended in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) containing 10 ng/mL recombinant GM-CSF (PeproTech, Hamburg, Germany), 5 ng/mL recombinant TNF (PeproTech, Hamburg, Germany) or 15 ng/mL recombinant IL-4 (PeproTech, Hamburg, Germany), 0.055 mM 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA), 10% fetal calf serum (Biochrom, Berlin, Germany) and 1% antibiotics/antimycotics (Sigma-Aldrich, St. Louis, MO, USA) and BMDCs cultured in 150 mm petri dishes (Sarstedt, Nümbrecht, Germany) (2x per bone sampled) for 7-8 days with half of the medium changed at day 3 and 5.

BMDCs ($2x10^5$ cells/well) were stimulated in cell culture-treated 12-well plates (Thermo Scientific, Roskilde, Denmark) for 24 hours with bacterial lysates (3 µg/mL or 15 µg/mL) from *E. faecalis* wild type OG1RF, *ΔepaB*, *Δlgt* or reconstituted mutant strains or LPS (150 ng/mL) as control (n=3-4 wells/treatment group) and supernatants taken for detection of cytokines.

2.3.3 Flow cytometry analysis

Stimulated BMDCs were transferred to a 96-well plate (Thermo Scientific, Roskilde, Denmark) (n=3-4 wells/treatment group) and washed once with PBS. Cells were stained with anti-CD11b, -CD11c, -MHCII and -CD86 antibodies (Table 6) diluted in FACS buffer (PBS containing 5% fetal calf serum (Biochrom, Berlin, Germany), 2 mM EDTA (AppliChem, Darmstadt, Germany)) according to the manufacturer's instructions and incubated for 15 min at 4°C. Cells were washed twice with PBS and finally resuspended in 20 µL FACS buffer. Per treatment group, $1x10^4$ cells were acquired with the LSR II flow cytometer (BD Bioscience, Sparks, MD, USA), gated and analyzed using BD FACSDiva software version 5.0.3 (BD Bioscience, Sparks, MD, USA).

Table 6: Antibodies used for flow cytometry analysis of BMDCs

Target	Fluorochrome	Manufacturer details (Cat. #)
CD11b	APC-Cy7	BD (Sparks, MD, USA) (#557657)
CD11c	PE	BD (Sparks, MD, USA) (#557401)
MHCII	FITC	BD (Sparks, MD, USA) (#553623)
CD86	APC	BD (Sparks, MD, USA) (#558703)

Bone marrow-derived dendritic cells (BMDC).

2.3.4 Antigen presenting cell-T cell co-culture

For dendritic cell (DC)-T cell co-cultures, BMDCs were generated from pooled IL-10-/- mice (n=4) and cultured in 150 mm petri dishes (Sarstedt, Germany) with RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) containing 10 ng/mL recombinant GM-CSF (PeproTech, Hamburg, Germany), 5 ng/mL recombinant TNF (PeproTech, Hamburg, Germany), 0.055 mM 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA), 10% fetal calf serum (Biochrom, Berlin, Germany) and 1% antibiotics/antimycotics (Sigma-Aldrich, St. Louis, MO, USA) for 7 days with medium changed at day 3 and 5. At day 7 non-adherent BMDCs were harvested, and $2.24x10^6$ cells per petri dish were pulsed with lysates from *E. faecalis* wild type OG1RF, *ΔepaB* or *Δlgt* mutant strain (15 µg/mL) for 24 hours. CD4+ T cells were isolated from pooled MLN of IL-10-/- mice (n=5 monoassociated with *E. faecalis* OG1RF for 16 weeks) using a CD4+ T cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). BMDCs pulsed with the *E. faecalis* lysates from different strains were washed three times with RPMI-1640 medium and adherent cells harvested. Finally, $8x10^4$ BMDCs were co-cultured with $2x10^5$ CD4+ T cells in triplicates in cell culture-treated 96-well plates (Thermo Scientific, Roskilde, Denmark) including appropriate controls in fresh medium (RMPI-1640, 10% fetal calf serum, 1% sodium pyruvate (Sigma-Aldrich, St. Louis, MO, USA), 0.1% 2-mercaptoethanol, 100 µg/mL gentamycin). After 72 hours supernatants were collected for cytokine measurements.

2.4 Cytokine and chemokine quantification

Detection of IFN-γ, IL-17A, IL-12p40, TNF, IL-6 and serum amyloid A in murine plasma or cell culture-supernatant specimens was performed using commercially available ELISA kits (eBioscience, San Diego, CA, USA) and 96-well plates (Thermo Scientific, Roskilde, Denmark).

2.5 Histological & immunohistochemical analysis

2.5.1 Histopathological analysis

Tissue sections taken from distal colon and caecum of wild type and IL-10-/- mice monoassociated with *E. faecalis* wild type OG1RF or *ΔepaB* or *Δlgt* mutant strains (n=8-10/group) were fixed in 4% PFA ON or in Carnoy's solution (60% methanol (Merck, Darmstadt, Germany), 30% chloroform (Roth, Karlsruhe, Germany), 10% glacial acetic acid (AppliChem, Darmstadt, Germany)) for 3 to 4 hours, dehydrated (for PFA: ascending amounts of ethanol followed by xylene (Fisher Scientific, Loughborough, UK) and paraffin)

(for Carnoy's solution: 3-6 washing steps with 100% ethanol, 2 hours in methanol (VWR Chemicals, Leuven, Belgium), 1 hour in fresh methanol, 2 x 1 hour in fresh xylene and ON in warm paraffin) and embedded in paraffin. For histopathological analysis sections were cut (5 µm), automatically deparaffinized and stained with hematoxylin (Medite, Burgdorf, Germany) and eosin (Medite, Burgdorf, Germany) by a staining device (Rehydration: 2 x 3 min xylene, 2 min 100% ethanol, 2 min 96% ethanol, 1 min 70% ethanol, 1 min dH2O; Staining: 4 min hematoxylin, 2 min dH2O, 2 min eosin; Dehydration: 1 min 70% ethanol, 1 min 96% ethanol, 1 min 100% ethanol, 1.5 min 100% ethanol, 1.5 min xylene/ethanol, 2 x 2 min xylene). A histological scoring was performed by the assessment of mononuclear cell infiltration into lamina propria, crypt hyperplasia, goblet cell depletion and ulcer formation resulting in a score from 0 (not inflamed) to 12 (highly inflamed) by a blinded independent examiner as previously described [220].

2.5.2 Immunofluorescence staining of tissue sections

For immunofluorescence staining 4% PFA-fixed (for staining of Ly6G, CD3, F4/80, E-cadherin, CCL-20) or Carnoy-fixed (for staining of MUC2, LTA) paraffin-embedded murine distal colon or *M. sexta* midgut samples were cut in 5 µm sections and fixed on SuperFrost Plus glass slides (VWR, Darmstadt, Germany). After drying for 15 min at 60°C, the slides were automatically deparaffinized (2 x 3 min xylene, 2 min 100% ethanol, 2 min 96% ethanol, 1 min 70% ethanol, 1 min dH2O) and rinsed twice in dH2O for 5 min each. For staining of Ly6G, CD3, E-cadherin or CCL-20 the antigen unmasking was achieved by boiling the specimens for 30 min in 10 mM sodium citrate buffer (pH=6) in a pressure cooker placed in a microwave (900W), followed by 30 min cooling at RT. For staining of F4/80 the antigen retrieval was performed by treating the tissue sections with 20 µg/mL proteinase K (Roth, Karlsruhe, Germany) for 16 min at 37°C. Staining of CD11c was performed in fresh tissue samples embedded in OCT (Sakura Finetek, Alphen aan den Rijn, Netherlands), which were cut in 5 µm sections, fixed for 15 min in water-free methanol (Merck, Darmstadt, Germany) and washed three times in PBS.

After antigen unmasking the slides were washed three times with dH2O for 5 min each and once washed in PBS for 5 min. Then slides were blocked by a blocking buffer (PBS containing 5% serum of the animal, in which the secondary antibody was produced, and 0.3% Triton X-100 (AppliChem, Darmstadt, Germany)) for 60 min at RT. Slides were aspirated and incubated with the primary antibody (Table 7) diluted (E-cadherin (intra- and extracellular domain), MUC2, CD11c: 1:200 from stock solution; F4/80: 1:300 from stock solution; Ly6G, CD3, CCL-20, LTA: 1:400 from stock solution) in antibody dilution buffer

(PBS containing 1% bovine serum albumin (AppliChem, Darmstadt, Germany) and 0.3% Triton X-100) ON at 4°C. The tissue specimens were rinsed three times in PBS for 5 min each and incubated with the secondary antibody (Table 7; 1:200 from stock solution in antibody dilution buffer containing DAPI (1:1000 from 1 mg/mL stock)) for 1-2 hours at RT. Finally, the slides were rinsed three times in PBS for 5 min each and covered with a glass slide (VWR, Darmstadt, Germany) using mounting medium containing additional DAPI (Vector Laboratories, Burlingame, CA, USA).

Pictures (n=3-8 mice/group) were acquired using an Olympus FluoView FV-1000 confocal microscope (Olympus, Hamburg, Germany) and further analyzed using Volocity 3D Image Analysis software version 5.4.1 (PerkinElmer, Waltham, MA, USA).

Table 7: Antibodies used for immunofluorescence staining

Target	Ab Species	Manufacturer details / Cat. #	Secondary antibody used Cat. #
CCL20	Rabbit	Abcam (Cambridge, UK) #ab9829	Goat-anti-rabbit AF594 (#A11012)*
CD3	Rabbit	Sigma-Aldrich (St. Louis, MO, USA) #C7930	Goat-anti-rabbit AF594 (#A11012)*
CD11c	Hamster	BD (Sparks, MD, USA) #550283	Goat-anti-hamster Cy3 (#127-165-160)**
E-cadherin (intracellular domain)	Mouse	Abcam (Cambridge, UK) #ab76055	Goat-anti-mouse AF488 (#A11001)*
E-cadherin (extracellular domain)	Rabbit	Santa Cruz Biotechnology (Dallas, TX, USA) #sc-7870	Goat-anti-rabbit AF488 (#A11008)*
F4/80	Rat	BMA Biomedicals (Augst, Switzerland) #T-2006	Goat-anti-rat AF546 (#A11081)*
LTA	Rabbit	[215]	Goat-anti-rabbit AF594 (#A11012)* Goat-anti-rabbit AF488 (#A11008)*
Ly6G	Mouse	BD (Sparks, MD, USA) #551459	Goat-anti-mouse AF546 (#A11003)*
MUC2 (mouse)	Rabbit	Santa Cruz Biotechnology (Dallas, TX, USA) #sc-15334	Goat-anti-rabbit AF488 (#A11008)*
MUC2 (human)	Rabbit	Sigma-Aldrich (St. Louis, MO, USA) #HPA006197	Goat-anti-rabbit AF488 (#A11008)*
MUC5AC	Mouse	Abcam (Cambridge, UK) #ab3649	Goat-anti-mouse AF546 (#A11003)*

*from Life Technologies by Thermo Scientific (Carlsbad, CA, USA); ** from BMA Biomedicals (Augst, Switzerland)

2.5.3 Fluorescence in-situ hybridization

For co-staining of immunofluorescence and fluorescence *in-situ* hybridization (FISH), Carnoy's solution-fixed samples embedded in paraffin were cut in 5 µm sections, transferred to SuperFrost Plus glass slides (VWR, Darmstadt, Germany), dried for 15 min at 60°C, manually deparaffinized (3 x 2 min xylene (Fisher Scientific, Loughborough, UK), 2 min 96% ethanol, 2 min 80% ethanol, 2 min 60% ethanol) and manually dehydrated (3 min 60% ethanol, 3 min 80% ethanol, 3 min 96% ethanol, 3 min air drying). Slides were put in hybridization chambers filled with dH2O, treated with permeabilization buffer (25 mL dH2O containing 20 mM Tris (Sigma-Aldrich, St. Louis, MO, USA), 2 mM EDTA (AppliChem, Darmstadt, Germany), 1.2% Triton X-100 (AppliChem, Darmstadt, Germany), filter sterilized and 40 mg/mL of lysozyme (Sigma-Aldrich, St. Louis, MO, USA) freshly added) and the closed chambers stored in an oven for 45 min at 37°C for antigen-retrieval. Specimens were washed with dH2O, manually dehydrated (3 min 60% ethanol, 3 min 80% ethanol, 3 min 96% ethanol, 3 min air drying) and washed once with PBS. Water from hybridization chambers was discarded and chambers filled with 5-8 mL of pre-warmed (46°C) hybridization buffer (50 mL dH2O containing 20 mM Tris (Sigma-Aldrich, St. Louis, MO, USA), 0.9 M NaCl (VWR Chemicals, Leuven, Belgium), 0.01% SDS-solution 20% (AppliChem, Darmstadt, Germany) adjusted to pH=7.3 and filter sterilized). The slides were stored again in hybridization chambers, 50 µL of hybridization buffer containing 0.5 ng/µL Cy5-labeled Eub338-probe [221] added to each tissue specimen and samples stored ON at 46°C.

After ON incubation, slides were washed twice with pre-warmed (48°C) FISH-washing buffer (25 mL dH2O containing 20 mM Tris, 0.064 M NaCl adjusted to pH=7.3 and filter sterilized). For staining of intestinal mucus layer the tissue sections were incubated with an anti-MUC2 antibody (Table 7) diluted (1:200) in FISH-washing buffer containing DAPI (1:1000 from 1 mg/mL stock) for 2 hours at 4°C. After washing twice with pre-warmed FISH-washing buffer, the secondary antibody (Table 7; diluted 1:200 in FISH-washing buffer) was added to the slides for 30 min at RT. Slides were washed again twice with pre-warmed FISH-washing buffer and covered with a glass slide (VWR, Darmstadt, Germany) using mounting medium containing additional DAPI (Vector Laboratories, Burlingame, CA, USA). Pictures (n=3-8 mice/group) were acquired using an Olympus FluoView FV-1000 confocal microscope (Olympus, Hamburg, Germany) and analyzed using Volocity 3D Image Analysis software version 5.4.1 (PerkinElmer, Waltham, MA, USA). For distance measurements of single bacterial cells to the intestinal epithelial cell surface, an automated tracking and distance measurement protocol was generated in the Volocity 3D Image Analysis software.

2.6 Cell culture assays

2.6.1 Biofilm formation on abiotic surfaces

Biofilm formation on polystyrene surfaces was assessed as described before [222]. *E. faecalis* strains from stationary phase ON cultures (20 µL) were incubated in tryptic soy broth (TSB) (Oxoid, Basingstoke, UK) supplemented with 1% glucose (180 µL/well) for 18-20 hours at 37°C in 96-well tissue culture plates (Thermo Scientific, Roskilde, Denmark) (n=4 wells/strain). Growth rates were assessed by measuring the OD at 630 nm. After three times washing carefully with PBS, plates were dried at 60°C for 1 hour and stained with 100 µL/well 2% Hucker's crystal violet (Sigma-Aldrich, St. Louis, MO, USA) for 2 min. Excess stain was removed by rinsing the plates thoroughly under tap water and plates were dried for 10-20 min at 60°C. OD was measured at 630 nm and biofilm formation was normalized to growth with the biofilm index (ODbiofilm x (0.5/ODgrowth)).

To determine biofilm thickness, *E. faecalis* strains from stationary phase ON cultures (diluted 1:100) were cultivated for 18-20 hours in TSB supplemented with 1% glucose (250 µL/well) in collagen-IV-coated 8-well chambers (ibidi, Munich, Germany) at 37°C with gentle shaking. Bacteria and liquid broth were removed carefully and carbohydrate structures containing terminal α-D-glucose in the biofilm were stained for 1 hour using Alexa Fluor-488 labelled concavalin A (Invitrogen, Carlsbad, USA) in PBS (1:200 from stock solution in antibody dilution buffer). After carefully washing twice with PBS, 250 µL of PBS were added to each well and images of stained biofilm were acquired (40x magnification) using an Olympus IX81 inverted confocal microscope (Olympus, Hamburg, Germany). Biofilm thickness measurements (n=5-7) and 3D-reassembling of acquired pictures were performed using Volocity 3D Image Analysis software version 5.4.1 (PerkinElmer, Waltham, MA, USA).

2.6.2 Biofilm and associated microcolony formation on biotic surfaces

The murine colonic epithelial cell line **Ptk6** has been described by *Whitehead et al.* [223]. Ptk6 cells were grown at 37°C in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) containing 5% fetal calf serum (Biochrom, Berlin, Germany) and 1 µg/mL Insulin-Transferrin-Selenium A (Invitrogen, Carlsbad, CA, USA). Biofilm assays on biotic surface of fixed epithelial cell monolayer were performed in collagen-IV-coated 8-well plates (ibidi, Munich, Germany) and conducted as described before [172], but adapted to *E. faecalis* routine growth conditions. First, Ptk6 cells ($0.5x10^5$ cells/well) were cultured until they were fully confluent and differentiated (5-7 days), fixed with 4% PFA (15 min) and after four times carefully washing with PBS, 250 µl of *E. faecalis* suspension from an stationary phase ON culture (diluted 1:100 in BHI) added to the wells. After incubation for 20-24 hours, the cell

monolayer was washed once with PBS and blocked with 5% goat serum diluted in antibody dilution buffer (30 min, 4°C). *E. faecalis* microcolonies were stained by an antibody raised in rabbit against purified enterococcal LTA from *E. faecalis* (Table 7; 1:400 from stock solution) and Ptk6 cells stained with an anti-E-cadherin antibody (Table 7; intracellular domain, 1:400 from stock solution) both diluted in antibody dilution buffer and incubated for 30 min at 4°C. The wells were carefully washed twice with PBS, incubated for 30 min at 4°C with secondary antibodies (Table 7; 1:200 from stock solution in antibody dilution buffer containing DAPI (1:1000 from 1 mg/mL stock)) and washed twice again. Finally, 250 µL of PBS were added to each well. Pictures were acquired at 40x magnification using an Olympus IX81 inverted confocal microscope (Olympus, Hamburg, Germany). 3D-reassembling and volume quantifications (n=4 pictures/strain, using a newly generated protocol that calculated the total volume of 3D-reassembled structures identified by a common threshold signal) were performed using Volocity 3D Image Analysis software version 5.4.1 (PerkinElmer, Waltham, MA, USA).

2.6.3 Adhesion to mucosal surfaces

For adhesion experiments to IEC surfaces, the human colonic epithelial cell line **Caco-2** was seeded in cell culture-treated 24-well plates (Thermo Scientific, Roskilde, Denmark) ($1x10^5$ cells/well) and cultured in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) containing 5% fetal calf serum (Biochrom, Berlin, Germany) until they were fully confluent and differentiated (14-21 days). To measure adhesion to the Caco-2 monolayer, fresh cell culture medium was added containing different *E. faecalis* strains (MOI 100:1; n=4 wells/strain). After co-incubation for 2 hours at 37°C, the medium was discarded and all wells gently washed three times with PBS. Cells were lysed with 200 µL/well trypsin/EDTA (Sigma-Aldrich, St. Louis, MO, USA) for 10 min at 37°C followed by 300 µL/well PBS containing 0.1% Triton X-100 (AppliChem, Darmstadt, Germany) for 15 min at 4°C. Cells were resuspended by pipetting up and down several times and the supernatant plated in serial dilutions at BHI agar to determine CFU counts.

The human colonic goblet cell line **LS174T** was obtained from the American Type Cell Culture Collection (Rockville, MD, USA) by the Chair of Microbial Ecology of the Technical University of Munich and all experiments performed at their facilities. LS174T cells were grown at 37°C in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) containing 10% fetal calf serum (Biochrom, Berlin, Germany) and 1% MEM non-essential amino acids (Sigma-Aldrich, St. Louis, MO, USA). To test bacterial adhesion to colon-like mucus surfaces, LS174T cells were seeded ($1x10^5$ cells/well) in cell culture-treated 12-well plates

(Thermo Scientific, Roskilde, Denmark) and grown for 7-10 days until a thick mucus layer attached to the monolayer was visible. Every second day fresh cell culture medium was added to the cells. *E. faecalis* strains were added for 2 hours (MOI 100:1; n=4 wells/strain) and after three washing steps with PBS, cells lysed with PBS containing 1% Triton X-100 (AppliChem, Darmstadt, Germany) and the supernatant plated in serial dilutions at BHI agar to determine CFU counts. Imaging of *E. faecalis* adhesion to the mucus-covered surface of LS174T cells was performed in collagen-IV-coated 8-well plates (ibidi, Munich, Germany) and conducted as described in section 2.6.2, but modified regarding incubation times with *E. faecalis*. First, LS174T cells ($0.5x10^5$ cells/well) were cultured until they were fully confluent and differentiated (7-10 days), fixed with 4% PFA (15 min) and after carefully washing with PBS for four times, 250 µl of *E. faecalis* suspension from an stationary phase ON culture (diluted 1:100 in BHI) was added to the wells. After incubation for 2 hours, the cell monolayer was washed once with PBS and blocked with 5% goat serum diluted in antibody dilution buffer (30 min, 4°C). *E. faecalis* microcolonies were stained by an antibody raised in rabbit against purified enterococcal LTA from *E. faecalis* (Table 7, 1:400 from stock solution) and the mucus produced by LS174T cells was stained with anti-MUC5AC and/or anti-MUC2 (human) antibody (Table 7; MUC5AC: 1:200; MUC2: 1:50 from stock solution) both diluted in antibody dilution buffer and incubated for 30 min at 4°C. The wells were carefully washed twice with PBS, incubated for 30 min at 4°C with secondary antibodies (Table 7; 1:200 from stock solution in antibody dilution buffer containing DAPI (1:1000 from 1 mg/mL stock)) and washed twice again. After adding 250 µL of PBS to each well, pictures were acquired at 40x magnification using an Olympus IX81 inverted confocal microscope (Olympus, Hamburg, Germany). 3D-reassembling of images was performed using Volocity 3D Image Analysis software version 5.4.1 (PerkinElmer, Waltham, MA, USA).

2.7 Statistical analysis

Data was analyzed by using Prism software version 6.02 (GraphPad Software, La Jolla, CA, USA). Data in figures are presented as mean with error bars indicating standard deviation. Survival experiments with infection models were statistically analyzed by log-rank test performed on Kaplan-Meier curves. Statistical differences due to mouse genotype or different time points and *E. faecalis* strain were analyzed by Two-way ANOVA with Bonferroni post-test. Differences between *E. faecalis* strains or different time points were statistically analyzed by One-way ANOVA following Bonferroni post-test. Differences were considered significant for *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Unless otherwise stated all infection model (except for the mouse colonization) and *in vitro* experiments were repeated three times and representative results are shown.

3. RESULTS

3.1 EpaB and lipoproteins promote virulence and colitogenic activity of *E. faecalis*

3.1.1 E. faecalis ΔepaB and Δlgt exert reduced virulence

To screen for expression of virulence traits of *E. faecalis* under conditions of chronic inflammation, we monoassociated germ-free wild type and IL-10-/- mice with *E. faecalis* wild type OG1RF and performed RNA-sequencing of *E. faecalis* isolated from colon content (Fig.2A). A focused screening for selected genes known to be involved in *E. faecalis* virulence (Table 3) confirmed our previous findings that *gelE* (OG1RF_11526) and related genes including *sprE* (OG1RF_11525) as well as the *fsr*-locus (OG1RF_11527, OG1RF_11528, OG1RF_11529), which controls the expression of these two proteases, were upregulated by *E. faecalis* in intestinal inflammation. Surprisingly, the expression levels of most investigated *E. faecalis* virulence

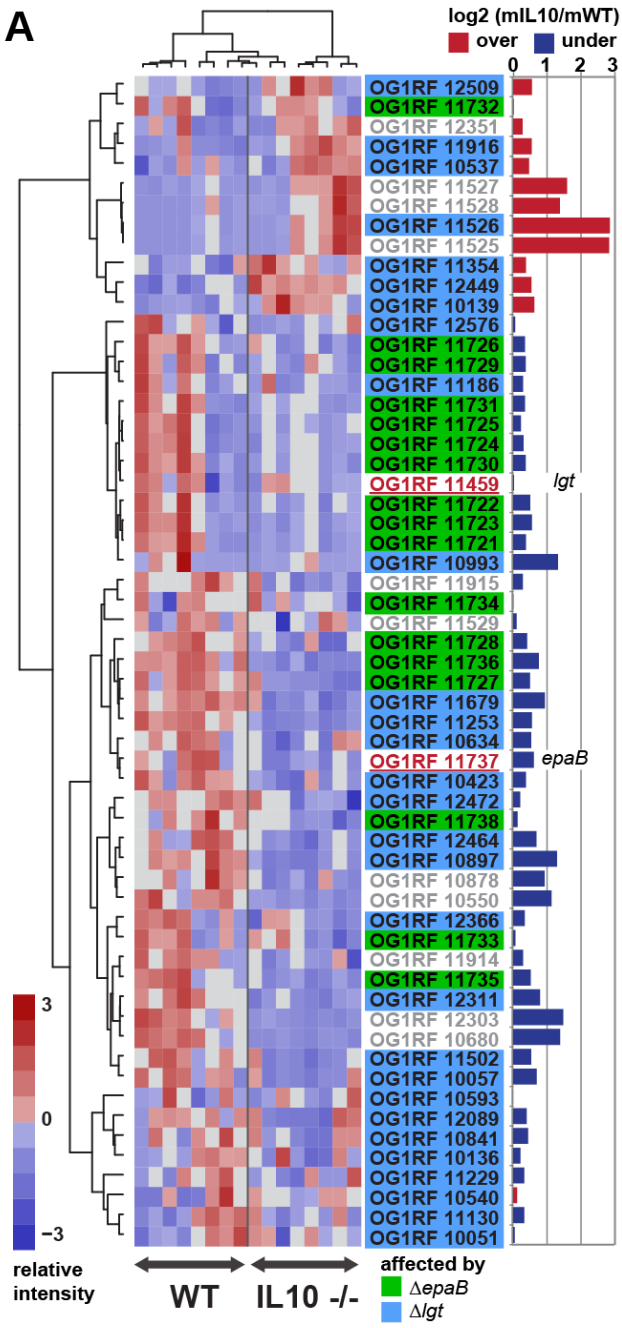

Figure 2: Expression of known virulence-related genes by *E. faecalis* under chronic colitis in IL-10-/- mice.

(**A**) Expression profile of selected virulence-related genes of *E. faecalis* wild type OG1RF isolated from colon content of monoassociated IL-10-/- mice vs. monoassociated wild type (WT) mice: differential expression of genes in relation to a chronically inflamed environment is shown for virulence-related genes including the *epa* cluster (green-labeled locus tags) and *lgt*-dependent (predicted) lipoproteins putatively involved in *E.* faecalis virulence (blue-labeled locus tags); *epaB* (OG1RF_11737) and *lgt* (OG1RF_11459) are highlighted by red letters. Samples and genes are hierarchically clustered according to Ward-Spearman correlation and log2 ratio of mean abundance (mIL10/mWT) of normalized expression levels is shown (up-regulation is indicated by red bar color, down-regulation is indicated by blue bar color). Please see table 3 for annotation of locus tags.

genes did not undergo substantial alterations in the inflamed environment (Fig.2A), raising questions about additional GelE-independent virulence factors contributing to colitogenic activity of *E. faecalis* in the IL-10-/- mouse model. To determine whether *E. faecalis* virulence factors also confer colitogenic activity, we selected two genes known to render *E. faecalis* virulence by different mechanisms. These include *epaB* (OG1RF_11737) from the enterococcal polysaccharide antigen cluster and *lgt* (OG1RF_11459), a prolipoprotein diacylglyceryl transferase.

After generation of *E. faecalis* isogenic mutant strains deficient for *epaB* or *lgt* and reconstituted mutant strains, we first tested the growth rates of all *E. faecalis* strains used in this study in BHI medium at standard conditions. All *E. faecalis* mutant and reconstituted mutant strains showed a similar growth at stationary phase as determined by optical density of bacterial cells (Fig.3A). The slightly slower growth of *E. faecalis ΔepaB* during the early exponential growth phase may originate from the reduced formation of cocci chains by this strain, which normally occurs for *E. faecalis* wild type OG1RF as demonstrated before [195]. However, all assays performed in this study use stationary phase ON cultures and defined numbers of bacteria as detected by counting of colony forming units (CFU).

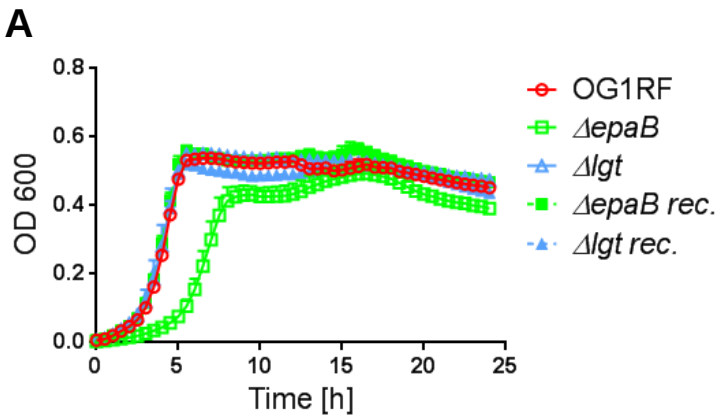

Figure 3: Growth of *E. faecalis* in the stationary phase is not affected by deficiency in *epaB* or *lgt*.

(**A**) Growth of *E. faecalis* OG1RF, *ΔepaB*, *Δlgt*, reconstituted *ΔepaB* or *Δlgt* mutant strain in BHI broth detected by automatic measurement of OD 600 every 30 min for 24 hours at standard conditions.

In a next step, we investigated how these bacterial structures impact on *E. faecalis* virulence. For this reason, we used the two invertebrate infection models *G. mellonella* and *C. elegans*, where the impact of single bacterial virulence factors can be easily monitored by survival of the model organism. Injection of *E. faecalis ΔepaB* or *Δlgt* into *G. mellonella* resulted in an increased survival of larvae (Fig.4A), while both reconstituted mutants exerted virulence

comparable to wild type *E. faecalis* (Fig.4B and C). Since we were interested in *E. faecalis*-host interactions that take place in the gut lumen, we next used the model organism *C. elegans* where bacteria are orally administered. In this infection assay *C. elegans* also revealed a significantly increased survival in the presence of the two mutant strains compared to *E. faecalis* wild type OG1RF (Fig.4D). Accordingly, *E. faecalis* virulence in these experimental model organisms with only innate immunity is mediated by both *epaB* and *lgt*.

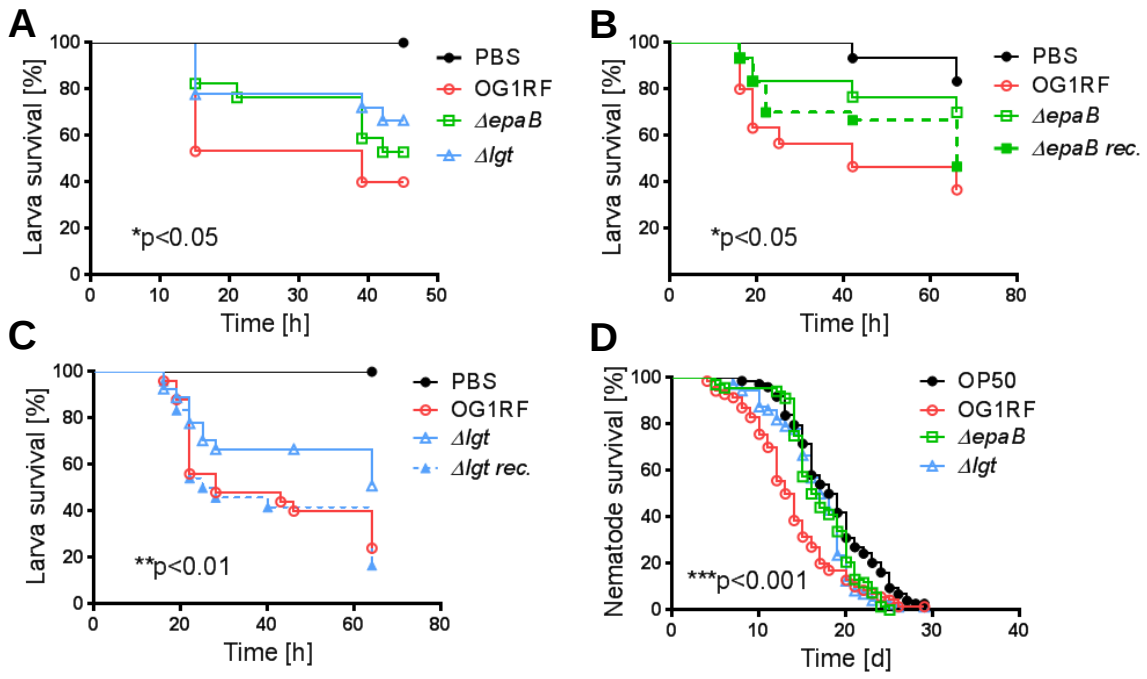

Figure 4: EpaB and Lgt promote virulence of *E. faecalis*.

(**A**) Survival of *G. mellonella* larvae after injection of *E. faecalis* OG1RF, *ΔepaB* or *Δlgt* strain and (**B**) *E. faecalis* OG1RF, *ΔepaB* or reconstituted *ΔepaB* mutant or (**C**) *E. faecalis* OG1RF, *Δlgt* or reconstituted *Δlgt* mutant. (**D**) Survival of *C. elegans* nematodes after oral administration of *E. faecalis* OG1RF, *ΔepaB* or *Δlgt* strains or *E. coli* OP50 as food control.

3.1.2 E. faecalis ΔepaB and Δlgt have impaired colitogenic activity

To investigate if virulence-relevant bacterial structures also direct the colitogenic activity of *E. faecalis*, germ-free wild type and IL-10-/- mice were monoassociated with *E. faecalis* wild type OG1RF, *ΔepaB* or *Δlgt* mutant strains for 16 weeks. While IL-10-/- mice monoassociated with *E. faecalis* OG1RF had severe inflammation in the distal colon (OG1RF histopathological score: 7.2±1.2), colitis was partially impaired in *E. faecalis ΔepaB* (*ΔepaB* score: 4.7±2.3; *p<0.05) and was almost completely abrogated in *E. faecalis Δlgt* monoassociated IL-10-/- mice (*Δlgt* score: 2.3±2.3; ****p<0.0001) (Fig.5A). In contrast, the inflammation persisted at low level in the cecum of IL-10-/- mice independent of which *E. faecalis* strain was used for monocolonization (Fig.5B). All monoassociated wild type mice remained disease free (Fig.5A and B). As demonstrated in figure 5C, the colonic inflammation in monoassociated IL-10-/- mice was characterized by massive infiltration of mononuclear cells into the lamina propria and loss of physiological crypt architecture that was gradually amended, when *E. faecalis* mutant strains were used for monoassociation.

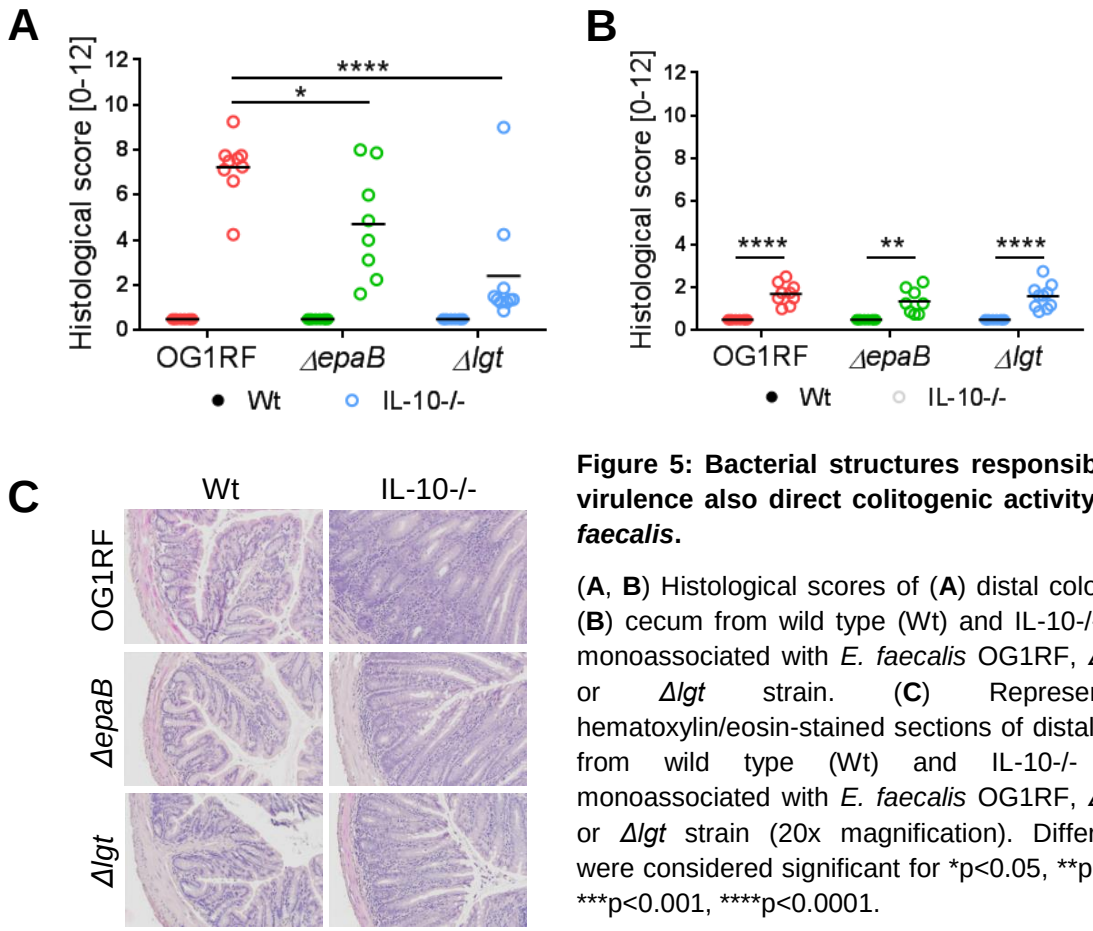

Figure 5: Bacterial structures responsible for virulence also direct colitogenic activity *of E. faecalis*.

(**A**, **B**) Histological scores of (**A**) distal colon and (**B**) cecum from wild type (Wt) and IL-10-/- mice monoassociated with *E. faecalis* OG1RF, *ΔepaB* or *Δlgt* strain. (**C**) Representative hematoxylin/eosin-stained sections of distal colon from wild type (Wt) and IL-10-/- mice monoassociated with *E. faecalis* OG1RF, *ΔepaB* or *Δlgt* strain (20x magnification). Differences were considered significant for *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

For wild type and IL-10-/- mice, the *E. faecalis* wild type OG1RF and both mutant strains showed a similar colonization density as detected by countable CFU in luminal contents from the ileum, cecum or colon (Fig.6A-C). A similar colonization pattern was detected when bacteria were isolated from mucosa-associated sites in the ileum or colon (Fig.6D and E).
Body and cecum weights of mice were not significantly altered according to the grade of intestinal inflammation or the *E. faecalis* strain used for monocolonization (Fig.7A and B). Compared to wild type mice, the spleen or mesenteric lymph nodes (MLN) to body weight ratio was significantly increased for IL-10-/- mice in general (Fig.7C and D). These weight

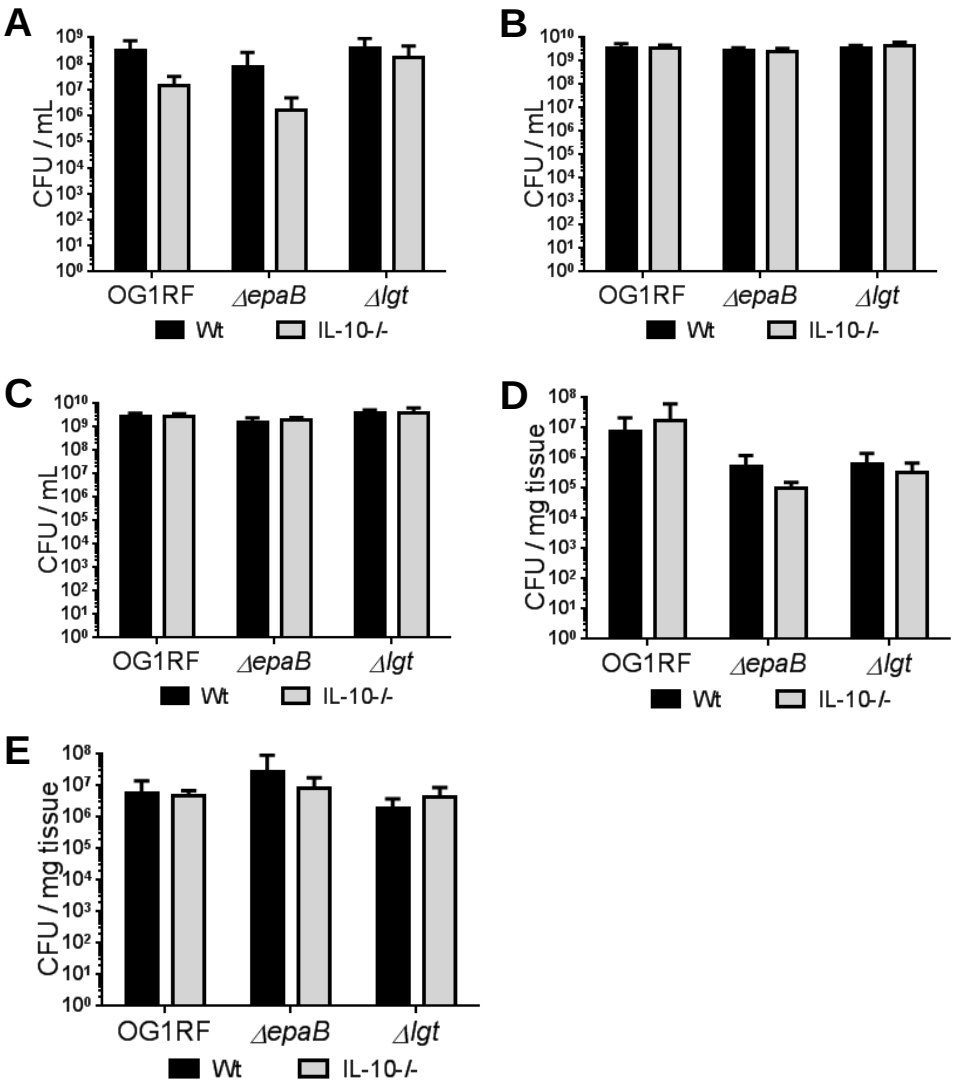

Figure 6: Deficiency in *epaB* or *lgt* does not affect intestinal colonization patterns of *E. faecalis* in wild type and IL-10-/- mice.

(**A**-**C**) *E. faecalis* presence in luminal contents from (**A**) ileum, (**B**) cecum and (**C**) colon of wild type (Wt) and IL-10-/- mice monoassociated with *E. faecalis* OG1RF, *ΔepaB* or *Δlgt* mutant strain according to CFU counts/mL. (**D**, **E**) Mucosa-associated *E. faecalis* presence in mucosal tissue from (**D**) ileum and (**E**) colon of wild type (Wt) and IL-10-/- mice monoassociated with *E. faecalis* OG1RF, *ΔepaB* or *Δlgt* mutant strain according to CFU counts/mg tissue.

ratios shifted closer to wild type level when the intestinal inflammation was decreased due to monoassociation with *E. faecalis ΔepaB* or *Δlgt* mutant strains. A similar trend was observed for length measurements of ileum or colon, where increased values for IL-10-/- mice also approximated to wild type levels when the chronic colitis was reduced in IL-10-/- mice monoassociated with *E. faecalis ΔepaB* or *Δlgt* (Fig.7E and F).

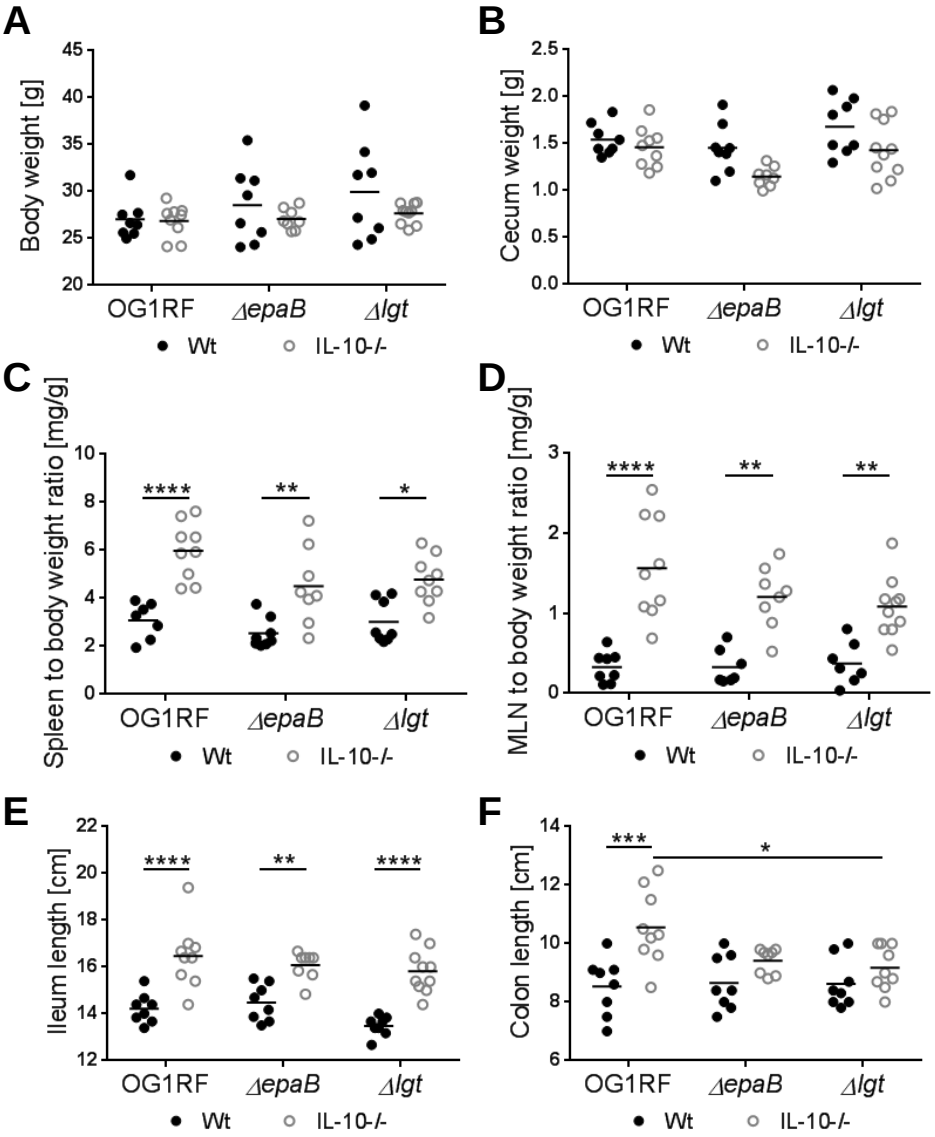

Figure 7: Attenuated colitogenic activity of *E. faecalis ΔepaB* or *Δlgt* does not impact on weight parameters in IL-10-/- mice.

(**A**) Body weight, (**B**) cecum weight, (**C**) spleen and (**D**) mesenteric lymph nodes (MLN) to body weight ratio, (**E**) ileum and (**F**) colon length of wild type (Wt) and IL-10-/- mice monoassociated with *E. faecalis* OG1RF, *ΔepaB* or *Δlgt* mutant strain. Differences were considered significant for *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

3.1.3 Reduced colitogenic activity of E. faecalis is directed by host immunity

Next, we examined how the loss of specific *E. faecalis* virulence factors affected cell infiltration and secretion of pro-inflammatory cytokines in monoassociated IL-10-/- mice. An immunofluorescence analysis revealed that infiltration of F4/80-positive (+) macrophages, Ly6G+ granulocytes and CD3+ T cells into the colonic mucosa of monoassociated IL-10-/- mice correlated with severity of inflammation in the distal colon (Fig.8A and B). However, the number of CD11c+ dendritic cells in the colonic mucosa was independent of disease activity or the strain used for monoassociation (Fig.8A and B).

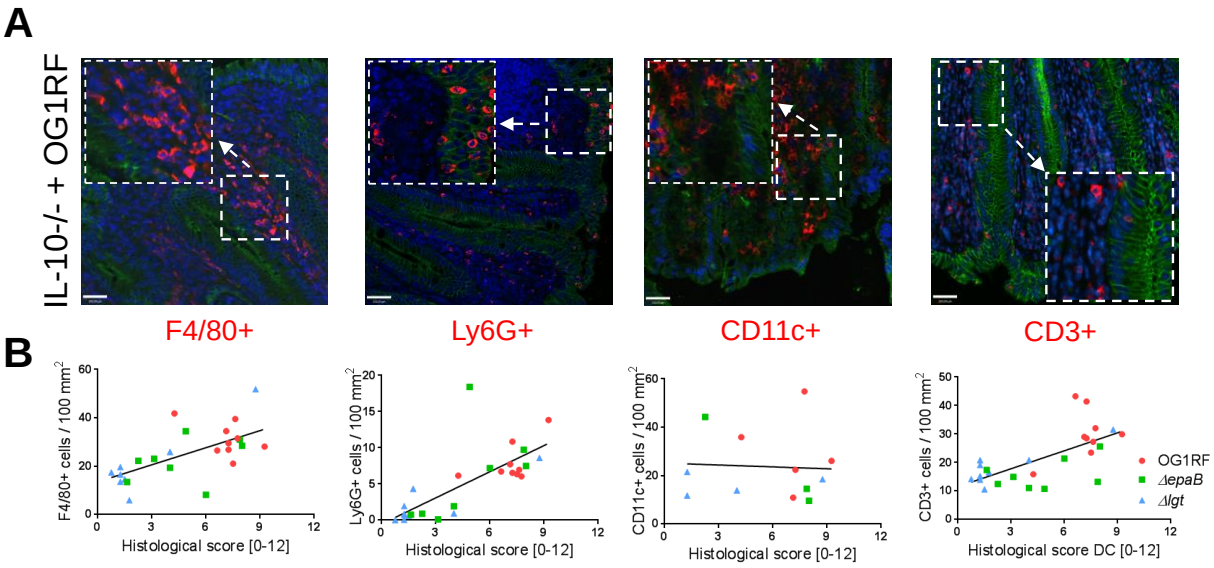

Figure 8: Colitogenic activity of *E. faecalis* is associated with infiltration of different immune cell subsets in the colon of IL-10-/- mice.

(**A**) Representative images of distal colon sections from IL-10-/- mice monoassociated with *E. faecalis* OG1RF stained by immunofluorescence for F4/80+ (red), Ly6G+ (red), CD11c+ (red) or CD3+ (red) cells, E-cadherin (intracellular domain, green), nuclei (blue) and magnifications of respective images as indicated by white frames (scale bar=100 µm). (**B**) Relationship between F4/80+, Ly6G+, CD11c+ or CD3+ cells infiltrating the distal colon of monoassociated IL-10-/- mice with respective histological scoring for distal colon was assessed by Pearson correlation coefficient test (F4/80+ cells: Pearson r=0.6460, ***p<0.001; Ly6G+ cells: Pearson r=0.7382, ****p<0.0001; CD11c+ cells: Pearson r=-0.05360, p>0.05; CD3+ cells: Pearson r=0.6661, ***p<0.001).

In an organ culture assay, where colonic tissue sections from wild type or IL-10-/- mice were incubated in cell culture medium to analyze spontaneous cytokine secretion, we detected increased levels of the pro-inflammatory cytokines IFN-γ and IL-17A for most IL-10-/- mice when compared to colon tissue from wild type mice (Fig.9A and B). No significant differences were detected with regard to the *E. faecalis* strain used for monoassociation. Since these colon sections were cut from the middle colon, the diverging results in the organ culture highlight the patchy distribution of colonic inflammation in IL-10-/- mice monoassociated with

E. faecalis, suggesting that different sites in the colon are exposed to a completely different local grade of inflammation.

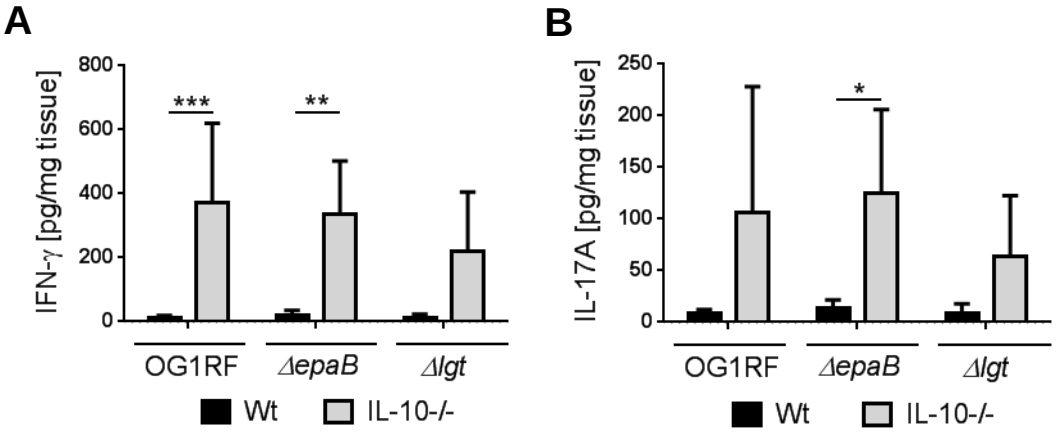

Figure 9: Impaired colitogenic activity of *E. faecalis ΔepaB* or *Δlgt* is not indicated by secretion of pro-inflammatory cytokines of colonic tissue from IL-10-/- mice.

(**A**) IFN-γ and (**B**) IL-17A cytokine secretion by whole colon tissue sections isolated from wild type (Wt) and IL-10-/- mice monoassociated with *E. faecalis* OG1RF, *ΔepaB* or *Δlgt* strain after incubation in RPMI medium for 24 hours *ex vivo*. Differences were considered significant for *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

In a next step, we analyzed the gene expression of pro-inflammatory cytokines in tissue sections, which were isolated from middle colon. Similar to the colon sections taken for the organ culture experiments, these tissue pieces did not reflect the grade of inflammation observed in the distal colon of monoassociated IL-10-/- mice. Accordingly, the expression of pro-inflammatory TNF, IL-6, IL-12p40 and IL-22 analyzed by qPCR from whole colonic tissue sections showed high inter-individual variation with non-significant trends towards higher expression levels in IL-10-/- mice, but no differences related to the *E. faecalis* strain used for monocolonization (Fig.10A-D).

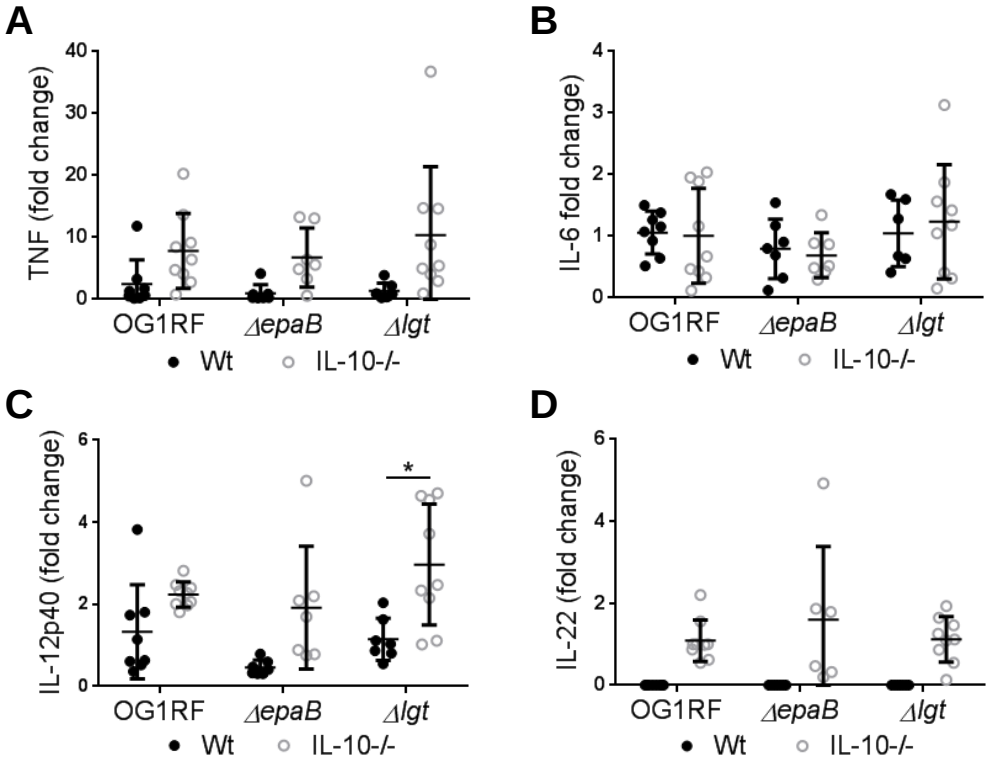

Figure 10: Impaired colitogenic activity of *E. faecalis ΔepaB* or *Δlgt* does not impact on expression of pro-inflammatory cytokines in the colon of IL-10-/- mice.

(**A**) TNF, (**B**) IL-6, (**C**) IL-12p40 and (**D**) IL-22 expression in whole colon tissue sections from wild type (Wt) and IL-10-/- mice monoassociated with *E. faecalis* OG1RF, *ΔepaB* or *Δlgt* mutant strain shown as fold change normalized to cytokine expression levels in wild type mice monoassociated with *E. faecalis* OG1RF. Differences were considered significant for *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

In addition, chemoattractant cytokines such as keratinocyte-derived chemokine (KC) and MIP3α/CCL-20 showed no differential expression between *E. faecalis* wild type OG1RF and the *E. faecalis* mutant strain lacking *lgt* (Fig.11A and B). This was confirmed by an immunofluorescence staining for MIP3α/CCL-20 in IECs of the distal colon of monoassociated IL-10-/- mice demonstrating no substantial alterations regarding the presence of MIP3α/CCL-20 in the murine colonic mucosa (Fig.11C). However, we detected a non-significant reduction in the expression of chemotactic KC and MIP3α/CCL-20 in the colon of IL-10-/- mice monoassociated with *E. faecalis* lacking *epaB* (Fig.11A and B). Representative immunofluorescence pictures confirmed the slightly decreased presence of MIP3α/CCL-20 in IL-10-/- mice monoassociated with *E. faecalis ΔepaB*, which may suggest that the secretion of chemotactic stimuli by IECs in the colon is dependent on sensing of *E. faecalis* in close proximity to the intestinal epithelium (see next chapter).

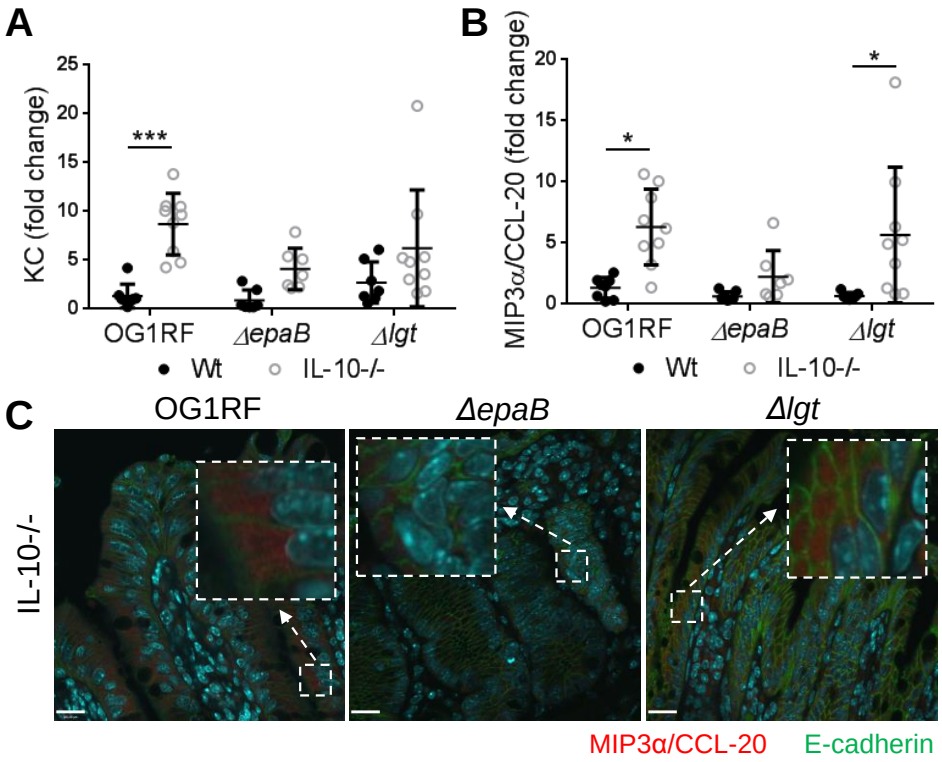

Figure 11: *E. faecalis* EpaB and lipoproteins do not affect expression of chemokines in the colon of IL-10-/- mice.

(**A**) Keratinocyte-derived chemokine (KC) and (**B**) MIP3α/CCL-20 expression in whole colon tissue sections from wild type (Wt) and IL-10-/- mice monoassociated with *E. faecalis* OG1RF, *ΔepaB* or *Δlgt* mutant strain shown as fold change normalized to cytokine expression levels in wild type mice monoassociated with *E. faecalis* OG1RF. (**C**) Representative images stained for MIP3α/CCL-20 (red) and E-cadherin (intracellular domain, green) in the distal colon of IL-10-/- mice monoassociated with *E. faecalis* OG1RF, *ΔepaB* or *Δlgt* mutant strain (scale bar=100 µm). Differences were considered significant for *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

3.2 EpaB mediates *E. faecalis* mucus penetration and adhesion to intestinal mucosa

3.2.1 E. faecalis ΔepaB exhibit defective mucus penetration

We next tried to identify how EpaB and bacterial lipoproteins direct *E. faecalis*-host interactions and by this impact on its colitogenic activity. For this reason, we performed co-staining of fluorescence *in-situ* hybridization (FISH)-labeled *E. faecalis* and MUC2 in the distal colon of monoassociated wild type and IL-10-/- mice to visualize distribution of bacteria at inflammation-relevant mucosal sites (Fig.12A). To quantify the overall proximity of luminal *E. faecalis* to the colonic epithelium or the bacterial penetration of colonic mucus, respectively, we measured the distance of single bacterial cells in acquired images as displayed by representative histograms for *E. faecalis* distribution (Fig.12B and C). In wild type mice, the bacterial distribution pattern and average distance to the epithelial surface

was similar for all *E. faecalis* strains used in this study (Fig.12B). But in IL-10-/- mice the lack of *epaB* significantly increased the average distance of *E. faecalis* from the epithelial surface when compared to *E. faecalis* wild type OG1RF and *Δlgt* mutant strain (Fig.12C), suggesting attenuated bacterial penetration of the outer colonic mucus in the absence of EpaB. As already demonstrated [44], the inner mucus layer in the colon of IL-10-/- mice was thicker compared to wild type mice (Fig.12A). However, we were not able to observe an increased bacterial penetration of the inner mucus in IL-10-/- mice by any *E. faecalis* strain (Fig.12C) as described before [44].

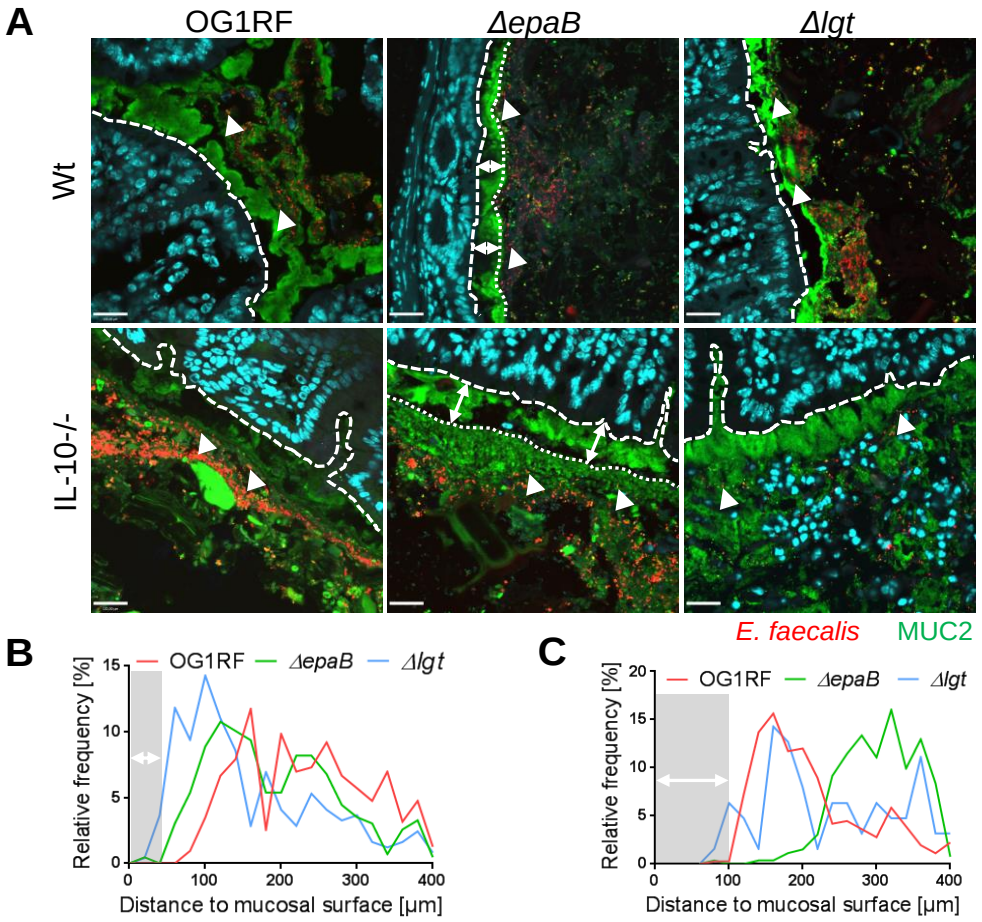

Figure 12: EpaB mediates *E. faecalis* adhesion to intestinal mucus *in vivo*.

(**A**) Representative images of distal colon sections from wild type (Wt) and IL-10-/- mice monoassociated with *E. faecalis* OG1RF, *ΔepaB* or *Δlgt* mutant strain, stained by immunofluorescence for MUC2 (green), nuclei (blue) and FISH for *E. faecalis* (red) (scale bar=120 µm). The epithelial cell surface is indicated by interrupted white line, white arrows indicate representative FISH-labeled *E. faecalis*. In the representative pictures for *E. faecalis ΔepaB* the inner-to-outer mucus interface is indicated by dotted white line with white long arrows indicating the distance from this interface layer to the epithelial cell surface. (**B**, **C**) Histograms showing the depth of penetration of the mucus layer by *E. faecalis* cells in the corresponding representative distal colon sections from (**B**) wild type and (**C**) IL-10-/- mice monoassociated with *E. faecalis* OG1RF, *ΔepaB* or *Δlgt* mutant strain (0 to 400µm distance from epithelial cell surface as indicated by interrupted white line in the representative pictures; grey areas indicate the average thickness of the inner mucus layer).

The defective adhesion of *E. faecalis ΔepaB* to mucus-covered surfaces was confirmed in an *in vitro* adhesion assay with LS174T cells. This human colon carcinoma cell line features a goblet cell-like phenotype and produces a thick and continuous mucus layer that resembles the properties of colonic mucus (Fig.13A and [224]). The co-incubation of monolayers of LS174T cells with *E. faecalis* resulted in bacteria attaching to the mucus-covered surface (Fig.13A and B). In this assay setup, only *E. faecalis* lacking *epaB* showed a significantly impaired adhesion to the colon-like mucus produced by the monolayer of LS174T cells (Fig.13C).

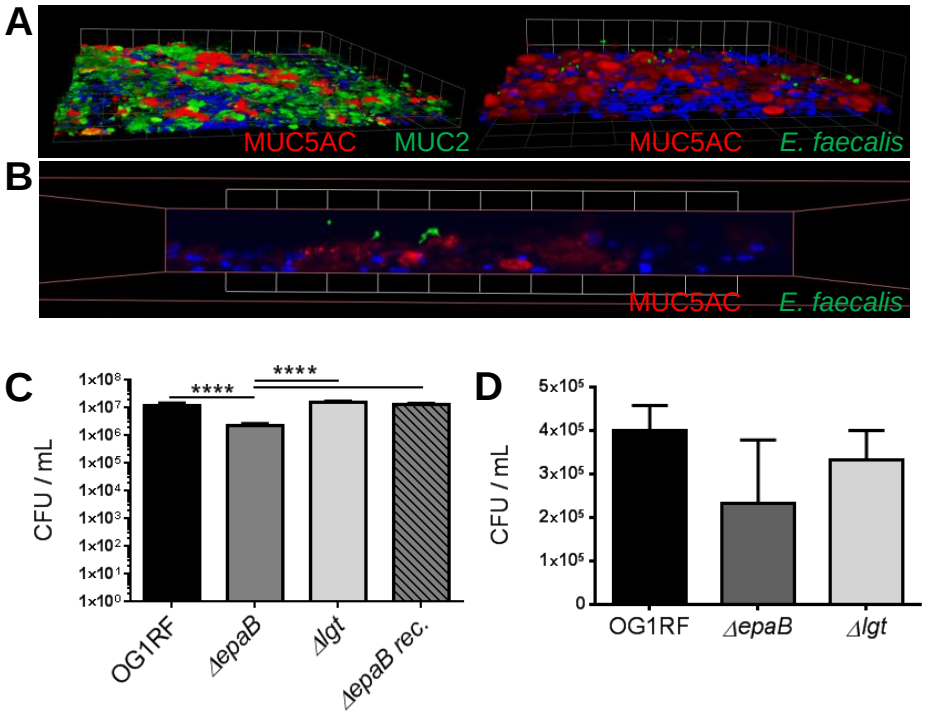

Figure 13: EpaB promotes adhesion of *E. faecalis* to colon-like mucus and mucosal surfaces *in vitro*.

(**A**, **B**) Mucus formed by LS174T cell monolayer stained by immunofluorescence for MUC5AC (red), MUC2 (green) or *E. faecalis* (green), respectively, and nuclei (blue). *E. faecalis* OG1RF adhesion to mucus formed by LS174T cell monolayer after 2 hours of incubation is shown by representative 3D-reassembled images from (**A**) plan and (**B**) cross-sectional view. (**C**) Adhesion of *E. faecalis* to mucus-covered surface of LS174T cell monolayer after 2 hours of incubation according to CFU counts/mL. (**D**) Adhesion of *E. faecalis* to Caco-2 cell monolayer after 2 hours of incubation according to CFU counts/mL. Differences were considered significant for *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

3.2.2 E. faecalis ΔepaB show altered adhesion to intestinal epithelium

In contrast, no significant difference was detected regarding the adhesion of all three *E. faecalis* strains to an IEC monolayer of Caco-2 cells (Fig.13D), suggesting that *E. faecalis*

adhesion is differentially mediated by host structures and cell types. We then performed monocolonization experiments with germ-free *Manduca* (*M.*) *sexta* larvae (Fig.14A) to analyze the impact of EpaB on *E. faecalis* adhesion to mucosal surfaces *in vivo*. Since *E. faecalis* is a natural commensal to *M. sexta* [225,226], this invertebrate represents a simplified model to study commensal-host interactions *in vivo*. It can be raised under germ-free conditions with an artificial wheat-based diet and can be monoassociated with *E. faecalis* (Fig.14B).

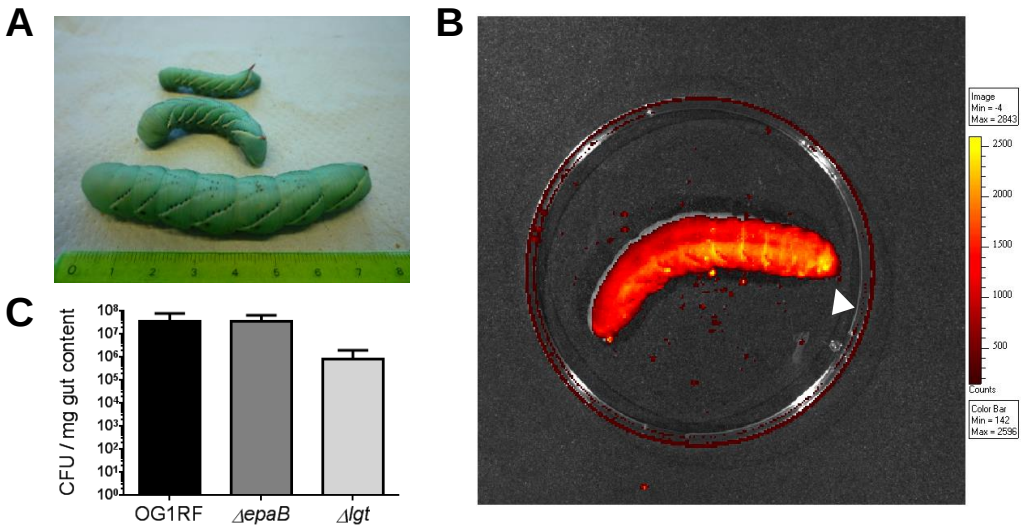

Figure 14: Monoassociation of germ-free *Manduca sexta* with *E. faecalis* to investigate commensal-host interactions.

(**A**) Different larval stages of *M. sexta* with the biggest one being in the 5th instar stage. (**B**) Representative picture from *in vivo* imaging analysis of *M. sexta* (anterior end indicated by white arrow) associated with *E. faecalis* OG1RF expressing a GFP-vector showing the colonization density and localization of *E. faecalis* by bioluminescence detection. (**C**) Colonization of the midgut epithelium of groups of *M. sexta* larvae monoassociated with *E. faecalis* OG1RF, *ΔepaB* or *Δlgt* strain according to CFU counts/mg gut content.

Despite equal colonization of *M. sexta* larvae (Fig.14C), only *E. faecalis* wild type OG1RF and *Δlg*t mutant strain were able to reach close proximity to the brush-border membrane of monoassociated *M. sexta* larvae, whereas the *ΔepaB* mutant strain failed to localize in direct contact to the midgut epithelium (Fig.15A). Interestingly, *M. sexta* were susceptible to DSS treatment resulting in significant growth retardation and higher mortality of larvae (Fig.16A and B). This was probably mediated by an abrogated peritrophic matrix formation (Fig.16C and D), which resembles mucus homologous functions in invertebrates [227–229] and also serves as habitat for *E. faecalis* in the midgut.

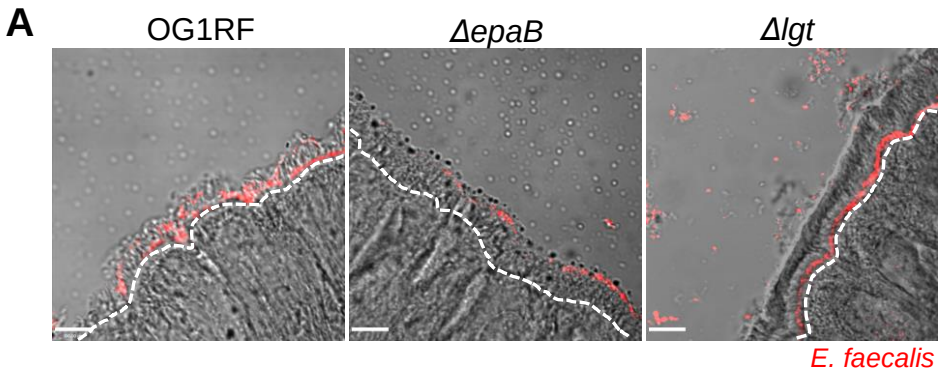

Figure 15: EpaB mediates adhesion of *E. faecalis* to intestinal epithelium *in vivo*.

(**A**) Adhesion of *E. faecalis* OG1RF, *ΔepaB* or *Δlgt* strains to the intestinal midgut epithelium of monoassociated *M. sexta* larvae as shown by representative bright-field images from sections stained for *E. faecalis* (red) by immunofluorescence (epithelial cell surface is indicated by interrupted white line; scale bar=100 µm).

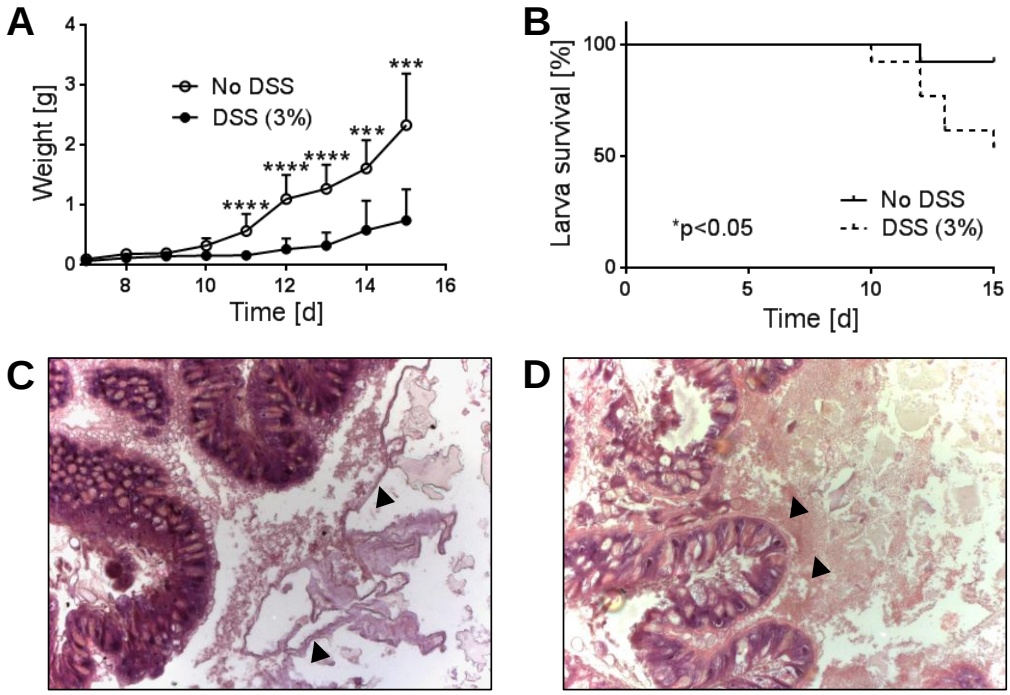

Figure 16: *M. sexta* is susceptible to DSS treatment.

(**A**) Weight development and (**B**) survival of *M. sexta* raised under conventional conditions on wheat-based artificial diet with/without 3% dextran sodium sulfate (DSS) added. (**C**, **D**) Representative hematoxylin/eosin-stained sections of the midgut epithelium of *M. sexta* fed a diet (**C**) without or (**D**) with 3% DSS added to the diet (20x magnification). The peritrophic matrix (indicated by black arrows), which retains the luminal content from the epithelium and confers “mucus-like functions” in invertebrates, is impaired when DSS is added to the diet. Differences were considered significant for *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

3.2.3 Loss of epaB has no effect on innate immunity in M. sexta

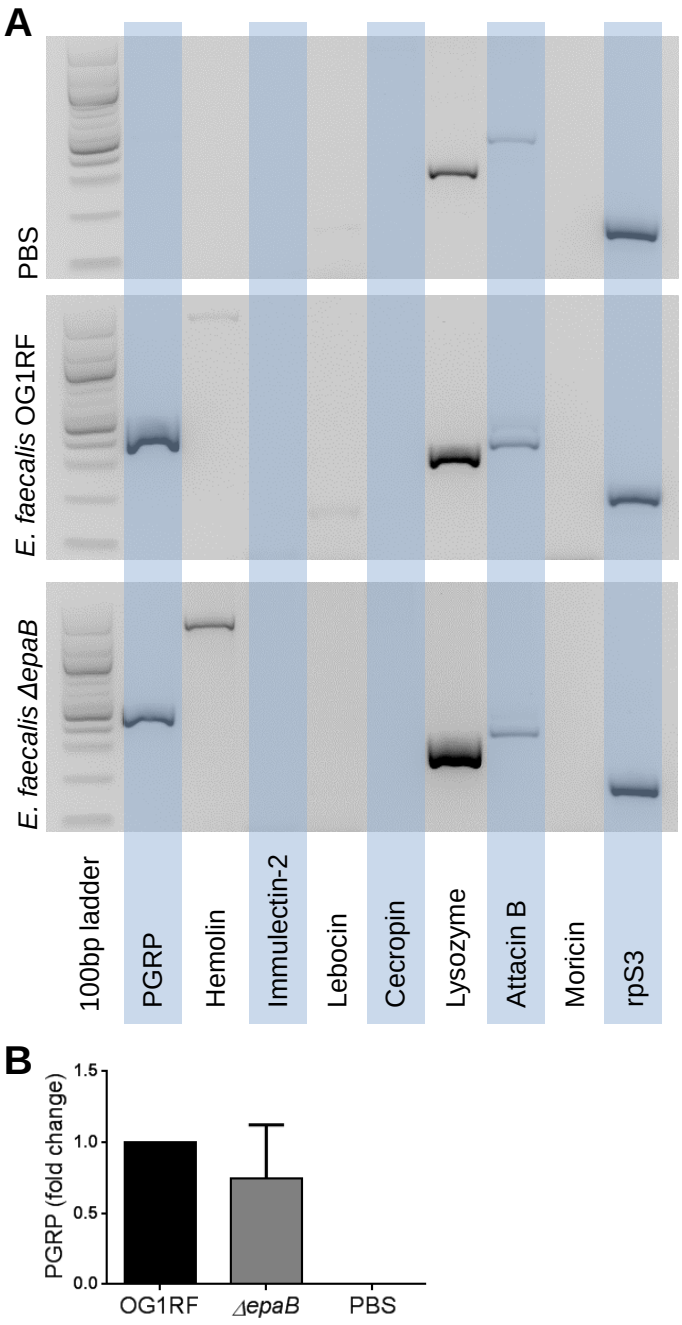

Figure 17: Altered adhesion of *E. faecalis* to intestinal epithelium does not impact on expression of innate immune-related proteins and peptides in *M. sexta*.

(**A**) Representative expression of pattern recognition proteins (PGRP, Hemolin, Immulectin-2), antimicrobial peptides (Lebocin, Cecropin, Lysozyme, Attacin B, Moricin) and ribosomal protein rpS3 in midgut tissue of germ-free *M. sexta* or monoassociated with *E. faecalis* OG1RF or *ΔepaB* mutant strain by PCR. (**B**) PGRP expression in the midgut tissue of germ-free *M. sexta* or monoassociated with *E. faecalis* OG1RF or *ΔepaB* mutant strain as fold change normalized to PGRP expression induced by *E. faecalis* OG1RF.

Next, we screened for the inducible expression of pattern recognition receptors (PRR) (Peptidoglycan recognizing protein (PGRP), Hemolin, Immulectin-2) [230] and antimicrobial peptides (AMP) (Lebocin, Cecropin, Lysozyme, Attacin B, Moricin) [218] (Table 5) in the midgut tissue of *M. sexta* larvae monoassociated with either *E. faecalis* wild type OG1RF or the *ΔepaB* mutant strain. Compared to larvae associated with *E. faecalis* wild type OG1RF, the expression of all host innate immune proteins detected by PCR (PGRP, Lysozyme, Attacin B) was reduced for germ-free larvae treated with PBS as control (Fig.17A). The ribosomal *M. sexta* protein rpS3 served as positive baseline control. When comparing *E. faecalis* OG1RF and the *ΔepaB* mutant strain, the most substantial shift in expression occurred for PGRP. Since peptidoglycan is known to be involved in the integration of secondary wall polysaccharides, such as the Epa rhamnopolysaccharide, into the cell wall of *E. faecalis* [192], we quantitatively determined the impact of *E. faecalis ΔepaB* on PGRP expression in monoassociated *M. sexta* by qPCR. The expression of

this PRR for bacterial peptidoglycan in the midgut tissue of *M. sexta* was similar for larvae monoassociated with either *E. faecalis* wild type OG1RF or *ΔepaB* mutant strain (Fig.17B). This suggests that altered distances of *E. faecalis* to the intestinal epithelium do not necessarily coincide with shifts in innate immune response in this experimental model.

3.3 EpaB promotes formation of biofilm and associated bacterial microcolonies

3.3.1 Deletion of epaB impairs biofilm and associated microcolony formation

Finally, *E. faecalis* wild type OG1RF and all mutant strains were tested for their capacity to form biofilm and associated multi-cellular aggregates ('microcolonies') measured as microcolony volume on a fixed monolayer of murine intestinal epithelial cells (Fig.18A). Immunofluorescence analysis demonstrated significantly reduced volume of visualized microcolonies for *E. faecalis ΔepaB* compared to wild type OG1RF, *Δlgt* or reconstituted *ΔepaB* mutant strains (Fig.18B).

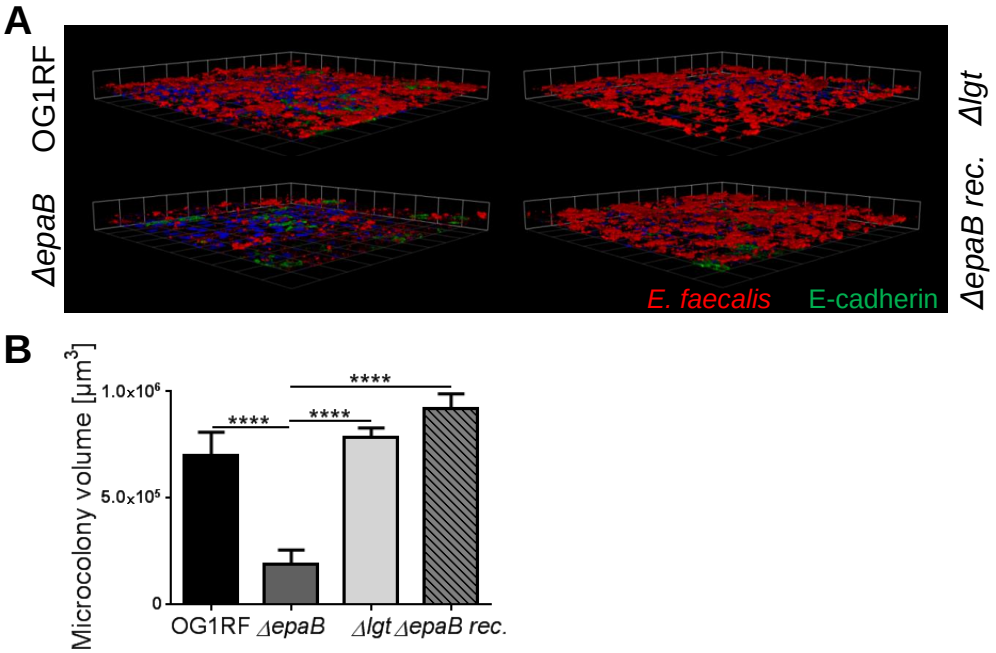

Figure 18: *E. faecalis* biofilm-associated microcolony formation is dependent on *epaB*.

(**A**) Microcolonies formed by *E. faecalis* OG1RF, *ΔepaB*, *Δlgt* or reconstituted *ΔepaB* strain *in vitro* after incubation for 20 hours on a fixed monolayer of murine Ptk-6 intestinal epithelial cells. Representative images stained by immunofluorescence for *E. faecalis* (red), E-cadherin (intracellular domain, green) and nuclei (blue) showing 3D-reassembling of single stacks and (**B**) quantitation of total microcolony biomass. Differences were considered significant for *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

The production of biofilms is known as virulence trait of *E. faecalis* [211] and provides a growth-promoting matrix for *E. faecalis* microcolonies. Hence, we investigated the ability of *E. faecalis* wild type OG1RF and both mutant strains to form biofilm. Here, only the *E. faecalis ΔepaB* mutant strain showed a significantly impaired capacity to form biofilm on polystyrene surfaces (Fig.19A) confirmed by reduced average thickness and total biomass of biofilms (Fig.19B and C).

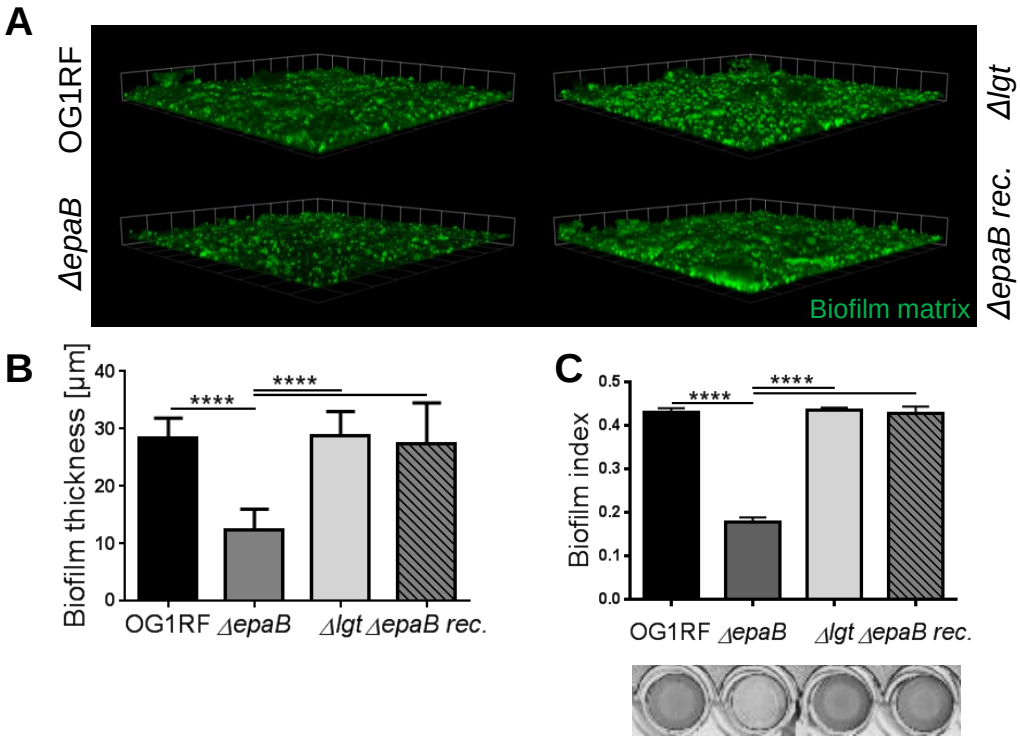

Figure 19: *E. faecalis* biofilm formation is mediated by *epaB*.

(**A**) Representative 3D-reassembled images and (**B**) average thickness of biofilms from *E. faecalis* OG1RF, *ΔepaB*, *Δlgt* or reconstituted *ΔepaB* strain on collagen-IV-coated polystyrene surface after 24 hours incubation, stained for total biomass (green). (**C**) Biofilm indices and representative wells showing total biofilm formation of *E. faecalis* OG1RF, *ΔepaB* or *Δlgt* or reconstituted *ΔepaB* strain on polystyrene surface after 20 hours incubation stained for biofilm matrix with Hucker's crystal violet. Differences were considered significant for *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

3.4 *E. faecalis* gelatinase E activity is not affected by *epaB* or *lgt* deficiency

3.4.1 E. faecalis ΔepaB and Δlgt show substantial gelatinase E activity

GelE is known as a bacterial virulence factor relevant for the colitogenic activity of *E. faecalis* [29] and upregulated by *E. faecalis* under conditions of chronic colitis in IL-10-/- mice. Thus, we analyzed how GelE activity is affected by *epaB* or *lgt* deficiency. As indicated by E-cadherin presence in a fixed monolayer of murine IECs, *E. faecalis ΔepaB* and *Δlgt* mutant strains exerted a similar GelE activity compared to *E. faecalis* wild type OG1RF (Fig.20A). In the same assay, the quantification of immunofluorescence revealed a slightly reduced GelE

activity for *E. faecalis* lacking *epaB in vitro* (Fig.20B), which may originate from the attenuated formation of microcolonies by *E. faecalis ΔepaB* in this setup (see also Fig.18A and B) resulting in less bacteria and less GelE present in close contact to epithelial cells.

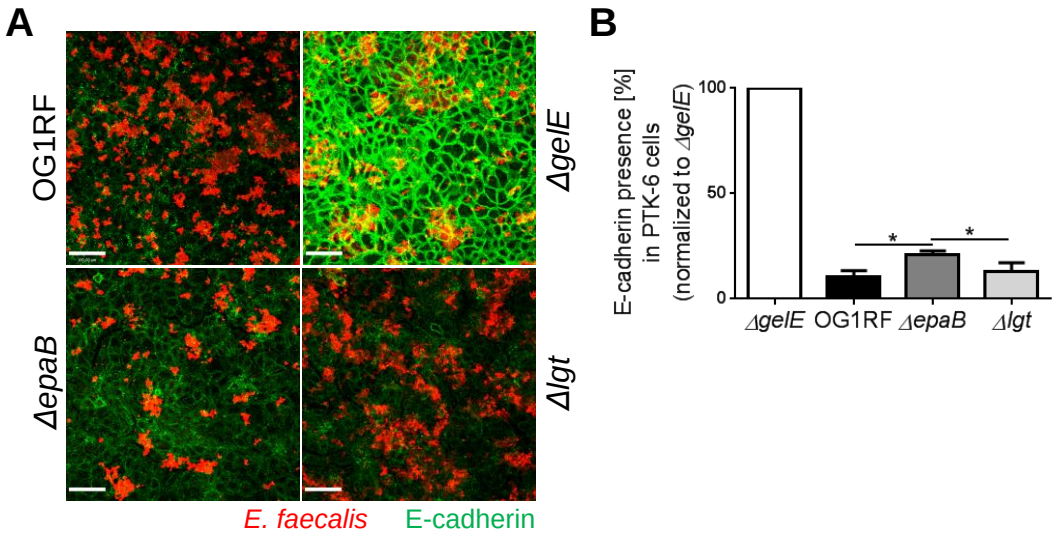

Figure 20: Gelatinase E activity of *E. faecalis* is not dependent on *epaB* or *lgt in vitro*.

(**A**) Presence of E-cadherin in a fixed monolayer of murine Ptk-6 intestinal epithelial cells incubated for 20 hours with *E. faecalis* OG1RF, *ΔepaB* or *Δlgt* strain or an *E. faecalis* mutant lacking gelatinase E (*ΔgelE*) as reference (scale bar=100 µm). Representative images stained by immunofluorescence for E-cadherin (intracellular domain, green) and *E. faecalis* (red) showing E-cadherin of the intestinal epithelial cell monolayer and (**B**) according quantification of E-cadherin (normalized to value for *E. faecalis ΔgelE*) as indicator for gelatinase E presence and/or activity secreted by *E. faecalis*. Differences were considered significant for *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

In addition, immunofluorescence staining showed substantial degradation of extracellular parts of E-cadherin for all three *E. faecalis* strains in the distal colon of monoassociated IL-10-/- mice independently of the inflammation level (Fig.21A). This emphasizes that GelE activity of *E. faecalis in vivo* is not affected by the deletion of *epaB* or *lgt* and suggests that

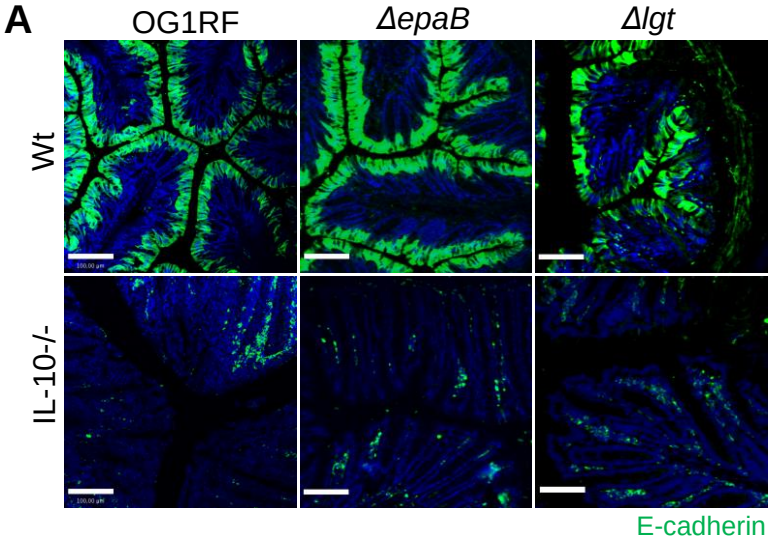

Figure 21: EpaB or Lgt do not affect *E. faecalis* gelatinase E activity in the inflamed colon of IL-10-/- mice.

(**A**) Representative images of distal colon sections from wild type (Wt) and IL-10-/- mice monoassociated with *E. faecalis* OG1RF, *ΔepaB* or *Δlgt* strain stained by immunofluorescence for E-cadherin (extracellular domain, green) and nuclei (blue) to visualize degradation of E-cadherin by gelatinase E *in vivo* (scale bar=100 µm).

GelE-mediated cleavage of E-cadherin is not crucial for the induction of chronic colitis in IL-10-/- mice.

3.5 *E. faecalis* deficient in lipoproteins show altered activation of immune cells

3.5.1 Loss of E. faecalis lipoproteins impairs activation of DCs, mediated by TLR2

Since the *E. faecalis Δlgt* mutant was associated with almost abrogated inflammation in the distal colon of monoassociated IL-10-/- mice, we further investigated the capacity of lipoprotein-deficient *E. faecalis* to trigger immune cell activation. First, bone marrow-derived dendritic cells (BMDC) from wild type mice secreted significantly less TNF (Fig.22A) and IL-6 (Fig.22B) in response to stimulation with lysates from *E. faecalis Δlgt* mutant strain vs. lysates from wild type OG1RF. The lipoprotein-dependent induction of TNF and IL-6

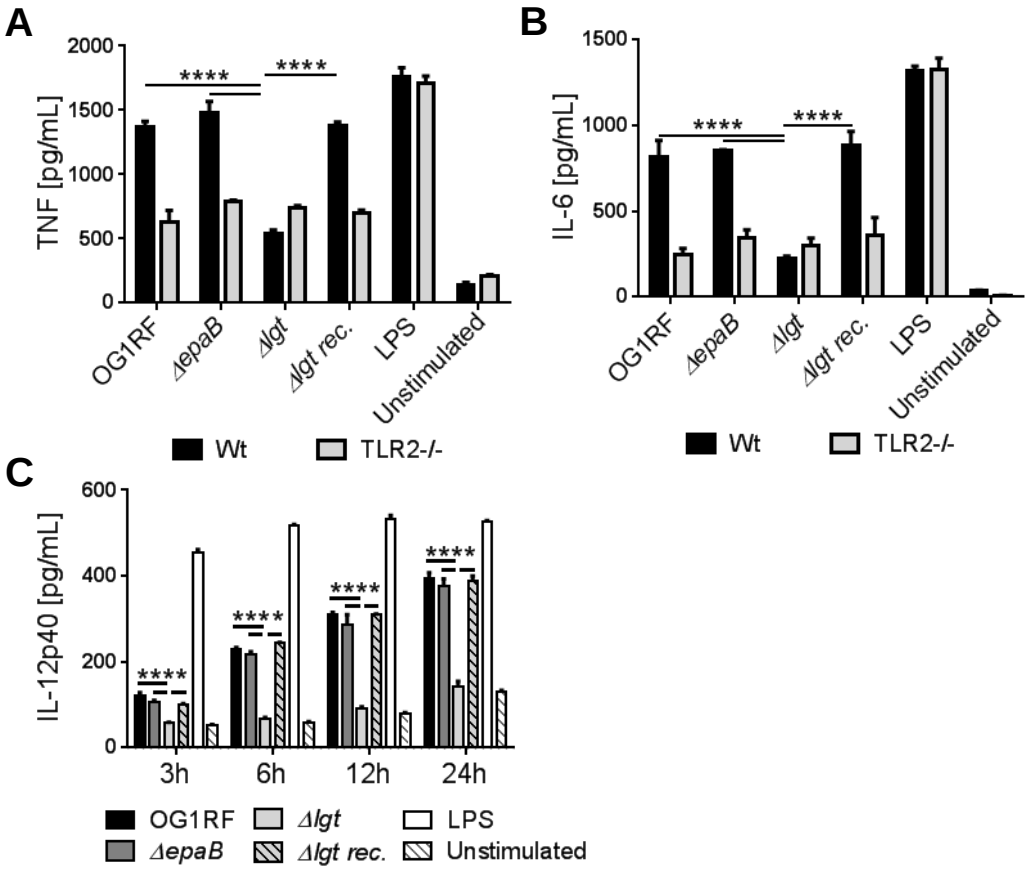

Figure 22: TLR-2 mediated activation of dendritic cells is dependent on *E. faecalis* lipoproteins *in vitro*.

(**A**) TNF and (**B**) IL-6 cytokine secretion by bone marrow-derived dendritic cells from wild type (Wt) and TLR2-/- mice after stimulation with lysates of *E. faecalis* OG1RF, *ΔepaB*, *Δlgt* or reconstituted *Δlgt* strain or LPS as control for 24 hours *in vitro*. (**C**) Time-dependent secretion of IL-12p40 by BMDCs isolated from IL-10-/- mice after stimulation with lysates of *E. faecalis* OG1RF, *ΔepaB*, *Δlgt* or reconstituted *Δlgt* strain or LPS as control for 3, 6, 12 or 24 hours *in vitro*. Differences were considered significant for *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

secretion was completely abrogated in BMDCs from TLR2-deficient (TLR2-/-) mice (Fig.22A and B). The secretion of IL-12p40 seen with wild type strain OG1RF was persistently reduced when BMDCs from IL-10-/- mice were stimulated with *E. faecalis Δlgt* lysates for 3, 6, 12 and 24 hours (Fig.22C).

When analyzed by flow cytometry, BMDCs stimulated with lysates from the *E. faecalis Δlgt* mutant strain showed no substantial differences in surface expression of DC activation marker MHC-II and CD86 compared to BMDCs stimulated with lysates from *E. faecalis* wild type OG1RF (Fig.23A).

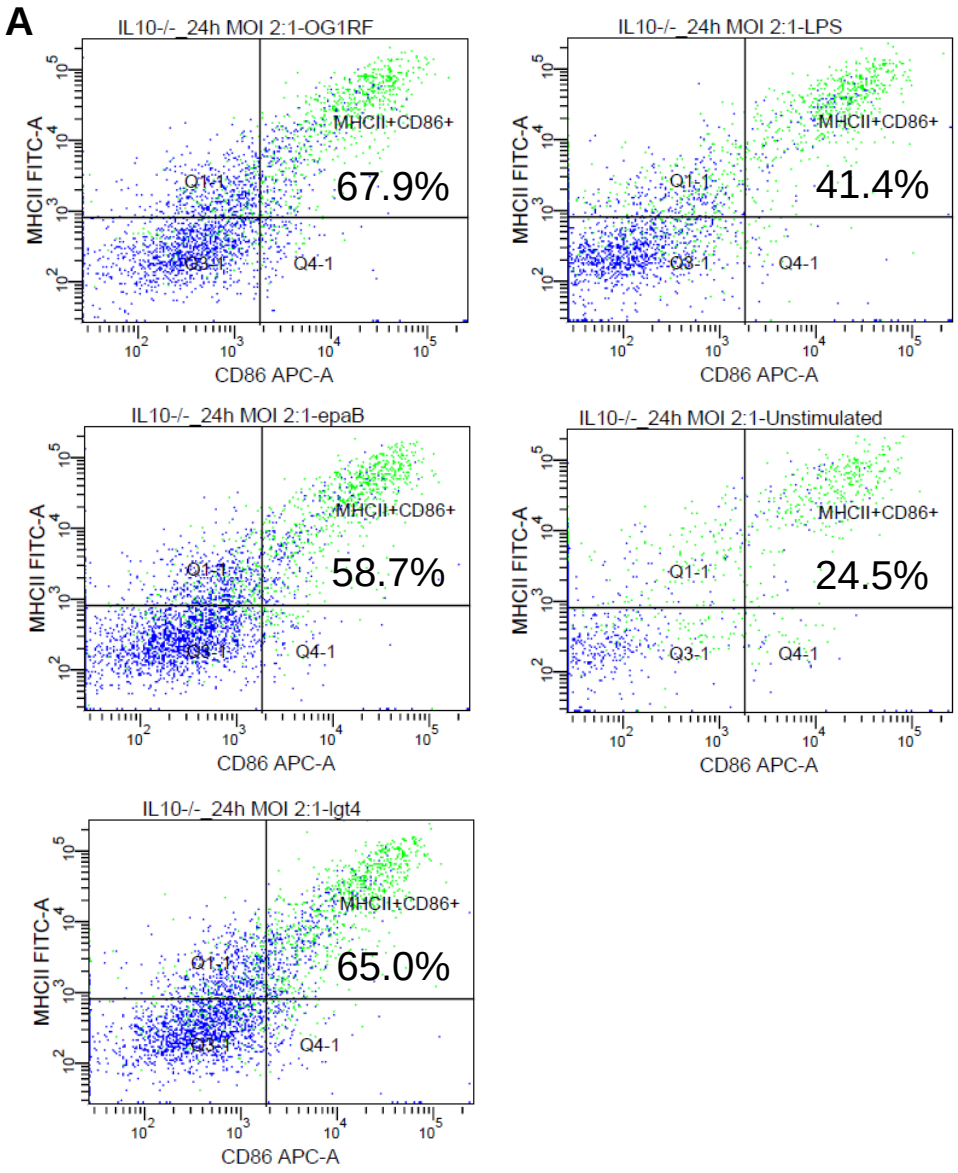

Figure 23: Activation markers of dendritic cells are not affected by bacterial lipoproteins *in vitro*.

(**A**) Percentages of MHCII+/CD86+ subpopulations in bone marrow-derived dendritic cells after stimulation with lysates from *E. faecalis* OG1RF, *ΔepaB* or *Δlgt* mutant strain or LPS or unstimulated control for 24 hours as analyzed by representative flow cytometry analysis (population gated for CD11b+/CD11c+ cells).

Also the phagocytic uptake of all *E. faecalis* strains by BMDCs was similar [212], suggesting that the TLR2-mediated induction of pro-inflammatory cytokine secretion was not caused by altered phagocytosis. Finally, Western blot analysis using polyclonal antibodies recognizing either enterococcal LTA or whole bacteria revealed that both, electrophoretic mobility and the amount of LTA as well as protein patterns, are not affected in the lysates of the *E. faecalis Δlgt* mutant strain [212] making it unlikely for this cell wall structure to modulate any of the effects observed in BMDCs.

3.5.2 E. faecalis Δlgt is able to (re-)activate colitogenic T cells

To investigate how differences in innate immune cell activation impact on the potential to reactivate colitogenic T cells from inflamed IL-10-/- mice, we performed DC-T cell co-cultures. BMDCs, isolated from IL-10-/- mice and stimulated with lysates from the different *E. faecalis* strains, were co-cultured with CD4+ T cells isolated from MLN of *E. faecalis* OG1RF monoassociated IL-10-/- mice (same mice as in Fig.5A). As shown in figure 24, there was no

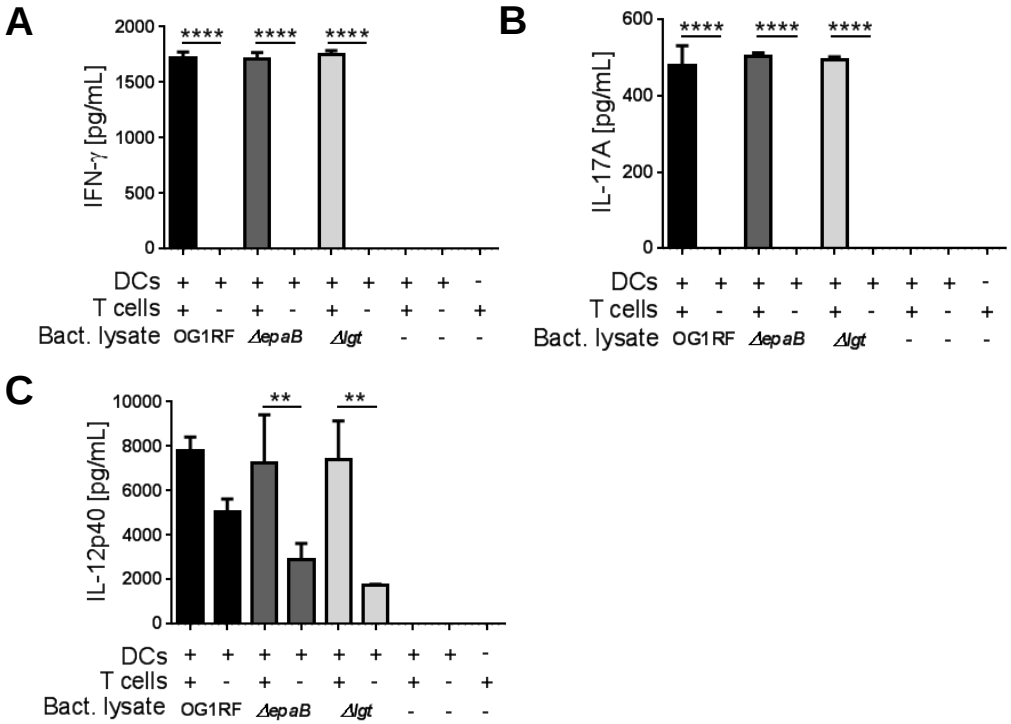

Figure 24: Activation of colitogenic T cells by antigen-presenting cells is not dependent on *E. faecalis* lipoproteins.

(**A**) IFN-γ, (**B**) IL-17A and (**C**) IL-12p40 secretion in an DC-T cell co-culture, where DCs isolated from bone-marrow of IL-10-/- mice were pulsed with lysates from *E. faecalis* OG1RF, *ΔepaB* or *Δlgt* strains and afterwards co-cultured for 72 hours with CD4+ T cells isolated and pooled from mesenteric lymph nodes of IL-10-/- mice monoassociated with *E. faecalis* OG1RF. Differences were considered significant for *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

difference in IFN-γ, IL-17A and IL-12p40 (Fig.24A-C) secretion in the presence of T cells, suggesting that both *E. faecalis* mutant strains were able to stimulate an antigen-dependent reactivation of colitogenic T cells. However, in the absence of T cells there was a non-significant trend towards lower IL-12p40 secretion detectable, when BMDCs were stimulated with *E. faecalis* mutant lysates (Fig.24C).

To determine whether the differences in colonic inflammation observed in IL-10-/- mice monoassociated with the different *E. faecalis* strains correlate with altered antigen-specific T cells responses *ex vivo*, we isolated MLN cells from IL-10-/- and wild type mice monoassociated with *E. faecalis* OG1RF, ***ΔepaB*** or ***Δlgt*** mutant strain and restimulated them with lysates of the corresponding *E. faecalis* strain. IFN-γ and IL-17A secretion from reactivated MLN cells was similar for all IL-10-/- mice (Fig.25A and B), suggesting that independent of colonic pathology MLN cells from these mice were fully capable of responding to the bacterial antigens. But there was a non-significant trend towards lower IL-17A secretion levels detected for MLN cells isolated from IL-10-/- mice monoassociated with the *E. faecalis* mutant strains (Fig.25B).

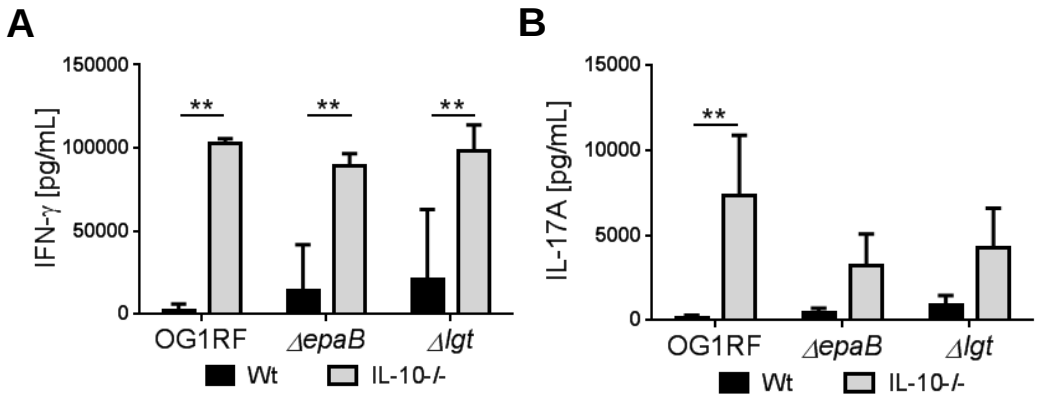

Figure 25: Reactivation of colitogenic T cells is not directed by *E. faecalis* lipoproteins.

(**A**) IFN-γ and (**B**) IL-17A secretion by mesenteric lymph node cells isolated from wild type (Wt) and IL-10-/- mice monoassociated with *E. faecalis* OG1RF, ***ΔepaB*** or ***Δlgt*** strains that were reactivated with the corresponding lysate for 72 hours. Differences were considered significant for *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Similarly, we detected no differences in IFN-γ in plasma from IL-10-/- mice colonized with the different *E. faecalis* strains (Fig.26A). However, the significantly reduced plasma levels of IL-17A for *E. faecalis* ***ΔepaB*** monoassociated IL-10-/- mice (Fig.26B) and of serum amyloid A (SAA) for *E. faecalis* ***Δlgt*** monoassociated IL-10-/- mice (Fig.26C) partially reflected the attenuated colonic inflammation observed for both *E. faecalis* mutant strains.

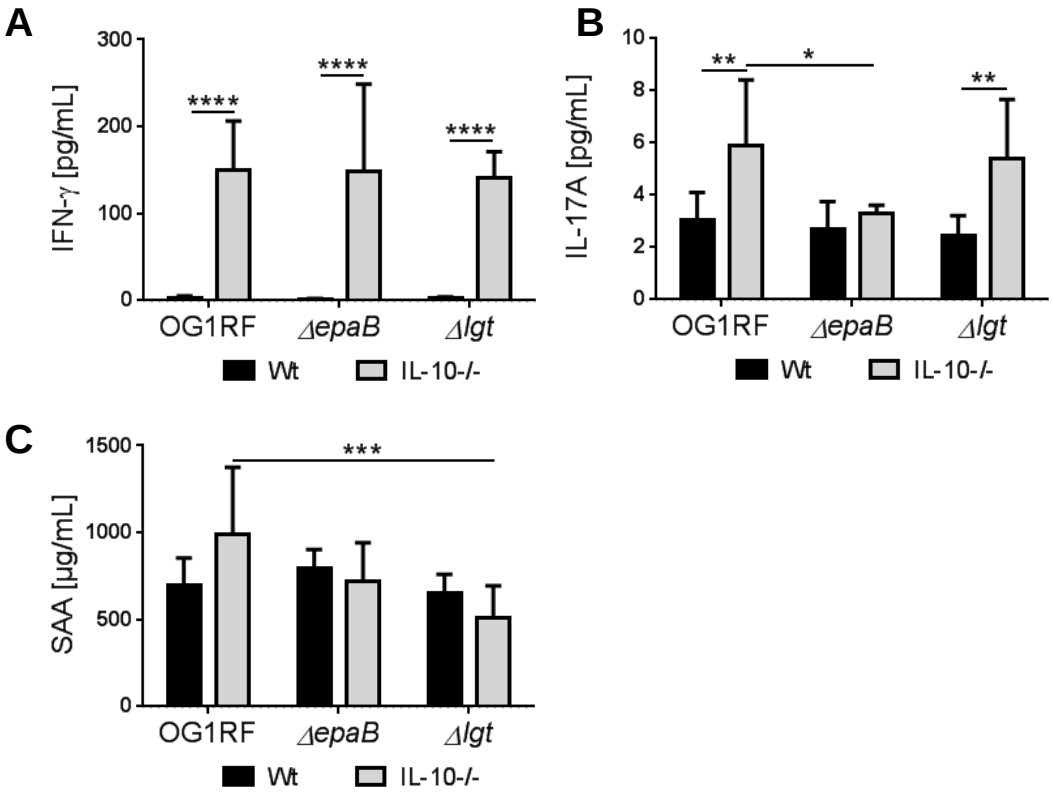

Figure 26: Secretion of pro-inflammatory cytokines is not dependent on *E. faecalis* lipoproteins.

(**A**) IFN-γ, (**B**) IL-17A and (**C**) serum amyloid a (SAA) cytokine levels in plasma of wild type (Wt) and IL-10-/- mice monoassociated with *E. faecalis* OG1RF, ***ΔepaB*** or ***Δlgt*** strain. Differences were considered significant for *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

3.5.3 Bacterial lipoproteins do not affect colonic T cell differentiation or chemokines

In a final attempt to determine how a lack of bacterial lipoproteins affect host immune factors that render the pathogenesis of chronic colitis in IL-10-/- mice, we screened for expression of T cell differentiation factors and chemokines in colonic tissue of wild type and IL-10-/- mice monoassociated with the different *E. faecalis* strains. Analysis by qPCR revealed no significant differences in the expression of T cell differentiation markers FOXP3, GATA-3, RORγt and T-bet related to monocolonization of IL-10-/- mice with different *E. faecalis* strains (Fig.27A-D). Again, these tissue sections were sampled from the middle colon, meaning that they only partially represent the scored intestinal inflammation in figure 5.

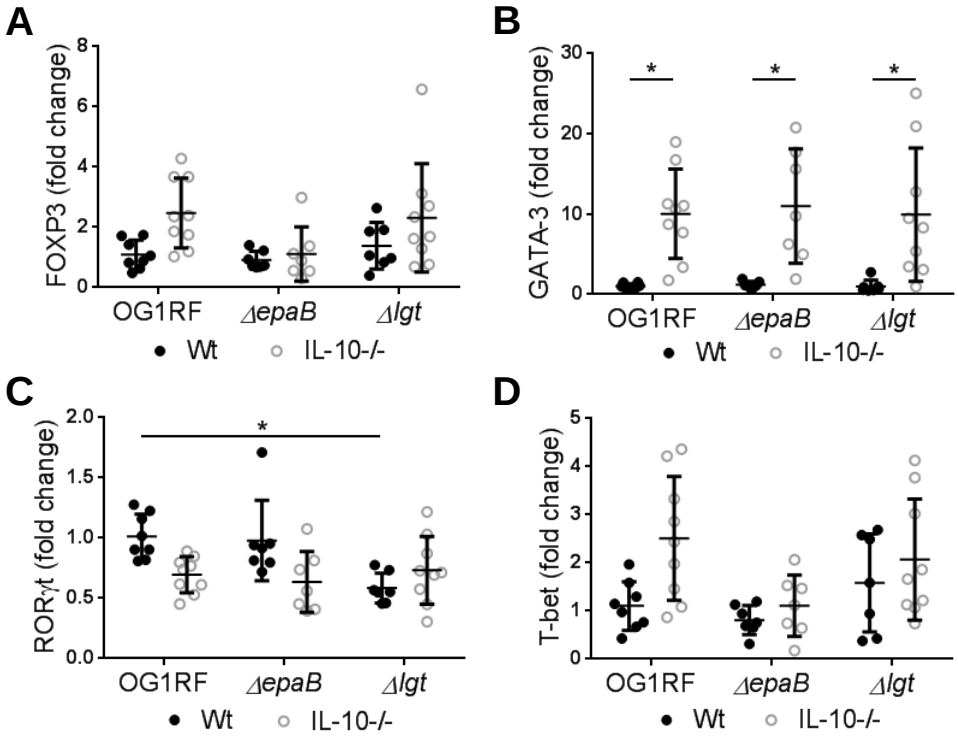

Figure 27: Differentiation of colonic T cells is not affected by *E. faecalis* lipoproteins.

(**A**) FOXP3, (**B**) GATA-3, (**C**) ROR-γt and (**D**) T-bet expression in whole colon tissue sections from wild type (Wt) and IL-10-/- mice monoassociated with *E. faecalis* OG1RF, *ΔepaB* or *Δlgt* mutant strain shown as fold change normalized to cytokine expression levels in wild type mice monoassociated with *E. faecalis* OG1RF. Differences were considered significant for *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

4. DISCUSSION

The colitogenic character of *E. faecalis* in genetically susceptible hosts was already demonstrated. However, the dynamic molecular relationship between disease-relevant host compartments and specific bacterial structures able to trigger intestinal inflammation remain unclear. Using *E. faecalis* as a model organism, we provide new insights regarding the significance of specific bacterial virulence-related factors in chronic colitis (Table 1). We identify a crucial role for bacterial cell wall-associated lipoproteins in the induction of experimental colitis, adding new knowledge into the complex interdependence of intestinal opportunistic pathogens and the genetically predisposed host (Fig.28A).

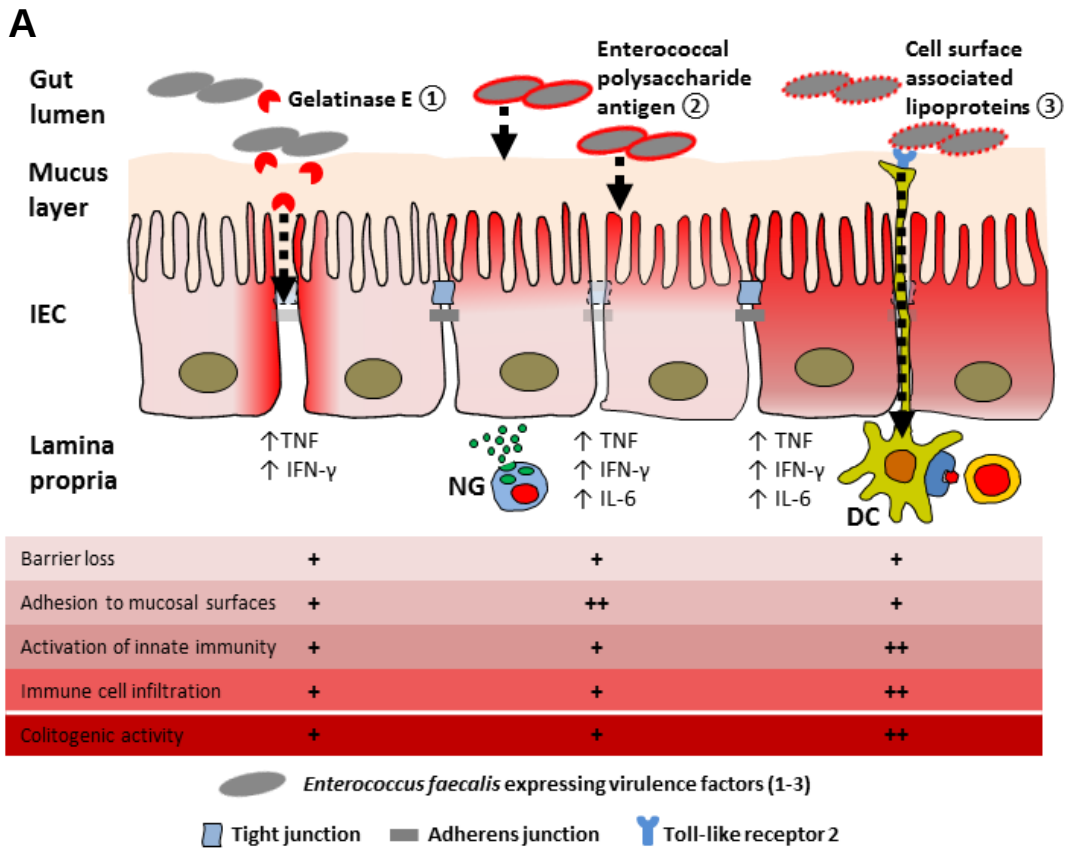

Figure 28: Proposed mechanisms of *E. faecalis* virulence factors responsible for colitogenic activity in the disease susceptible host.

(**A**) Differential contribution of virulence factors to colitogenic activity of *E. faecalis*: (**1**) Gelatinase E secreted by *E. faecalis* triggers degradation of E-cadherin in intestinal epithelial cells (IEC) impairing the intestinal barrier. (**2**) The enterococcal polysaccharide antigen mediates adhesion of *E. faecalis* to mucosal surfaces and facilitates resistance against lysozyme secreted by neutrophil granulocytes (NG) infiltrating the lamina propria. (**3**) Cell surface-associated lipoproteins are essential for colitogenic activity of *E. faecalis* promoting activation of innate immune cells through TLR2, such as dendritic cells (DC) for example, and infiltration of immune cells.

4.1 Linking bacterial virulence to colitogenic activity: Insight from invertebrate models

Since bacterial structures that are involved in virulence may be also implicated in colitogenic activity of an opportunistic pathogen/pathobiont, it is essential to detect common underlying mechanisms that trigger infection or inflammation, respectively. In this context, invertebrate models are efficient and elegant experimental systems to study the contribution of single bacterial structures to virulence of pathogenic bacteria and interaction with host innate immunity *in vivo* [231,232]. The simple genetic modification, conserved patterns in innate immunity, cheap and - compared to vertebrate models – simple maintenance predestine invertebrate models for microbe-host interaction studies [233]. Virulence mechanisms of several human pathogenic bacteria have been examined using *C. elegans* including *Pseudomonas aeruginosa* [234] and *Staphylococcus aureus* [235] as well as the IBD-relevant opportunistic pathogens AIEC [236] and *E. faecalis* [209]. In addition to intestinal colonization and killing assays, the interaction of *E. faecalis* with other opportunistic pathogens was investigated in *C. elegans* [237,238]. Hence, we first confirmed the importance of Epa and bacterial lipoproteins as *E. faecalis* virulence traits in *G. mellonella* and *C. elegans* and subsequently proceeded with investigating underlying mechanisms involved in the interaction with the host. For this reason, we established *M. sexta* as a model system to analyze intestinal commensal-host interactions *in vivo*.

4.1.1 Manduca sexta as experimental model for microbe-host interactions

The caterpillar *M. sexta* has a simplified intestinal morphology [239–241], well described innate immunity [218,230] and microbiota [242,243] with *E. faecalis* being one naturally occurring bacterium [225,226]. To date *M. sexta* was predominantly used to study the impact of gut microbiota on *Bacillus thuringiensis* toxin insecticidal activity [244] and as infection model for insect pathogens such as *Photorhabdus luminescens* [245]. Importantly, we were able to maintain germ-free and monoassociated *M. sexta* larvae that were used to unravel several aspects of *E. faecalis*-host interaction: bacterial adhesion to midgut epithelium and intestinal colonization, as well as expression of PRRs and AMPs (Table 5). Strikingly, only *E. faecalis* lacking *epaB* showed an impaired adhesion to mucosal surfaces in *M. sexta* confirming our results in the IL-10-/- mouse model and suggesting that *M. sexta* is a reasonable experimental model to analyze commensal-host interactions. The fact that we did not observe altered innate immune responses (by expression of PRRs and AMPs) in *M. sexta* due to monoassociation with *E. faecalis ΔepaB* may highlight the commensal character of *E. faecalis* in this model – especially considering the reduced expression of PRRs and AMPs in germ-free PBS control larvae.

4.2 *E. faecalis* EpaB: Bacterial adhesion to mucosal surfaces as colitogenic factor

4.2.1 Bacterial adhesion to mucosal surfaces as colitogenic factor

We demonstrate that proximity of bacteria to the intestinal epithelium is a contributing prerequisite for *E. faecalis*-induced colitis in monoassociated IL-10-/- mice and represents a significant colitogenic feature that is shared by IBD-relevant pathobionts such as AIEC and *E. faecalis* [169,188]. Bacteria that occur adjacent to the intestinal epithelium need to pursue a cascade of several steps including penetration of mucus, establishment of growth-promoting structures as well as biofilm formation and adhesion to mucosal surfaces. In contrast to other opportunistic pathogens implicated in IBD pathogenesis (AIEC, *Fusobacterium spp.*), invasion of IECs does not seem to be a substantial factor for colitogenic activity of *E. faecalis* and we did not detect significant translocation of *E. faecalis* to MLN or plasma of IL-10-/- mice. However, bacterial adhesion to the intestinal epithelium is a critical factor for mucosal immune modulation. Being an example, segmented-filamentous bacteria (SFB) adhere to the intestinal epithelium in a wide range of animals [246]. Despite the fact that SFB were recently described to occur in increased numbers in UC patients [247], their presence and functional role in the human intestine is still unclear. However, in mice SFB induce pro-inflammatory Th17 cell differentiation in the lamina propria and promote the development of organized gut lymphoid tissue that regulate Th17 cell and IgA responses [248,249], highlighting the general relationship of bacterial adhesion to the intestinal epithelium and host immune modulation.

4.2.2 Mucus penetration by bacteria

Representing the first step in bacterial adhesion to the intestinal epithelium, microbes need to penetrate the intestinal mucus layer. As mentioned before, the colonic mucus bilayer exhibits critical functions in IBD pathogenesis mediating microbe-host crosstalk and limiting access of bacteria to the underlying epithelium [44,51]. A functional mucus layer confers epithelial protection in the colon, as a deletion of *Muc2* results in severe colitis of mice [38]. Resembling conditions in UC patients, the colonic mucus of IL-10-/- mice is thicker than in wild type mice, but more penetrable to bacteria and allows gut microbes to attach to the epithelium [44]. While we observed no substantial penetration of the inner mucus layer in IL-10-/- mice, the *E. faecalis* mutant lacking *epaB* fails to penetrate even the outer mucus area. Most importantly, this EpaB-mediated defective adhesion of *E. faecalis* to the outer colonic mucus occurred exclusively in the susceptible milieu of inflamed IL-10-/- mice, where bacterial penetration of / adhesion to mucus is an important feature contributing to chronic colitis pathogenesis. Accordingly, the attenuated inflammation in IL-10-/- mice

monoassociated with *E. faecalis ΔepaB* most likely originated from impaired penetration of the colonic mucus and a defective adhesion to intestinal epithelial surfaces.

The impaired mucus adhesion was confirmed *in vitro*, where only the *E. faecalis* mutant lacking *epaB* demonstrated an attenuated attachment to colon-like mucus on an LS174T cell monolayer. LS174T have higher expression rates of MUC2 compared to other cell lines such as Cacao-2 or HT-29 [224] and the mucus produced by LS174T monolayers resembles conditions found in the colon [250], making these cells an excellent model to mimic colonic bacteria-mucus interactions *in vitro*. The impact of an intact mucus layer on bacterial virulence was demonstrated for *Pseudomonas aeruginosa*, where mucin biopolymers prevented bacterial adhesion to underlying surfaces and retained bacterial aggregation by maintaining bacteria in a motile state [251]. Although *E. faecalis* are non-motile bacteria, this points out that an efficient adhesion to intestinal mucus may be a prerequisite for biofilm formation and establishment of multicellular growth structures within outer mucus layers in the colon of IL-10-/- mice.

4.2.3 Biofilm formation & associated microcolonies

In fact, it was demonstrated that mucins (and IgA) may facilitate biofilm formation by enteric bacteria in the gut [252,253]. Enterococci form biofilm structures with associated microcolonies in close proximity to the UC mucosa, whereas these multi-cellular aggregates are not found in crypts of colon biopsies from healthy controls [254]. Consistently, we here demonstrate an impaired formation of microcolonies on epithelial cell surfaces and attenuated biofilm generation for *E. faecalis* lacking *epaB* being associated with attenuated colonic inflammation. In contrast to healthy controls, *E. faecalis* isolates from IBD patients exhibited higher biofilm formation [188], which was also identified as a pathobiont feature of AIEC [171]. These results suggest that under conditions of experimental colitis the abilities to adhere to colonic mucus, to form biofilms and/or to grow in microcolonies adjacent to the intestinal mucosa are pathobiont features of *E. faecalis* contributing to its colitogenic activity.

4.2.4 Bacterial adhesion to epithelial cells

Adhesion to mucosal surfaces is a feature shared by several pathobionts that are implicated in the pathogenesis of IBD and experimental colitis. For example, the adhesion of AIEC to IECs is mediated by the type 1 pili adhesion FimH and flagella [176,255,256]. Both of these bacterial structures are known to be relevant for bacterial virulence, but similarly they also confer colitogenic activity of AIEC in genetically susceptible hosts [257], since mutants lacking *fimH* or flagellin exert defective adhesion to IECs and reduced induction of colitis in

mouse models [83,256,258]. Analogous to the higher adhesion capacity of AIEC to IECs, *E. faecalis* strains isolated from IBD patients exhibit increased attachment to Caco-2 cell monolayers compared to isolates from healthy controls [188] and show increased mucosal growth in UC patients [186]. In contrast to other commensal bacteria, significantly increased levels of systemic IgA specific for *E. faecalis* are reported in IBD patients [54], pointing at a major role for the contact of *E. faecalis* with the intestinal epithelium under chronic inflammatory conditions. In our study, *E. faecalis* lacking *epaB* did not only show less induction of colonic inflammation in IL-10-/- mice, but also exhibit impaired adhesion to the intestinal epithelium of *M. sexta*, supporting the idea of a mechanistic link between adhesion capacity to mucosal surfaces and colitogenic activity. However, in *M. sexta* the attenuated adhesion of *E. faecalis ΔepaB* to the intestinal epithelium did not result in an altered innate immune response against commensal *E. faecalis*, suggesting that the colitogenic impact of bacterial adhesion evolves under predisposed host conditions.

4.2.5 Role of Epa in E. faecalis adhesion capacity and biofilm formation

We can only speculate about the pleiotropic effects of the Epa rhamnopolysaccharide on *E. faecalis* virulence and colitogenic activity by promoting mucus penetration, adhesion to mucosal surfaces, biofilm and associated microcolony formation. As central part of the bacterial cell envelope, the Epa rhamnopolysaccharide renders cell wall integrity, cell shape and diverse interactions with the environment [194,195]. It was recently demonstrated that *epaB* is implicated in cellular stress resistance induced by high NaCl concentrations, bile acids, AMPs or antibiotics [193,194,210]. As proposed by *Solheim et al.* [193], the Epa rhamnopolysaccharide may be considered as an envelope fitness factor conferring stress resistance rather than a classical virulence factor, since many of these stress factors are encountered by *E. faecalis* in the gastrointestinal tract. Accordingly, the reduced colitogenic activity of *E. faecalis ΔepaB* mediated by impaired adhesion to mucosal surfaces may derive from attenuated stress protection in the inflamed intestinal milieu of IL-10-/- mice. This would likely affect the colonization capacity of *E. faecalis* deficient for *epaB*, but in our study all monoassociated wild type and IL-10-/- mice are similarly colonized with *E. faecalis* being not affected by *epaB* deletion. In a mouse model of transient colonization, *E. faecalis* lacking parts of the *epa* gene cluster showed reduced intestinal colonization rates [194], but this might be explained by a difference in the experimental setup (transient colonization after pretreatment with antibiotics vs. long-term monoassociation of germ-free mice), selection of differing *E. faecalis* deletion mutants (*epaX* vs. *epaB*) or different *E. faecalis* strains (OG1RF vs. V583) and emphasizes that the impaired colitogenic activity of *E. faecalis ΔepaB* in the IL-10-/- mouse model is not dependent on colonization rates.

In this context, the role of EpaB in lysozyme resistance is interesting, since *E. faecalis* lacking *epaB* is not only more susceptible to lysozyme [212], but also to neutrophil killing compared to *E. faecalis* OG1RF [199]. We might speculate that the lack of EpaB also facilitates the killing of *E. faecalis* cells translocating to or in close proximity to intestinal epithelium, via lysozyme produced by infiltrating neutrophils in the inflamed colonic mucosa of IL-10-/- mice [212]. Despite of unknown molecular mechanisms in *E. faecalis*, the Epa rhamnopolysaccharide reveals multifaceted structure-function relationships regarding the colitogenic activity of *E. faecalis* and by this delineates bacterial adhesion to mucosal surfaces as a pathobiont feature in chronic intestinal inflammation.

4.3 Differential interaction of colitogenic virulence factors in *E. faecalis*

Still factors other than adhesion ability are required for full colitogenic activity of *E. faecalis*, since Epa-deficiency resulted only in partial reduction of intestinal inflammation. Noteworthy, the colitogenic activity of EpaB is GelE-independent. The impaired adhesion of *E. faecalis ΔepaB* to the intestinal epithelium does not substantially impact on GelE activity *in vitro* and in the IL-10-/- mouse model (all three *E. faecalis* strains show the same degradation of E-cadherin in the colon of monoassociated IL-10-/- mice), suggesting that close proximity of *E. faecalis* to the intestinal epithelium is not a crucial prerequisite for GelE activity at inflammation-relevant sites. In this context, the diverging roles of GelE are remarkable and highlight the complexity of contributing and causal factors for colitogenic activity of *E. faecalis*. Both *E. faecalis ΔepaB* and *Δlgt* show substantial GelE activity without full colitogenic evolvement, especially *E. faecalis Δlgt* potently degrades E-cadherin without any subsequent induction of colitis in IL-10-/- mice. In contrast, *gelE* is the only of these virulence factors overexpressed in *E. faecalis* OG1RF under inflammatory conditions. Recalling the intermediate effects of *gelE* deficiency in *E. faecalis* on chronic colitis in the IL-10-/- mouse model [29], GelE can be considered as a factor contributing to, but not sufficient for colitogenic activity of *E. faecalis*. In contrast, the loss of *E. faecalis* cell surface-associated lipoproteins resulted in almost complete abrogation of intestinal pathology, despite the presence of both Epa and GelE. Hence, bacterial lipoproteins represent fundamental key structures in this complex of virulence factors rendering and limiting colitogenic activity of *E. faecalis*.

4.4 Interaction of *E. faecalis* lipoproteins and host immunity

4.4.1 Lipoproteins-dependent virulence factors of E. faecalis

Similar to previous studies using Gram-positive bacterial mutants of the *lgt* gene, we also identified TLR2 as prime PRR responding to bacterial lipoproteins. It remains unclear whether the specific interaction of *E. faecalis* lipoproteins with TLR2 is solely responsible for the exerted colitogenic effects, since about 40% of all predicted mature lipoproteins processed by Lgt have been linked to virulence of *E. faecalis* or Gram-positive bacteria in general [216]. This includes lipoproteins belonging to peptidylprolyl cis-trans isomerases [259], involved in capsular carbohydrate synthesis [260] or ABC transporters such as the endocarditis-specific antigen (EfaA) [261]. However, expression levels of none of these putatively virulence-relevant lipoproteins were substantially up-regulated in *E. faecalis* OG1RF in the chronically inflamed intestine of IL-10-/- mice. This argues for a mechanism involving TLR2 signaling occurring after lipidation of immature lipoproteins via Lgt rather than a modified expression of individual lipoproteins that are relevant for *E. faecalis* virulence.

4.4.2 Impact of bacterial lipoproteins on colitis pathogenesis in IL-10-/- mice

The number of F4/80+ macrophages and Ly6G+ granulocytes infiltrating the colon correlated with disease activity and were massively reduced in IL-10-/- mice monoassociated with lipoprotein-deficient *E. faecalis*. This coincides with a similar observation for lysozyme-positive cells [212], since macrophages and granulocytes are major subpopulations of cells in the inflamed mucosa that contain lysozyme-positive granules. We observed similar numbers of CD11c+ cells in the colonic mucosa of *E. faecalis* OG1RF and *Δlgt* monoassociated IL-10-/- mice despite of different tissue pathology. However, the number of CD3+ T cells infiltrating the colonic mucosa of IL-10-/- mice was consistently reduced when *E. faecalis* lipoproteins were lacking. Most importantly, we showed that lipoprotein-deficient *E. faecalis* are fully capable of reactivating MLN-derived colitogenic T cells despite their significantly impaired potential to induce BMDC activation. In this context, it is remarkable that lipoprotein-deficient *E. faecalis* lysates are able to induce IFN-γ secretion in MLN cells isolated from minimally-inflamed IL-10-/- mice colonized with the *E. faecalis Δlgt* mutant strain. Together with the observation that IFN-γ plasma levels were increased in all monoassociated IL-10-/- mice, we might speculate that lipoprotein-deficient *E. faecalis* induce full maturation of colitogenic T cells, but obviously fail to activate and/or recruit a sustained inflammatory immune response in the colonic mucosa. The contribution of TLR2 in this context remains unclear.

4.4.3 The role of toll-like receptor 2 in chronic intestinal inflammation

Emerging evidence from experimental mouse models delineates an essential contribution of TLR2 agonists such as bacterial lipoproteins in propagation and/or modulation of chronic intestinal inflammation [81]. Considering the diminished epithelial barrier under inflammatory conditions, it is tempting to speculate that the increased contact of immune cells from the lamina propria to highly abundant bacterial components, such as cell-wall associated lipoproteins, triggers mucosal immunopathology. Here we demonstrate that a lack of TLR2 stimulants consistently abrogates chronic colitis in IL-10-/- mice monoassociated with a Gram-positive bacterium. Since a dual-association of IL-10-/- mice with *E. faecalis* and *E. coli* induces chronic colitis dependent on TLR downstream signaling [114], an additional colonization in our setting with a Gram-negative microbe such as *E. coli* would be interesting to investigate, if TLR4 or TLR5 override the defective TLR2 signaling and exacerbate chronic colitis [107,113,262]. Both, TLR2 and TLR4 signaling depend on the same downstream adaptor molecule MyD88, which is implicated in the development of spontaneous colitis in IL-10-/- mice, since mice lacking IL-10 and MyD88 do not develop intestinal pathology [125]. MyD88 activation also plays a role in the restriction of adhesion of Gram-positive bacteria to the intestinal epithelium, as the secretion of the antimicrobial C-type lectin RegIIIγ in the distal ileum of mice is promoted by TLR4 signaling via MyD88 [263–265]. Although the action of AMPs in limiting bacterial access to the mucosal surface is primarily relevant for the small intestine, this exemplifies TLR functionality beyond innate immune recognition linking TLR signaling and bacterial adhesion.

Interestingly, the development of ovalbumin-induced colitis in mice deficient in NOD2 depends on TLR2-derived signals. Compared to NOD2-deficient mice, NOD2xTLR2 double-deficient mice showed attenuated ovalbumin-induced colitis with impaired CD4+ T cell infiltration into the mucosa and reduced production of IFN-γ in MLN cells [75]. It was demonstrated that TLR2 is expressed by activated T cells as costimulatory receptor mediating T cell development and expression of IFN-γ [266] and promotes Th1 cell differentiation and effector functions [267]. Strikingly, we still observe full (re-)activation of colitogenic T cells and INF-γ secretion despite of defective TLR2 stimulation due to missing bacterial lipoproteins. Since we demonstrate a potent effect for functional TLR2-signaling in BMDCs triggered by bacterial lipoproteins, it is tempting to hypothesize that the bacterial lipoprotein-TLR2-signaling in intestinal DCs is responsible for conferring colitogenic activity of *E. faecalis* cell surface-associated lipoproteins. In fact, pro-inflammatory characteristics of lamina propria effector monocytes can be triggered by TLR2 ligands in DSS-induced murine colitis [268] and TLR2, as well as TLR4, is highly expressed by colonic DCs and intestinal macrophages isolated from patients with active IBD compared to cells isolated from healthy

mucosa [95,96]. Taken together, these findings emphasize the importance of a balanced TLR2 signaling in the mucosal innate immune compartment directing subsequent mucosal immunopathology.

Still, it remains to be elucidated how bacterial lipoproteins *de facto* render colitogenic activity of *E. faecalis* by modulating the interaction with host immune compartments. We propose that a sustained TLR2-dependent activation of lamina propria DCs by bacterial lipoproteins might play a crucial role in directing T cells responses in the colonic mucosa. However, more analyses are needed to confirm the impact of the bacterial lipoprotein-TLR2-signaling axis in lamina propria mononuclear cells on the pathogenesis of chronic colitis in IL-10-/- mice and chronic inflammation in human IBD.

5. CONCLUSION AND PERSPECTIVE

Beyond *E. faecalis* and IL-10-/- mice as model organisms

This study demonstrates novel colitogenic function for two *E. faecalis* envelope structures that are also relevant for bacterial virulence. We provide first evidence that bacterial cell surface-associated lipoproteins are essential in mounting colitogenic activity of antigen-primed T cells in the colonic mucosa of IL-10-/- mice. Despite the fact that bacterial adhesion and close proximity to the intestinal epithelium as well as intestinal barrier disruption contribute to the colitogenic activity of *E. faecalis*, bacterial lipoprotein-mediated immune cell activation most likely through TLR2 is critical for the development of chronic intestinal inflammation in the genetically susceptible host (Fig.28A).

Beyond the ‘pathobiont model organism’ *E. faecalis*, different conclusions arise regarding the extrapolation to the human IBD scenario: Our results confirmed observations that identify bacterial adhesion to mucosal surfaces as an important factor in IBD pathogenesis. Considering the role of bacterial lipoproteins, this is the first study demonstrating that this group of structures, originally linked to bacterial virulence, also exhibits colitogenic activity and is almost solely responsible for inducing chronic intestinal inflammation in a susceptible mouse model. This raises interesting questions regarding the role of TLR2 in IBD pathogenesis, especially the impact of TLR2 signaling in lamina propria mononuclear cells, but it also illustrates the variety of bacterial structures with potential colitogenic activity: Are bacterial lipoproteins critical drivers of human IBD? Or do bacterial lipoproteins, as structures highly conserved in Gram-positive bacteria, just highlight the fundamental concept of IBD pathogenesis as being an aberrant immune response against the endogenous commensal microbiota? If so, which bacterial lipoproteins from species other than *E. faecalis* have the same colitogenic effects? And is TLR2 a reasonable target for treating chronic intestinal inflammation by selectively blocking TLR2 signaling in specific cell populations? Beyond basic characterization of microbe-host interactions, this also has fundamental implications for the treatment of IBD, since novel approaches with selective introduction of symbiotic bacteria or fecal transplantations need to consider possible highly conserved colitogenic bacterial structures, such as bacterial cell surface-associated lipoproteins, for example. Hence, the identification of virulence traits of the commensal microbiota with critical detrimental effects in IBD pathogenesis is necessary - as well as the assignment of colitogenic key structures of commensal bacteria. By this, further targeted characterization of bacterial structures relevant for the development of chronic inflammation will help to identify the most essential steps in IBD-related microbe-host interactions.

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ADDENDUM

LIST OF FIGURES

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ABBREVIATIONS

AIEC	Adherent-invasive *E. coli*
AMP	Antimicrobial peptide
APC	Antigen-presenting cell
BHI	Brain heart infusion
BMDC	Bone marrow-derived dendritic cell
CD	Crohn's Disease
CEACAM	Carcinoembryonic antigen-related cell adhesion molecule
CED	Chronisch-entzündliche Darmerkrankungen
CFU	Colony forming units
COX	Cyclooxygenase
DC	Dendritic cell
DSS	Dextran sodium sulfate
DTT	Dithiothreitol
DUOX	Dual oxidase
EfaA	Endocarditis-specific antigen
Epa	Enterococcal polysaccharide antigen
ER	Endoplasmic reticulum
IBD	Inflammatory bowel diseases
IEC	Intestinal epithelial cell
GelE	Gelatinase E
Ig	Immunoglobulin
IL-10	Interleukin-10
KC	Keratinocyte-derived chemokine
Lgt	Prolipoprotein diacylglyceryl transferase
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
MD	Muramyl dipeptide

MLN	Mesenteric lymph nodes
Mn	Manganese
NGM	Nematode growth medium
NOD2	Nucleotide oligomerization domain 2
OD	Optical density
ON	Over night
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PFA	Phosphate-buffered formaldehyde
PGRP	Peptidoglycan recognition protein
PRR	Pattern recognition receptor
PTPN2	protein tyrosine phosphatase N2
RT	Room temperature
SAA	Serum amyloid A
SFB	Segmented-filamentous bacteria
SNP	Single nucleotide polymorphism
TLR	Toll-like receptor
TNBS	Trinitrobenzene sulfonate
TSB	Tryptic soy broth
Wt	Wild type
UC	Ulcerative Colitis

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