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Lactocepin as a protective microbial structure in the context of IBD

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Probiotics have been shown to exert beneficial effects in the context of different diseases including inflammatory bowel diseases (IBD). However, clinical use of probiotics is hampered by lack of understanding of the protective mechanisms and by safety concerns regarding the application of high numbers of live bacteria in patients. The identification of protective microbial structure-function relationships might enable to overcome these restraints and might lead to innovative therapies using the isolated active microbial structures. In our study, we aimed to characterize the protective mechanisms of VSL#3, a clinically relevant probiotic mixture in IBD. We found *Lactobacillus casei/paracasei*-produced lactocepin to selectively degrade pro-inflammatory chemokines, resulting in reduced immune cell infiltration and reduced inflammation in experimental IBD models. As immune cell recruitment is a major proinflammatory mechanism our findings suggest that lactocepin might be of broad therapeutic relevance in an array of inflammatory diseases like IBD, allergic skin inflammation and psoriasis.

Keywords: lactocepin, *prtP*, CEP, IBD, probiotic, inflammation, chemokines, protease

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Introduction

An extensive bulk of experimental data and an array of clinical studies indicate that specific probiotics might be a good alternative or adjunct therapy in the context of inflammatory bowel disease (IBD). However, the therapeutical application of probiotics is still hampered by uncertainties concerning appropriate strain selection, timing and dosage in the context of different IBD indications (ulcerative colitis, Crohn disease or pouchitis, acute inflammation or remission, location and severity of the inflammation). In addition, there are still safety concerns regarding the uptake of huge amounts of live bacteria in IBD patients that are characterized by immune dysregulation and compromised intestinal barrier functions.[1] Besides the lack of extensive strain specific clinical data, all these problems boil down to the lack of mechanistic understanding of probiotic efficacy. In contrast to the use of defined pharmaceuticals like corticosteroids, NSAIDs, immunosuppressive drugs or biologics that are known or even designed to target specific immune functions of the host, the protective structure-function relationships underlying the observed anti-inflammatory effects of specific probiotics are largely unknown. The identification of probiotic structure-function relationships is therefore a clear prerequisite for the targeted clinical use of probiotics or isolated probiotic structures in the future.

Recent studies already identified various microbial structures (e.g., proteins, cell surface components, metabolites, DNA) that beneficially affect the health of the host via highly diverse mechanisms.[2-4] These microbial structures were found to either affect other microbes or to modulate host functions. The exact nature of these probiotic structure-function relationships has important implications for the potential therapeutic use of the respective microbe or microbial structure. Microbial structure-microbe relationships are exemplified by the finding that a *Lactobacillus salivarius* UCC118-produced bacteriocin (Abp118) mediates the observed prevention of systemic *Listeria monocytogenes*

infection by *Lactobacillus salivarius*.[4] This finding clearly indicates that the protective effect of potential *Lactobacillus salivarius* applications is restricted to the prevention of infections with Abp118-sensitive pathogens, demonstrating the major relevance of probiotic structure-function relationships for the development of effective probiotic therapies. Concerning direct microbe-host relationships, microbial structures were found to affect various intestinal barrier and immune functions. Bifidobacteria-produced acetate was found to reduce the mortality of EHEC-infected gnotobiotic mice by strengthening the intestinal epithelial barrier, resulting in reduced translocation of Shiga toxin into the systemic circulation. Importantly, this protective effect could be reproduced by feeding acetylated starch to germfree mice, proving that isolated microbial structures can be sufficient to mediate protection.[5] In the context of IBD, polysaccharide A (PSA) of the intestinal commensal *Bacteroides fragilis* was also found to be sufficient to prevent and cure experimental colitis, probably via the induction of IL10-producing regulatory CD4+T cells.[6-8] Furthermore, a secreted protein of *Lactobacillus (L) rhamnosus* GG, p40, was identified to reduce IEC apoptosis by epidermal growth factor receptor activation.[9,10] Feeding isolated and encapsulated p40 to mice suffering from chemically-induced colitis resulted in significant reduction of IEC apoptosis and colonic inflammation.[11] This result shows that isolated microbial proteins like p40 have the potential to exert protective functions in the intestine, provided that they are protected from the harsh conditions in the upper gastrointestinal tract. In summary, these findings demonstrate that tracking down probiotic effects to the mechanistic level is not only necessary to enable rational use of probiotics but can also result in the detection of promising new agents for the treatment of IBD.

PrtP-Encoded Lactocepin as Anti-Inflammatory Microbial Structure in the Context of IBD

In our study, we aimed to elucidate the protective mechanisms underlying the anti-inflammatory effects of the probiotic mixture VSL#3 that has been proven to be clinically relevant in the prevention and treatment of pouchitis[12] as well as in the treatment of ulcerative colitis.[13,14] Initial screening experiments using VSL#3 or the eight single bacterial strains (*Bifidobacterium (B) breve*, *B. infantis*, *B. longum*, *Streptococcus thermophilus*, *L. acidophilus*, *L. bulgaricus*, *L. paracasei* (L.p), *L. plantarum*) revealed that the complete mixture and the single strain L.p reduce TNF-induced secretion of the proinflammatory chemokine interferon-induced protein 10 (IP-10) in intestinal epithelial cells (IEC).[15] The T-cell recruiting chemokine IP-10 is not only known to be strongly upregulated in inflamed intestinal tissue of IBD patients[16,17] but has been shown to play a major proinflammatory role in experimental IBD. The neutralization of IP-10 via anti-IP-10 antibodies resulted in significantly reduced inflammation in DSS-treated mice and IL10$^{-/-}$ mice.[18-20] The selective inhibitory effect of L.p on IP-10 was therefore thought to be of significant anti-inflammatory relevance. Subsequent stimulation experiments in order to analyze the underlying protective structure-function relationship revealed that the reduction of TNF-induced IP-10 is mediated by a cell surface attached and secreted protein of the probiotic strain. Mechanistically, we were not able to detect any impact of L.p on TNF-induced IP-10 expression in IEC, whereas the active protein in L.p supernatants (CM) was found to mediate loss of existing IEC-surface attached and secreted IP-10 in TNF-pre-activated IEC. These findings clearly indicated that the observed loss of IP-10 is due to an IEC-independent, direct effect of the active probiotic protein on the chemokine. Indeed, cell free experiments with CM and recombinant IP-10 revealed that the observed loss of IP-10 is due to IP-10 degradation by an L.p-derived serine protease. Further experiments revealed that the probiotic protease targets an array of additional proinflammatory chemokines like I-TAC and Eotaxin. In contrast, cytokines like IL-6 and IL-10 as well as IEC viability and barrier function were unaffected. This selective anti-inflammatory substrate profile suggested that the probiotic protease might potentially be used as a safe and efficient anti-inflammatory agent in vivo. In order to identify the active protease, chromatographic fractionation of L.p supernatants and subsequent LC-MS-MS analysis of the fractions were performed, suggesting *prtP*-encoded lactocepin to be the active anti-inflammatory structure. *PrtP*-encoded lactocepins are cell envelope proteases (CEPs) of lactobacilli and lactococci that are mainly known for the degradation of caseins and that play an important role in cheese production. The hypothesis that this long known protease is the anti-inflammatory structure of L.p was finally proven by IP-10 cleavage assays using immunoprecipitated L.p lactocepin. The selective degradation of proinflammatory chemokines by *prtP*-encoded lactocepin was therefore identified as anti-inflammatory structure-function relationship of L.p (**Fig. 1**). In order to test the physiological relevance of this newly identified probiotic mechanism, we generated a lactocepin-deficient mutant (L.c *prtP*^{dis}) of a transformable human fecal *L. casei* (L.c) isolate that had been found to secrete *prtP*-encoded lactocepin with analogous anti-IP-10 activity as L.p. Feeding studies in inflamed T cell transferred RAG2$^{-/-}$ mice revealed significantly reduced cecal IP-10 levels, significantly reduced T cell and neutrophil infiltration (CD3^{+} and MPO-positive cells) and significantly reduced histopathological cecal inflammation in L.c fed mice compared with L.c *prtP*^{dis}-fed mice.[21] These results demonstrate that *prtP*-encoded lactocepin is indeed a physiologically relevant anti-inflammatory bacterial protease in the context of IBD that may, at least in part, be responsible for the observed anti-inflammatory effects of VSL#3 in experimental and clinical studies.

However, the characterization of this anti-inflammatory structure-function relationship raises several questions concerning the mode of action of *prtP*-encoded lactocepin in the intestine. Considering the fact that the intestinal lumen harbours large amounts of various bacterial and host proteases that degrade IP-10 (unpublished data) the underlying reasons for the specific anti-inflammatory effect of *prtP*-encoded lactocepin remain to be elucidated. One hypothesis is based on the assumption that *prtP*-encoded lactocepin degrades tissue distributed chemokines in the mucosa and therefore, the protective effect is dependent on the ability of *prtP*-encoded lactocepin to penetrate into the

inflamed target tissue. The penetration might be facilitated by bacterial carriers that adhere to the intestinal mucosa and secrete *prtP*-encoded lactocepin in close proximity to the target tissue. Interestingly, the expression of *prtP*-encoded lactocepin seems to positively affect mucosal adherence in itself, as L.c showed better mucosal adherence than L.c *prtP*^{dis} in monocolonized mice.[21] The possible penetration of bacteria-produced proteins into inflamed intestinal mucosal tissue has already been proven in a study using TNF-nanobody-secreting *Lactococcus lactis*[22] and is presumably due to disturbed intestinal barrier functions in inflammation.

Furthermore, it is unclear why L.c-produced lactocepin was found to be protective in the cecum whereas the inflammation of the distal colon did not differ between L.c and L.c *prtP*^{dis}-fed T cell transferred RAG2$^{-/-}$ mice.[21] This discrepancy in responsiveness had already been observed in studies using VSL#3 in IL10$^{-/-}$ mice[15] and might be due to the different morphology, different fecal passage kinetics and/or the reduced water content in the colon.[23] It remains to be determined whether these factors impair the proteolytic activity of *prtP*-encoded lactocepin, reduce the accessibility of mucosal attachment sites for lactocepin-expressing bacteria or hamper the expression of *prtP*-encoded lactocepin, resulting in insufficient protective activity of *prtP*-encoding bacteria in the colon.

In light of these questions, it will be highly interesting to investigate the anti-inflammatory impact of isolated encapsulated lactocepin on experimental IBD. On the one hand, the application of the isolated protective protease allows the uptake of defined high amounts of *prtP*-encoded lactocepin, circumventing the potential problem of insufficient *prtP*-encoded lactocepin expression of the probiotic bacteria in the intestinal tract. On the other hand, appropriate bacterial carriers might be necessary for the protective effect. However, the outcome of these experimental studies will answer the question whether isolated *prtP*-encoded lactocepin can be considered as a promising new anti-inflammatory agent in the context of IBD (**Table 1**). If bacterial carriers are found to be necessary for the protective effect, *prtP*-encoded lactocepin might also be applied via defined bacterial carriers, e.g., *Lactococcus lactis*, that are approved for the application in humans.[24]

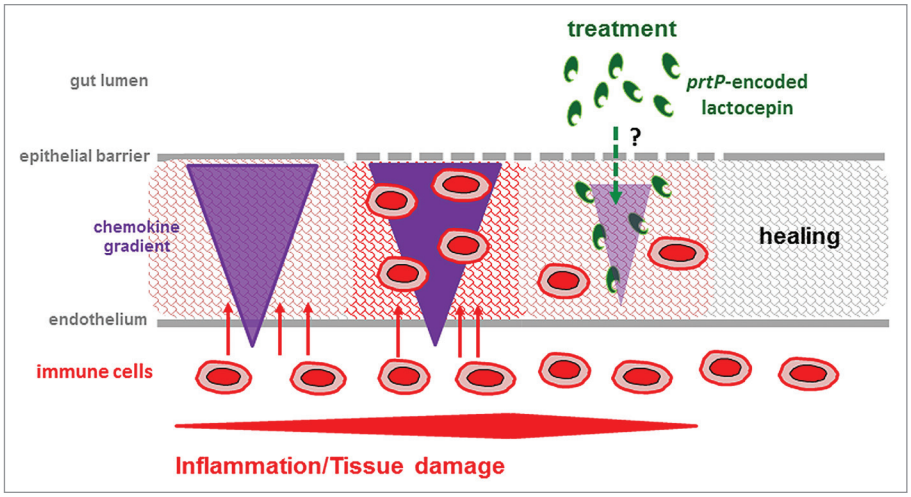

Figure 1. Anti-inflammatory mechanism of *prtP*-encoded lactocepin. Chronic inflammatory diseases like IBD, allergy and autoimmune diseases are characterized by a vicious circle of chemokine secretion, immune cell recruitment and activity and tissue damage including barrier disruption. *PrtP*-encoded lactocepin produced by *L. casei/L. paracasei* interferes with this vicious circle by specifically degrading proinflammatory chemokines, resulting in reduced infiltration of immune cells and potentially healing. However, the location of luminal lactocepin production and the mechanisms underlying its penetration into the mucosal tissue remain to be elucidated.

Table 1. Questions in the context of the newly identified anti-inflammatory activity of lactocepin.

Summary of open questions
Intestinal *prtP*-expression?
· localization (luminal, mucosal, intestinal compartments)
· expression level (health, inflammation, probiotic supplementation)
Substrate specificity of *prtP*?
· substrate binding residues, folding
· lactobacillus vs. lactococcus *prtP*
· related CEPs (*prtB*, *prtH*, *prtR* and *prtS*)
Therapeutical relevance of isolated *prtP*-encoded lactocepin?
· oral application in IBD
· topical application in inflammatory skin diseases
· injections (i.v., joints and subcutaneous)

Substrate Profiles and Expression Levels of *prtP*-Encoded Lactocepin are Strain-Specific

The newly observed anti-IP-10 activity of L.p and L.c *prtP*-encoded lactocepin raises questions concerning the substrate specificity of highly similar *prtP*-encoded lactocepins from other lactobacilli as well as lactococci. In addition, the anti-chemokine activity of less similar CEPs like *prtB*, *prtH*, *prtR* and *prtS*, which are expressed by other *Lactobacillus* species like *L. delbrueckii*, *L. rhamnosus* and *L. helveticus*, remain to be elucidated (**Table 1**).[25,26] First analysis in this context revealed that most tested *L. casei*/*L. paracasei* strains encode functional IP-10-degrading *prtP*-encoded lactocepin (**Fig. 2**) whereas the anti-IP-10 activity of lactococcal *prtP*-encoded lactocepin seems to be strain specific (unpublished data). The amino acid residues that determine whether a specific *prtP*-encoded lactocepin degrades IP-10 remain to be determined. Earlier work in the context of casein cleavage already indicated that the catalytic (PR), insertion (I) and A-domain contribute to the

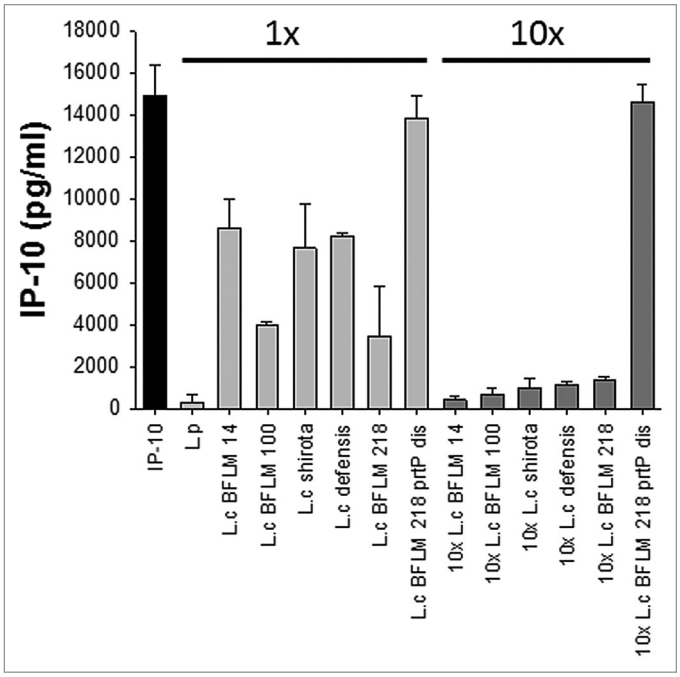

Figure 2. Screening of lactobacilli for anti-IP-10 activity. Human fecal *L. casei* isolates (L.c BFLM14, L.c BFLM 100 and L.c BFLM 218) and commercial strains (L.c shirota and L.c defensis) were screened for their ability to degrade murine IP-10. All tested strains secrete low amounts of IP-10-degrading *prtP*-encoded lactocepin compared with L.p. The anti-IP-10 activity was strongly increased after cultivation in higher cell densities (1 × = 5×10^7 cfu/ml, 10 × = 5×10^8 cfu/ml). L.c BFLM 218 $prtP^{dis}$ served as negative control.

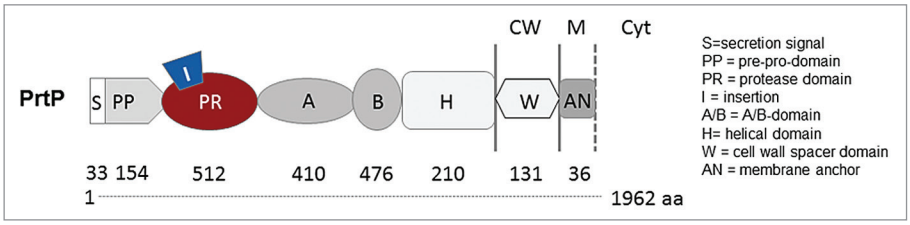

Figure 3. Domain structure of *prtP*-encoded lactocepin. *PrtP*-encoded lactocepins from lactobacilli and lactococci are highly similar but show different substrate specificities even within the same species. It has been found that the catalytic domain (PR), the insertion domain (I) and the A domain play a role in the casein specificity of certain *prtP*-encoded lactocepins. However, it is not known which residues of the active secreted protease (PR to H domain) determine the anti-inflammatory substrate profile of L.p-derived *prtP*-encoded lactocepin.

substrate specificity of *prtP*-encoded lactocepin (**Fig. 3**).[26]

Regarding the expression level of *prtP*-encoded lactocepin, most strains were found to be less active than L.p and higher bacterial numbers are required to produce similar anti-IP-10 activity (**Fig. 2**). Besides this high intraspecies variability, environmental factors were found to play an important role in the regulation of *prtP* expression, as e.g., L.p BL23 does not secrete detectable amounts of lactocepin in CM whereas it produces proteolytically active lactocepin in milk.[21] The mere presence of *prtP* does therefore clearly not serve as an indicator for the anti-inflammatory potential of a specific bacterial strain. The enormous complexity of *prtP*-regulation and substrate specificity is underlined by the bulk of work that has already been published in the field of food technology.[27-29] The newly identified anti-inflammatory effect of *prtP*-encoded lactocepin in IBD raises additional questions concerning the regulation of *prtP* expression in the intestinal tract (**Table 1**). Strains of *L. casei*/*L. paracasei* are present in the human intestinal microbiota[30-32] and it will therefore be interesting to determine intestinal *prtP* expression levels in health and disease as well as in different intestinal compartments before vs. after the additional administration of bacteria like L.p, that are known to express high levels of *prtP*-encoded lactocepin in vitro.

Therapeutic Application of *prtP*-Encoded Lactocepin in Extraintestinal Inflammation

The identification of selective chemokine degradation by *prtP*-encoded lactocepin allows the assumption that *prtP*-encoded lactocepin might be protective in an array of chronic inflammatory diseases. The reason for this hypothesis is that the vicious circle of dysregulated high secretion of proinflammatory chemokines that induces increased recruitment and therefore activity of immune effector cells in the tissue, which in turn results in tissue destruction and even more chemokine secretion (**Fig. 1**), is thought to drive the inflammation in chronic inflammatory diseases like allergic diseases, psoriasis, lupus erythematosus and arthritis. As *prtP*-encoded lactocepin specifically degrades an array of proinflammatory chemokines that play an important role in these diseases,[33-37] the purified bacterial protease might potentially be used as a new anti-inflammatory pharmaceutical agent. *PrtP*-encoded lactocepin might be applied topically to reduce inflammatory skin diseases or it might even be injected e.g., to reduce arthritis. To date, these possible applications of *prtP*-encoded lactocepin are merely hypothetical and require extensive studies regarding safety and efficacy in the respective physiological context before they can be considered as a real therapeutic option. It will also be of major importance to determine the accessibility of the respective target tissue as well as the stability of the selective proteolytic activity of *prtP*-encoded lactocepin under physiological conditions using appropriate model systems. Our proof of concept study in the context of IBD revealed that intraperitoneally injected *prtP*-encoded lactocepin

reduces the infiltration of immune cells into the inflamed ileum,[21] underlining the significant anti-inflammatory potential of isolated *prtP*-encoded lactocepin.

Summary

The characterization of the specific degradation of pro-inflammatory chemokines by *prtP*-encoded lactocepin as anti-inflammatory probiotic structure-function relationship opens the door toward the development of anti-inflammatory therapies based on *prtP*-expressing bacteria or isolated *prtP*-encoding lactocepin in a broad range of chronic inflammatory diseases. The study therefore serves as an example for how the large array of already observed probiotic effects could be used in order to identify protective microbial structures which, in the best of all cases, might be used as new pharmaceutical agents in the future.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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