RESEARCH ARTICLE

Impact of a probiotic *Enterococcus faecalis* in a gnotobiotic mouse model of experimental colitis

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Scope: IL-10-deficient (IL-10$^{-/-}$) mice are susceptible to the development of chronic intestinal inflammation in response to the colonization with commensal *Enterococcus faecalis* isolates. The aim of this study was to characterize the impact of a probiotic *E. faecalis* strain in germ-free, wild-type (WT), and disease-susceptible IL-10$^{-/-}$ mice.

Methods and results: The probiotic *E. faecalis* and the colitogenic control strain OG1RF induced IL-6 and IFN-γ inducible protein-10 secretion in the murine intestinal epithelial cell line Mode K. Epithelial cell activation involved nuclear factor κ B, p38 and extracellular signal-regulated kinase 1/2-dependent pathways. Mouse embryonic fibroblasts from WT and toll-like receptor-2-deficient (TLR-2$^{-/-}$) mice confirmed that both *E. faecalis* strains trigger pro-inflammatory responses *via* the pattern recognition receptor TLR-2. Monoassociation of germ-free IL-10$^{-/-}$ mice with the probiotic *E. faecalis* strain revealed pro-inflammatory epithelial cell activation and colonic tissue pathology. The non-pathogenic nature of *E. faecalis* was confirmed in monoassociated WT mice. 2-DE and MALDI-TOF MS identified the ER stress chaperone Hspa5 (glucose-regulated protein 78) and 3-mercaptopyruvate sulfurtransferase as key targets in the epithelium from IL-10$^{-/-}$ and TLR-2$^{-/-}$ mice.

Conclusion: This study shows the potential of probiotic bacteria to initiate pro-inflammatory responses in the disease-susceptible but not the normal host.

Keywords:
Colitis / *Enterococcus faecalis* / IL-10-deficient mice / Inflammatory bowel disease / Probiotic

1 Introduction

Inflammatory bowel disease (IBD) including the distinct idiopathic pathologies of ulcerative colitis (UC) and Crohn's disease (CD) are spontaneously relapsing, immunologically mediated disorders of the gastrointestinal tract. The etiology of these chronic inflammatory diseases is not known. However, the combined action of genetic polymorphisms as well as environmental factors dramatically increases the susceptibility for disease development [1–3]. Different animal models of chronic intestinal inflammation demonstrate that the pathogen-free enteric microbiota plays a major role in the initiation and perpetuation of these chronic inflammatory processes in the gut mucosa [4–6]. However, the selective

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Abbreviations: $^{-/-}$, deficient; **CD,** Crohn's disease; **dn,** dominant negative; **Erk,** extracellular signal-regulated kinase; **grp78,** glucose-regulated protein 78; **Hmgc2,** hydroxymethylglutaryl-CoA synthase; **IBD,** inflammatory bowel disease; **IEC,** intestinal epithelial cell; **IKKβ,** IκB kinase β; **IP-10,** inducible protein-10; **JNK,** c-Jun NH2-terminal kinase; **MAPK,** mitogen-activated protein kinase; **MEF,** mouse embryonic fibroblast; **moi,** multiplicity of infection; **Mpst,** 3-mercaptopyruvate sulfurtransferase; **NFκB,** nuclear factor κB; **TLR,** toll-like receptor; **UC,** ulcerative colitis; **Ugdh,** uridine diphasphate-glucose dehydrogenase; **WT,** wild-type

colonization of germ-free rodent models for experimental colitis with defined non-pathogenic bacterial species shows that enteric bacteria are not equal in their capacities to induce or protect from chronic intestinal inflammation [7]. The activation of pattern recognition receptor signaling through members of the nucleotide-binding oligomerization domain (NOD) and toll-like receptor (TLR) family effectively triggers inflammatory defense mechanisms required to alert and protect the host, but in the absence or functional loss of anti-inflammatory feedback signals the host may turn these physiological response mechanisms towards enteric bacteria into a pathological situation including failure of bacterial clearance and development of chronic inflammation [8–10]. A meta-analysis of genome-wide association studies defines more than 30 susceptibility loci for CD patients [11]. Variations in genes that are responsible for mucosal barrier maintenance (MAGI2) [12], bacterial recognition and defence (NOD2/CARD15, ATG16L1) as well as immune regulation (IL23R, IL12B) are correlated with the risk of developing IBD [13], underlining the importance of bacteria–host interactions in health and disease.

Enterococcus faecalis strains are frequently isolated from traditional Mediterranean food products and some strains are used as probiotics [14–16]. However, *E. faecalis* strains are also regarded as nosocomial pathogens and play a role in several infections, *e.g.* endocarditis [17], bacteremia [18], root canal [19] and urinary tract infections [20]. The role of *E. faecalis* strains in the pathogenesis of IBD is unknown. However, in IL-10$^{-/-}$ mice monocolonization with the well-described human oral isolate *E. faecalis* OG1RF was sufficient to induce severe colitis after 14 wk [21, 22]. Recently, we were able to show that monocolonization with an endogenous *E. faecalis* isolate derived from an IL-10$^{-/-}$ mouse induced only moderate colitis after 14 wk [23]. Therefore, we investigate here whether monocolonization with a probiotic *E. faecalis* strain leads to significantly reduced pathology scores in disease-susceptible IL-10$^{-/-}$ mice compared to *E. faecalis* OG1RF. Further, we investigated the capacity of the probiotic *E. faecalis* to induce proinflammatory cytokine secretion in intestinal epithelial cell (IEC) lines. Therefore, we used Mode K cells. Mode K cells are an undifferentiated cell line that has the advantage that it does not derive from tumor tissue and that it is not responsive to LPSs. LPS frequently contaminates stimulation agents. As endpoints we chose the inflammatory cytokines IL-6 and inducible protein-10 (IP-10) because they are known to be secreted by IEC and because they play a role in the pathogenesis of IBD. IL-6 expression is elevated in IBD [24] and gram-positive bacteria have the potential to induce IL-6 expression in IEC [25]. IP-10, a chemokine to attract activated TH1 lymphocytes and phagocytes, is an interesting target since its production was found to be associated with colitis disease activity in UC patients [26], supporting the pathological relevance of IP-10 expression for chronic intestinal inflammation. In addition, the use of IP-10 antibodies abrogated colitis in IL-10-deficient (IL-10$^{-/-}$) mice [27].

2 Materials and methods

2.1 Bacteria

E. faecalis OG1RF (in figures designated as OG1, a generous gift from M. Huycke, University of Oklahoma, Oklahoma City, OK) is a human oral isolate that is well described in the literature. The probiotic *E. faecalis* (in figures designated as pro) is a component of the pharmaceutical preparation Symbioflor® 1, which is produced by SymbioPharm (Herborn). *Enterococcus* strains were cultured as described previously [23]. Cell cultures including the IEC line Mode K and mouse embryonic fibroblasts (MEFs) were stimulated with *E. faecalis* using a multiplicity of infection (moi; bacteria to epithelial cell ratio) of 30.

2.2 Mice, bacterial monoassociation and histology

Germ-free 129 SvEv TAC wild-type (WT) mice and germ-free IL-10 gene-deficient 129 SvEv TAC mice (derived by E. Balish, University of Wisconsin, Madison, WI) were monoassociated at 12–16 wk of age with one of the *E. faecalis* strains. Gnotobiotic mice were maintained in the National Gnotobiotic Rodent Resource Center at the University of North Carolina, Chapel Hill. Mice were monoassociated by gavage feeding and rectal swabbing with cultured bacteria. The absence of contamination by other bacterial species was confirmed by plating and culturing ten-fold dilution series of cecal contents of all mice under aerobic and anaerobic conditions at necropsy. Animal-use protocols were approved by the Institutional Animal Care and Use Committee (IACUC), University of North Carolina, Chapel Hill. Mice were killed 14 wk after initial bacterial colonization using carbondioxide asphyxiation. Germ-free mice were used as controls.

For histology, sections of distal colon were fixed in 10% v/v neutral buffered formalin (five mice in each group). The fixed tissue was embedded in paraffin. Histology scoring (0–4) was analyzed by blindly assessing the degree of lamina propria mononuclear cell infiltration, crypt hyperplasia, goblet cell depletion and architectural distortion in hematoxylin and eosin-stained sections as described by Berg *et al.* [28]. A score of 0–1 is regarded as no inflammation, 1–2 as mild, 2–3 as moderate and 3–4 as severe inflammation.

2.3 Isolation of primary mouse epithelial cells

Primary IECs were isolated and purified as described previously [23].

2.4 Cell culture and cell stimulation

For cell-culture experiments we used the mouse IEC line Mode K (a generous gift by I. Authenrieth, University of

Tübingen, Germany) and WT and toll-like receptor-2-deficient (TLR-$2^{-/-}$) MEFs (a generous gift from C. J. Kirschning, University Duisburg-Essen, Germany). Mode K cells are adherent nondifferentiated cells that were initially generated from enterocytes derived from C3H/HeJ mice and were immortalized by SV40 large T-gene transfer [29]. Because of a missense mutation in one of the exons of the TLR-4 gene in C3H/HeJ mice [30], Mode K cells do not respond to LPS and are therefore not sensitive to LPS contaminations. Mode K cells (passage 15–35) were cultured in 25 or 75 cm^2 plastic flasks or six-well culture plates (all Greiner Bio-One) in a humidified 5% CO_2 atmosphere at 37°C. The culture medium consisted of DMEM (Invitrogen, without sodium pyruvate, high glucose, with pyridoxine chloride), supplemented with 10% v/v FBS, 2 mM L-glutamine and 0.8% v/v antibiotic/antimycotic solution (all Invitrogen) and was replaced every 2–3 days. Cells were seeded at a cell density of approximately 2×10^5 cells/mL. For experiments sub-confluent cells (*ca.* 90%) were used.

The culture medium for MEF cells consisted of DMEM (without sodium pyruvate, high glucose, with pyridoxine chloride), supplemented with 10% v/v FBS, 2 mM L-glutamine, 0.0003% v/v β-mercaptoethanol and 0.8% v/v antibiotic/antimycotic solution (Invitrogen). The pharmacological inhibitors PD98059 (Mek1 Inhibitor; extracellular signal-regulated kinase (Erk)1/2-cascade) and SB203580 (p38 Inhibitor) were used in a 20 μM final concentration, D-c-Jun NH2-terminal kinase (JNK)-I-1 (JNK Inhibitor) in a 1 μM final concentration. Pharmacological inhibitors were pre-incubated for 1 h before bacterial stimulation. Cells were stimulated with *E. faecalis* (moi 30) or with IFN-γ (50 ng/mL) or IL1β (10 ng/mL).

2.5 Adenoviral infection

We used adenoviral infection to inhibit the (NFκB) pathway. Therefore, we transfected Mode K cells with a dominant negative (dn) form of IκB kinase β (IKKβ) using adenoviral delivery (Ad5). IKKβ is a part of the IκB kinase complex, which phosphorylates IκB, leading to ubiquitination and degradation of IκB and subsequent translocation of NFκB into the nucleus. Mode K cells were infected overnight with the adenoviral dn IKKβ (Ad5dnIKKβ) vector (a generous gift from C. Jobin, University of North Carolina, Chapel Hill, NC) in serum-reduced (2% v/v) cell culture medium in the absence of antibiotics at an moi of 50. The adenovirus was removed by washing, and fresh cell culture medium was added. Cells were then stimulated with the *E. faecalis* strains (moi 30) for 24 h.

2.6 ELISA analysis

For ELISA we used an IFN-γ IP-10 and an IL-6 ELISA Kit (R&D Systems, Minneapolis, USA) according to the manufacturer's protocol.

2.7 Protein from primary IEC

Protein from primary IEC was purified and quantified as describe previously [23].

2.8 Identification of differentially regulated proteins

2-DE and identification of differentially regulated proteins was carried out as described earlier [23]. Briefly, IEF was performed with an IPGphor 2, using anodic cup loading of 500 μg protein *per* IPG strip. For the second dimension 12.5% v/v SDS-PAGE gels were cast. After reduction and alkylation of the proteins on the IPG strips electrophoresis was carried out at 4 mA *per* gel for 1 h followed by 12 mA *per* gel overnight. After fixation and staining with Coomassie, the gels were scanned and analyzed by Proteome Weaver software. Reference gels from pooled IEC samples of all five mice in the control group were generated and compared with five single gels from *E. faecalis*-monoassociated mice. Spots with at least two-fold differences in protein intensity present in at least three out of five gels were submitted to MALDI-TOF MS. Spots were digested with 6 μL of 0.02 μg/μL trypsin at 37°C overnight and peptide mass fingerprints were generated using an Autoflex 1 mass spectrometer and the Flex Control as well as Flex Analysis software (Bruker Daltonics). Proteins were identified by using the MASCOT server 1.9 based on mass searches within murine sequences only. The search parameters allowed the carboxyamidomethylation of cystein and one missing cleavage. The mass accuracy was set to 100 ppm. As criterion for the positive identification of proteins we used the significant score value of 61 defined by MASCOT. This score value equals a p value of 0.05 and proteins with a higher score value can be regarded as significantly identified. Only proteins that were at least two times identified by MALDI-TOF MS using two different spots are listed.

The differentially regulated proteins in TLR-$2^{-/-}$ mice were identified in the same way as previously published [31].

2.9 Statistical analyses

Data are expressed as the mean ± SD. Statistical analysis was performed by using Sigma Plot 11.0 software as described in the respective figure legends.

3 Results

3.1 *E. faecalis*-induced IL-6 and IP-10 secretion in IEC requires NFκB and mitogen-activated protein kinase-dependent signals

We used the colitogenic *E. faecalis* OG1RF and the probiotic *E. faecalis* to characterize the bacteria-induced pro-inflammatory response of the IEC line Mode K and first

investigated *E. faecalis*-induced cytokine secretion. Preliminary experiments showed a time- and dose-dependent secretion of IL-6 and IP-10 showing full induction after 24 h stimulation with *E. faecalis* when using moi 30 (data not shown). IL-6 and IP-10 are inflammatory mediators that can be regulated by the NFκB and the mitogen-activated protein kinase (MAPK) signal transduction pathways. The MAPK pathways are complex signaling pathways that have diverse cellular functions [32]. Since *E. faecalis* strains were capable of inducing MAPK and NFκB phosphorylation (data not shown), we used specific pharmacologic MAPK inhibitors to evaluate the impact of these pathways on *E. faecalis*-induced IL-6 and IP-10 expression. To inhibit the NFκB pathway we transfected Mode K cells with a dn form of IKKβ using adenoviral delivery (Ad5). As shown in Fig. 1, *E. faecalis*-induced IL-6 and IP-10 expression was found to be dependent on the NFκB and the p38 MAPK, but not the JNK MAPK pathway. In addition, the Erk inhibitor PD98059 blocked *E. faecalis*-induced IL-6 but not IP-10 secretion.

3.2 *E. faecalis* induces IL-6 secretion in MEF cells *via* TLR-2

Gram-positive bacteria are likely to mediate signals *via* the pattern recognition receptor TLR-2. It has been recently shown for *E. faecium* and *E. faecalis* strains that pro-inflammatory mechanisms were mediated through TLR-2-dependent signals [33, 34]. To investigate the role of TLR-2 in mediating signals from a probiotic and a colitogenic *E. faecalis* strain, we used WT and TLR-$2^{-/-}$MEFs. As shown in Fig. 2, IL-6 expression in response to *E. faecalis* was completely abrogated in TLR-$2^{-/-}$ MEF cells compared to WT MEF, supporting the hypothesis that the pattern recognition receptor TLR-2 is necessary for *E. faecalis*-induced IL-6 expression, regardless of the probiotic or the colitogenic nature of the *E. faecalis* strain. IL-1β-induced IL-6 expression in TLR-$2^{-/-}$ MEF confirmed the responsiveness of the MEF cells.

3.3 Monocolonization of germ-free IL-$10^{-/-}$ mice with the probiotic *E. faecalis* revealed severe colitis

To further investigate the impact of the different *E. faecalis* strains on the development of chronic intestinal inflammation and to better understand the interaction of distinct *E. faecalis* strains with the intestinal epithelium, we mono-associated germ-free WT and IL-$10^{-/-}$ mice with the probiotic and colitogenic *E. faecalis* strain for 14 wk. Histopathological analysis of formalin-fixed paraffin-embedded

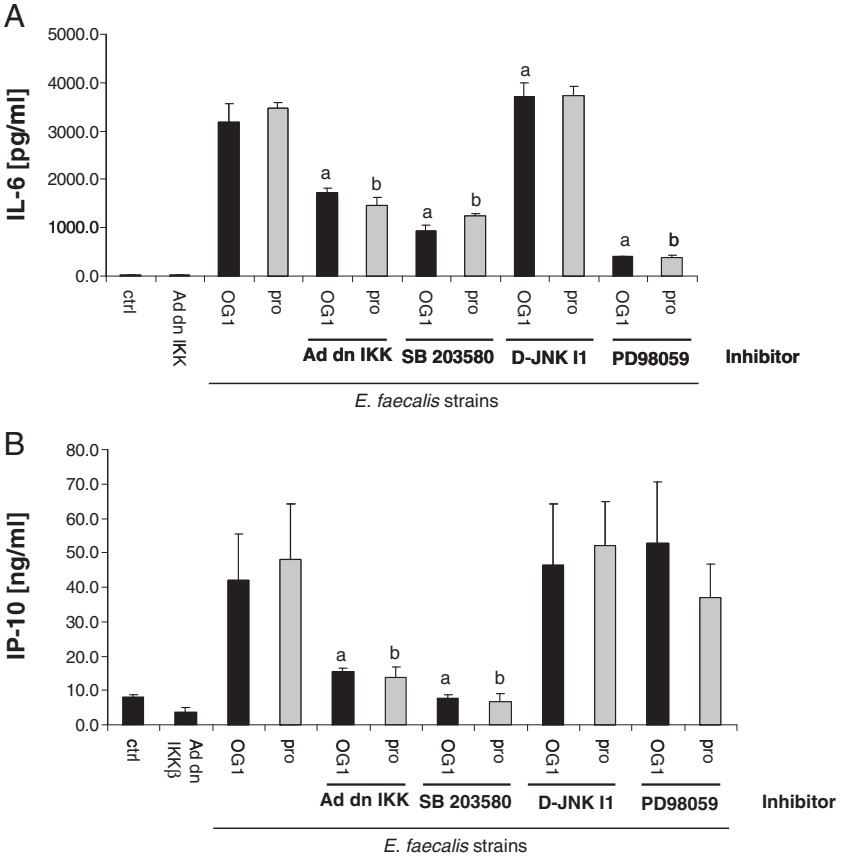

Figure 1. Impact of NFκB and MAPK inhibitors on *E. faecalis*-induced (A) IL-6 and (B) IP-10 secretion in IEC. Mode K cells were infected with Ad dn IKK (Adenovirus dn for IKKβ) or pre-treated with the following inhibitors: SB203580 (p38 Inhibitor), D-JNKI1 (JNK inhibitor), PD98059 (Mek1 inhibitor; Erk1/2-cascade). Cells were then stimulated with the *E. faecalis* strains (moi 30) for 24 h as indicated. Culture supernatants were analyzed by ELISA technique with specific antibodies for IL-6 and IP-10. Results shown represent the mean ± SD of triplicate samples. Statistics were analyzed using ANOVA and Holm Sidak method: a = $p < 0.05$ *versus E. faecalis* OG1 without inhibitor; b = $p < 0.05$ *versus E. faecalis* pro without inhibitor. There were no significant differences between the strains.

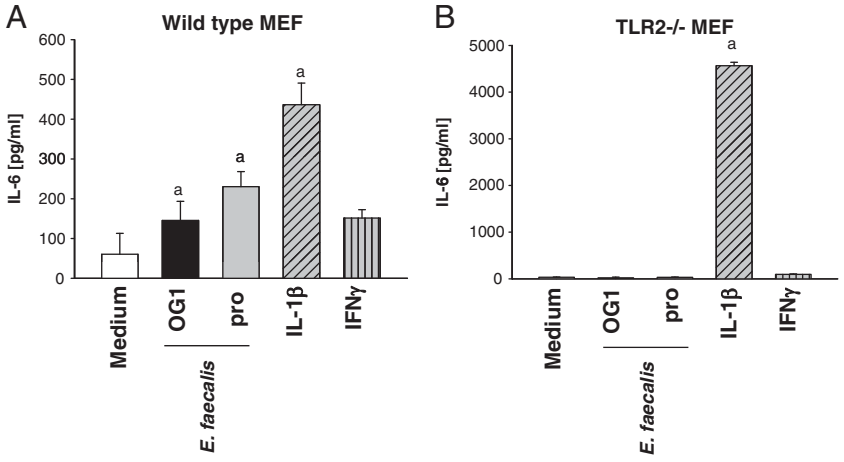

Figure 2. *E. faecalis*-induced IL-6 secretion is TLR-2 dependent. (A) WT MEF cells and (B) TLR-$2^{-/-}$ MEF cells were stimulated with the *E. faecalis* strains (moi 30) or with the cytokines IL-1β or IFN-γ (as controls) for 24 h as indicated. Culture supernatants were analyzed by ELISA technique for secreted IL-6. Results shown represent the mean ± SD of triplicate samples. Statistics were analyzed using ANOVA and Holm Sidak method: a = $p < 0.05$ *versus* medium.

tissue sections from the distal colon of *E. faecalis*-monoassociated IL-$10^{-/-}$ mice revealed severe colitis for the probiotic (Histology Score: 3.1 ± 0.6; $n = 5$) as well as the colitogenic control strain OG1RF (Histology Score: 3.6 ± 0.2; $n = 5$) (Fig. 3). The level of tissue pathology was not significantly different between the two *E. faecalis* strains. WT mice were not inflamed, supporting the non-pathogenic nature of both *E. faecalis* strains. Equal colonization of the monocolonized mice was confirmed by plating ten-fold dilution series of cecal contents of all mice (all values depicted in log10(CFU/g)(± SD); WT mice: OG1RF: 9.62 ± 0.05, probiotic: 9.63 ± 0.40; IL-$10^{-/-}$ mice: OG1RF: 9.69 ± 0.15, probiotic: 9.79 ± 0.30).

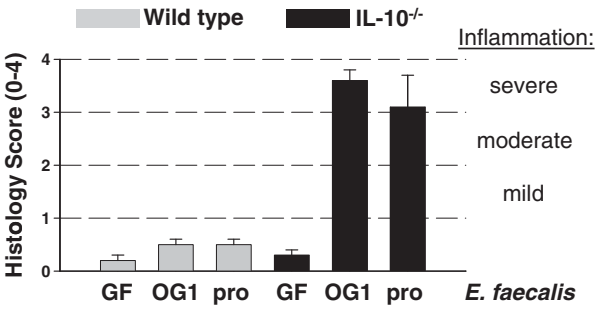

Figure 3. Probiotic *E. faecalis* induces severe colitis in IL-$10^{-/-}$ mice. Germ-free (GF) WT and IL-$10^{-/-}$ mice were mono-associated with the *E. faecalis* strains at 12–16 wk of age (five mice in each group). Mice were killed 14 wk after bacterial monoassociation. Blinded histopathological analysis (score 0–4) was performed in formalin-fixed paraffin-embedded tissue sections of the distal colon, as described in Section 2. Statistics were done using ANOVA on Ranks and Tukey test. There were no significant differences between the strains

3.4 Protein profiling in primary IEC derived from IL-$10^{-/-}$ mice monoassociated with the probiotic *E. faecalis*

We previously started to characterize the epithelial cell proteome in germ-free WT and IL-$10^{-/-}$ mice after mono-association with *E. faecalis* OG1RF [22]. To further evaluate the epithelial cell proteome in WT and IL-$10^{-/-}$ mice after monoassociation with the probiotic *E. faecalis* strain, we applied 2-D SDS-PAGE and MALDI-TOF MS analysis as described inSection 2. Compared to germ-free IL-$10^{-/-}$ mice we identified ten differentially up- and 11 down-regulated proteins in the probiotic *E. faecalis*-mono-associated IL-$10^{-/-}$ mice (Tables 1 and 2). Figures 4 and 5 illustrate the localization and the spot intensity of the regulated proteins.

Interestingly, we were able to identify the glucose-regulated protein (grp78) (gene name: Hspa5) to be strongly up-regulated (mean fold change 3.41) in probiotic *E. faecalis*-monoassociated IL-$10^{-/-}$ mice compared to germ-free animals, confirming the previously published results for *E. faecalis* OG1RF [22]. Furthermore, the proteins hydroxymethylglutaryl-CoA synthase (Hmgc2), uridine diphosphate-glucose dehydrogenase (Ugdh), 3-mercaptopyruvate sulfurtransferase (Mpst) and retinal dehydrogenase 1 (Aldh1a1) were found to be differentially regulated for *E. faecalis* OG1RF and the probiotic *E. faecalis* (Fig. 6), indicating general *E. faecalis*-induced mechanisms in monoassociated IL-$10^{-/-}$ mice.

Since the probiotic *E. faecalis* and the colitogenic control strain induced pro-inflammatory epithelial cell activation through TLR-2-dependent signaling mechanisms, we next compared the epithelial cell proteome from the probiotic *E. faecalis* with previously published proteins regulated in IEC derived from TLR-$2^{-/-}$mice [31] and from *E. faecalis* OG1RF monocolonized IL-$10^{-/-}$ mice [22]. As shown in Fig. 6, we included 53 differentially regulated proteins from all three comparisons (probiotic, colitogenic, TLR-2-dependent). Interestingly, the comparison between the colitogenic and probiotic groups revealed only five overlapping target proteins, suggesting *E. faecalis* strain-dependent effects at the epithelial interface independent of the chronic inflammatory processes. These five overlapping proteins were

Table 1. Differentially up-regulated proteins comparing IEC derived from germfree IL-10$^{-/-}$ mice (reference) *versus* probiotic *E. faecalis*-monoassociated

No.	Gene name	Accession number	Name of protein	Mass[a] (Da)	p*I*[b]	Mean score[c]	SD score[d]	Mean SCov (%)[e]	Mean fold change[f]	SD fold change[g]	Frequency[h]
Up-regulated proteins:											
1	Calml4	Q8R1P1	Calmodulin-like 4	17 783	7.7	76.5	29.2	37.5	3.21	0.64	5/5
2	Hspa5	A37048	DnaK type molecular chaperone grp78	72 491	5.1	330.7	85.1	47.0	3.41	0.31	5/5
3	Tpt1	S00775	Translationally controlled tumor protein	19 564	4.8	73.0	10.8	26.0	3.06	0.65	5/5
4	Aco2	BC004645	Aconitate hydratase, mitochondrial precursor	86 151	8.1	125.3	50.8	25.0	2.51	0.78	3/5
5	Mrps22	AK013925	Mitochondrial ribosomal protein S22	41 281	8.6	91.0	36.0	33.9	2.40	0.82	3/5
6	Bcat2	BC017688	Branched chain amino-transferase 2, mitochondrial	44 669	8.6	80.3	23.7	25.8	2.06	0.52	3/5
7	Nucb1	AK004886	Nucleobindin	53 376	5	129.3	46.3	42.0	3.63	1.21	3/5
8	Pdia3	Q8C2F4	Glucose-regulated protein, 58 kDa	57 103	5.8	218.6	65.4	47.8	2.35	0.57	4/5
9	Golph3l	Q8R088	Golgi phosphoprotein 3-like	32 999	5.7	104.0	27.0	35.2	2.58	0.65	4/5
10	G6pdx	A56686	Glucose-6-phosphate 1-dehydrogenase	59 681	6.0	212.3	54.4	42.5	4.16	1.24	5/5

a) Theoretical protein mass according to the MASCOT search result.
b) Theoretical p*I* of the proteins according to the MASCOT search result.
c) Mean MASCOT Mowse score derived from the MASCOT search result.
d) Standard deviation of the mean MASCOT Mowse score.
e) Mean sequence coverage of the proteins in percentage derived from the MASCOT search result.
f) Mean fold change in spot intensity of the regulated proteins derived from the Proteome Weaver software comparison.
g) Standard deviation of the mean fold change.
h) Frequency: number of mice in which the protein was differentially regulated/number of mice investigated.

regulated in the same direction (grp78 (= Hspa5) up; Hmgc2, Ugdh, Aldh1a1 and Mpst down). Of note, we included 31 TLR-2-regulated proteins independent of inflammatory processes and, consistently, all overlapping proteins regulated in severely inflamed *E. faecalis* monocolonized mice were regulated in opposite directions compared to TLR-2$^{-/-}$mice, except Mpst. In conclusion, we identified two target proteins (grp78 (= Hspa5) and Mpst) that were differentially regulated in response to TLR-2-mediated signals from probiotic and colitogenic *E. faecalis*.

4 Discussion

Although the selective colonization of germ-free IL-10$^{-/-}$ mice with *E. faecalis* may only reflect a simplistic mouse model to study the colitogenic nature of these commensal bacteria, we showed in this study that probiotic bacteria harbor the potential to initiate experimental colitis in the disease-susceptible but not the normal host. Recent studies with human volunteers clearly demonstrated that probiotic bacteria trigger NFκB-dependent gene expression in the normal mucosa [35], supporting our findings on *E. faecalis*-mediated TLR-2-dependent pro-inflammatory epithelial cell activation.

The role of IP-10, a chemokine to attract activated TH1 lymphocytes and phagocytes, is not completely described in the pathophysiology of IBD. IP-10 is secreted by endothelial cells, epithelial cells, fibroblasts, keratinocytes and monocytes [36]. We were able to measure high levels of IP-10 secretion in response to the *E. faecalis* strains. Compared to IL-6 secretion, *E. faecalis*-induced IP-10 secretion in IEC was mediated by the NFκB and the p38 MAPK but not by the Erk1/2 pathway. Since no differences were found between the two *E. faecalis* strains the mechanisms of IP-10 secretion in IEC are probably similar for all *E. faecalis* strains. In contrast *Lactobacillus casei* and *Streptococcus thermophilus* were not able to induce IP-10 secretion in Mode K cells (data not shown). We were even able to show that *L. casei* derived from the probiotic mixture VSL♯3 is capable of inhibiting TNF-induced IP-10 secretion by post-translational degradation [37]. These findings suggest that probiotic bacteria have different mechanisms to influence epithelial IP-10 secretion.

The relevance of probiotics for the treatment of human IBD is still controversial [38, 39]. In mild-to-moderate UC,

Table 2. Differentially down-regulated proteins comparing IEC derived from germ-free IL-10$^{-/-}$ mice (reference) versus probiotic *E. faecalis*-monoassociated

No.	Gene name	Accession number	Name of protein	Mass[a] (Da)	p*I*[b]	Mean score[c]	SD score[d]	Mean SCov (%)[e]	Mean fold change[f]	SD fold change[g]	Frequency[h]
Down-regulated proteins:											
1	Glod4	Q9CPV4	Glyoxalase domain-containing protein 4	33 581	5.28	165.67	48.63	52.3	0.33	0.07	5/5
2	Hmgcs2	B55729	Hydroxymethylglutaryl-CoA synthase, mitochondrial	53 115	7.46	201.25	42.45	47.25	0.49	0.13	3/5
3	Anxa4	Q7TMN7	Annexin A4	36 192	5.4	180.50	63.92	50.0	0.56	0.33	3/5
4	Ak2	Q9WTP6	Adenylate kinase isoenzyme 2, mitochondrial	25 686	7.2	75.00	16.46	37.2	0.39	0.05	5/5
5	Prdx6	O08709	Peroxiredoxin 6	24 838	5.7	150.67	34.41	60.4	0.45	0.05	5/5
6	Hao3	Q8JZR9	Hydroxyacid oxidase 3	39 145	7.6	66.60	13.28	27.0	0.36	0.11	4/5
7	Ugdh	AF061017	uridine diphosphate-glucose dehydrogenase	55 482	7.49	148.17	63.70	37.5	0.40	0.07	5/5
8	Aldh1a1	JQ1004	Retinal dehydrogenase 1	55 131	7.89	185.00	55.13	42.0	0.46	0.09	3/5
9	Mpst	Q99J99	3-Mercaptopyruvate sulfurtransferase	33 100	6.12	159.00	20.66	51.3	0.46	0.09	3/5
10	Aldh2	I48966	Aldehyde dehydrogenase 2, precursor, mitochondrial	57 015	7.53	130.75	47.54	33.0	0.43	0.09	4/5
11	Eef2	P58252	Elongation factor 2	96 091	6.42	148.25	34.54	21.8	0.77	0.43	3/5

a) Theoretical protein mass according to the MASCOT search result.
b) Theoretical p*I* of the proteins according to the MASCOT search result.
c) Mean MASCOT Mowse score derived from the MASCOT search result.
d) SD of the mean MASCOT Mowse score.
e) Mean sequence coverage of the proteins in % derived from the MASCOT search result.
f) Mean fold change in spot intensity of the regulated proteins derived from the Proteome Weaver software comparison.
g) SD of the mean fold change.
h) Frequency: number of mice in which the protein was differentially regulated/number of mice investigated.

Escherichia coli Nissle or VSL#3, a mixture of eight lactic acid bacteria and Bifidobacteria, have been shown to induce remission or to be as effective as Mesalazine [40, 41]. Also, the treatment of pouchitis using VSL#3 is effective [42]. However, in CD there is only a limited number of studies and different probiotics did not show efficacy in preventing recurrence of CD [43, 44]. Further, placebo-controlled studies with high numbers of participants and standardized study designs would be necessary to confirm these results.

In human IBD, the use of a probiotic *E. faecalis* strain has not been investigated. In conventionally housed IL-10$^{-/-}$ mice probiotics like *E. coli*, Nissle 1917 or VSL#3 were sufficient to reduce colitis [45, 46]. In germ-free IL-10$^{-/-}$ mice it was shown that monocolonization with *E. faecalis* OG1RF is sufficient to induce severe colitis after 14 wk [21]. Our study did not support a beneficial effect of the probiotic *E. faecalis*. Probiotic strains induce specific mechanisms in the host and, similar to pharmaceutical substances, can probably only be beneficial for specific medical conditions. Our study suggests that the probiotic *E. faecalis* is not appropriate to treat colitis in mice. However, it needs to be mentioned that monocolonized IL-10$^{-/-}$ mice are a simplified model and the translation to the human situation requires further studies. It has been shown that dual association of IL-10$^{-/-}$ mice with *E. faecalis* and *E. coli* results in severe distal colitis already after 7 wk [47]. Furthermore, the lack of IL-10 itself is an immense immunological deficiency. To further evaluate the beneficial effects of the probiotic *E. faecalis* on colitis, this strain has to be applied in other animal models of IBD. Monoassociation of IL-10$^{-/-}$ mice with the commensal bacteria *E. faecalis*, *E. coli* or *B. vulgatus* differentially triggered intestinal inflammation in the presence of *E. faecalis* and *E. coli* but not *B. vulgatus* and resulted in distinctly different kinetics and anatomic distribution in the same host [47, 48], demonstrating the species-dependent capability of commensal bacteria to induce experimental colitis in a susceptible mouse model.

For both *E. faecalis* strains, mitochondrial Hmgc2 and Ugdh were found to be down-regulated in IL-10$^{-/-}$ mice compared to germ-free mice. Of note, we previously found both enzymes to be down-regulated in IL-10$^{-/-}$ mice before the onset of colitis after 2 wk of monoassociation with *E. faecalis* OG1RF [49]. Therefore, the regulation of these enzymes seems to be an early *E. faecalis*-induced effect on IEC. Ugdh is an enzyme involved in the biosynthesis of glycosaminoglycans, hyaluronan, chondroitin sulfate and heparan sulfate [50]. These substrates might serve as

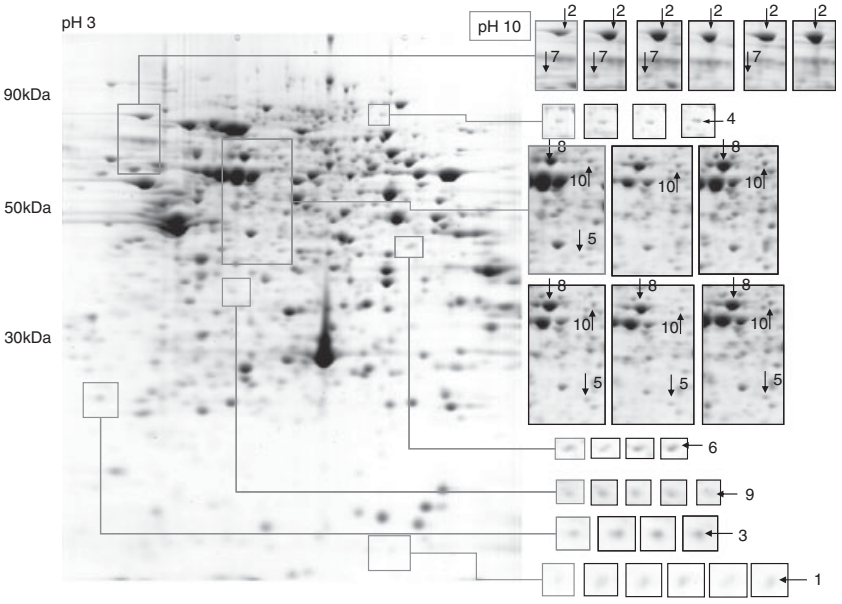

Figure 4. Proteome analysis of primary IEC derived from germ-free *versus* probiotic *E. faecalis*-monoassociated IL-10$^{-/-}$ mice – up-regulated proteins. Germ-free IL-10$^{-/-}$ mice were monoassociated with the probiotic *E. faecalis* for 14 wk. Primary IEC were purified from germ-free and *E. faecalis*-monoassociated animals. Two-dimensional SDS-PAGE and proteome analysis was performed as described in Section 2. The reference gel (left side) was obtained with pooled IEC samples from germ-free IL-10$^{-/-}$ mice ($n = 5$) and compared with each of the five IEC samples from the probiotic *E. faecalis*-monoassociated mice using the Proteome Weaver software. Localization and regulation of the numbered differentially up-regulated spots is shown. Identified proteins with at least two-fold changes in expression level confirmed in at least three out of the five mice were considered as differentially regulated.

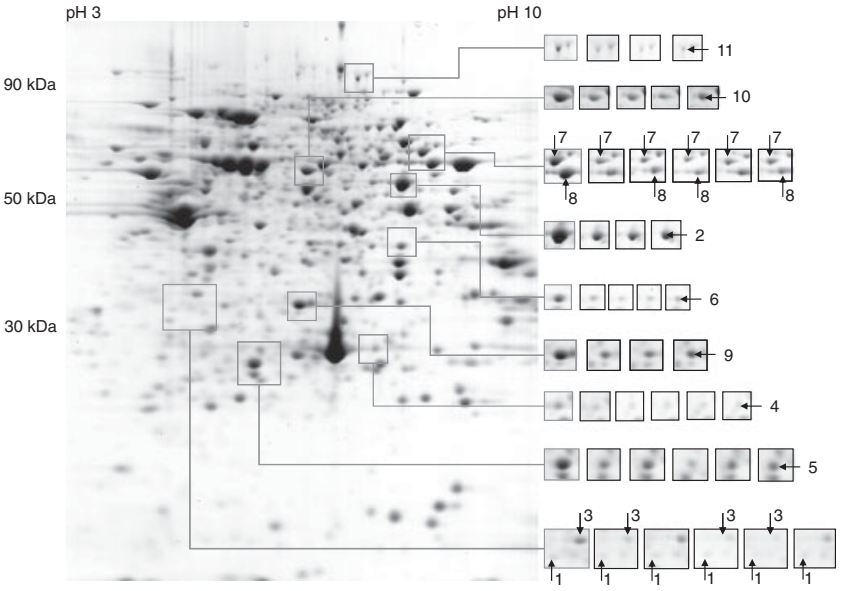

Figure 5. Proteome analysis of primary IEC derived from germ-free *versus* probiotic *E. faecalis*-monoassociated IL-10$^{-/-}$ mice–down-regulated proteins. Germ-free IL-10$^{-/-}$ mice were monoassociated with *E. faecalis* for 14 wk. Primary IEC were purified from germ-free and *E. faecalis*-monoassociated animals. Two-dimensional SDS-PAGE and proteome analysis was performed as described in Section 2. The reference gel (left side) was obtained with pooled IEC samples from germ-free IL-10$^{-/-}$ mice ($n = 5$) and compared with each of the five IEC samples from *E. faecalis*-monoassociated mice using the Proteome Weaver software. Localization and regulation of the numbered differentially down-regulated spots is shown. Identified proteins with at least two-fold changes in expression level confirmed in at least three out of the five mice were considered as differentially regulated.

nutrients for *E. faecalis*. Interestingly, Baldassarri *et al.* showed that glycosaminoglycans mediate invasion and survival of *E. faecalis* into macrophages [51]. Furthermore, glycosaminoglycans, especially heparin and/or heparan sulfate, play an important role in the binding of enterococci to human colon cells [52]. Therefore, the down-regulation of Ugdh might be an early counter-regulation of IEC against bacterial adhesion.

Grp78 (gene name: Hspa5) was found to be up-regulated for the colitogenic and the probiotic *E. faecalis* strains in IL-10$^{-/-}$ mice compared to germ-free mice. This protein is a chaperone in the ER and the major inducer of the unfolded protein response, which is a result of mis- or unfolded protein accumulation in the ER. We recently demonstrated that primary IEC from IL-10$^{-/-}$ mice and from IBD patients revealed increased expression levels of grp78 under conditions of chronic inflammation. Interestingly, we showed that IL-10 modulates the grp78 expression levels by blocking the recruitment of ATF6 to the grp78 promoter in IEC [22]. These results clearly demonstrated that immune mediators have the potential to modulate inflammation-driven ER stress responses. The up-regulation of grp78 in *E. faecalis* monoassociated IL-10$^{-/-}$ mice and in human patients suggests that, in the context of a chronic inflammatory response, the inadequate resolution of ER stress mechanisms may induce tissue pathology in the intestinal mucosa,

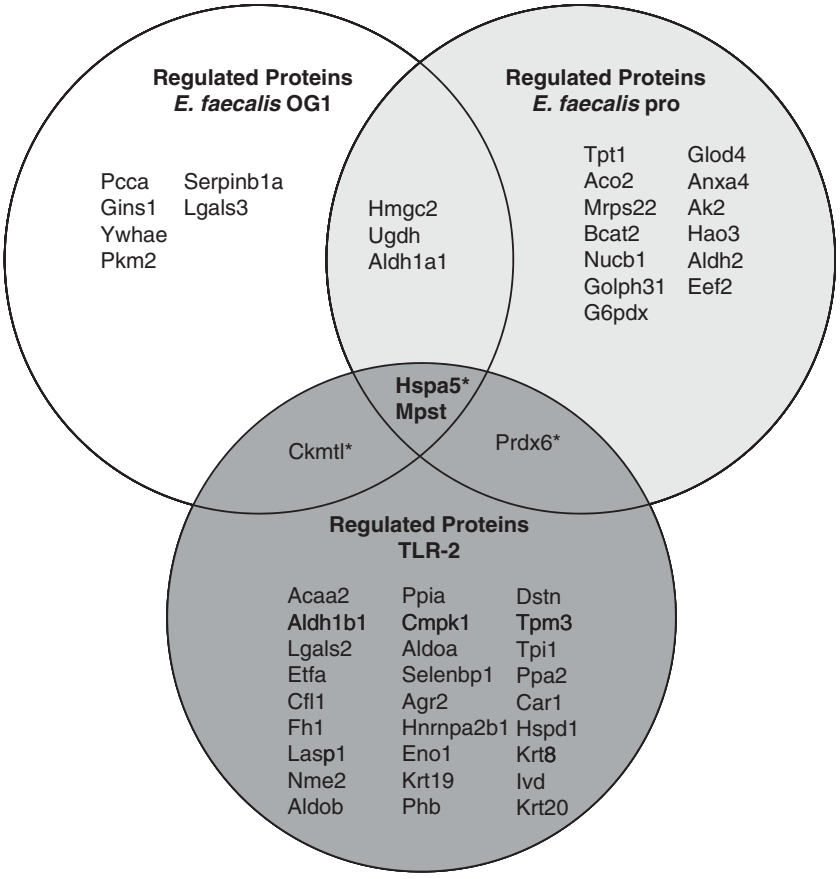

Figure 6. Overlap of proteins differentially regulated by the probiotic *E. faecalis* and *E. faecalis* OG1RF in monoassociated IL-10$^{-/-}$ mice as well in TLR-2$^{-/-}$mice (abbreviated using gene names). TLR-2 mice were not inflamed. * = Proteins regulated in opposite directions (*e.g.* up/down) when comparing *E. faecalis*-regulated proteins and TLR-2-regulated proteins. All overlapping proteins comparing the two *E. faecalis* strains were regulated in the same direction.

probably because of the lack of IL-10. Recently, IBD patients were associated with variations in the *xbp1* gene. XBP1 is an immature transcription factor of the UPR that becomes activated by ER-anchored IRE1 under conditions of chronic inflammation [53]. In TLR-2$^{-/-}$ mice grp78 was also found to be differentially regulated [31], however not up-regulated but strongly down-regulated, supporting the role of TLR-dependent signals in regulating the ER stress response program [54].

The intensity and abundant expression of the Mpst-spot in our 2-D gels suggests an important physiological role of Mpst in IEC. Mpst is an enzyme that is implemeted in cyanide detoxification [55] and in cysteine catabolism to form pyruvate. Recently, we found Mpst to be down-regulated (2.5-fold) in severe *E. faecalis* OG1RF-induced colitis [49]. Consistently, we here demonstrate here that this protein is down-regulated in probiotic *E. faecalis*-induced colitis. Ramasamy *et al.* [56] showed in the colonic mucosa of UC patients that Mpst expression is lost under conditions of chronic inflammation, suggesting that the decreased Mpst expression levels might be a general feature of IBD and not an *E. faecalis* specific effect.

In summary, the probiotic *E. faecalis* strain harbors the potential to initiate disease-relevant mechanisms in a susceptible but not a normal host. We may speculate that the induction of pro-inflammatory processes are key mechanisms of the innate immune response to alert the host, not only towards harmful but also towards beneficial microbes.

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