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Structure Function Analysis of Probiotic Bacteria in Mouse Models of Chronic Intestinal Inflammation

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ABSTRACT

Interferon-inducible protein (IP)-10 is a major chemokine for lymphocyte recruitment and is upregulated in inflammatory bowel diseases (IBD). Previously it was suggested that *Lactobacillus paracasei* (L.p) from the clinically relevant probiotic mixture VSL#3, expresses a probiotic isotype of the prtP-encoded serine protease lactocepin that mediates post-translational loss of the pro-inflammatory chemokine IP-10 in intestinal epithelial cells. The aim of the present study was to confirm this hypothesis, elucidate the underlying molecular mechanism of this effect and assess the anti-inflammatory effects of prtP-encoded lactocepin in mouse models of chronic intestinal inflammation.

Unexpectedly, the protease-mediated loss of IP-10 was found to be independent of cellular signal transduction processes but to be due to direct degradation of the chemokine. Immunoprecipitation experiments clearly confirmed prtP-encoded lactocepin as the IP-10 degrading protease in conditioned media of L.p (CM L.p). Subsequent experiments showed that prtP-encoded lactocepin of L.p is not restricted to IP-10 but selectively degrades an array of pro-inflammatory chemokines without exerting negative effects on barrier function. Explant culture experiments in inflamed murine tissue demonstrated accessibility of CM L.p to tissue-distributed IP-10, suggesting a therapeutic potential of the probiotic protease in inflammatory diseases. Consistently, intraperitoneal injection of CM L.p in inflamed $TNF^{\Delta ARE/+}$ mice, an experimental ileitis model, resulted in loss of ileal IP-10 and significantly reduced lymphocyte infiltration into ileal tissue. A human *Lactobacillus casei* (L.c) isolate was found to produce IP-10-degrading, prtP-encoded lactocepin *in vitro* as well as in the cecum of L.c mono-associated healthy mice. Therapeutic feeding studies in T cell transferred $Rag2^{-/-}$ mice, an experimental colitis model, revealed that feeding of L.c results in significantly reduced IP-10 tissue levels, T cell infiltration and inflammation in cecal tissue, compared to mice fed with the lactocepin-deficient isogenic mutant L.c $prtP^{dis}$.

Our study characterizes the selective degradation of pro-inflammatory chemokines by probiotic prtP-encoded lactocepins as a protective microbial structure-function relationship in the context of chronic intestinal inflammation, suggesting lactocepin-based therapies as effective treatment for chemokine-mediated diseases in general.

ZUSAMMENFASSUNG

Chronisch entzündliche Darmerkrankungen (CED) sind immunvermittelte Krankheiten, die u.a. durch erhöhte IP-10 Spiegel im Gewebe gekennzeichnet sind. IP-10 ist ein Chemokin, das über Chemotaxis entzündungsfördernde Lymphozyten an den Entzündungsherd rekrutiert. Dieser Arbeit vorrausgehende Studien haben gezeigt, dass *Lactobacillus paracasei* (L.p) aus der klinisch-evidenten Mischung VSL#3 eine probiotische Isoform der - durch den Genlocus prtP kodierten - Serinprotease Lactocepin exprimiert. Es wurde vermutet, dass diese Protease eine posttranslationale Reduktion des entzündungsfördernden Chemokins IP-10 in intestinalen Epithelzellen (IEZ) vermittelt. Ziel der vorliegenden Arbeit war es, die Hypothese der Lactocepin-vermittelten IP-10 Reduktion in IEZ zu bestätigen, den zugrundeliegenden Mechanismus aufzuklären und das anti-inflammatorische Potential von Lactocepin in CED-Mausmodellen zu evaluieren.

In vitro Studien zeigten, dass sowohl sekretiertes, als auch an die IEZ-Oberfläche assoziiertes IP-10 direkt und ohne Beteiligung zellulärer Signaltransduktionsmechanismen durch Lactocepin degradiert wird. Der Einsatz von immunpräzipitiertem Lactocepin in Inkubationsstudien bestätigte Lactocepin schließlich als IP-10 degradierende Protease. Weitere Inkubationsstudien zeigten, dass Lactocepin selektiv IP-10 und andere entzündungsfördernde Chemokine degradiert, ohne dabei die Barrierefunktion des intestinalen Epithels zu beeinträchtigen. Zudem zeigten Experimente mit intestinalem Gewebe, dass Lactocepin auch gewebeständiges IP-10 degradiert. Die intraperitoneale Injektion Lactocepin-haltiger Überstände führte im Ileum entzündeter $TNF^{\Delta ARE/+}$-Mäuse zu einer Reduktion der IP-10 Spiegel und der Lymphozytenrekrutierung. Ein aus humanem Fäzes isolierter *Lactobacillus casei* (L.c) wurde ebenfalls positiv auf die Fähigkeit IP-10 zu degradieren getestet. Monoassoziierungsexperimente in BALB/c-Mäusen mit L.c bestätigten die *in vivo* Expression von funktionalem prtP-kodierten Lactocepin im Darminhalt des Zäkums. Fütterungsstudien in T-Zell-transferierten $Rag2^{-/-}$ Mäusen, einem Kolitis-Mausmodell, zeigten, dass mit L.c behandelte Mäuse niedrigere IP-10 Spiegel, eine verringerte T-Zell-Infiltration und eine reduzierte Entzündung im Zäkum aufwiesen, als Mäuse, die mit der Lactocepin-defizienten, isogenen Mutante L.c $prtP^{dis}$ gefüttert wurden.

Diese Arbeit beschreibt eine anti-inflammatorische Struktur-Funktions-Beziehung für probiotisches, prtP-kodiertes Lactocepin im Zusammenhang mit CED und bietet somit eine Grundlage für die Entwicklung und Erforschung Lactocepin-basierter Therapeutika in Chemokin-vermittelten Krankheiten.

TABLE OF CONTENT

1 INTRODUCTION

1.1 Inflammatory Bowel Disease

During the last decades, lifestyle changes in the industrialized world were associated with an increasing prevalence of metabolic pathologies such as diabetes mellitus type 2 as well as chronic inflammatory diseases including inflammatory bowel diseases (IBD). In terms of IBD, a recent study revealed Europe to have the highest prevalence rates among industrialized regions worldwide (e.g. up to 505 cases of ulcerative colitis per 100,000 inhabitants in Norway; up to 322 cases of Crohn`s disease per 100,000 inhabitants in Italy), with tendency to rise.[1] Although only few data are available, a rising incidence of IBD in developing countries becomes apparent, too.[1] IBD, comprising the idiopathic pathologies ulcerative colitis (UC) and Crohn´s disease (CD), are immune mediated chronic disorders of the gastrointestinal tract. UC pathology arises in the rectum, spreading continuously in proximal direction without being prominent in the small intestine. In contrast, CD is characterized by a patchy and transmural inflammation involving small and/or large intestine.[2, 3]

1.2 Etiology of IBD

Present hypothesis assume that genetic susceptibility and environmental factors are crucial parameters in the pathogenesis of IBD. A genetically susceptible host reacts with an errant and excessive immune response towards the commensal microbiota.[2] This hypothesis is supported by so far 99 disease susceptibility loci (71 loci associated with CD and 47 loci associated with UC, including overlapping loci) that have been identified in genome-wide association studies in IBD (status as of april 2012).[4, 5] These numbers also show, that a noticeable moiety of susceptibility genes is shared by both diseases, whereas the major part of these loci remains unique for either CD or UC.[6] The susceptibility loci for CD and/or UC range from genes that play a role in barrier function (e.g. UC-associated gene locus HNF4A)[7], autophagy (e.g CD-associated gene locus ATG16L1)[6, 8] to the innate immune

response (e.g. CD-associated gene locus NOD2)[9, 10] and the adaptive immunity (UC- and CD-associated gene locus IL-23R).[6, 11] Very few of the susceptibility genes can be associated with a distinct disease phenotype. For example mutations in NOD2 are linked to a severe stricturing/penetrating ileal disease phenotype in CD.[12] However, twin studies in monozygotic twins showed that the genetic setup accounts for only approx. 35% of heredity in CD and approx.15% of heredity in UC. This clearly shows that disease etiology cannot soley be credited to genetic predisposition but that environmental factors, especially the intestinal microbiota, play a major role.[13, 14] T-bet$^{-/-}$ × RAG2$^{-/-}$ deficient mice develop spontaneous colitis resulting from a pro-inflammatory response to the commensal microbiota that is driven by dendritic cells (DC) and TNF.[15] Interestingly, treatment with antibiotics ameliorated colitis in T-bet$^{-/-}$ × RAG2$^{-/-}$ deficient mice and wildtype (wt) mice developed colitis upon transfer of intestinal flora from T-bet$^{-/-}$ × RAG2$^{-/-}$, both indicating that intestinal inflammation driven by dysregulated innate immunity can select a bacterial flora with pro-inflammatory potential.[16, 17] Experimental studies using gnotobiotic IBD models supported the hypothesis that both, genetic susceptibility as well as environmental microbial factors trigger the loss of intestinal homeostasis. Intestinal epithelial cells (IEC) of non-inflamed germfree IL-10$^{-/-}$ mice showed no activation, whereas after mono-association with *Enterococcus feacalis* the mice exhibited inflammation and showed activation of IEC. In contrast, wt mice show only transient IEC activation when mono-associated with *Enterococcus feacalis*.[18] The excessive IEC activation leads to an increased cytokine and chemokine secretion in order to recruit and activate immune effector cells and contributes to the development of a IBD-like pathology.[19-23]

1.3 Composition and function of the mucosal immune system

The intestinal mucosa harbors specialized immune compartments that orchestrate tolerance towards the commensal or symbiotic microbiota in order to perpetuate intestinal homeostasis. In contrast, presence or translocation of pathogens evokes the host`s defense mechanisms. First line of defense in antagonizing pathogens is the mucus layer as a biochemical barrier, where one can find viscous mucus, antimicrobial defensins and

neutralizing secretory IgA (sIgA).[24] Adjacent to the mucus layer follows the epithelial barrier which controls intestinal permeability via the expression of tight and adherence junctions, thus separating the “inner” organism from the “outer” intestinal microbiota. For example in IBD patients an upregulation of pore-forming claudin-2 has been observed, suggesting increase of barrier permeability.[25] In addition, this single cell layer that consists of several different cell types was found to play an important role in immune regulation. Most IEC are enterocytes that are not only responsible for nutrient transport but also for the polymeric Ig receptor-mediated secretion of plasma cell derived sIgA into the lumen. The sIgA neutralizes bacterial toxins and prevents antigens from entering the mucosa.[26] Enterochromaffine cells secrete neurotransmitters, such as gut motility affecting serotonin, or hormones, such as pancreatic and biliary bicarbonate secretion stimulating secretin. Paneth cells are located in the crypts and secrete antimicrobial defensins in order to exclude pathogens that would perturb proliferating IEC and stem cells.[27] In IBD patients, microbial clearance is disturbed. The CD susceptibility gene NOD2/CARD15 is strongly associated with reduced secretion of human defensin 5 by ileal Paneth cells.[28] The major cell type responsible for mucus production is the goblet cell. Secreted mucins are connected via glycosylation and sulfate bonds and form a viscous layer in order to minimize direct contact of bacteria with the intestinal epithelium. In the context of IBD, inflamed tissue of UC patients was shown to be characterized by a decrease of goblet cells and impaired mucin production compared to controls.[29] IEC are able to sense bacteria by the expression of toll like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD) molecules (see 1.8)[30] resulting in stimuli-dependent downstream signaling that involves pathways such as NFKB or mitogen activated protein kinases (MAPK). In the case of a strong activating signal, IEC respond via the secretion of chemokines (e.g. IL-8, IP-10) and cytokines (e.g IL-6, IL-10, TGFβ, IFN, TNF). In the case of commensals, IEC exhibit only transient activation that is ceased by negative regulators of pro-inflammatory signaling, such as A20 or Toll-interactin protein in order to maintain homeostasis.[31]

Intestinal epithelial M cells are located in the follicle-associated epithelium (FAE) of the Peyers`s patches (PP). M cells take up luminal antigens and transport them via transcytosis to the antigen presenting cells (APC) such as dendritic cells (DC), macrophages and T cells. PP are part of the gut-associated lymphoid tissue (GALT) and are mainly located in the distal ileum. They harbor DC, macrophages, T cells as well as B cell follicles and are prone to induce tolerance towards antigens.[24] The GALT plays a key role in distinguishing food or microbiota-derived harmless antigens from dangerous pathogens. Antigens are collected in the inductive sites of the GALT, such as PP or isolated epithelial lymphoid follicles and APC initiate the differentiation of T cells into effector T cell subsets (T helper (Th)1, Th2 and Th17) or a regulatory T cell subset (Treg). Treg express IL-10 and TGFβ and play an important role in oral tolerance, thereby contributing to maintenance of intestinal homoeostasis. A balance between the composition of the commensal bacteria, epithelium-derived antimicrobial molecules, epithelial barrier function and innate and adaptive immune cell functions (e.g. physiological inflammation, dentritic cell function, Treg-mediated oral tolerance) is essential in maintaining homeostasis in a healthy subject.[26] In IBD, genetic susceptibility for defects in immunological development, mucosal barrier and/or impaired regulatory mechanisms contribute in combination with environmental factors to loss of intestinal homeostasis. UC is characterized by a modified Th2 response driven by IL-5[21] and IL-13,[19] whereas CD exhibits a Th1- and Th17-mediated response with elevated levels in IL-12, IL-23, IL-17 and IFNγ.[22] To study IBD-like inflammation, different mouse models are used that are either characterized by excessive pro-inflammatory responses (type 1) or by defective regulatory mechanisms (type 2). A type 1 mouse model to study CD-like transmural ileitis is the $TNF^{\Delta are/+}$ mouse model. This mouse model was generated by a deletion in the adenosine-rich element (ARE) in the untranslated regulatory region of the TNF gene resulting in increased stability of TNF mRNA.[32] The $IL\text{-}10^{-/-}$ mouse model is a type 2 model based on the absence of the regulatory cytokine IL-10 and results in a Th1-mediated colitis. Another well established type 2 model is the adoptive transfer model in which colitis is induced by transfer of naïve T cells from e.g. $IL\text{-}10^{-/-}$ mice into recipient mice that lack B and T cells, e.g. $RAG2^{-/-}$ mice. After the transfer,

these mice develop a Th1-mediated colitis that is characterized by high levels of TNF and IFNγ.[33, 34]

1.4 Chemokines and IBD

Recruitment and activation of immune cells play a pivotal role in the onset and progression of inflammation. Immune cell recruitment is orchestrated by chemokines that are expressed by various immune and non-immune cells in order to chemotactically attract other immune cells by binding the cognate chemokine receptors. So called homeostatic and constitutive chemokines are involved in physiological immune cell maturation and trafficking whereas inflammatory and inducible chemokines are crucial in recruiting effector cells to the site of inflammation. In mouse models, many chemokines and their cognate receptors have been identified to be involved in the development of IBD.[35]

Chronic intestinal inflammation is characterized by an increased expression of pro-inflammatory cytokines.[36] One of these cytokines is IFN-γ, a strong inducer for chemokines that bind to CXCR3. Studies have shown that CXCR3 and its ligands like monokine induced by gamma IFN (Mig/ CXCL9), IFN-γ inducible protein 10 (IP-10/CXCL10) and IFN-γ inducible T cell chemoattractant (I-TAC/CXCL11) are upregulated in IBD patients.[37-40] IP-10, for example, is secreted by immune cells (leukocytes, neutrophils, eosinophils and monocytes) and tissue-related cells (epithelial, endothelial, keratinocytes and stromal cells) in order to attract manifold immune cells, such as T cells (in particular subtype Th1), natural killer cells, DCs and macrophages to the site of inflammation. Mouse experiments revealed that therapy with an anti-IP-10 antibody resulted in reduced colitis mediated by decreased Th1 recruitment to the mesenteric lymph nodes and the colon in piroxicam treated IL-10$^{-/-}$ mice.[41] The treatment of UC patients with MDX1100, an anti-IP-10 antibody, was therefore investigated in a phase II study. The results of that study demonstrated a weak but significant disease reduction due to MDX1100 treatment.[42] In this context, Hörmannsperger and colleagues described that a cell-surface protein of *Lactobacillus paracasei* derived from the

clinically relevant probiotic mixture VSL#3 is able to mediate physiologically relevant selective loss of IP-10 in IEC.

1.5 Intestinal microbiota in health and IBD

The human intestine comprises an estimated total of 10^{14} microbes. The amount of microbes ranges from about 10^2 colony forming units/gram gut content (g^{-1}) microbes in the stomach, over 10^7-10^9 g^{-1} in the terminal ileum to 10^{11}-10^{12} g^{-1} in the large intestine.[43] More than 90% of the intestinal microbes belong to the two phyla Bacteroidetes, representing the majority of gram-negative bacteria, and Firmicutes, representing the majority of gram-positive bacteria.[44] Proteobacteria (including the genera *Escherichia* and *Helicobacter*) and Actinobacteria as well as viruses, protists, and fungi fill up the remaining 10%.[44, 45] The proceedings in the microbial analysis by means of culture-independent microbiome analysis (16S rRNA) and metagenomic approaches have already delivered new insights into the human microbiome. A recent study divided the human microbiome into three functionally distinct enterotypes, either dominated by the genera Bacteroides, Prevotella or Ruminococcus, that were found to be independent of the geographical origin of the analysed cohorts.[46] Furthermore, the intestinal microbiota of IBD patients differs from the one of healthy individuals in terms of bacterial species abundance and diversity.[47-49] For instance, the intestinal microbiota of CD patients often lacks the species *Feacalibacterium prausnitzii*, which was reported to exhibit anti-inflammatory properties.[50, 51] A recent twin study by Lepage *et al.* gave insight into the dysbiotic microbiota of UC patients, which was characterized by a reduced bacterial diversity.[52] They observed two UC-related microbiota clusters, the one rather rich in the phylum Actinobacteria, mostly *Rodococcus erythropolis* (low Bacteroides and low Prevotella) and the other showed high abundance of the phylum Proteobacteria, mainly represented by the genera Escherichia/Shigella. The species *Rodococcus erythropolis* as well as the genera Escherichia/Shigella belong to potentially pathogenic genera. Potentially preventive genera, which were low abundant in the microbiota of UC patients when compared to their healthy sibling were phylotypes from the

Rumincoccaceae and Lachnospraceae family. Transcriptional profiles of the mucosa were correlated with the colonic microbiota and revealed a reduced correlation in healthy siblings of a discordant UC twin pair compared to healthy individuals, and an even more reduced correlation in the UC sibling. Most interestingly, in terms of microbial diversity and microbe-host crosstalk, the study observed a gradual shift from healthy, unrelated individuals over healthy, but genetically susceptible siblings of UC discordant twin pairs to diseased siblings of twins.[52] Whether an altered intestinal microbiota is cause or consequence of IBD, or even both, is not yet completely revealed. However, the study of Lepage *et al.* gives strong evidence, that a dysbiotic microbiota may be partially causative in the pathogenesis of UC.

The predominant composition of ingested food seems to involve establishment of a distinct enterotype. Long-term food intake, rich in protein and fat was associated with an enterotype characterized by *Bacteroides* whereas long-term food intake rich in carbohydrates was associated with an enterotype characterized by *Prevotella*. However, short-term changes (10 days) in food intake lead to rapid but transient changes of the microbiota that did not have long-term effects on the enterotype.[46, 53] These studies allow us to differentiate between transient microbes that arise from the environment as well as stable microbes that determine the enterotype and suggest differences in the host interactions between these two populations.[54] Furthermore, these findings at least do not exclude the hypothesis of external manipulation of the microbial ecosystem as a supposed mode of action for probiotics.

1.6 Probiotics in health and disease

According to the WHO probiotics were defined in 2001 as “live microorganisms which when administered in adequate amounts confer a health benefit on the host/consumer”. But speculations about beneficial effects of certain bacteria have been observed decades earlier. Starting at beginning of the 20th century when Elie Metchnikoff stated, that intake of yoghurt bacteria supports the longevity of Bulgarian population. Later on, Henry Tissier observed different morphological compositions in the gut microbiota of healthy breast-fed babies compared to the one from formula-fed babies that suffered from diarrhea. These differently

shaped bacteria later turned out to be Bifidobacteria.[43, 55] Until today, most probiotics belong to the group of lactic acid bacteria such as Lactobacilli or Bifidobacteria, although also other probiotic microorganisms (*E. coli* Nissle 1917, *Saccharomyces boulardii*) are used.[56]

Probiotic use can be roughly divided into two application areas: they are either used as dietary supplement (sole or in food) for the maintenance of health or they are used for therapeutic purposes. In case of the first application area, proof of effectiveness has often not been accomplished in the past. At least so called “functional food” products now have to prove efficacy in order to get a health claim. In terms of therapeutic purposes, probiotics are supposed to be used in a wide range, but therapeutic approaches are so far merely focused on gastrointestinal diseases, such as antibiotics associated diarrhea, *Clostridium difficile* infection, infectious diarrhea, necrotizing enterocolitis in premature infants, inflammatory bowel syndrome (IBS) and IBD.[57, 58]

Table 1-1: Effect of probiotics in gastrointestinal diseases (adapted from Gareau[57])

Disease	Effect
Crohn`s disease	Little or no benefit[59-62]
Ulcerative Colitis	Induction of remission and increased maintenance of remission[63-68]
Pouchitis	Prolonged remission and reduced onset of pouchitis[69-71]
Inflammatory bowel syndrome	Better quality trials required to confirm benefit on clinical symtoms (abdominal pain, bloating, stool consistency)[72]
***Clostridium difficile* infection**	May prevent infection and recurrence of infection[73]
Antibiotic-associated diarrhea	Prevention of diarrhea and reduction of symptoms[73-76]
Infectious diarrhea	Decreases the severity and duration of diarrhea[76]
Necrotizing enterocolitis	Reduces mortality of preterm neonates[77, 78]

It is supposed that the interplay between the microbial ecosystem and the host also influences extra-intestinal organ systems with effects on the metabolism,[79-83] the respiratory tract, and even the brain. The discovery of these interactions will promote the development of new probiotic research areas. Furthermore, probiotic research needs to emphasis on the

revelation of microbial structure-function relationship in the context of disease pathogenesis on a molecular level.[58]

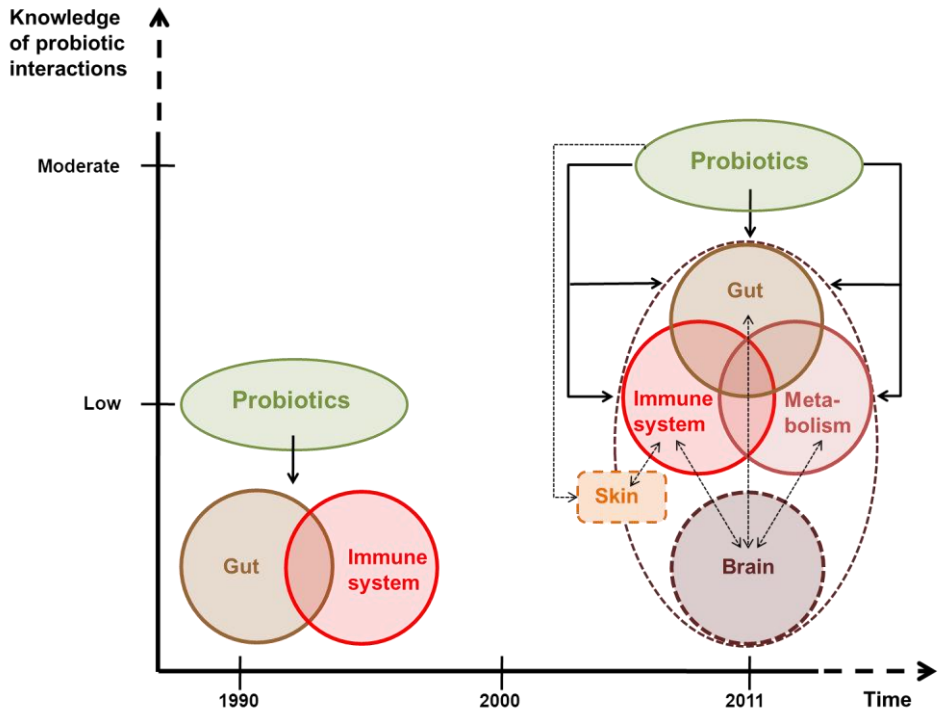

Figure 1-1: Development of probiotic research areas (adapted from von Schillde[58]).

Probiotic research developed from a rather descriptive field of science with low knowledge of probiotic-host interactions to a mechanistically-oriented field of science with moderate knowledge of probiotic interactions. As a consequence to the recent findings that the microbial ecosystem interacts with extraintestinal organ systems, probiotic research may expand to research areas outside the gut. (black dashed lines).

1.7 Probiotics in IBD

In the context of IBD, two probiotic products are mainly used as either sole or supportive medication. These two products are single strain product Mutaflor (containing *E. coli* Nissle 1917) and VSL#3, a formula comprising eight different strains (*Lactobacillus* (L.) *plantarum*, *L. delbrueckii subsp. bulgaricus*, *L. paracasei*, *L. acidophilus*, *Bifidobacterium* (B.) *breve*, *B. longum*, *B. infantis* and *Streptococcus salivarius subsp. thermophilus*). Supporting or replacing conventional therapies clinical studies report effectiveness of specific probiotics, mainly *E. coli* Nissle 1917 and VSL#3, in reducing intestinal inflammation, preventing flares and maintaining remission in cases of pouchitis and ulcerative colitis. (of note: the term pouchitis refers to recurrent inflammation that may occur in IBD-patients that underwent ileal-

pouch-anal anastomosis after colectomy).[63-71] A recent double-blind, randomized, placebo-controlled study included 131 adult patients with mild-moderate relapsing UC under 5-aminosalicylic acid (5-ASA) treatment and/ or immunosuppressive treatment and lasted for 8 weeks. However, patients receiving immunosuppressive treatment were excluded from the study later in order to increase homogeneity in the study group. Tursi et al. reported, that 63.1% in the VSL#3-group ($3.6x10^9$ cfu/day) reached remission compared to 40.8% in the control group.[66] In another recent study, Sood et al. have chosen a longer treatment period (12 weeks). In this double blind, placebo controlled randomized study, 147 patients suffering from mild to moderate ulcerative colitis under either 5-ASA treatment and/ or immunosuppressive treatment were included. Also this study reported that a significant higher proportion of VSL#3-treated patients achieved remission compared to 15,7% in the placebo group.[65] However, this promising study suffers from high drop out rates and inhomogeneity in the VSL#3-treated group compared to the placebo-group regarding concomitant medication (significantly more people in the placebo-group received 5-ASA and immunosuppressive medication).

The usage of probiotics in the context of IBD is discussed controversely. A recent published review states that probiotics may even act more efficiently in the less intense colonized regions, such as the small intestine, as competition to other bacteria is limited to the large intestine.[56] At least in the case of CD, this statement cannot be approved, because several trials showed no or little attenuation after probiotic therapy in CD.[59-62] The reasons for this ineffectiveness are not clear and might be due to the prevalently ileal affection as well as the transmural histopathology of CD compared to UC.

1.8 Probiotic, commensal and pathogen sensing

Although pathogens, commensal or probiotic bacteria can elicit completely different immune responses in the intestine of the host, both use the same signal transduction pathways to communicate with the host. In general, all bacteria-derived effector molecules,

either probiotic-, commensal-, or pathogen-derived, are summarized as so called microorganism-associated molecular pattern (MAMPs). These MAMPs are widespread and conserved among bacteria and mainly located on the cell surface of bacteria. MAMPs get in contact with luminal front line cells (such as IEC and DC) after overcoming the mucus layer in order to interact with highly specific pattern recognition receptors (PRRs).[30] PRRs are evolutionary conserved receptors that sense the presence of either MAMPs or endogenous danger signals, including tissue damage, cellular transformation or metabolic disturbances, and mediate an appropriate innate immune response by the expression of cytokines, chemokines and defensins.[30, 84] The best investigated PRRs in this context were over years the toll like receptors (TLR), which occur as transmembrane proteins either on the cell surface (e.g. on IEC and DC) or in the membrane of intracellular endosomes. TLRs get activated via ligand binding to leucin rich repeat (LRR) domain, resulting in homodimerisation (in case of TLR2 heterodimerisation with TLR1 or TLR6) of TLR-ectodomains. Upon activation, recruitment of adaptor molecules, such as MyD88, TRIF, TIRAP, and TRAM, involves signal transmission that finally ends with the induction or suppression of mediators of innate immunity. There are also other microbial motifs sensing MAMPs, for example the extracellularly occurring C-type lectin receptors (CLRs), the retinoic acid inducible gene I (RIG-I)-like receptors (RLR) and the intracellular nucleotide-binding oligoerization domain-containing protein (NOD)-like receptors (NLRs).[30] The importance of the latter is given by the fact, that the NOD2 related CARD15 gene is a susceptibility locus for CD. In contrast to TLRs, NOD1 and NOD2 are located in the cytosol of IEC and antigen-presenting cells. NOD receptors are activated via binding of peptidoglycan-derived motifs (NOD2: muramyl dipeptide, NOD1: γ-D-glutamyl-meso-diaminopimelic acid) to the LRR domain. This is followed by activation of effector molecule receptor-interacting serine/threonine kinase (RICK) resulting in activation of the NFκB pathway as well as other effector pathways.[85] MAMP-PRR interactions are often not restricted to a single interaction but involve activation of co-receptors. These co-receptors allow differentiated responses to either commensal/probiotic derived MAMPs or pathogen- derived MAMPs (also called pathogen

associated molecular patterns, PAMPs). In addition, different MAMPs can induce different activation intensities via the same PRR. Several studies reported NFκB activation after beneficial probiotic exposure.[86-88] However, this NFκB activation did not result in inflammation. Low grade activation of NFκB signaling seems to be important for the maintenance of IEC homeostasis. Transgenic mice lacking functional IκB kinase-γ, the major IκB kinase essential for NF-κB activation, in IEC spontaneously develop colitis. In addition, NF-κB deficiency led to apoptosis of colonic epithelial cells, impaired expression of antimicrobial peptides and translocation of bacteria into the mucosa. The additional knockout of MyD88 rescued this phenotype, demonstrating involvement of the intestinal microbiota.[89] In addition, the distinction between pathogen and commensal is linked to the ability of pathogens to invade and cause damage to the host cells and tissues. This leads to the presence of so called damage-associated molecular pattern (DAMP) that are also recognized by PRR, inducing local inflammatory responses, recruitment of leukocytes by chemokines and attraction of migrating DC.[90] In summary, different MAMPs are often recognized via the same PRR, but minor structural differences,[91] lack of co-activation, co-presence of DAMPs[90] as well as location of stimulus can lead to completely different host response. For example TLR9 was reported to induce NFκB activation in IEC as response to basolateral, but not to apical stimulation.[92] Another example is the presence of motility associated flagella that are known to mainly interact with TLR5. However, the flagella of commensal *E. coli* was even found to support homeostasis via NOD2-based bacterial clearance, suggesting that whether a bacterial structure may be considered as a pathogenicity factors depends on the context.[93, 94] Probiotic *E. coli* Nissle 1917 was reported to express a flagellin, that induces human ß-defensin 2 in IEC via NFκB and AP-1 dependent pathways.[95] On the other hand, pathogen *Salmonella* Typhimurium (S. Typhimurium) derived flagellin initiates a pro-inflammatory host response by binding to TLR5. The difference between *S.* Typhimurium and *E. coli* dependent activation may lay in the intensity or the location of activation. S. Typhimurium expresses the virulence factor Salmonella Pathogenicity Island-2 that is involved in transcytosis and increases the access of *S.*

Typhimurium derived flagellin to mainly basolaterally expressed TLR5 in IEC.[96-98] In contrast, commensals from the genus *Bacteroides* lack this virulence factor. These three examples of commensal, probiotic and pathogenic origin clearly demonstrate that one microbial motif (flagellin) may excite entirely different responses in the presence or absence of co-factors (e.g. virulence factors) and are intensity dependent.[30]

1.9 Mechanisms of probiotics

From a mechanistic point of view, probiotic research underwent radical change over the past years. Selection of potential new probiotics was often based on merely physico-chemical properties (e.g. resistance to bile acid) and subsequent testing for effectiveness resembled a “try-and-error” procedure/approach. Nowadays, more and more studies focus on elucidating probiotic mechanisms, comprising bacterial effector molecules and target structures of the host. First recent studies identified secreted proteins, glycan ligands, flagella and other structures as probiotic effectors.

Proposed mode of actions of probiotics are often summarized to three main mechanisms.[30] First, competitive inhibition or exclusion of pathogens is one mechanism by which probiotics are supposed to act. This may be performed by probiotics competing with pathogens for nutrients and mucosal adhesion sites as well as by probiotics secreting antimicrobials, supporting clearance of pathogens. *In vitro*, probiotic *Lactobacillus casei* DN-114 00 was found to inhibit the ability of adherent-invasive *Escherichia coli* isolated from CD patients to adhere and invade IEC.[57, 99] As a high occurrence of adherent-invasive *Escherichia coli* has been observed in the ileal mucosa of CD patients, the latter finding suggests that *L. casei* DN-11400 might be used in order to counteract adherent-invasive *Escherichia coli*.[100] Another example, though not related to IBD, is the probiotic strain *Lactobacillus salivarius* UCC118, which was shown to antagonize the foodborne pathogen *Listeria monocytogenes* (*L. monocytogenes*) by production of the bacteriocin Abp118.[101] This finding is a very good example with regard to the importance of knowledge about probiotic mechanisms, showing that bacteriocin Abp118-based probiotic treatments may be highly

efficient in the prevention of infections with *L. monocytogenes* but not in the treatment of an established infection.

The second proposed mechanism for probiotic action is the enhancement of epithelial barrier function.[57] This may be for example achieved by augmenting defensin and/or mucus expression, by reducing IEC apoptosis or by protecting/enhancing IEC tight junctions.[56] The probiotic mixture VSL#3 was shown to prevent a rise in epithelial permeability in murine DSS- colitis by the reduction of epithelial cell apoptosis and increased the maintenance of tight junction protein expression.[102] In addition VSL#3 was shown to mediate an increase of biochemical barrier functions by inducing human β-defensin expression in Caco-2 cells, involving a physiological, low-grade activation of NFκB and AP-1 signaling pathways.[88] However, the bacterial effector molecule/s and the host receptor/target structure that are responsible for the VSL#3 mediated up-regulation of β-defensin in Caco-2 cells are so far not known. In contrast, *E. coli* Nissle 1917 was found to induce human ß-defensin production in Caco-2 cells via its flagellin (see chapter 1.8).[95] With regard to active bacterial structures, Fukuda et al. reported, that high levels of *Bifidobacterium longum subsp. longum* JCM 1217-derived acetate mediate reduced lethality of enterohaemorrhagic *Escherichia coli* O157:H7 infections by preventing apoptosis induced barrier loss and reducing translocation of Shiga toxin 2. However, many bifidobacteria produced acetate, but only distinct strains were able to produce sufficient high levels of acetate to mediate this preventive effect. Using an elegant "omics" approach, they identified an ATP-binding-cassette-type carbohydrate transporter to be only present in the high acetate producing, protective bifiobacteria.[103] The study could identify acetate as bacterial effector molecule, but how acetate reduces translocation of shiga toxin and epithelial apoptosis is not yet known.

The third proposed mechanism of probiotic action is the modulation of the immune system of the host.[56, 57] Kwon *et al.* screened for probiotics that induce Foxp3 expression, high IL-10 and low IL-12 levels *in vitro*. Strains tested positive were composed to a probiotic mixture (including *L. acidophilus*, *L. casei*, *Lactobacillus reuteri*, *Bifidobacterium bifidium and Streptococcus thermophilus*) and adminstered to a murine colitis model (TNBS-induced

colitis). The application of the probiotics led to an enrichment of CD4+Foxp3+ Tregs in the inflamed lesions that mediated reduced progression of the inflammation.[104] However, the bacterial structure-function relationship underlying this probiotic effect is not known. Nevertheless, this study nicely demonstrates an effect-based pre-selection of potentially protective strains, increasing the chance to observe similar effects *in vivo*.

In this context, Yan *et al.* recently proposed delivery of probiotic bacteria-derived soluble proteins as new probiotic-related mechanism, enabling usage also in immunocompromised patients.[105] Probiotic LGG-derived, soluble protein p40 reduces dextran sulphate sodium (DSS)- and oxazolone-induced colitis via epidermal growth factor receptor (EGFR)-activation, resulting in decreased intestinal apoptosis and increased epithelial barrier functions. However, with regard to the clinical relevance of p40 in the treatment of IBD, a trial in children suffering from CD demonstrated, that the administration of LGG in addition to standard medication did not prolong length of remission compared to the placebo group.[106] This discrepancy clearly shows, that due to various reasons such as species differences and the highly complex and highly different disease pathogenesis, results deriving from experimental studies cannot necessarily be transferred to the clinical situation. However, clinical use of probiotics in the context of CD showed dissatisfying results anyway, suggesting that the so far investigated probiotics do either not modulate CD-relevant mechanisms or do not reach the respective intestinal site in sufficient amounts. Administration of LGG in UC patients after ileal pouch-anal anastomosis in order to prevent occurrence of pouchitis resulted in a significantly delayed onset of pouchitis after surgery,[107] whereas another trial, analyzing effectiveness of LGG in treating pouchitis showed no effect of the probiotic.[108] In summary, although LGG showed effectiveness in the treatment of infectious diarrhea[109] and antibiotic-associated diarrhea,[73, 109, 110] the probiotic strain failed to convincingly demonstrate clinical evidence in IBD. Therefore further testing of sole and concentrated LGG-derived p40 in the context of IBD may be of great interest.

Although it has been already shown that probiotic mixture VSL#3 acts as live bacteria, bacterial surface structures[111] and bacterial DNA[112], the molecular mechanisms underlying

the protective effects are not yet fully elucidated. However, Pagnini et al. showed that VSL#3 mediated prevention of ileitis in the SAMP1/YitFc murine model was associated with elevated TNF levels, suggesting protective effects via local stimulation of an innate immune response in the epithelium and again demonstrating that the induction of controlled pro-inflammatory mechanisms might result in reduced inflammation in the end.[86] A very recent publication indicates that conjugated linoleic acid, produced by VSL#3 decreases DSS-induced colitis in mice in a macrophage-derived PPAR-γ dependent mechanism.[113] Hörmannsperger G. proposed prtP-encoded lactocepin (see 1.10) expressed by VSL#3 derived *Lactobacillus paracasei* (L.p) to mediate anti-inflammatory effects by the reduction of IP-10 levels in IEC.[114]

Finally, one has to bear in mind that all described suggested mechanisms are strain and case specific. A strain exerting protective effects in the context of a certain disease may be completely ineffective in another disease and *vice versa*. Comprehension of probiotic structure-function relationships is essential for selecting adequate probiotic strains dependent on the respective disease. Therefore, on the one hand bacterial effector molecules and on the other hand the targeted host cells/receptors have to be elucidated. *In vitro* studies should be used for screening and mechanistic elucidation whereas final evalution of probiotics must be performed *in vivo*. These studies need to be randomized and placebo controlled clinical studies, using disease relevant surrogate marker or end points. In addition, these high quality studies need to analyse an adequately sized and homogeneous (e.g. in terms of additional medication) study population.[115]

1.10 PrtP-encoded lactocepin as potential probiotic structure

A previous study suggested that prtP-encoded lactocepin from L.p mediates anti-inflammatory effects in VSL#3-treated IL-$10^{-/-}$ mice.[114] Procaryotic and eucaryotic serine proteases, having all in common a catalytic triade characterized by the amino acid serine, are involved in many processes including coagulation, inflammation and digestion. Their expression and activity is tightly regulated, e.g by serine protease inhibitors, so called serpins.[116] Serine proteases are divided into different families. One of them is the family S8,

the so called subtilisin-like proteases. It consists of subtilisin and its homologous proteases and are characterized by their catalytic triad in the order Asp, His and Ser in the sequence, which differs from other families such as family S1, the so called chymotrypsin-like proteases (e.g. trypsin).[117] One group of subtilisin-like proteases comprise the lactocepins, a group of cell-envelope proteases (CEP) that are mainly expressed by lactic adicd bacteria including lactococci, lactobacilli and streptococci, and represent their major proteolytic system. Physiologically, lactocepins play an important role in the acquisition of amino acids in lactic acid bacteria. In the case of casein, lactocepins show a high preference for hydrophobic caseins and degrade them into oligopeptide varying from 4-30 amino acids. These casein fragments are then processed and taken up by cell-wall associated transporters and further processed by intracellular peptidases.[118] So far five different types of lactocepin have been mainly characterized, including prtP-encoded lactocepin from *Lactococcus lactis*, *L. plantarum, L. casei* and *L. paracasei*[118-120], prtB-encoded lactocepin from *L. delbrueckii subsp. bulgaricus*[121], prtH-encoded lactocepin from *L. helveticus*[122], prtR-encoded lactocepin from *L. rhamnosus*[123, 124], and prtS-encoded lactocepin from *Streptococcus thermophilus* (Figure 1-2).[125]

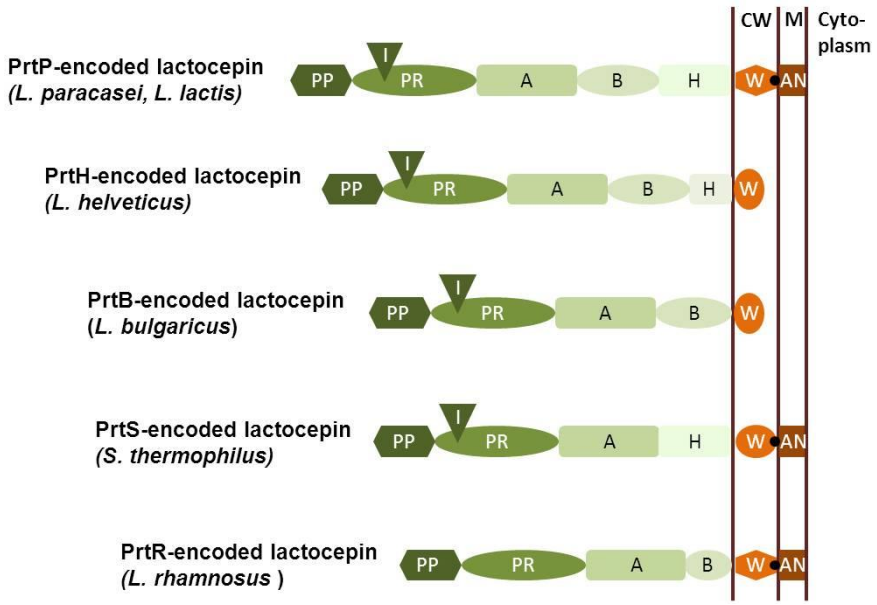

Figure 1-2: Functional domains of lactocepins of different lactic acid bacteria (model according to[126] and adapted from[118]).

"CW, Cell wall; M, membrane; C, Cytoplasm; PPD, preprodomain, required for secretion and is removed via autocatalytic processing; PR, catalytic domain; I, insert domain; A, A domain; B, B domain; H, helix domain; W, cell wall spacer domain; black dot represents sorting signal and AN, anchor domain."[118] Lactocepins are expressed as pre-pro-proteins with a size of approximately 2000 amino acids. The N-terminal preprodomain comprises signal sequence that is required for secretion and the prosequence that is eliminated via autocatalytic processing in order to activate the enzyme. The catalytic domain is a subtilisin-like serine protease domain and includes an internal insert domain that is supposed to account for substrate specificity. The A-domain forms a β-strand structure and is supposed to be involved in regulation of activity and specificty. The B-domain, exhibiting β-sheet secondary structure, may be involved in the stabilisation modulation of substrate specificity and activity of lactocepin. Helix domain may function as a long single-helix spacer between cell surface and A-/B-domain. The W-domain is a hydrophilic domain that exhibits cell wall spacer functions; The C-terminal cell wall anchor domain comprises a hydrophobic putative membrane-spanning α-helix, a charged tail and a sorting signal (LPXTG; only prtR: MPQAG) that is cleaved after translocation at the threonine residue to allow covalent linkage to cell surface peptidoglycan.[126]

Among them, the prtP-encoded lactocepin from *Lactococcus lactis* is the best studied. Regarding the specificity, prtP-encoded lactocepins from lactococci mainly target hydrophobic caseins. In consequence, a lot of research has been performed in order to clarify the activity and highly complex substrate specificity of different prtP-encoded lactocepins. It is known that although prtP-encoded lactocepins show high sequence homology, some of them need a prtM encoded lipoprotein for maturation whereas others (e.g. *L. helveticus, L. delbrueckii* spp. *bulgaricus*- and *S. thermophilus*-derived lactocepins) do not.[121, 125-128] In addition, some strains express exclusively the cell-wall anchored isoform whereas others shed high amounts of differentially cleaved or autoproteolytically processed

isoforms of the protease. Lactococcal derived prtP-encoded lactocepins are divided into PI-type and PIII-type proteases according to their substrate specificity for the different group of caseins (α_{s1}-, α_{s2}-, β– and κ-casein). Furthermore, although prtP proteases are known to be rather unspecific with regard to cleavage sites, prtP-encoded lactocepins from lactococci are classified into seven groups (a-g) according to their cleavage site preferences towards alpha$_{s1}$-casein [118, 129]. From a food technological point of view, lactocepin-mediated cleavage of casein plays an important role in the ripening process of cheese. The different oligopeptides resulting from the degradation of lactocepin are flavor contributing compounds and vary in their taste. Especially proline-dominated peptides cause obejectionable bitter taste. Thus casein-specificity of lactocepin plays a pivotal role in dairy industry.[130] Apart from cheese production lactic acid bacteria are widely used in yoghurt as well in probiotic food and drugs. In this context, expression of lactocepin was rarely associated with beneficial effects of probiotic. Merely the production of the casein-fragment Ile-Pro-Pro after lactocepin hydrolysis has been reported. This tripeptide has been shown to inhibit angiotensin I-converting enzyme and to exert anti-hypertensive effects.[131, 132]

2 AIMS OF THE WORK

IBD pathology is accompanied by various disturbances in cellular immune functions, defective barrier function and alterations in the intestinal microbiota. This altered microbiota, in turn, interacts with likewise dysfunctional epithelial cells of the host.[133, 134] In this context, probiotics are hypothesized being beneficial by either modulating the intestinal microbiota or by directly influencing the host immune response.[57] Although recent studies report progress in identifying probiotic mode of action in terms of limited selected strains, little is known about the molecular mechanisms of the probiotic mixture VSL#3. Previous studies reported that *L. paracasei* from VSL#3 expresses a serine protease that mediates post-translational loss of IP-10 in IEC. The protective probiotic protease was finally identified to be an isotype of the family of prtP-encoded lactocepins, comprising subtilisin-like proteases that have long been known and used in the context of food processing but that have never been investigated with regard to their anti-inflammatory potential. The aim of this thesis was to elucidate the underlying molecular mechanism by which prtP-encoded lactocepin provokes the reduction of IP-10 in IEC and to analyse the physiological relevance of this probiotic effect in $TNF^{\Delta are/+}$ mice, an experimental ileitis model, and in T cell transferred $Rag2^{-/-}$ mice, an adoptive transfer model of colitis.

3 MATERIAL AND METHODS

3.1 Bacterial culture and preparation of conditioned media

L. paracasei VSL#3 (L.p) (classified as *L. casei* until 2009) (a generous gift from Dr. DeSimone, L'Aquila, Italy), *L. paracasei* BL23 (BL23)[135] and *L. paracasei* BL23 prtP^{dis} (L.p BL23 prtP^{dis})(prtP-disruption mutant) (both generous gifts from Dr. Pérez-Martínez, Valencia, Spain), *L. casei* BFLM 218 (L.c) (isolated of human feces; institute-internal classification) and *L. casei* BFLM 218 prtP^{dis} (L.c prtP^{dis}) (prtP-disruption mutant, institute-internal classification) were grown at 37°C in MRS broth (Fluka, Sigma-Aldrich, Taufkirchen, Germany) containing 0.05% L-cysteine (Roth, Karlsruhe, Germany) under anaerobic conditions using anaerogen packages (Anaerogen, Basingstoke, Oxoid, UK). For the growth of the transformed strains *L. c* prtP^{dis} and *L. p* BL23 prtP^{dis} 5 µg/ml erythromycin (Fluka, Sigma-Aldrich, Taufkirchen, Germany) was added to the medium. Fixed bacteria (5% formaldehyde, 3 hours, 4°C) were washed three times with sterile Dulbecco's Phosphate-Buffered Saline (PBS) (Invitrogen, Carlsbad, USA) before use. To generate concentrated conditioned media (CM), bacteria (1x: 5x10^7 cfu/ml) from an overnight culture were transferred to DMEM (1% glutamine, 20 mM HEPES (both Invitrogen, Carlsbad, USA)) and cultivated anerobically overnight at 37°C. Bacteria and bacterial supernatant (CM) were separated after centrifugation (4300 g, 15 min, RT). CM was adjusted to pH 7.4, filter sterilized (0.22 µm), diafiltrated in PBS for buffer exchange and concentrated (200x) using Vivacell filter systems with an exclusion size of 100 kDa (satorius stedim biotech, Goettingen). Inhibition of serine protease activity was achieved by adding phenylmethylsulfonylfluoride (PMSF) (1 mM) to CM L.p. In order to remove free PMSF in case of the intraperitoneal injection experiments, CM L.p and PMSF-inhibited CM L.p were again diafiltrated in PBS buffer. Inhibition of matrix metalloproteases was achieved by adding Marimastat (Marim.) (0.5 mM) (Tocris Biosience, Ellisville, USA) to CM L.p. Agar plates were obtained by adding 1.4% of agar-agar, kobe (Roth, Karlsruhe, Germany) to the above described respective medium.

3.2 Bacterial culture of *Lactococcus lactis* (*L. lactis*) PH and *L. lactis* PHa

Lactococcus lactis (L. lactis) PH (lactocepin negative strain) and *L. lactis* PHa (lactocepin positive strain) (both generous gifts from Dr. Broadbent, Department of Nutrition and Food Sciences, Utah State University) were grown under aerobic conditions at 30°C in M17 broth containing 0,5% lactose (both Oxoid, Basingstoke, UK). In the case of L. lactis PHa, 5 µg/ml chloramphenicol (Sigma-Aldrich, Taufkirchen, Germany) was added to the medium.

3.3 Generation of cell surface extracts (cse)

An overnight culture of L.p (conditions of cultivation → see section 3.1) is centrifuged (4500 g, 15min, RT), the pellet is washed in an equal volume of Ca/Mg-containing PBS and subsequently re-suspended in an equal volume of PBS (desired concentration: $5x10^9$ cfu L.p / ml PBS) without CaMg (Sigma-Aldrich, Taufkirchen, Germany) and incubated for 1 h (1000 rpm at 37°C). Thereafter, the bacterial cell solution was centrifuged (4500g, 15 min) and the supernatant (cse) was retained and stored at -20°C. Co-incubation assay were performed using the indicated dilution.

3.4 Site targeted disruption of prtP ($prtP^{dis}$):

An internal fragment for prtP gene disruption (484bp) (5`-caagcaaaggcggctatctcgcagcaaactaaaggatcatcactcgcaaatacggttaaggccacgactgctaagcaagcggccactgacacaaccgcagcgacaacgaatcaagcgattgccacacagttggcggctaaaggtattgattacaataagctgaataaagttcagcagcaagatacttatgttgacgtcatagttcaaatgagcgcagcgcctgcctctgaaaacggcactttaagaactgattactccagcacggcggagattcagcaggaaaccaataaagtgatcgcggctcaggcaagcgttaaggcagctgttgaacaagtcacccaacaaactgccggtgaaagttatggctatgtcgttaacggcttttcaactaaagttagggtagttgatatccctaaactgaaacaaattgccggagttaaaacagtcacattggcgaaagtttattatccgactgatgctaagg-3`) of prtP from *L. paracasei* BL23 was cloned into the integrative and erythromycin resistance coding vector pRV300 at the pBluescript polylinker (between EcoRI and SacI)[136] using *Escherichia coli* DH5a (grown in M17 medium, 0,5% glucose, 100 mg/ml ampicillin) as cloning host (generous gift from Dr. Vincente Monedero, Instituto de Agroquimica y Tecnologia de

Alimentos, Valencia, Spain). The vector was isolated using the DNA purification system Wizard® Plus, SV MiniPrep (Promega, Madison, USA). Electrocompetent cells were obtained as described by Posno et al., 1991.[137] Briefly, MRS medium containing 1% glycine (AppliChem GmbH, Darmstadt, Germany) was inoculated with L.c and grown until an OD_{600} of 0.4-0.6 at 37°C. The culture is immediately placed on ice to cool down and pelletized (4500 g, 4°C, 10 min). Supernatant is discarded and the pellet washed twice in equal volume with ice cold sterile washing solution comprising 0.3 M sucrose (AppliChem GmbH, Darmstadt, Germany), 5 mM sodium phosphate pH 7.4 (Merck KGaA, Darmstadt, Germany) and 1 mM $MgCl_2$ (added after autoclaving procedure) (J.T. Baker, Avantor Performance Materials, Center Valley, USA). After each washing step the solution is pelletized via centrifugation (4500 g, 4°C, 10 min) and after the last washing step the pellet is resuspended in 1/100 of the initial volume using the above mentioned washing solution and electrocompetent cells stored in aliquots à 50 µl stored at -80°C. For transformation, cells were thawed on ice and up to 10 µg purified vector DNA containing the prtP-disruption construct (volume in at most 5 µl) was added. Subsequently, the cells were electroporated (1250 V). Immediately after electroporation, cells were resuspended in 1 ml of MRS medium containing 0,3 M sucrose, (AppliChem GmbH, Darmstadt, Germany), 2 mM $CaCl_2$ (Merck KGaA, Darmstadt, Germany), 20 mM $MgCl_2$. (J.T. Baker, Avantor Performance Materials, Center Valley, USA) (cave: additives are added after autoclaving procedure), incubated at 37°C for 1h and plated on MRS plates supplemented with 5 µg/µl erythromycin (Fluka, Sigma-Aldrich, Taufkirchen, Germany). Erythromycin resistant colonies were picked and chromosomal DNA was isolated using DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The integration of the vector at the correct locus was verified by PCR on chromosomal DNA isolated from erythromycin-resistant colonies using one primer positioned within the T7 promotor of the construct (T7s: 5`-atacgactcagagacc-3`) and the other primer positioned further downstream outside the inserted construct (1r (=nr. 1156): 5`-agcaactgtggtcgctcctc-3`). Primers for loading control were positioned downstream the disrupting vector, but within the prtP gene (prtP 774seq

(=nr. 1155): 5`-agtgccatatgggtttaattacgc-3` and prtP 1907rev (=nr. 1158): 5`-gcttgtttcaacaatgcttgtga-3`) (all primers purchased from Biomers, Ulm, Germany).[138]

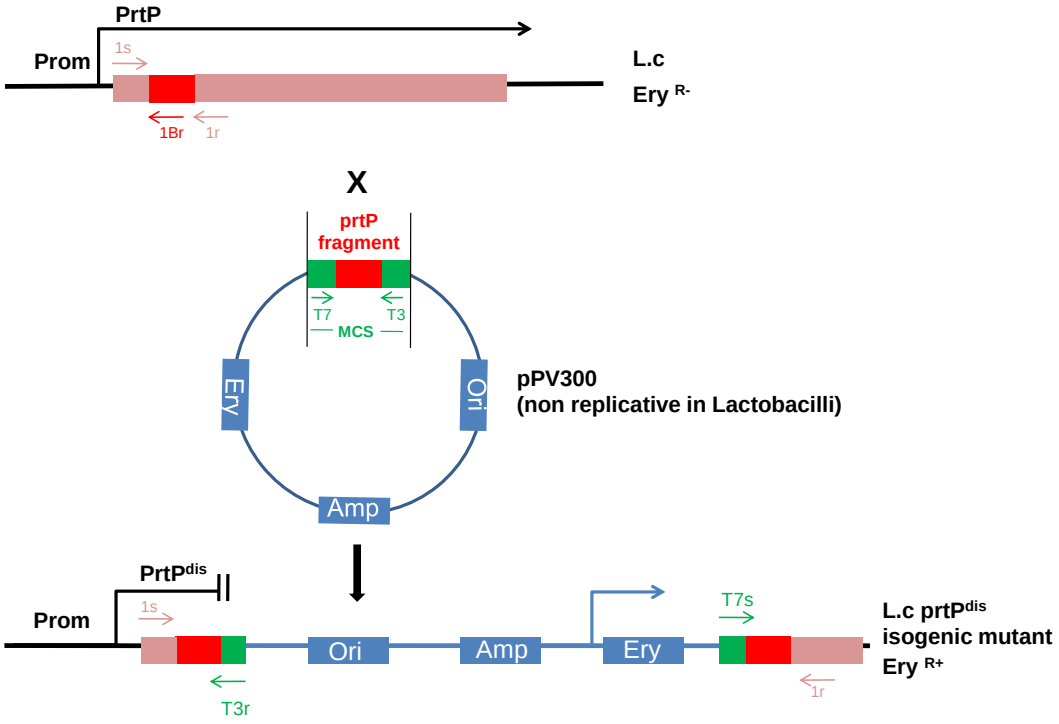

Figure 3-1: Schematic presentation of vector construct and homologous recombination in L.c prtP^{dis}

Prom, promotor; prtP, gene encoding for L.c lactocepin; EryR^{-}, strain sensitive to erythromycin; T7, T7 promotor, T3, T3 promotor, Ori, origin of replication, Amp, ampicillin resistance cassette; Ery, erythromycin resistance cassette; EryR^{+}, strain resistant to erythromycin.

3.5 Cleavage Assay

0.5 µl CM L.p (≈ 0,22 µg) were incubated with 250 ng of the particular recombinant (r) protein for 1h and 24h, respectively. These samples were subsequently analyzed using either MALDI-TOF or SDS-PAGE.[138]

Table 3-1: List of recombinant proteins

M, murine; h, human.

Recombinant protein	**species**	**manufacturer**
IP-10	m	R&D Systems, Wiesbaden-Nordenstadt, Germany
	h	PeproTech GmbH, Hamburg, Germany
MIG	h	PeproTech GmbH, Hamburg, Germany
SDF-1α,	m	PeproTech GmbH, Hamburg, Germany
	h	
I-TAC	m	PeproTech GmbH, Hamburg, Germany
	h	
GROα	m	PeproTech GmbH, Hamburg, Germany
ENA-78	m	PeproTech GmbH, Hamburg, Germany
Fractalkine	h	PeproTech GmbH, Hamburg, Germany
Eotaxin	h	PeproTech GmbH, Hamburg, Germany
GROβ	h	PeproTech GmbH, Hamburg, Germany
MIP-2	m	PeproTech GmbH, Hamburg, Germany
GCP-2	h	PeproTech GmbH, Hamburg, Germany
IL-8	h	PeproTech GmbH, Hamburg, Germany
RANTES	m	PeproTech GmbH, Hamburg, Germany
MIP-3α	h	PeproTech GmbH, Hamburg, Germany
TNF	m	PeproTech GmbH, Hamburg, Germany
	h	
IFNγ	m	PeproTech GmbH, Hamburg, Germany
IL-6	m	PeproTech GmbH, Hamburg, Germany
IL-10	m	PeproTech GmbH, Hamburg, Germany
E-Cadherin	m	Leinco, St. Louis, USA
	h	

3.6 ELISA

Murine IP-10 concentration was either analyzed in recombinant murine IP-10 or in supernatants of ieal explant from wt or $TNF^{\Delta are/+}$ mice, using a murine IP-10 ELISA kit (R&D Systems, Wiesbaden-Nordenstadt, Germany). The ELISA was performed using Nunc MaxiSorp® flat-bottom 96 well plates (Greiner Bio-One GmbH, Frickenhausen, Germany).
TNF concentration was measured in ileal explant derived supernatants from wt mice using the appropriate TNF ELISA kit (R&D Systems, Wiesbaden-Nordenstadt, Germany). All kits were used according to the manufacturer`s instructions.[114, 138]

3.7 Immunoprecipitation of lactocepin.

CM L.p (150x) was incubated with and without anti-lactocepin-antibodies directed against a lactococcal lactocepin (kindly provided by Dr. Harry Laan, Menz Engineering, Netherlands)[139] (3 h, 4°C) in a shaker. Samples were then incubated with protein A/G beads (Santa, Cruz, Europe) (o.n., 4°C) in a shaker. Beads were separated from CM via centrifugation (2 min, 8000 g) and washed four times with 1 ml 1xPBS. The IP-10 degrading capacity of the beads was subsequently determined via an overnight incubation step (37°C) followed by ELISA. The remaining beads were again washed four times with 1ml 1xPBS, resuspended in 20 µl 1xPBS, heated (95°C, 15 min) and the supernatant was subsequently analysed via LC-MS/MS analysis.[138]

3.8 Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

Samples were mixed with NuPAGE LDS buffer (Invitrogen, Carlsbad, USA), reduced and alkylated using 10 mM DTT (Roth, Karlsruhe, Germany) for 10 min at 95°C and 50 mM iodoacetamide (Roth, Karlsruhe, Germany) for 45 min at room temperature. The samples were subjected to a short gel electrophoresis run (5 min) using 4-12% NuPAGE gels, and stained with colloidal Coomassie blue. The in-gel digest was performed according to Shevchenko et al.[140] using trypsin (Promega, Madison, USA). Alternatively, a non-gel-based digestion was performed.[141] LC-MS/MS measurements were performed on an amaZon ETD

mass spectrometer (Bruker Daltonik, Bremen, Germany) coupled to an easy-nLC (Thermo Scientific, Waltham, USA). Peptides were separated on a self packed 0.075x15 cm reversed-phase column (Reprosil, Dr. Maisch, Ammerbuch, Germany) using a 110 minutes linear gradient (0-35 % acetonitrile in 0.1 % formic acid, flow rate 300 nl/min). Intact masses of eluting peptides were determined in enhanced scan mode and the five most intense peaks were selected for further fragmentation by collision-induced dissociation (CID) and acquisition of fragment spectra in ultra scan mode. Singly charged ions as well as ions with unknown charge state were rejected. Dynamic exclusion was enabled and dynamic exclusion duration was set to 10 seconds. Peaklist files were generated with DataAnalysis 4.0 (Bruker Daltonik, Bremen, Germany) and database searches were performed using the Mascot search engine version 2.3.01 (Matrix Science, London, UK) with a tolerance of 0.3 Da for peptide and fragment ions against the firmicutes-subset of the Swiss-Prot database (release 57.15) considering oxidation of methionine (15.99 Da) and carbamidomethylation of cystein residues (57.01 Da) as variable modifications. Search result files were imported into Scaffold 3.0 (Proteome Software, Portland, USA) and all peptide-spectrum matches with a Mascot ion score greater than 30 were accepted.[114, 138]

3.9 Preparation of protein spots and MALDI-TOF-MS

Samples were prepared as described above (see 3.5) and acidified with trifluoroacetic acid (TFA) (final conc. 0.5%) (Roth, Karlsruhe, Germany), before 1 µl of the sample was mixed with an equal volume of sinapinic acid solution (10 mg/ml in 30% acetonitrile, 0.1 % TFA) on a MTP 384 ground steel MALDI target (Bruker Daltonik, Bremen, Germany). Protein Calibration standard I ranging from approx. 4-20 kDa (Bruker Daltonik, Bremen, Germany) was used to calibrate MALDI-TOF MS spectra. MALDI-TOF MS spectra were acquired on an Ultraflex eXtreme MALDI-TOF/TOF mass spectrometer (Bruker Daltonik, Bremen, Germany) equipped with a 1 kHz Smartbeam II laser and controlled via FlexControl software (version 3.3.69). Briefly, a full MS spectrum (m/z 3000 to 20,000) was acquired in positive mode with 10,000 laser shots randomly distributed over the MALDI spot. MS spectra were processed

with FlexAnalysis version 3.3.65 (Bruker Daltonik, Bremen, Germany) using default parameters.[138]

3.10 SDS-PAGE electrophoresis and flamingo staining

Samples were prepared as described above, mixed with 5 µl of 1x Laemmli buffer and subjected to a 10% SDS-gel. After electrophoresis, the proteins were fixed by placing the gel for one hour in a solution comprising 40% ethanol (Roth, Karlsruhe, Germany) and 10 % acetic acid (Fluka, Sigma Alrich, Taufkirchen) and subsequently stained in 1x Flamingo™ fluorescent gel stain (BioRad, Hercules, USA) over night. The protein staining was visualized in the Typhoon Trio+ variable mode imager (GE Healthcare Life Sciences, Chalfon St. Giles, UK).[138]

3.11 Cell culture experiments

The small intestinal epithelial cell line Mode-K (passage 10–25) was grown to confluency in a humidified 5% CO2 atmosphere at 37°C in Cellstar™ tissue culture flasks (Greiner bio-one, Frickenhausen, Germany) using Dulbecco's modified Eagle's medium (DMEM; Gibco Invitrogen, Carlsbad, CA) containing 10% fetal calf serum (FCS) (Biochrom, Berlin, Germany), 1.0% glutamine and 0.8% of an antibiotic antimycotic mixture (Gibco Invitrogen, Carlsbad, CA) as cell culture medium. $2x10^6$ cells were seeded in a 12-well Nunclon™Δ Surface plate (Nunc, Roskilde, Denmark) and grown to 80% confluency prior stimulation. Cells were stimulated with TNF (10ng/ml) (R&D Systems, Wiesbaden-Nordenstadt, Germany) and/or concentrated CM (diluted to 1x) as indicated. Table 3-2 lists all stimulants used in cell culture experiments.[114, 138]

Table 3-2: Reagents used for stimulation in cell culture experiments

Stimulant	Used concentration	manufacturer
TNF	10 ng/ml	R&D Systems, Wiesbaden-Nordenstadt, Germany
CM L.p	diluted to 1x ≈0,6 µg/ml	In-house production
NaCl	1 M	Roth, Karlsruhe, Germany
Heparin Sodium	10 µg/ml	Braun Melsungen AG, Melsungen, Germany
Heparinase	1 U/ml	Sigma Aldrich, Taufkirchen, Germany
IFN-γ	50 ng/ml	R&D Systems, Wiesbaden-Nordenstadt, Germany

3.12 Western Blot analysis

Ileal tissue or Mode-K cells were lysed in 1× Laemmli buffer and 20 µg of protein was applied to 15% SDS polyacrylamide gels and subsequetly subjected to electrophoresis (SDS-PAGE). Respective antibodies except for anti-β-actin, were diluted in Tris-buffered saline tween (TBST)-20 solutution. Anti-β-actin was diluted in 5% skimmed milk (Roth, Karlsruhe, Germany). Diluted antibodies were applied to detect immunoreactive IP-10, TNF, p-p38, p-Akt, p-RelA and β-actin respectively, using an enhanced chemiluminescence light-detecting kit (Amersham, Arlington Heights, Illinois, USA). Table 3-3 and Table 3-4 list antibodies and solutions used for Western Blotting.[138]

Table 3-3: Antibodies used for Western Blotting

1st antibody	dilution	manufacturer	2nd antibody	dilution	manufacturer
Anti-IP-10	1:2000	R&D Systems, Wiesbaden-Nordenstadt, Germany	Anti-goat	1:5000	Dianova GmbH, Hamburg
Anti-TNF	1:1000	R&D Systems, Wiesbaden-Nordenstadt, Germany	Anti-goat	1:5000	Dianova GmbH, Hamburg
Anti-p-p38	1:1000	NEB, Frankfurt, Germnay	Anti-rabbit	1:5000	Dianova GmbH, Hamburg
Anti-pAkt	1:1000	NEB, Frankfurt, Germnay	Anti-rabbit	1:5000	Dianova GmbH, Hamburg
Anti-pRelA	1:1000	NEB, Frankfurt, Germnay	Anti-rabbit	1:5000	Dianova GmbH, Hamburg
Anti-β-actin	1:5000	MP Biomedicals Illkirch, France	Anti-mouse	1:5000	Dianova GmbH, Hamburg

Table 3-4: Solutions used for Western Blotting

Solution	Composition	Manufacturer
1x TBST	2,42, g Tris	Roth, Karlsruhe, Germany
	8 g Sodium Chloride	Roth, Karlsruhe, Germany
	1 ml tween-20	Sigma-Aldrich, Taufkirchen, Germany
	Fill up to 1L with dH_2O	In-house production
1x Laemmli	12,5 ml 1,5M Tris	Roth, Karlsruhe, Germany
	8 ml 10% SDS	Merck, Darmstadt, Germany
	7,9 g 99% glycerol	Roth, Karlsruhe, Germany
	772 mg DDT	Roth, Karlsruhe, Germany
	Bromphenole blue	Roth, Karlsruhe, Germany
	Filled up to 50 ml with dH_2O	In-house production
15% stacking gel	2,5 ml 30% Bis-acrylamide solution	Roth, Karlsruhe, Germany
	1,25 ml 0,5 M Tris-Cl (pH6,8)	Roth, Karlsruhe, Germany
	50 µl 10 % SDS	Merck, Darmstadt, Germany
	25 µl 25% ammoniume persulphate	Roth, Karlsruhe, Germany
	5 µl TEMED	Roth, Karlsruhe, Germany
	1,2 ml dH_2O	In-house production
15% separation gel	2,5 ml 30% Bis-acrylamide solution	Roth, Karlsruhe, Germany
	1,25 ml 1,5 M Tris-Cl (pH8,8)	Roth, Karlsruhe, Germany
	50 µl 10 % SDS	Merck, Darmstadt, Germany
	25 µl 25% ammoniume persulphate	Roth, Karlsruhe, Germany
	5 µl TEMED	Roth, Karlsruhe, Germany
	1,2 ml dH_2O	In-house production

3.13 Measurement of the transepithelial electrical resistance

Ptk6 cells (kindly provided by Robert Whitehead, Vanderbilt University, Nashville, Tennessee) were cultivated as described previously.[142] Briefly, cells were grown at 33°C in RPMI-1640 medium (Invitrogen, Carlsbad, USA) plus 5% FCS (Biochrom, Berlin, Germany), 1 µg/ml Insulin-Transferrin-Selenium A (Invitrogen, Carlsbad, USA), 10 Units/ml murine IFN-γ and 0.8% antibiotic-antimycotic mixture (Invitrogen, Carlsbad, USA). For transwell experiments Ptk6 cells ($3x10^5$ cells/ml) were seeded on 0.4 µm Polyester Transwell filters (Costar Corning, Corning, USA) in cell culture medium at 37°C leaving out the IFN-γ in the medium. Transepithelial electrical resistance (TER) was measured using a Volt-Ohm-meter by Millipore. Apical or basolateral stimulation of the cells took place three days after reaching a stable TER. Translocation was analysed by replacing the medium in the apical compartment with Krebs solution containing 500 µg/ml sodium fluorescein (NaF) (Sigma-

Aldrich, Taufkirchen, Germany) and exchanging the medium in the basolateral compartment for plain Krebs solution. After 1h of incubation under gentle agitation (100 rpm) at 37°C the amount of translocated NaF was subsequently quantified in the basolateral compartment via analysis in a fluorimeter (Thermo Scientific, Waltham, USA).[141]

3.14 Ileal explant culture

0.5 cm sections were taken from the terminal ileum of 14 to 16-week-old wt mice (n=3) and TNF$^{\Delta ARE/+}$ (n=3). The gut was opened and placed with the luminal side to the top on a netwell™ polyester membrane with 500 µm mash size (Corning, New York, USA). The inserts (24 mm diameter) were placed in a six-well plate, each well containing 1.8 ml of culture medium. Lying on the membrane, the tissue piece is incubated in basolateral contact with the respective medium (o.n.).After incubation, each ileal explant was mashed in 100 µL lysis buffer containing 7 M urea, 2 M thiourea, 2% CHAPS, 1% DTT (all from Roth, Karlsruhe, Germany), complete mini and PhosSTOP (Roche Diagnostics, Mannheim, Germany), homogenized by 10 ultrasonic pulses (cycle 0.5; amplitude 80) and centrifuged at 18000 g for 20 min. The supernatant was sampled for ELISA. Total protein concentrations were determined using the Roti-Quant protein assay (Roth, Karlsruhe, Germany).[138]

3.15 Animal trials

3.15.1 Intraperitoneal injection trials in TNF$^{\Delta are/+}$ mice

Wt mice (background: C57BL/6n) and heterozygous TNF$^{\Delta ARE/+}$ mice[32] (background: C57BL/6n (a generous gift from Kollias, G., Institute for Immunology, Biomedical Sciences Research Center “Al. Fleming”, Greece) were conventionally raised on ssniff® R/M-H diet (ssniff, Soest, Germany). Inflamed heterozygous TNF$^{\Delta ARE/+}$ mice (all mice female and eight weeks old) (n=3 per group) got intraperitoneal injections of 200 µl CM L.p or PBS every second day for three weeks. In a second experimental setup, inflamed heterozygous TNF$^{\Delta ARE/+}$ mice (all mice female and nine weeks old) (n=3 per group) received intraperitoneal injections of 200 µl CM L.p or PMSF-inhibited CM every second day for three weeks. All mice

were killed by cervical dislocation and ileal tissue was removed for protein isolation, H&E staining, histopathological evaluation and the determination of immune cell infiltration. The animal experiments were performed as approved by the animal trial application number 55.2-1-54-2531-88-09.[138]

3.15.2 Feeding trial in Rag2$^{-/-}$ mice and T cell transferred Rag 2$^{-/-}$ mice

Lymphocyte-deficient 129 SvEv recombination-activating gene (Rag) 2$^{-/-}$ mice were raised under SPF conditions using ssniff®R/M-H diet. CD4^{+} T cells were obtained using fresh spleens from SPF housed IL-10$^{-/-}$ mice and the CD4^{+} T cell-isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to manufactuer`s instruction.[143] Ten Rag2$^{-/-}$ mice were reconstituted at 10-13 weeks of age by intraperitoneal injection of 5 x 10^5 CD4^{+} T cells suspended in 300µl of PBS (see 3.16). From six weeks until ten weeks after the transfer, control and T cell transferred Rag2$^{-/-}$ mice (n=5 per group) were gavaged with 2x10^{10} cfu L.c or L.c prtP^{dis} three times a week. As a control served age-mapped Rag2$^{-/-}$ mice (n=5 per group) that did not undergo T cell reconstitution, but were gavaged with 2x10^{10} cfu L.c or L.c prtP^{dis} three times a week. At week 10 after the transfer, all Rag2$^{-/-}$ mice were killed by CO_2. The animal experiments were performed as approved by the animal trial application number 55.2-1-54-2531-88-09.[138, 143]

3.15.3 Mono-association trial in germfree BALB/c mice

Germfree female BALB/c-mice (kindly provided by Dr. Tomas Hrncir, Institute of Microbiology, Czech Republic, Department of Immunology and Gnotobiology Academy of Sciences of the Czech Republic) were monoassociated with a single gavage of 1x10^9 cfu L.c or L.c prtP^{dis} (n=5 per group). Ten days after the initial bacterial exposure the mice were killed by cervical dislocation and cecal samples for the determination of luminal and mucosa-attached bacterial numbers as well as the analysis of luminal prtP expression were taken. The experiments were approved by the institutional animal care and use committee at the Academy of Sciences of the Czech Republic (approval number 9890/2009-17210).[138]

3.16 Isolation of mRNA and PCR

Cecal gut content of L.c or L.c prtP^{dis} monoassociated BalbC-mice was resuspended in sterile 1xPBS (w/v=100 mg/ml) (Gibco Invitrogen, Carlsbad, USA) and strongly agitated. Suspension was first purged from nutrient debris (3 min, 350 g) and cecal gut content derived bacteria were obtained by subsequent centrifugation (5 min, 5000 g). Bacterial cell were lysed via incubation with lysozyme (20 mg/ml), mutanolysin (100 units/ml; 200 µl per sample) for 15 minutes at 37°C. Total RNA was isolated using the NucleoSpin RNA kit, including provided DNAse treatment (Macherey-Nagel, Düren, Germany). Concentration and purity (A_{260}/A_{280} ratio) of isolated total RNA was determined by spectrophotometric analysis using a ND-1000 spectrophotometer (NanoDrop Technologies, Willigton, USA). Reverse transcription was performed using 250 ng total RNA and random hexamers (Invitrogen, Carlsbad, USA). The resulting copy DNA (cDNA) was subjected to PCR using primers (Biomers, Ulm, Germany) specific for the prtP gene and spanning the disruption vector of L.c prtP^{dis} (nr. 1153: 5`-cttgttagccggtacagtcgct-3` and nr. 1156: 5`-agcaactgtggtcgctcctc-3`). Primers for loading control were positioned downstream the disrupting vector of L.c prtP^{dis}, but within the prtP gene (nr. 1157: 5`-gctggttgactgggtcacagc-3` and nr. 1160: 5`-gattggtggaactgctgagagtc-3`) (see section 3.4). The PCR products (8 µl) were subjected to electrophoresis on 2% agarose (Roth, Karlsruhe, Germany) gels to visualize the amplicon and to determine amplicon specificity.[138]

3.17 Quantification of total cecal bacteria

Cecal content of mice monoassociated with L.c or L.c prtP^{dis} was resuspended in sterile 1xPBS (w/v=100 mg/ml) (Gibco Invitrogen, Carlsbad, USA) and plated on the appropriate agar plate. Thereby L.c was plated on MRS and L.c prtP^{dis} was plated on MRS with and without 5 µg/ml erythromycin (Fluka, Sigma-Aldrich, Taufkirchen, Germany).[138]

3.18 Cecal water activity

Cecal content of mice monoassociated with L.c or L.c prtP^{dis} was resuspended in sterile 1xPBS (w/v=100 mg/ml) (Gibco Invitrogen, Carlsbad, USA). Cecal water was separated from nutrient debris and bacteria via centrifugation (10 min, 5000 g) and subsequently incubated with IP-10 (o/n., 37°C, dilution 1:500). The remaining IP-10 concentration was determined via ELISA analysis.[138]

3.19 Determination of luminal and mucosa-attached bacteria

Cecal tissue was prepared as described elsewhere.[144] Briefly, segments were washed once in PBS containing 0.016% dithioerythritol and subsequently washed three times in PBS in order to take off the mucus as well as to prevent analysis of luminal bacteria. Cecal tissue was placed in distilled water and vortexed for 30 min for hypotonic lysis. After preparation, bacterial and tissue pellets were obtained by centrifugation (8000 g, 3 min) and quantified by plating on appropriate agar plates (L.c on MRS and L.c prtP^{dis} on MRS with and without 5 µg/ml erythromycin (Fluka, Sigma-Aldrich, Taufkirchen, Germany)).[138]

3.20 Isolation of splenic T cells

CD4$^+$ T cells were isolated from fresh spleens deriving from IL-10$^{-/-}$ 129 SvEv mice housed under specific pathogen-free (SPF) conditions. By means of the magnetic bead-based CD4$^+$ T cell-isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) theCD4$^+$ T cells were isolated according to the manufacturer's instructions. In short, single cell suspension from splenic tissue was gained by mildly squeezing the donor spleens. Via the mechanism of negative selection CD4$^+$ T cells were enriched. This was achieved by using anti-biotin microbeads coated with lineage-specific biotin-conjugated antibodies against non-CD4$^+$ T cells and special columns that are designed for cell depletion (LD columns, Miltenyi Biotec, Bergisch Gladbach, Germany). Unlabeled cells contained in the flow-through were collected for adoptive transfer (see section 3.15.2). The purity of the CD4$^+$ T cells was determined by flow cytometric analysis and was >90%.[143]

3.21 Quantification of immune cell infiltration

The number of infiltrated immune cells in the muscularis of $TNF^{\Delta ARE/+}$ mice was determined via counting all mononuclear cells in the H&E stained muscularis at three representative regions of interest (roi) per mouse (roi= rectangles with a baseline of 100 µm).[138]

3.22 Histopathological scoring

Tissue sections were fixed in 10% neutral buffered formalin (Roth, Karlsruhe, Germany), dehydrated and paraffin embedded. Tissue slides (5 µm) were deparaffinised and stained with hematoxylin and eosin. Histopathological scores were determined by blindly assessing the degree of mononuclear cell infiltration and architectural distortion using a score from 0-12.[138, 145]

3.23 Immunohistofluorescence staining

Standard immunohistofluorescence procedures were performed to stain IP-10 (Anti-IP-10 sc-1406) (Santa Cruz, Heidelberg, Germany), CD3 (Santa Cruz, Heidelberg, Germany), myeloperoxidase (MPO) (Thermo Fisher Scientific, Schwerte, Germany), E-Cadherin (Abcam, Cambridge, UK) and ß-catenin (#9587S) (NEB, Frankfurt, Germany) in paraffin embedded tissue sections. Antigen-unmasking was performed by cooking samples in sodium citrate buffer (10mM, pH 6). Unspecific binding was blocked by incubation with either 5% normal goat serum or 5% bovine serum albumin. Alexa Fluor 488 goat-anti-mouse IgG, Alexa Fluor 546 goat-anti-rabbit IgG or Alexa Fluor 488 rabbit-anti-goat IgG (Invitrogen Carlsbad, USA) or Cy3 donkey-anti-rabbit IgG were used to detect IP-10, ß-catenin, CD3, MPO or E-Cadherin, respectively. Nuclei were stained with DAPI (Invitrogen, Carlsbad, USA). Confocal microscopy was performed using an Olympus BX61 confocal microscope (Olympus, Hamburg, Germany). Quantification of CD3 positive cells, MPO-positive cells and E-Cadherin was performed using Volocity® 3D Image Analysis Software. The intensity of CD3, MPO or E-Cadherin, respectively, was quantified via determining the intensity of the

respective signal at three representative regions of interest (roi) per mouse (roi= rectangle of 40000 μm^2).[138]

3.24 Statistics

Data are expressed as mean values ± standard deviation (SD). Statistical tests were performed using Sigma Plot 11.0 software. Data comparing two groups were analyzed using unpaired t test. Data comparing more than two groups were analyzed with one-way analysis of variance or two-way analysis of variance. If data were not normally distributed or comprised discontinuous data, non-parametrical tests (Mann-Whitney/Rank sum test, ANOVA on ranks) were used. In all computations, differences were considered significant if p-values were < 0.05 (*) or < 0.01 (**).

Each experiment (except animal trials) was performed using technical triplicates and was repeated not less than twice. Data are expressed as mean values ± SD. In terms of animal trials, the group sizes are given in the respective material and method section (see 3.13) as well as in the respective figure caption. Histopathology scores and quantification of immunofluorescent stainings are depicted as box plots (representing median and 25^{th}-75^{th} percentile or median and 10^{th}-90^{th} percentile). The remaining data are expressed as mean values ± SD.

4 RESULTS

4.1 CM L.p derived serine protease lactocepin directly cleaves chemokine IP-10.

Previous studies suggested that CM Lp derived lactocepin mediates loss of IP-10 in IEC.[111, 114] As lactocepin is a serine protease, we hypothesized that this effect is either mediated by the activation of protease-activated receptors or by direct degradation of the chemokine. To test the latter hypothesis, cell-free assays in the absence of intestinal epithelial cells were performed. Indeed, incubation of recombinant murine IP-10 (IP-10) with CM L.p in a cell-free system resulted in reduced IP-10 levels (Figure 4-1 A). To validate the hypothesis that the observed degradation is due to the serine protease lactocepin, CM L.p was pre-incubated with PMSF, a specific serine and cysteine protease inhibitor and Marimastat, a matrix metalloprotease inhbititor, respectively. PMSF-inhibited CM L.p failed to reduce IP-10 levels, whereas Marimastat-pretreated CM L.p was still able to reduce IP-10 levels (Figure 4-1 A). The PMSF-sensitive reduction of IP-10 levels by CM L.p suggests that IP-10 is indeed directly cleaved by CM L.p derived lactocepin. Lactocepin exists in secreted as well as cell surface bound isoforms. Therfore, we extracted cell surface proteins of L.p and assessed the IP-10 degrading capacity of these cell surface extracts (cse). As expected, co-incubation of cse with IP-10 in a cell free system led to reduced levels of IP-10. This effect could be reverted by PMSF inhibiton of cse, further substantiating lactocepin to be the IP-10 cleaving protease (Figure 4-1 B).[138]

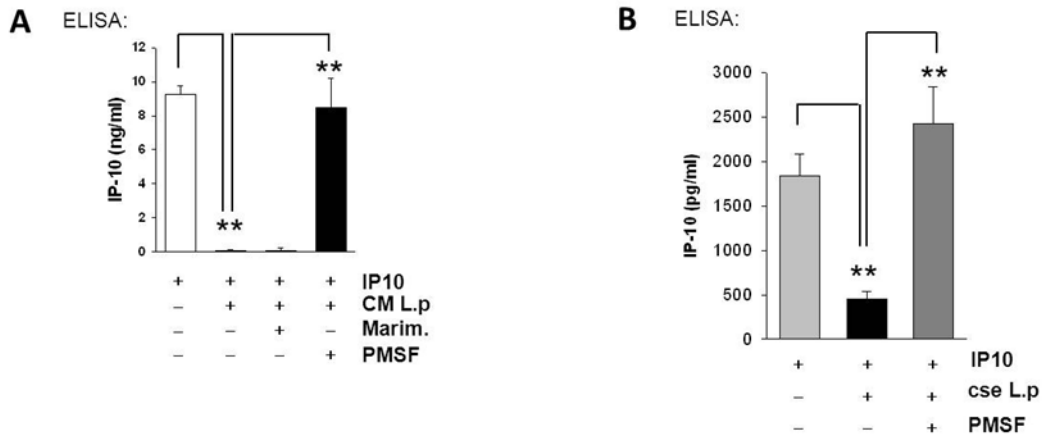

Figure 4-1: CM L.p derived serine protease lactocepin directly cleaves IP-10.

CM L.p, PMSF (1 mM) or Marimastat (Marim.) (0,5 mM)-pretreated CM L.p was co-incubated with recombinant murine IP-10 (IP-10). IP-10 concentrations were analyzed by ELISA (B). Cell surface extracts of L.p were either pre-incubated with PMSF (1 mM) or not and co-incubated with IP-10 over night. IP-10 levels were measured via ELISA (B). (*) $p < 0.05$ or (**) $p < 0.01$.

4.2 *L. casei* BL23 does not express lactocepin in DMEM.

Recent studies have shown, that *L. casei* BL23-derived CM (CM BL23) fails to mediate loss of IP-10 in IEC.[114] In addition, CM BL23 fails to degrade IP-10 in a cell free system (Figure 4-2 A, upper panel). These findings raised the question why BL23 is inactive although it is known to encode lactocepin. However, BL23 showed no expression of lactocepin, neither on RNA level (Figure 4-2 B) nor on protein level (Figure 4-2 A, lower panel; Table 6-2, appendix). LC-MS/MS analysis revealed lactocepin to be the only detectable serine protease in CM L.p (Table 6-1, appendix), again strengthening the assumption that lactocepin is the IP-10 degrading protease in CM L.p. In accordance with the missing expression of lactocepin in BL23, fixed cells of BL23 also failed to cleave IP-10 in comparism to fixed cells of L.p (Figure 4-2 C). In addition, these experiments demonstrate that cell-surface bound lactocepin is able to degrade IP-10.[138]

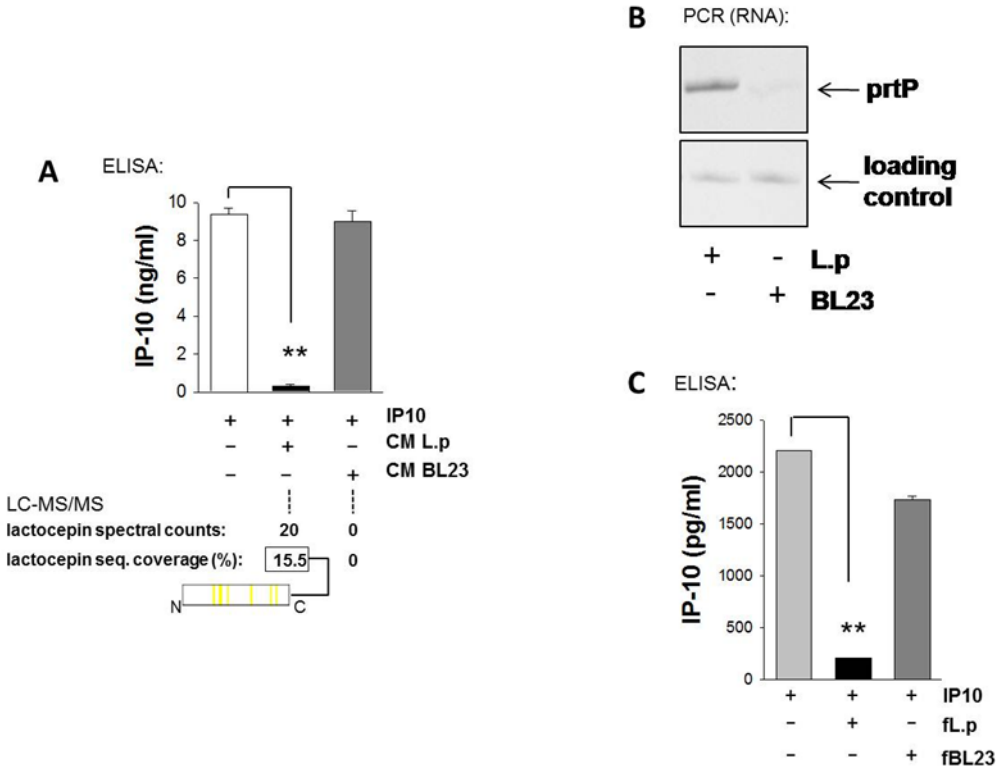

Figure 4-2: *L. casei* BL23 expresses no lactocepin and fails to cleave IP-10.

Murine IP-10 (IP-10) was co-incubated with CM L.p or CM BL23 (24h). IP-10 concentration was subsequently measured by ELISA (A, upper panel). CM L.p and CM BL23 were subjected to LC-MS/MS analysis in order to identify the protein content (Table 1+2). The lower panel of Fig. A indicates the result of the LC-MS/MS analysis with regard to the identification of prtP-encoded lactocepin. Spectral counts display the n° of identified lactocepin peptides. Sequence coverage displays the percentage of the prtP-sequence that is covered by the detected peptides. The localization of identified peptides within the lactocepin protein sequence is presented using yellow and green (modified peptides) marks in the bar chart (C: C-terminus, N: N-terminus) (A). Transcriptional analysis of lactocepin in L.p and BL23 using specific primers of prtP-encoding region (B). Fixed bacteria (fL.p, fBL23) were co-incubated with rmIP-10 and IP-10 levels were measured via ELISA (C). (*) $p < 0.05$ or (**) $p< 0.01$.

4.3 Immunoprecipitation analysis confirms lactocepin as IP-10 cleaving protease.

In order to confirm the identification of lactocpin as the serine protease that is responsible for degradation of IP-10 we performed an immunoprecipitation experiment using anti-lactocepin antibodies.[139] A/G beads were coated with anti-lactocepin antibodies (denoted by immunobeads) and co-incubated with CM L.p. Non-coated A/G beads (denoted by control beads) served as a control (Figure 4-3 A, lower panel). LC-MS/MS analysis confirmed successful immunoprecipitation of lactocepin (Table 4-1). Subsequently, the control beads as well as the lactocepin coated immunobeads were tested for their capacity to degrade IP-10. As expected, lactocepin-coated beads showed a strong IP-10 degrading capacity (Figure 4-3

A, upper panel), which was found to be abrogated by PMSF pre-treatment (Figure 4-3 B). These results clearly identified prtP-encoded lactocepin to be the IP-10 degrading protease in CM L.p.[138]

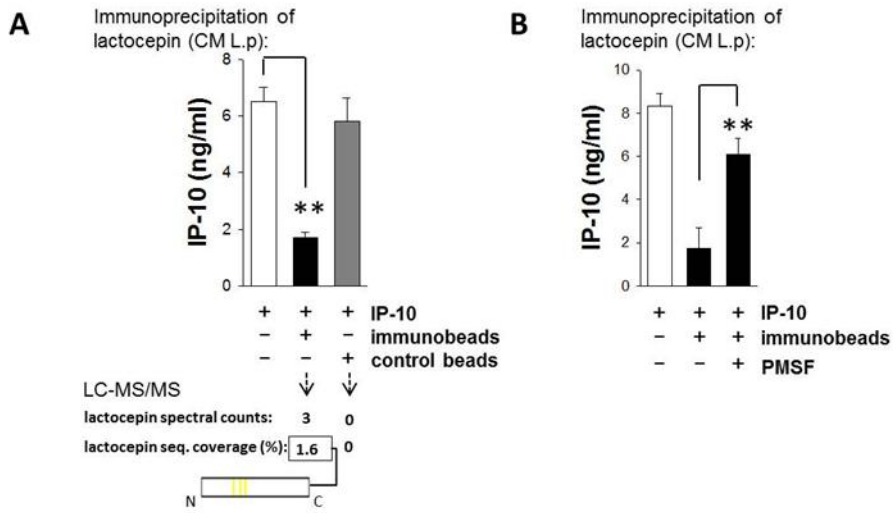

Figure 4-3: Immunoprecipitation analysis confirms lactocepin as IP-10 cleaving protease.

Immunoprecipitation of lactocepin was performed by using an anti-lactocepin antibody and protein A/G beads. IP-10 was incubated with lactocepin-loaded beads or control beads (24h) and IP-10 concentration was subsequently determined via ELISA analysis (A, upper panel). The lactocepin-loaded beads and the control beads were subjected to LC-MS/MS analysis. The lower panel of Fig. A indicates the result of the LC-MS/MS analysis with regard to the identification of prtP-encoded lactocepin. Spectral counts display the number of identified lactocepin peptides. Sequence coverage displays the percentage of the prtP-sequence that is covered by the detected peptides. The localization of identified peptides within the lactocepin protein sequence is depicted by yellow and green (modified peptides) marks in the bar chart (C: C-terminus, N: N-terminus) (A). Immunoprecipitation of lactocepin as above, followed by exposure of the lactocepin-loaded beads to PMSF and co-incubation with mIP-10. Subsequently, IP-10 concentration was determined via ELISA analysis (B). (*) $p < 0.05$ or (**) $p < 0.01$.

Table 4-1: LC-MSMS analysis of the precipitate after immunoprecipitation of lactocepin

+ or - indicates presence or absence of respective protein. Number in brackets indicates the number of identified spectra. n.d., not detectable.

Protein name	Immunobeads	Control beads
ALBU_BOVIN	+ (11)	+ (15)
Protein G' [Streptococcus sp. 'group G']	+ (2)	+ (1)
immunoglobulin G binding protein A [Staphylococcus aureus]	+ (6)	+ (2)
subtilisin-like serine protease [Lactobacillus casei ATCC 334]	+ (3)	- (n.d.)

4.4 Stability and activity of CM L.p and cse L.p

Enzymatic activity of proteases may be affected by heat and pH. CM L.p derived lactocepin maintained limited capacity to degrade IP-10 after 3 h heat treatment (95°C).

However, heat treatment exceeding 3 h resulted in loss of IP-10 degrading capacity (Figure 4-4 A). Stability of CM L.p derived lactocepin towards pH was tested by 1) adjusting CM L.p to desired pH, 2) maintaining this pH for 1h and 3) re-adjusting pH to a physiological level of 7.4 by either adding HCl or NaOH. In a subsequent experiment, CM L.p was co-incubated with IP-10. ELISA analysis demonstrated that CM L.p derived lactocepin remains its activity over a wide pH range. However, when reaching a pH below 3, thereby approaching physiological acidity of the stomach, IP-10 degrading capacity cannot be restored and is strongly decreased (Figure 4-4 B). A comparable loss of activity can be observed when exceeding a pH of 10. Nevertheless, these experiments revealed a high stability of CM L.p against stress factors such as heat and pH.

By default, we generated CM L.p out of $0.5*10^8$ cfu/ml L.p, which we defined as 1x concentration (relates 1:1 dilution). In order to titrate the activity of L.p-derived lactocepin we used a) different concentrations of L.p to generate CM L.p or cse L.p and b) different dilutions of 1x CM L.p/1x cse L.p (as indicated in the Figure 4-4 C). Figure 4-4 C shows that the capacity of CM L.p to degrade IP-10 increases with higher amounts of used bacteria. In addition, the activity of CM L.p and cse L.p correlates negatively with increasing dilution. However, CM L.p shows higher activity in degrading IP-10 than cse L.p, as fewer bacteria are necessary for a complete degradation of IP-10 (Figure 4-4 C).

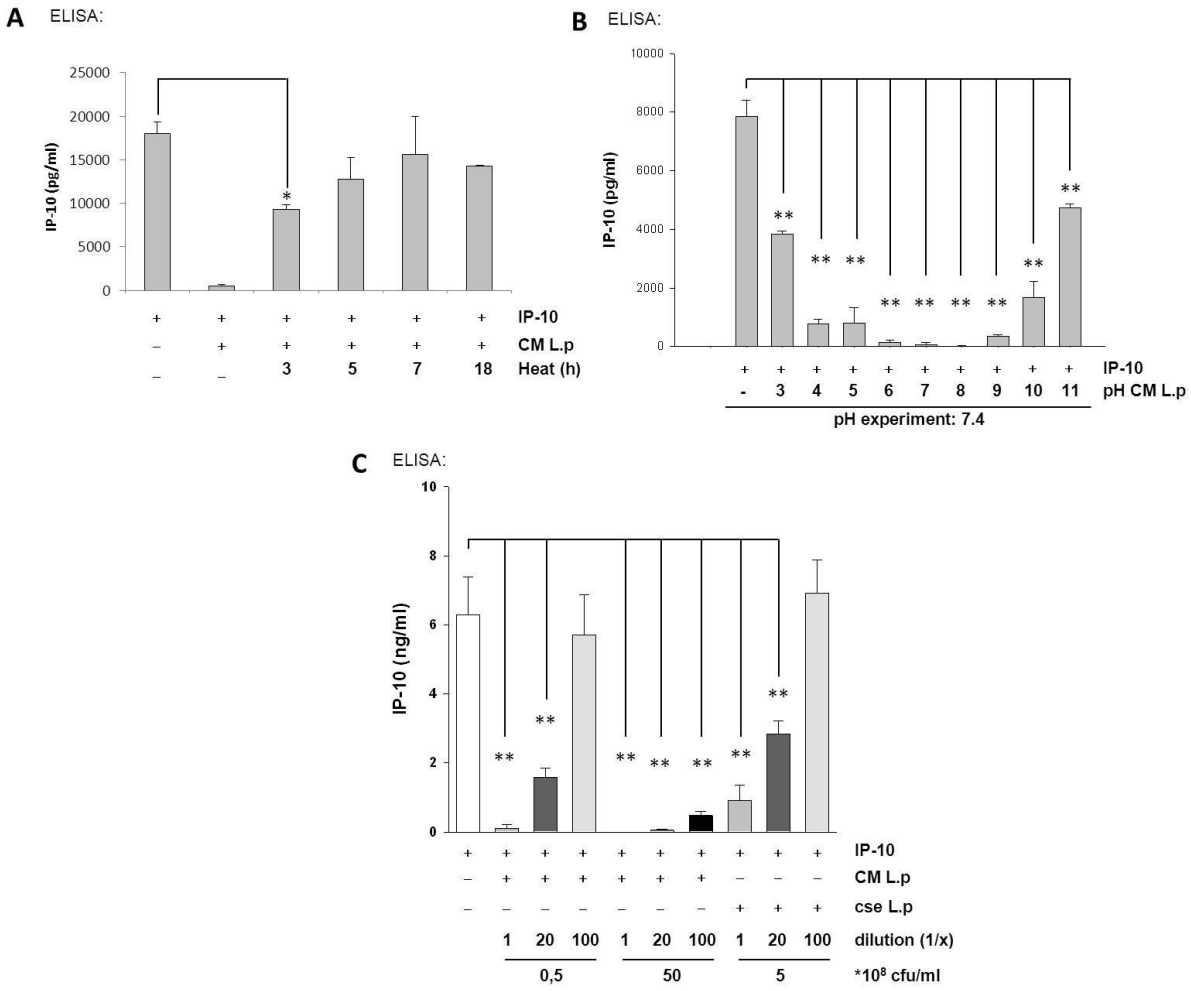

Figure 4-4: Stability and activity titration of lactocepin

CM L.p was heated (95°C) for the indicated length of time (hours, h) and subsequently co-incubated with murine IP-10 (IP-10). IP-10 levels were measured via ELISA (A). CM L.p was adjusted to the indicated pH for 1h, then re-adjusted to a physiological pH of 7.6 and subsequently co-incubated with IP-10. IP-10 levels were measured via ELISA (B). IP-10 was co-incubated with either CM L.p or cell surface extracts (cse) from L.p. Cfu/ml indicates the amount of bacteria used for the generation of the CM L.p or cse L.p., respectively. Dilution refers to the respective dilution of CM L.p or cse L.p used in the assay (C). (*) $p < 0.05$ or (**) $p < 0.01$.

4.5 Cleavage site specificity and substrate specificity of CM L.p derived lactocepin

In order to determine the cleavage site and substrate specificity of lactocepin, we aimed to purify the protease. Unfortunately, attempts to purify lactocepin from CM L.p failed because the chromatographic fractions lacked purity (data not shown). Hence we characterized CM L.p derived lactocepin in more detail using MALDI-TOF analysis. CM L.p derived lactocepin was found to cleave mIP-10 at various sites, resulting in complete degradation of the chemokine (Figure 4-5 A).

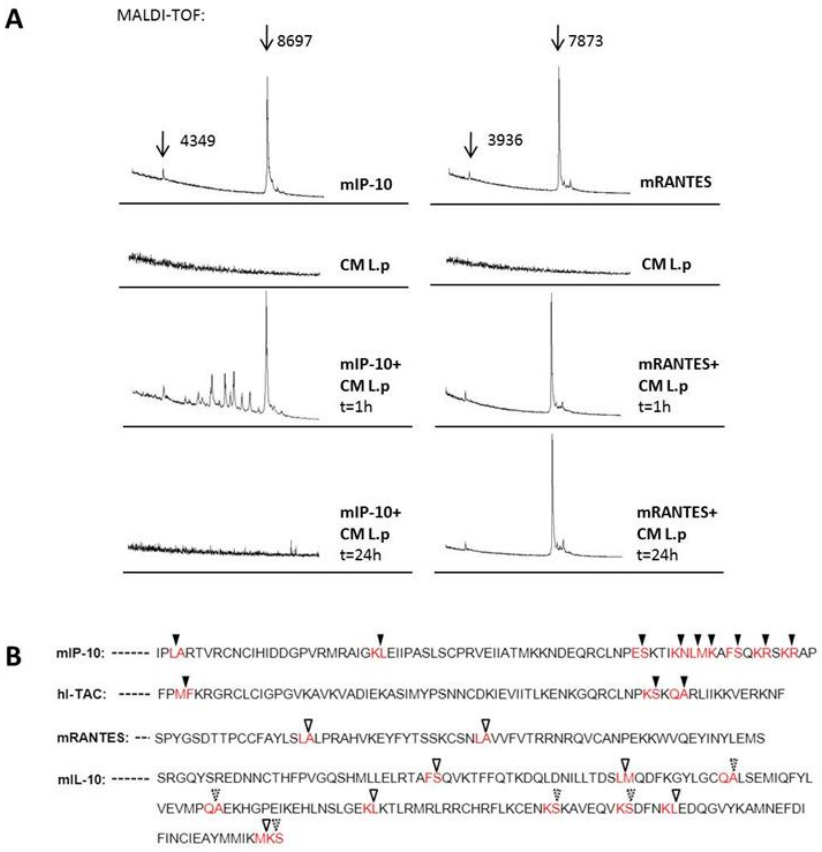

Figure 4-5: Cleavage site analysis of CM L.p derived lactocepin

CM L.p was co-incubated with murine (m) IP-10 or mRANTES (1h, 24h). The relative molecular mass of mIP-10 and mRANTES before and after co-incubation with CM L.p was determined by MALDI-TOF MS (respective mass (Da) is indicated by an arrow) (A). CM L.p was co-incubated with mRANTES, human (h) I-TAC, mIP-10 or mIL-10 for 0.5h, 1h or 24h and subjected to MALDI-TOF-MS. After 24h, the chemokine suspension and the respective chemokine/CM L.p suspension were subjected to SDS gel-electrophoresis and Flamingo staining (Figure C, left panel). Amino acid sequences (one letter code) of mIP-10, hI-TAC, mRANTES and mIL-10 are displayed and all detected cleavage sites of the serine protease in CM L.p are indicated (▼). Sites that were targeted in mIP-10 (▽) or in hI-TAC (▽) but that are not targeted in mRANTES or mIL-10 are indicated. Actual and theoretical cleavage sites are highlighted in red (B).

In addition, SDS-Page analysis confirmed complete degradation (Figure 4-6 A). In contrast to the low specificity regarding cleavage sites, we found that CM L.p derived

lactocepin is highly selective for an array of pro-inflammatory chemokines whereas all tested cytokines as well as the adherens junction protein E-Cadherin (Table 4-2 and Figure 4-6 B) were unaffected. Since the latter proteins are uncleaved although they contain several target cleavage sites of CM L.p derived lactocepin (Figure 4-5 B), we assume that the three-dimensional structure of the respective proteins determines accessibility of cleavage sites and thereby, whether a protein is cleaved or not.[138]

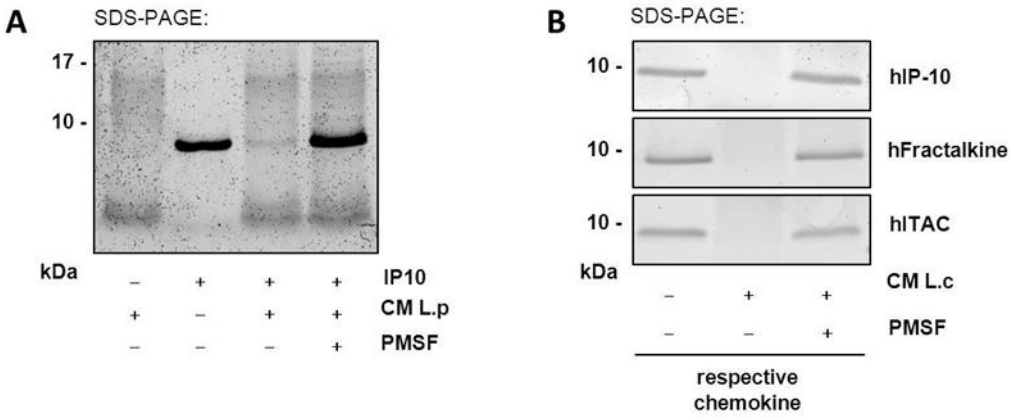

Figure 4-6: L.p lactocepin degrades an array of pro-inflammatory chemokines.

CM L.p or PMSF pre-treated CM L.p were co-incubated with murine (m) IP-10 (IP-10) (A), human (h) IP-10, hFractalkine or hITAC (B) overnight, subjected to SDS-Page analysis and protein was visualized by flamingo dye.

Table 4-2: Substrate specificity of CM L.p

Murine, m; human, h; + indicates degradation of the respective protein; – indicates no degradation of the respective protein. CM L.p was co-incubated with the respective protein (24h) and degradation was analysed by MALDI-TOF or SDS-PAGE/flamingo staining.

protein	species	degradation
MIG/CXCL9	h	+
IP-10 /CXCL10	m/h	+
I-TAC/CXCL11	m/h	+
SDF-1α/CXCL12	m/h	+
Fractalkine/CX3CL1	h	+
Eotaxin/CCL11	h	+
GROβ/CXCL2	h	-
MIP-2/CXCL2	m	-
GCP-2/CXCL6	h	-
IL-8/CXCL8	h	-
RANTES/CCL5	m	-
MIP-3α/CCL20	h	-
TNF	m/h	-
IFNγ	m	-
IL-6	m	-
IL-10	m	-
E-Cadherin	m/h	-

4.6 CM L.p does not impair barrier function

Proteolytic activity of commensal *E. faecalis*-derived gelatinase has recently been associated with impaired intestinal barrier integrity.[146] In order to test the impact of L.p derived lactocepin on barrier integrity we performed incubation experiments in polarized Ptk6 cells, a colonic intestinal epithelial cell line, via assessment of the transepithelial electrical resistance (TER). Consistent with the highly selective substrate spectrum, no negative impact of CM L.p derived lactocepin on the TER of IEC was observed. In addition, CM L.p did not affect translocation of sodium fluorescein (NaF) across the IEC layer. These findings show that CM L.p derived lactocepin does not exert adverse effects on IEC barrier function (Figure 4-7).

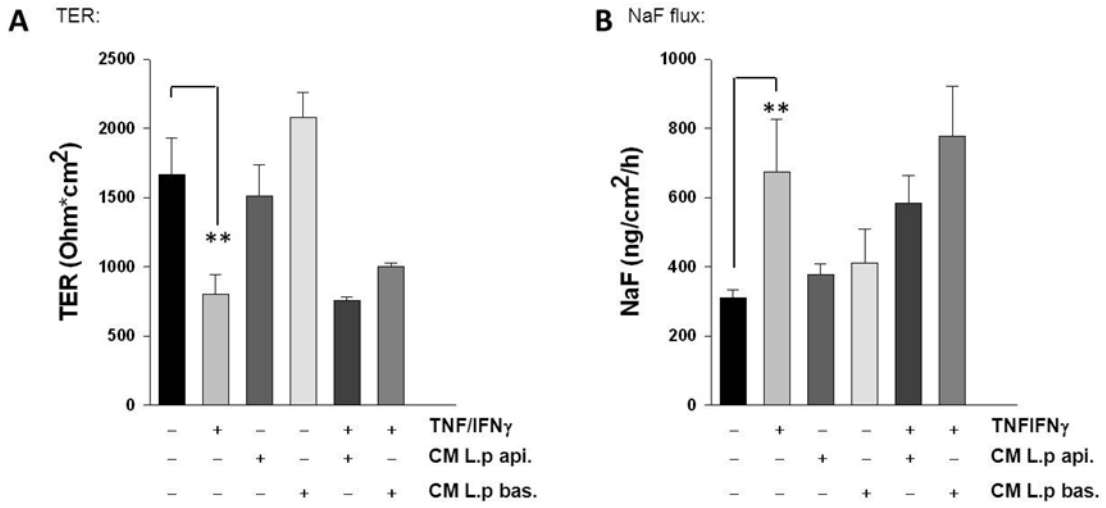

Figure 4-7: Effect of CM L.p on barrier integrity in Ptk6 cells.

Ptk6 cells were stimulated with TNF (20 ng/mL) /IFN (50 ng/mL) in the basolateral compartment. CM L.p was added either to the apical (api) or basolateral (bas) compartment. Barrier function was assessed by measuring the TER (A) and translocation of sodium fluorescein (NaF) (B) after 48h. Values represent triplicates from one of two independent experiments. (*) $p < 0.05$ or (**) $p< 0.01$.

4.7 Accessibility of cell-surface bound IP-10 to proteolytic degradation

Incubation of TNF-activated IEC with L.p or CM L.p results not only in degradation of secreted IP-10 but also in loss of IEC-associated IP-10.[111, 114] These findings suggest that IEC-associated IP-10 is not localized within IECs, as assumed before, but is localized at the cell surface like it has been described for endothelial cells (of note: in endothelial cells IP-10 is mainly bound to heparansulphate proteoglycans on the cell surface).[147, 148] If this hypothesis is valid, addition of heparin to TNF-activated IEC (small intestinal cell line Mode K) would competitively bind IP-10, abrogating binding of IP-10 to heparansulphate proteoglycans. Indeed, a competitive binding assay showed that IEC-association of IP-10 could be antagonized by adding heparin to TNF-activated IEC (Figure 4-8 A).[138] The binding of cationic IP-10 to anionic heparansulphate proteoglycans at neutral pH can be antagonized by addition of NaCl. Therefore we performed an elution assay by washing TNF-activated IEC with NaCl, resulting in reduced levels of IEC-associated IP-10 (Figure 4-8 B). Furthermore, addition of heparinase to TNF-activated Mode K cells led to increased detection of IP-10 in

the supernatant of IEC (Figure 4-8 C). These results suggest that IEC-associated IP-10 detected via Western Blot is mainly located at the epithelial cell surface and not stored within IEC, making it potentially accessible to L.p lactocepin.

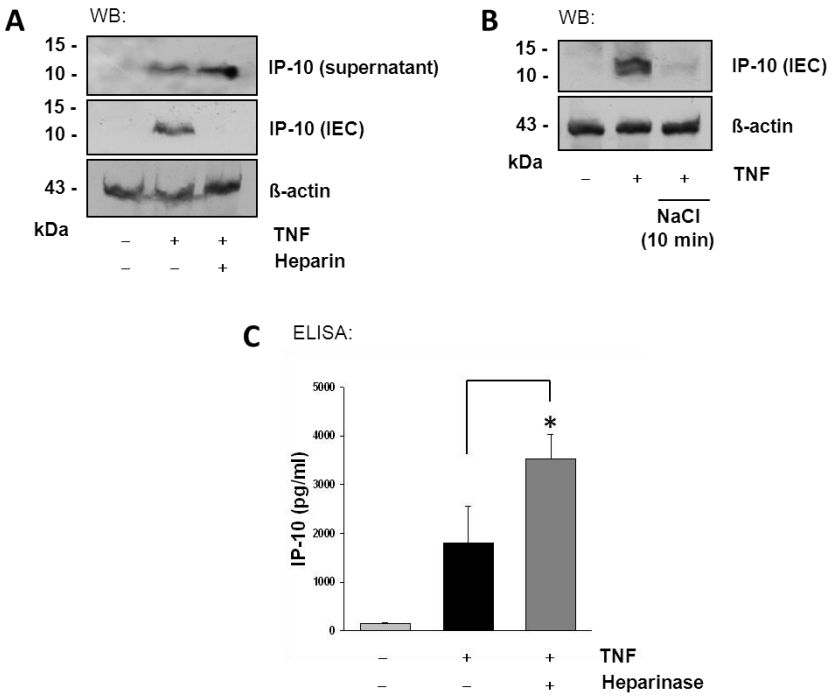

Figure 4-8: IP-10 is bound to the cell surface of intestinal epithelial cells.

TNF-activated Mode K cells (10 ng/ml) were incubated with heparin (10 ng/ml). IP-10 bound to the intestinal epithelial cells (IEC) as wells as IP-10 levels in the concentrated cell supernatants (supernatant) were measured via Western Blot analysis (A). TNF-activated IEC were washed with 1 M sodium chloride (NaCl) and cell-associated IP-10 levels measured via Western Blot analysis (B). TNF-activated Mode K cells were co-incubated with heparinase and IP-10 levels determined via ELISA (C). (*) $p < 0.05$ or (**) $p< 0.01$.

4.8 CM L.p derived lactocepin reduces IP-10 levels in explant cultures

To address the physiological relevance of CM L.p-mediated IP-10 degradation, we performed *ex vivo* tissue culture experiments using ileal specimens from healthy wt mice, inflamed $TNF^{\Delta ARE/+}$ mice (an experimental model for Th1-mediated ileitis)[32] or inflamed $IL\text{-}10^{-/-}$ mice (a colitis model).[149,150]

Activation of the ileal tissue of healthy wt mice was achieved by incubation with IFN-γ, a strong inducer of IP-10 expression that is not degraded by lactocepin (Figure 4-9 A and

Table 4-2). Figure 4-9 B shows that CM L.p is highly potent to degrade IFN-γ-induced secreted and tissue-distributed IP-10 in ileal tissue of wt mice.[138]

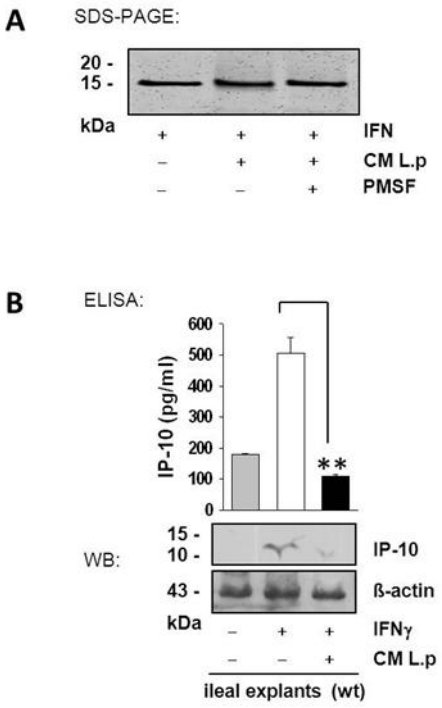

Figure 4-9: CM L.p derived lactocepin effectively reduces secreted and tissue-distributed IP-10 levels in IFNγ-activated intestinal ileal tissue.

CM L.p and PMSF pre-treated CM L.p, respectively, were co-incubated with hIFNγ (24h), then the suspension was subjected to SDS-Page analysis and protein in the gel was visualized via staining with fluorescent flamingo dye (A). Ileal explants from healthy wt mice were stimulated to produce IP-10 with IFNγ (50 ng/ml) in the presence or absence of CM L.p (3x, o.n, 37°C). The amount of secreted IP-10 in the supernatants of the ileal explants was subsequently determined by ELISA analysis and the amount of tissue-distributed IP-10 was determined by WB analysis (B). (*) $p < 0.05$ or (**) $p < 0.01$.

CM L.p was also found to degrade secreted IP-10 from inflamed ileal tissue explants of $TNF^{\Delta ARE/+}$ mice (Figure 4-10 A, upper panel). The loss of IP-10 in the tissue was confirmed to be due to selective degradation by the probiotic protease in CM L.p, since PMSF abrogated this effect (Figure 4-10 A, upper panel). In accordance with the substrate profile of CM L.p derived lactocepin, TNF secretion was unaffected by the probiotic treatment (Figure 4-10 A, lower panel and Table 4-2). Consistent with the tissue explants from wt mice, CM L.p was found to be highly potent in degrading tissue-derived IP-10 in inflamed ileal tissue explants of $TNF^{\Delta ARE/+}$ mice (Figure 4-10 B) and inflamed colonic tissue explants of $IL\text{-}10^{-/-}$ mice (Figure 4-10 C). These explant culture experiments clearly demonstrate the accessibility of tissue-

distributed IP-10 to lactocepin and consequently the physiological relevance of this probiotic protease.[138]

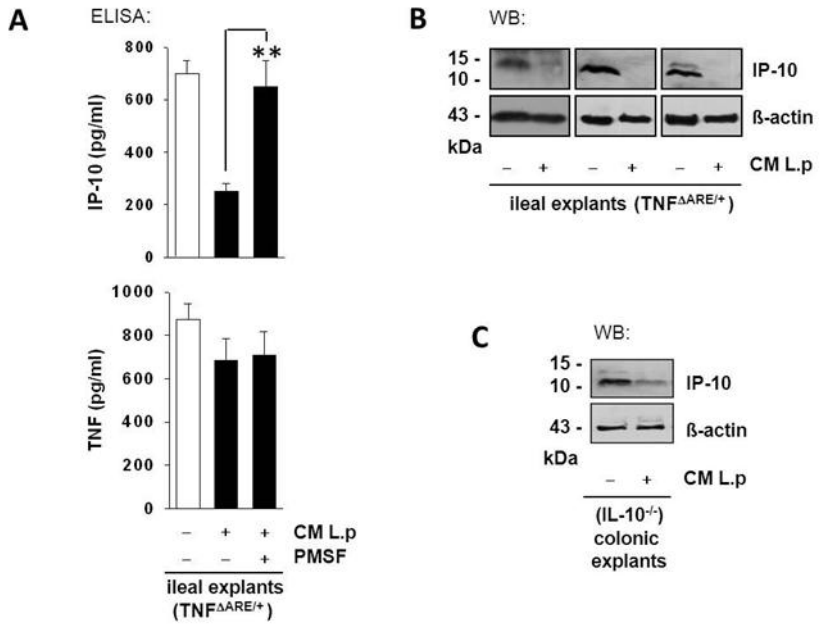

Figure 4-10: CM L.p derived lactocepin degrades tissue-distributed IP-10 in intestinal tissue explants from mouse models of chronic intestinal inflammation.

Terminal ileal specimen of TNF$^{\Delta ARE/+}$ mice (n=4) were incubated with CM L.p (3x) or with PMSF pre-treated CM L.p (3x) for 24h. The concentration of TNF and IP-10 in the explant supernatant was measured by ELISA (A). Terminal ileal specimen of TNF$^{\Delta ARE/+}$ mice (n=3) were incubated with CM L.p (3x) for 24h. IP-10 levels in the tissue were subsequently analyzed by Western Blot analysis (B). Distal colonic specimen of IL-10$^{-/-}$ mice (n=2) were incubated with CM L.p (3x) for 24h. IP-10 levels in the tissue were subsequently analyzed by Western Blot analysis (C). (*) $p < 0.05$ or (**) $p< 0.01$.

4.9 I.p injection of lactocepin-containing CM L.p reduces IP-10 tissue levels and lymphocyte recruitment in TNF$^{\Delta ARE/+}$ mice.

In order to analyze whether the *in situ* observed CM L.p-mediated degradation of IP-10 in the tissue can be validated *in vivo* , inflamed TNF$^{\Delta ARE/+}$ mice were treated with intraperitoneal (i.p) injections of CM L.p or PBS (Figure 4-11).

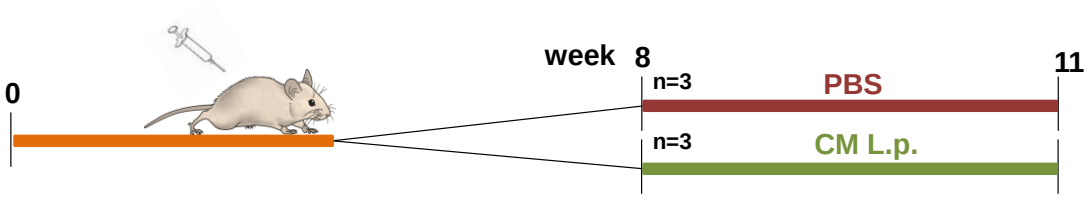

Figure 4-11: Experimental setup and timeline of intraperitoneal injection of CM L.p or PBS in TNF$^{\Delta ARE/+}$ mice.

200 µl of sterile filtered 150x concentrated CM L.p or PBS were injected i.p into TNF$^{\Delta ARE/+}$ mice three times a week over a period of 3 weeks, starting at the age of 8 weeks. (n=3 per group).

Western Blot analysis revealed that CM L.p treatment of TNF$^{\Delta ARE/+}$ mice results in selective loss of ileal IP-10 (Figure 4-12 A). In addition, activation of IBD-relevant pro-inflammatory signaling pathways such as NFκB, p38 MAPK and PI3P/Akt were reduced (Figure 4-12 B).

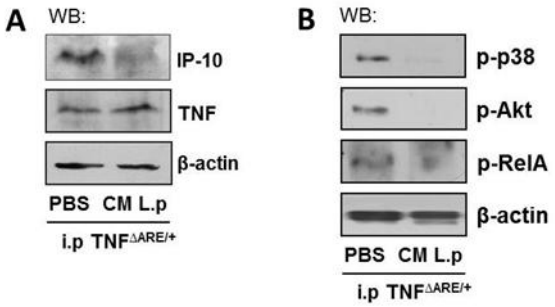

Figure 4-12: The serine protease in CM L.p degrades tissue-distributed IP-10 resulting in reduced inflammatory activation in the inflamed ileal tissue of TNF$^{\Delta ARE/wt}$ mice.

Inflamed TNF$^{\Delta ARE/+}$ mice (n=3 per group) were injected i.p (every other day) with PBS or CM L.p (150x) for 3 weeks. The levels of IP-10 and TNF (A) as well as the levels of phosphorylated NFκB, p38 and Akt (B) in the terminal ileal tissue of these mice were analyzed by Western Blot.[138]

Moreover, i.p injection of CM L.p reduced the infiltration of mononuclear cells (denoted by immune cells), which was most prominent in the ileal muscularis (Figure 4-13 A). In addition, myeloperoxidase (MPO)-positive cells were reduced in ileal tissue of CM L.p treated mice (Figure 4-13 C). Consistent with the fact that IP-10 mainly recruits activated T cells[151], the injection of CM L.p resulted in significantly reduced T cell infiltration (CD3+ cells) into the ileal tissue of TNF$^{\Delta ARE/+}$ mice (Figure 4-13 B). In accordance with the results of the cleavage site analysis (compare Table 4-2), no adverse effects on E-Cadherin levels were observed (Figure 4-13 D).[138]

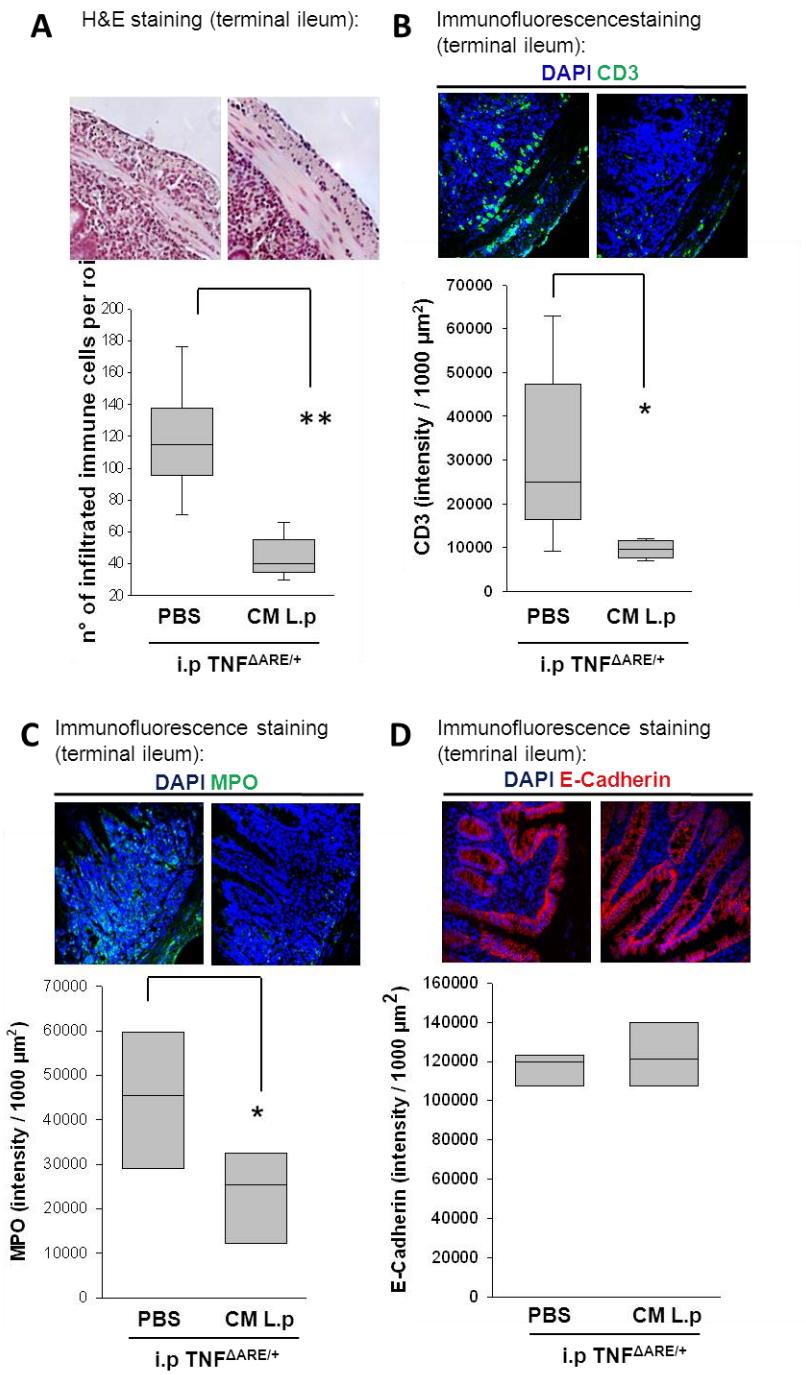

Figure 4-13: Intraperitoneal injection of CM L.p reduces immune cell infiltration and ileal inflammation in TNF$^{\Delta ARE/+}$ mice without affecting E-Cadherin levels.

Inflamed TNF$^{\Delta ARE/+}$ mice (n=3 per group) were injected i.p (every other day) with PBS or CM L.p (150x) for 3 weeks. Immune cell infiltration into the muscularis of TNF$^{\Delta ARE/+}$ mice was determined via counting mononuclear cells in the H&E stained muscularis at three representative regions of interest (roi) per mouse (A). The level of T cell infiltration in the ileal tissue was determined by immunofluorescence staining of CD3+ cells (green) and DAPI (blue) followed by CD3+ signal quantification using volocity (B). Box plots depict 10th-90th percentile (A,B). Representative pictures and signal intensity of the immunofluorescence staining of MPO (green) and DAPI (blue) in terminal ileal sections of TNF$^{\Delta ARE/+}$ mice treated with PBS or CM L.p (C). Representative pictures and signal intensity of the immunofluorescence staining of E-Cadherin (red) and DAPI (blue) in terminal ileal sections of TNF$^{\Delta ARE/+}$ mice treated with PBS or CM L.p (D). Box plots depict 25th -75th percentile (C, D). (*) $p < 0.05$ or (**) $p< 0.01$.

4.10 Anti-inflammatory effects of i.p injection of CM L.p are dependent on the proteolytic activity of lactocepin.

In order to analyze whether the reduced recruitment of immune cells was due to proteolytic activity of CM L.p derived lactocepin, inflamed TNF$^{\Delta ARE/+}$ mice were treated with i.p injections of CM L.p or PMSF-inactivated CM L.p (Figure 4-14).

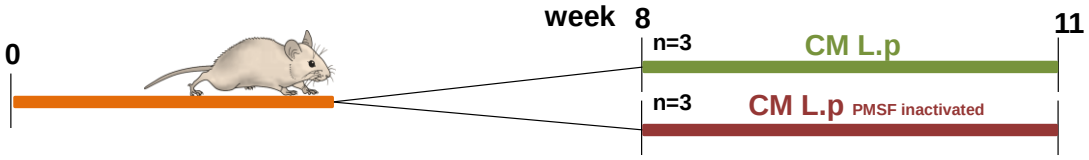

Figure 4-14: Experimental setup and timline of i.p injection of PMSF-treated CM L.p in TNF$^{\Delta ARE/+}$ mice.

Compared to PMSF-inactivated CM L.p, injection of CM L.p resulted in reduced numbers of tissue infiltrating mononuclear cells (denoted by immune cells) (Figure 4-15 A), CD3+ T cells (Figure 4-15 B) as well as MPO-positive cells (Figure 4-15 C). These findings suggest that the PMSF-sensitive proteolytic activity of lactocepin mediates the anti-inflammatory effect. Again, E-Cadherin levels were not affected by the active protease (Figure 4-15 D).[138]

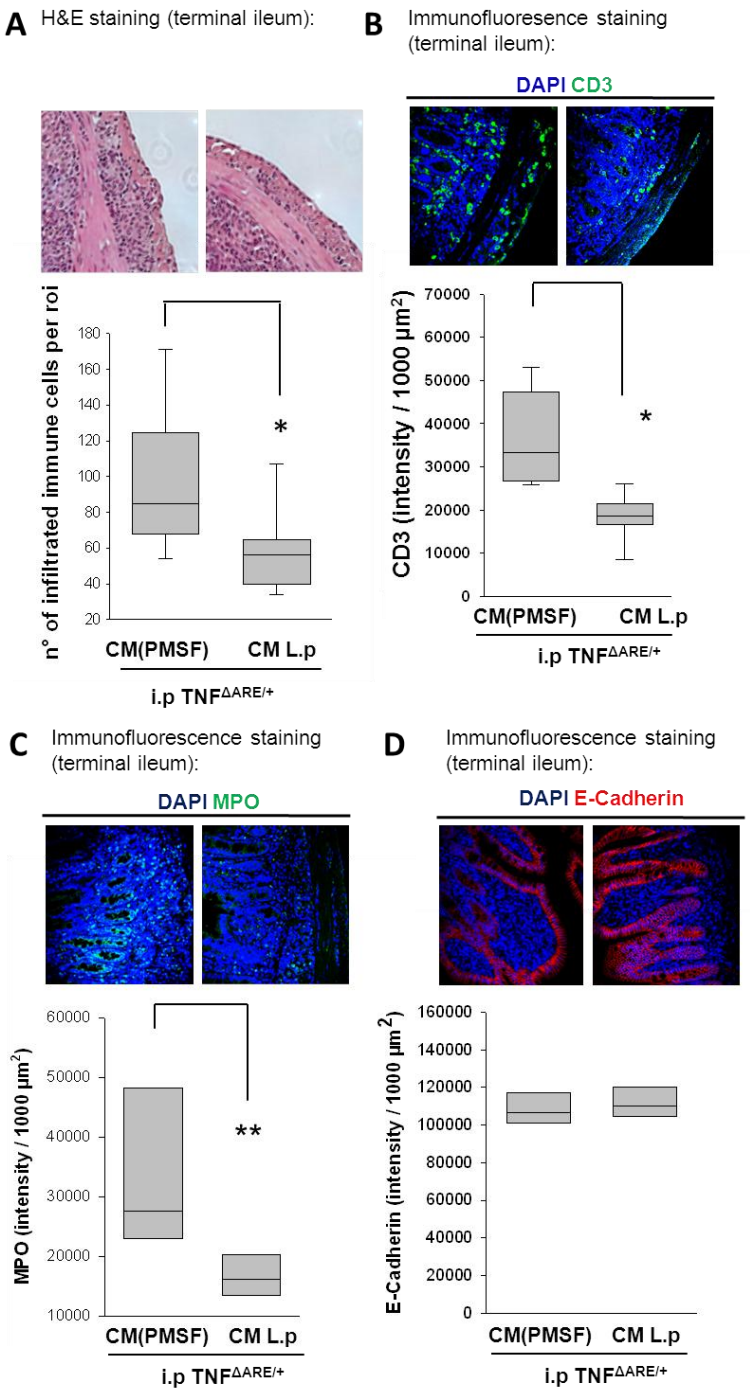

Figure 4-15: Intraperitoneal injection of PMSF-inhibited CM L.p fails to mediate anti-inflammatory effects in TNF$^{\Delta ARE/+}$ mice.

Inflamed TNF$^{\Delta ARE/+}$ mice (n=3 per group) were injected i.p (every other day) with CM L.p or PMSF pre-treated CM L.p (CM(PMSF)) (both 150x) for 3 weeks. Immune cell infiltration into the muscularis of TNF$^{\Delta ARE/+}$ mice was determined via counting mononuclear cells in the H&E stained muscularis at three representative regions of interest (roi) per mouse (A). Levels of T cell infiltration in the ileal tissue was determined by immunofluorescence staining of CD3+ cells (green) and DAPI (blue) followed by CD3+ signal quantification using volocity (B). Box plots depict 10th-90th percentile (A,B). Representative pictures and signal intensity of the immunofluorescence staining of MPO (green) and DAPI (blue) in terminal ileal sections of TNF$^{\Delta ARE/+}$ mice treated with CM L.p or CM(PMSF) (C). Representative pictures and signal intensity of the immunofluorescence staining of E-Cadherin (red) and DAPI (blue) in terminal ileal sections of TNF$^{\Delta ARE/+}$ mice treated with PBS or CM(PMSF) (D). Box plots depict 25th - 75th percentile (C, D).[138] (*) $p < 0.05$ or (**) $p < 0.01$.

4.11 Comparison of bacterial isogenic mutants that differ only with regard to the presence of functional prtP

In order to analyze the physiological relevance of orally applied prtP-encoded lactocepin in the context of the IBD pathogenesis, we tried to generate a prtP-disruption mutant of L.p to be applied in a mouse model of experimental colitis. However, the transformation efficacy of L.p was insufficient.

As lactocepin has been intensively investigated in the context of dairy research, lactocepin-lacking mutant strains have already been described. We kindly received some of these strains: *Lactococcus (L.) lactis* PHa expressing a prtP encoded lactocepin group a (see 1.10), and its isogenic prtP-deficient mutant *L. lactis* PH^{130} (for both see 3.2) as well as BL23 (see 4.2) and its isogenic prtP-deficient mutant L.p BL23 $prtP^{dis}$ (for both see 3.1). CM from all these strains has been generated in order to find an adequate model strain for feeding studies. *L. lactis* Pha expressing a prtP encoded lactocepin type a (see 1.8), as well as its isogenic prtP-deficient mutant. CM *L. lactis* Pha showed no IP-10-degrading capacity, although LC-MSMS analysis revealed expression of prtP-encoded lactocepin. However, *L. lactis* Pha was able to clot milk within 12 h compared to lactocepin-lacking *L. lactis* Ph when cultivated in milk culture, suggesting indeed an expression of functional prtP-encoded group a lactocepin but a missing substrate specificity of group a lactocepin for IP-10. As stated already above, when grown in DMEM (including 1% glutamine, 20 mM HEPES, pH 7.4) BL23 did not express lactocepin (compare 4.2). Nevertheless, in milk culture, BL23 clotted milk much faster (within 12 h) compared to its isogenic mutant (at least two days). Rapid milk clotting (within 24 h) is a strong indicator for the expression of active lactocepin, as milk clotting correlates with bacterial growth in milk, which is in turn dependent on the lactocepin-mediated capability of the strain to degrade caseins in order to generate amino acids that are necessary for growth.[130, 138] The results of these efforts have been summarized together with results of the following section (4.12) in Table 4-3 (see section 4.12).

4.12 Isolation of *L. casei* BFLM218 and generation of an isogenic lactocepin mutant *L. casei* prtP^{dis}

We screened fecal lactobacilli isolates from healthy human volunteers for strains showing anti-IP-10 activity. As shown in Figure 4-16 CM *L. casei* BFLM218 (L.c) was tested positive for the direct, PMSF-sensitive degradation of IP-10 and for the presence of prtP-encoded lactocepin.

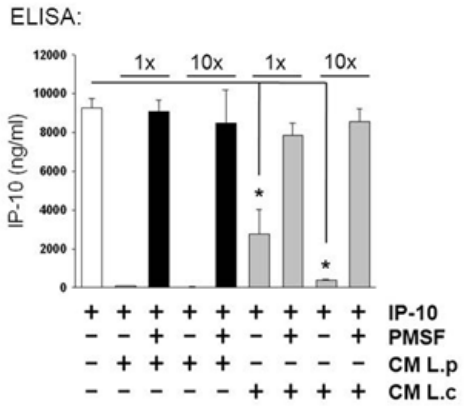

Figure 4-16: Human fecal isolate L.c shows analogous anti-IP-10 capacity like L.p.

CM L.p and CM *L. casei* BFLM 218 (CM L.c), respectively were either pre-treated with PMSF (500µM) or not. Respective CM (1x and 10x) was co-incubated with mIP-10 (37°C, o.n.) and subsequently IP-10 levels analyzed via ELISA. (*) $p < 0.05$ or (**) $p< 0.01$.

Sequence analysis revealed that L.c-encoded prtP is 99% homologous to L.p-derived prtP (Table 4-3) (Table 6-1 and Table 6-2, appendix). We then generated a prtP disruption mutant of L.c (L.c prtP^{dis}) by insertion of an internal fragment into the prtP gene locus via homologous recombination thus perturbing translation of the gene (Figure 4-17 A).[138] The disruption of the prtP gene locus seems to affect formation of chains by the bacteria. L.c tends to build short and straight chains whereas L.c prtP^{dis}, missing its major proteolytic system for protein degradation, reacts on this stress condition with the formation of longer and more curved chains (Figure 4-17 B). In addition, the disruption of the prtP gene locus decelerates growth in the initial growth period, which is compensated in the late growing phase (Figure 4-17 C).

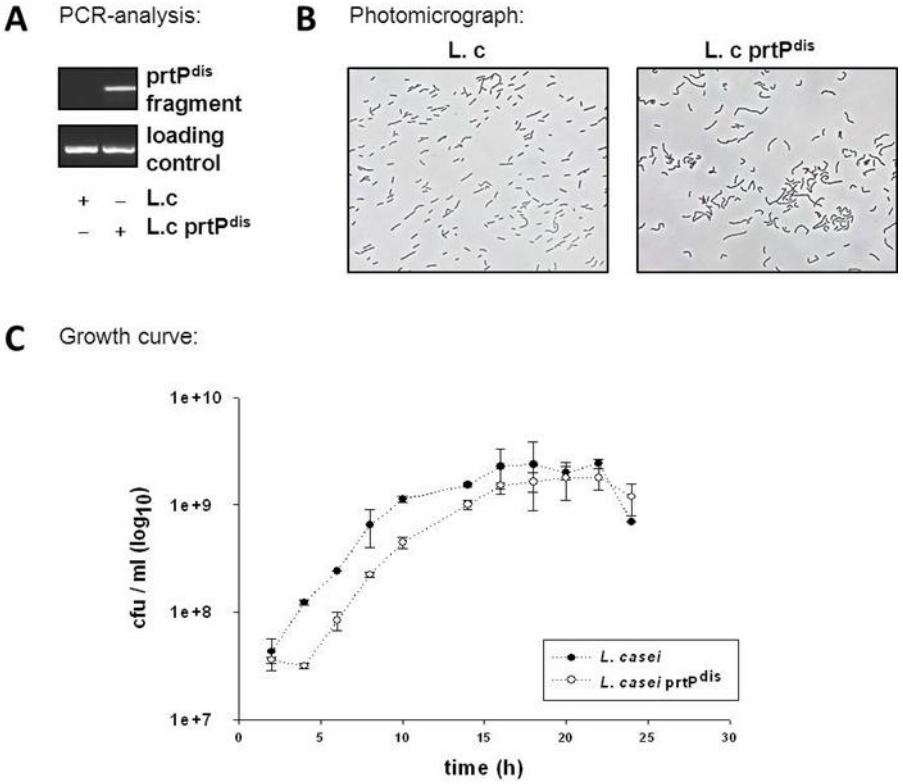

Figure 4-17: Generation of the prtP disruption mutant *L. casei* BFLM218 prtP^{dis}.

Site targeted disruption of prtP gene in *L. casei* BFLM218 (L.c prtP^{dis}) by insertion of an internal fragment was confirmed via amplification of the prtP region using one primer positioned within the T7 promotor of the construct and the other primer positioned further downstream outside the inserted construct (A). Overnight culture of L.c or L.c prtP^{dis} (magnification: 400x) (B). Growth kinetic (0-24h) of L.c and L.c prtP^{dis} cfu/ml was calculated by plating bacteria (C).

The site targeted disruption of the prtP gene locus resulted in the absence of lactocepin in CM L.c prtP^{dis} which could be confirmed via LC-MS/MS analysis (Figure 4-18 A). As expected, CM L.c prtP^{dis} and fixed L.c prtP^{dis} failed to degrade IP-10, confirming our hypothesis that prtP-encoded lactocepin is the chemokine degrading protease of L.c (Figure 4-18 B/C and Table 4-3).[138]

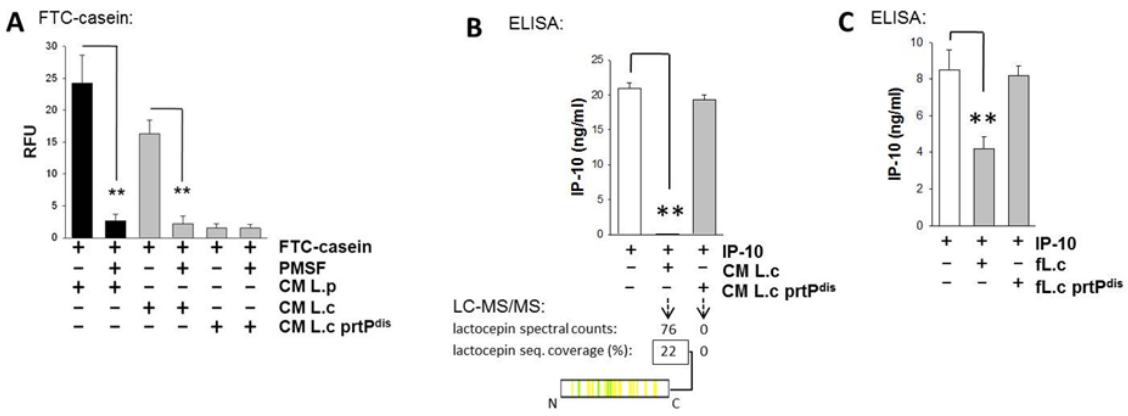

Figure 4-18: Site targeted disruption of prtP results in loss of anti-IP-10 capacity.

Proteolytic activity of CM (L.p, L.c or L.c prtP^{dis}) was assessed via co-incubation of respective CM with FTC-casein (o.n., 37°C) and intensity of fluorescence signal (relative fluorescence units, RFU) emitted by cleaved FTC-casein was determined (A). mIP-10 was co-incubated with CM L.c or CM L.c prtP^{dis} (24h). IP-10 concentration was subsequently measured by ELISA (B, upper panel). CM L.c and CM L.c prtP^{dis} were subjected to LC-MS/MS analysis. The lower panel of Fig. A indicates the result of the LC-MS/MS analysis with regard to the identification of prtP-encoded lactocepin. Spectral counts display the n° of identified lactocepin peptides. Sequence coverage displays the percentage of the prtP-sequence that is covered by the detected peptides. The localization of identified peptides within the lactocepin protein sequence is presented using yellow and green (modified peptides) marks in the bar chart (C: C-terminus, N: N-terminus) (B). Fixed bacteria (fL.c, fL.c prtP^{dis}) were co-incubated with rmIP-10 and mIP-10 levels measured via ELISA (C). (*) $p < 0.05$ or (**) $p < 0.01$.

Table 4-3: Differences and homologies between different prtP-encoding strains.

The homology of the prtP sequence from the analysed bacterial strains to L.p VSL#3 prtP was determined using NCBI blastn algorithm ((L.p) BL23: gi23191636824 (L.c BFLM prtP is 100% identical to BL23 prtP), *L. lactis* PHa: gi76574915). Lactocepin expression in dependence of the growth medium was analysed by the generation of CM of the respective strain and subsequent LC-MS/MS analysis as well as milk clotting assays. Anti-IP-10 activity refers to the ability of the respective lactocepin to degrade rmIP-10 (as tested with the respective CM).

Bacterial strain	Presence of prtP (gene)	Homology to L.p prtP	Activity of prtP (milk clotting)	Presence of prtP in CM (protein)	Anti-IP-10 activity of prtP (CM)
L.p VSL#3	+	100%	+	+	+
L.c BFLM 218	+	99%	+	+	+
L.c BFLM 218 prtP^{dis}	-	-	-	-	-
L.p BL23	+	99%	+	-	-
L.p BL23 prtP^{dis}	-	-	-	-	-
L. lactis PHa	+	98%	+	+	-
L. lactis PH	-	-	-	-	-

4.13 Functional expression-analysis of prtP-encoded lactocepin in mono-associated wt mice

In order to assess expression of prtP-encoded lactocepin in the gastrointestinal tract *in vivo*, we performed a mono-association study with L.c and L.c prtP^{dis} (Figure 4-19). Germfree wt mice (background: BALB/c) were mono-associated with either L.c or L.c prtP^{dis} and sacrificed ten days post colonization.

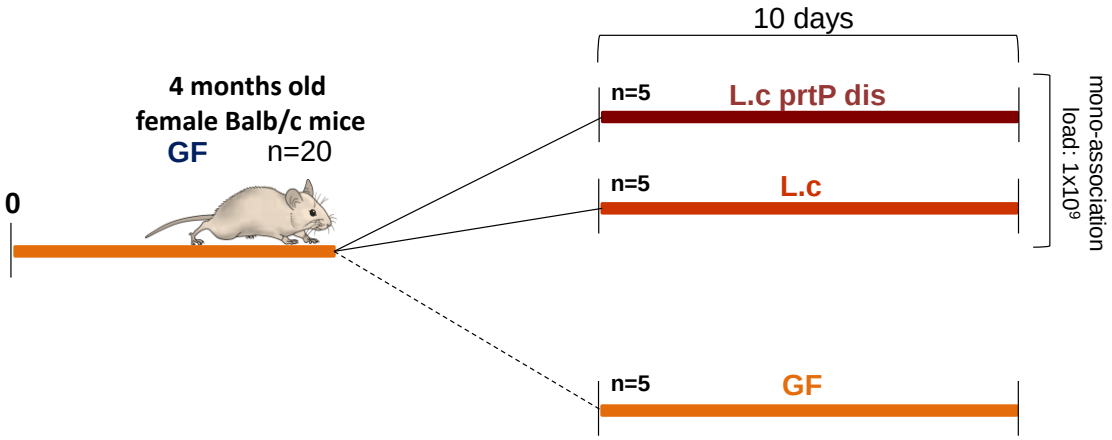

Figure 4-19: Experimental setup and timline of mono-association of wt mice with L.c or L.c prtP^{dis}.

Germfree (GF) wt mice (background: BALB/c) remained either germfree or were mono-associated with L.c or L.c prtP^{dis}, respectively. After ten days, mice were sacrificed and sampled. (n=5 mice per group).

4.13.1 Luminal and mucosal colonization of *L. casei* and its isogenic mutant strain

In order to compare the colonization ability of L.c and Lc prtP^{dis}, cecal content was sampled for cultivation analysis. The results of the cultivation analysis are shown in Figure 4-20 and revealed no differences regarding number of residing bacteria due to the presence or absence of lactocepin (Figure 4-20 A). The cecal content of mice mono-associated with L.c prtP^{dis} was plated on MRS agar with or without erythromycin. Comparable numbers of L.c prtP^{dis} were detected in both cases, suggesting genetic stability of the prtP disruption *in vivo*. Figure 4-20 A only depicts the number of L.c prtP^{dis} grown on erythromycin containing agar plates.[138]

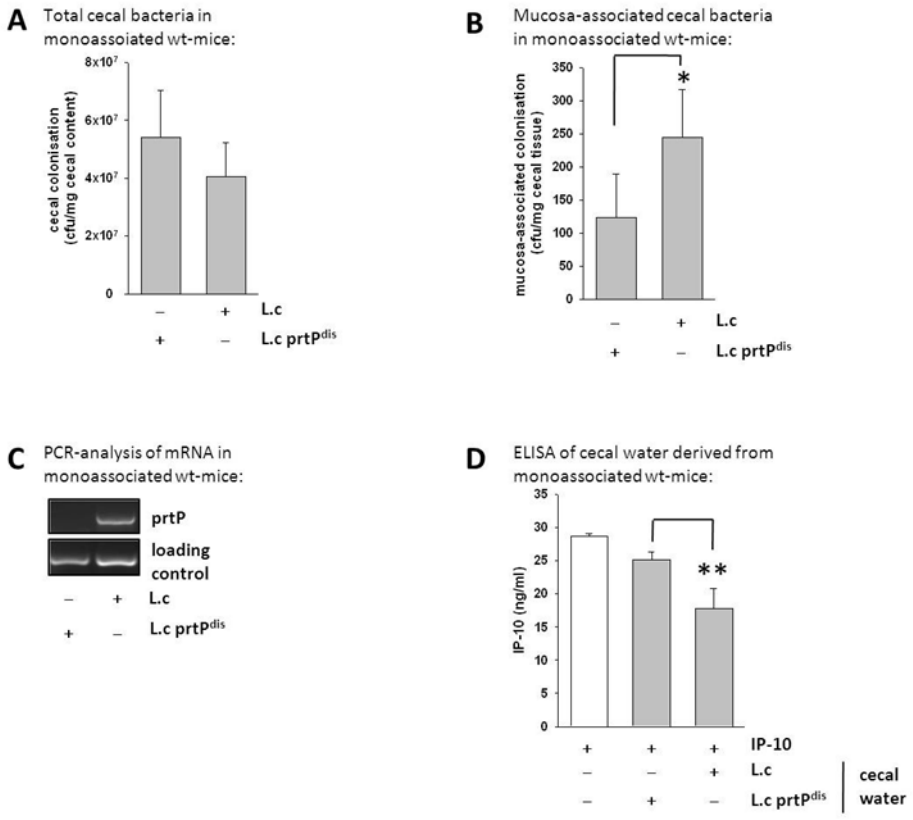

Figure 4-20: PrtP-encoded lactocepin is luminally expressed and functionally active in healthy, mono-associated wt mice.

The number of luminal (A) as well as mucosa-associated (B) L.c and L.c prtP^{dis} in the cecum of the monoassociated mice (n=5 per group) was determined via plating. RNA isolated from L.c and L.c prtP^{dis} residing in the cecum of mono-associated mice was subjected to reverse transcription and subsequently analysed by PCR with regard to the presence of prtP transcripts (C). IP-10 was incubated with cecal water from L.c and L.c prtP^{dis}-monoassociated mice for 24 h and the remaining IP-10 concentration was determined via ELISA (D). (*) $p < 0.05$ or (**) $p < 0.01$.

As the cecal gut content is characterized by a high content of water which may differ between the groups and thus falsify our cultivation results, we determined the water content of cecal content biomass via weighing and lyophilization. We could not observe different water contents among the colonized mice (Table 4-4). Surprisingly, we also could not observe changes between germfree and colonized mice (Table 4-4).

Table 4-4: Dry mass in gut content of germfree and mono-associated wt mice.

Percentage, %; germfree housed mice, GF; L.c-monoassociated mice, L.c; L.c prtP^{dis}-mono-associated mice.

cecal gut content	dry mass (%)	water fraction (%)	std. dev (%)
GF	67,8	32,2	±3,7
L.c	67,0	33,0	±2,7
L.c prtP^{dis}	70,6	29,4	±2,1

Mode of action of probiotic bacteria may not only depend on luminal colonization, but also on their ability to reach high proximity to the mucosa. Therefore we isolated the bacteria associated to the cecal mucosa and plated them. As shown in Figure 4-20 B, significantly higher numbers of L.c were found to be attached to the cecal mucosa compared to L.c prtP^{dis}.[138]

4.13.2 *In vivo* expression of prtP-encoded lactocepin

In Figure 4-2 A and Figure 4-18 B *in vitro* expression of lactocepin was shown. In order to show *in vivo* expression of lactocepin by L.c, mRNA of cecal derived gut content was isolated and the expression of prtP-encoded lactocepin analyzed. This transcriptional analysis of cecum-derived bacteria revealed that L.c indeed expresses prtP-encoded lactocepin. (Figure: 4-20 C).

In addition, although total bacterial numbers in the cecum did not differ between L.c and L.c prtP^{dis}-monoassociated mice (Figure 4-20 A), cecal water deriving from mice mono-associated with L.c was significantly more potent in degrading IP-10 compared to L.c prtP^{dis} mono-associated mice (Figure 4-20 D), suggesting that lactocepin retains at least part of its activity when it is produced and secreted in the gastrointestinal tract *in vivo*.[138]

4.14 PrtP-encoded lactocepin mediates significantly reduced cecal inflammation in an experimental colitis model.

To address the physiological relevance of oral prtP-encoded lactocepin treatment in the context of IBD, feeding studies with L.c and its isogenic lactocepin-lacking mutant were performed. Thereby we followed a prevention approach and a treatment approach. In the prevention approach, Rag2$^{-/-}$ were reconstituted with CD4+ T cells from IL-10$^{-/-}$ mice and

were given $2x10^{10}$ cfu L.c or L.c prtP^{dis} for 6 weeks, starting directly after the T cell transfer (Figure 4-21A). Plating on selective agar showed no differences in lactobacilli count between the T cell transferred groups (L.c prtP^{dis}: 2.3 $x10^6$ +/- $0.2x10^6$ cfu/mg cecal gut content; L.c: $2.2x10^6$ +/- $0.2x10^6$ cfu/mg cecal gut content; PBS: $2.3x10^6$ +/- $0.2x10^6$ cfu/ mg cecal gut content). Furthermore, oral administration of L.c did not prevent or reduce the establishment of the inflammation in the T cell transferred Rag2$^{-/-}$ compared to mice that were treated with lactocepin-deficient L.c prtP^{dis} (data not shown).

The second approach intended a probiotic treatment in an already established inflammation. Therefore, L.c or L.c prtP^{dis} were fed to inflamed Rag2$^{-/-}$ mice that were reconstituted with CD4+ T cells deriving from IL-10$^{-/-}$ mice (Figure 4-21 B).

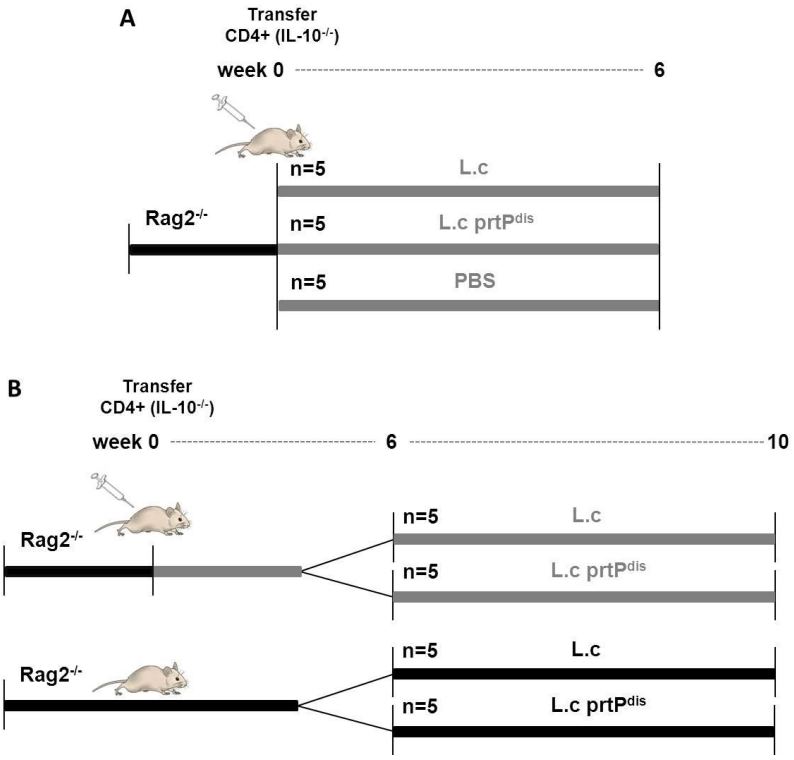

Figure 4-21: Experimental setup and timeline of oral administration of prtP-encoded lactocepin in T cell transferred Rag2$^{-/-}$ mice.

$2x10^{10}$ cfu L.c, L.c prtP^{dis} or sterile PBS were orally administered to T cell transferred Rag2$^{-/-}$ mice three times a week over a period of 6 weeks, starting directly after transfer (n=5 mice per group) (A). Rag2$^{-/-}$ and inflamed T cell transferred Rag2$^{-/-}$ mice received orally administered $2x10^{10}$ cfu L.c or L.c prtP^{dis} three times a week over a period of 4 weeks, starting in week 6 after transfer, when the mice are already inflamed (n=5 mice per group) (B).

As in the case of the prevention approach, the plating of cecal content revealed no differences in lactobacilli count between the T cell transferred groups (L.c prtP^{dis}: 1.1x10^{7} +/- 0.3x10^{7} cfu/mg cecal gut content; L.c: 1.9x10^{7} +/- 0.1x10^{7} cfu/mg cecal gut content). Systemic inflammatory parameters such as spleen weight did not differ within the T cell transferred groups and thus were independent of the administration of prtP-encoded lactocepin. Control Rag2$^{-/-}$ mice also did not exhibit any differences in the spleen weight (Figure 4-22 A). The inflammation of the distal colon (Figure 4-22 B) in T cell transferred Rag2$^{-/-}$ mice did not differ between the treatment groups but the cecal inflammation of the L.c treated group was significantly reduced compared to the mice that were treated with lactocepin-deficient L.c prtP^{dis} (Figure 4-22 C/D).[138]

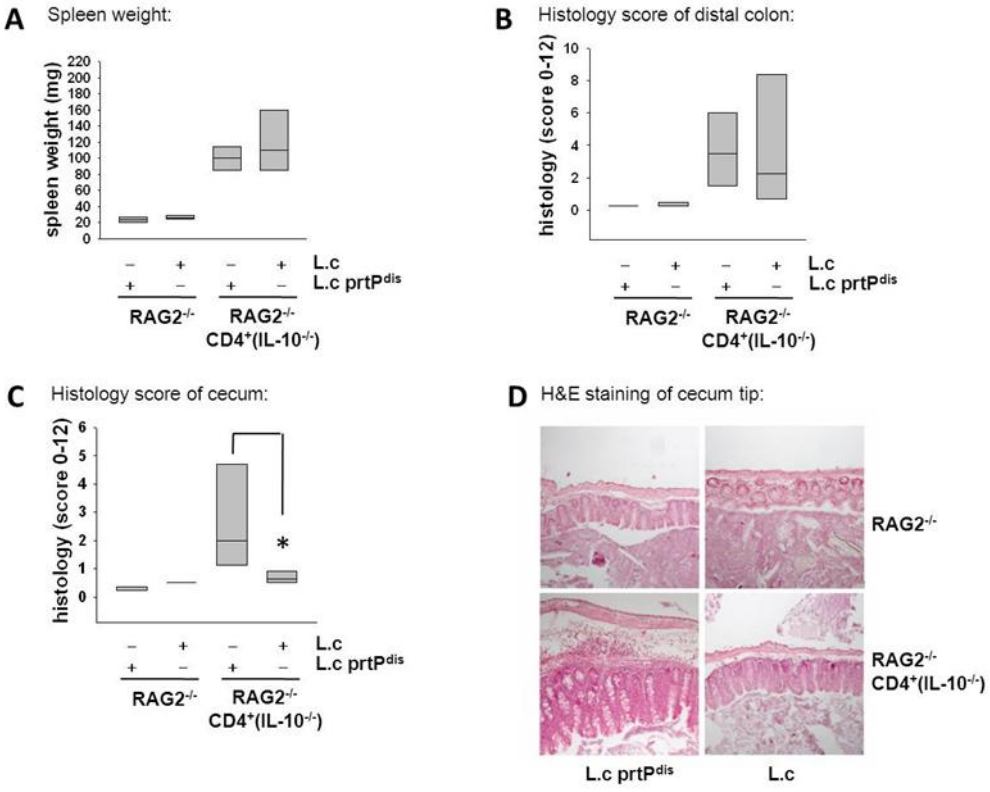

Figure 4-22: Orally administered L.c reduces cecal inflammation in T cell transferred Rag2$^{-/-}$ mice compared to L.c prtP^{dis}.

Control and T cell transferred Rag2$^{-/-}$ mice (n=5 per group) were fed L.c BFLM218 (L.c) or its isogenic lactocepin deficient mutant L.c prtP^{dis} for 4 weeks starting 6 weeks after T cell transfer. The spleen weight (A) and the histopathological score of the distal colon (B) and histopathological score of cecum tip (C) were assessed. Figure D shows representative H&E staining of cecal segments (D). Box plots depict 25th -75th percentile (A, B, C). (*) $p < 0.05$ or (**) $p< 0.01$.

Immunofluorescence staining revealed that the reduced cecal inflammation in L.c-fed mice correlated with strongly reduced IP-10 tissue levels (Figure 4-23 A/B) and significantly reduced T cell infiltration in the cecal tissue, as shown in the staining for CD3+ cells (Figure 4-23C/D). Analogous to the i.p injection of CM L.p, oral administration of lactocepin-expressing L.c did not affect cecal E-Cadherin levels (Figure 4-23E/F), confirming the highly specific substrate profile of lactocepin *in vivo*.[138]

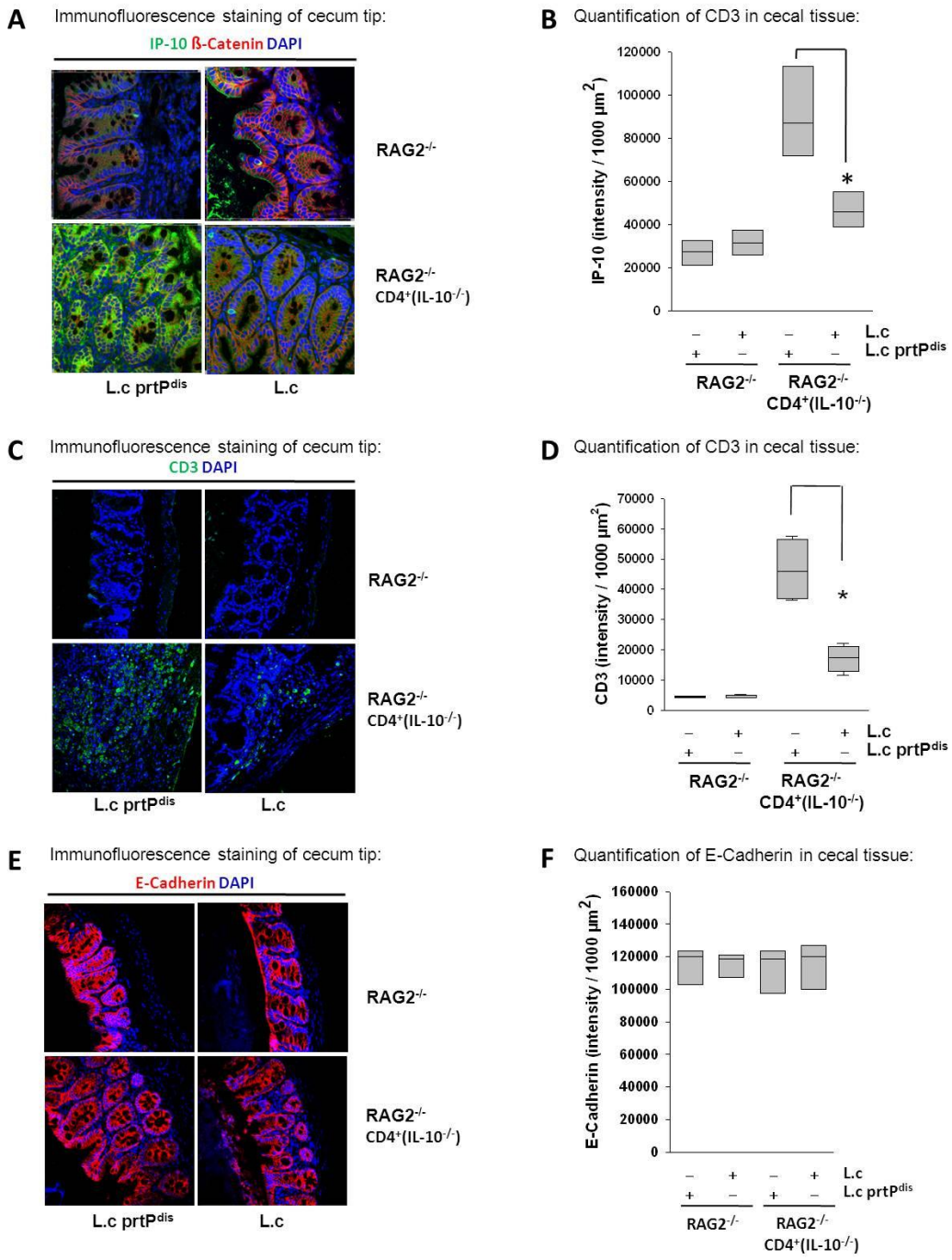

Figure 4-23: Oral administration of prtP-encoded lactocepin expressing L.c results in reduced IP-10 levels and diminished immune cell infiltration compared to L.c prtP^{dis}, whereas E-Cadherin is not affected.

Control and T cell transferred Rag2$^{-/-}$ mice (n=5 per group) were treated with oral application of L.c BFLM218 (L.c) or its isogenic lactocepin deficient mutant L.c prtP^{dis} for 4 weeks starting 6 weeks after T cell transfer. Representative immunofluorescence stainings of IP-10 (green), ß-catenin (red) and DAPI (blue) (A) as well as the mean intensity of IP-10 signal in the cecal tissue of the respective treatment groups (B). Representative immunofluorescence stainings of CD3+ cells (green) and DAPI (blue) (C) as well as the mean intensity of the CD3+ cells derived signal in the cecal tissue of the respective treatment groups (D). Representative immunofluorescence stainings of E-Cadherin (red) and DAPI (blue) (E) as well as the mean intensity of E-Cadherin signal in the cecal tissue of the respective treatment groups (F). Box plots depict 25th -75th percentile (B, F) or 10th-90th percentile (D). (*) $p < 0.05$ or (**) $p< 0.01$.

5 DISCUSSION

This is the first study identifying the molecular mechanism underlying the anti-inflammatory effect of probiotic prtP-encoded lactocepins. Probiotic prtP-encoded lactocepins were found to exert anti-inflammatory effects via selective degradation of an array of pro-inflammatory chemokines, notably IP-10.

There are some indications that the secretion of chemokines by IEC occurs not only in the basolateral direction, but also in the apical direction. It was reported, that CXCR1, the cognate receptor of chemokine CXCL-8, is expressed constitutively and apically in the villi of human, duodenal biospies, but not in the crypts, supporting the hypothesis of luminally secreted chemokines as an autocrine function loop. In human colon biopsies, the CXCR1 receptor was expressed in both directions, apically as well as basolaterally.[152] Under conditions of inflammation, MIP-2, the murine form of human IL-8 (an ELR^+ chemokine), could be detected in the feces of a murine model for traumatic intestinal inflammation.[153] This observation of an ELR^+ chemokine, may be used to explain from an evolutionary point of view, the anitmicrobial capacity of ELR^- chemokines (such as IP-10) to be an ancient innate defence mechanism.[154-156] Lactocepin-mediated degradation of these chemokines may then have been a survival factor to escape ELR^- chemokine-mediated killing or, and still valid today, promote colonization and adherence to the mucosa in homeostasis and inflammation. In a steady state, an apical secretion of chemokines by IEC into the lumen may function as an autocrine loop to maintain homeostasis,[153] whereas in an inflammatory state a luminal secretion of chemokines may amplify immune response. These hypotheses are supported by our observation, that CM L.p mainly targets ELR^- CXC chemokines, notably CXCR3 ligands. CXCR3 and its ligands are associated with a number of chronic inflammatory diseases such as rheumatoid arthritis,[157-159] multiple sclerosis[160, 161] and psoriasis,[160, 162, 163] but also with allograft rejection.[35, 164] Extrinsic, therapeutic-intended manipulation of CXCR3 signaling has been already shown. For example, UV-induced skin pigmentation and erythema are associated with CXCR3 signaling and elevated levels of its ligands CXCL9, CXCL10 and CXCL11. Suppression of CXCR3 signaling by pretreatment of the skin with extracts from

Paeonia suffruticosa ameliorated erythema formation and pigmentation in volunteers by transcriptional inhibiton of CXCR3 mediated melanin synthesis.[165] The identification of the direct anti-inflammatory structure function relationship between lactocepin and the respective target chemokines, notably CXCR3 ligand IP-10, enables further research of lactocepin-based therapies in other chemokine mediated diseases besides chronic intestinal inflammation.

Proteolytic modification of ELR^- chemokines is an important regulator of chemokine activity as e.g NH_2-terminal truncation of Mig, IP-10 and I-TAC by dipeptidyl peptidase (DDP) IV caused reduced CXCR3-A binding and impaired chemotactic potency, without affecting angiostatic activity of the chemokines. However, chemokine cleavage by DPP IV is highly specific and targets penultimate proline residues[166] whereas L.p derived lactocepin was found to target a broad spectrum of cleavage sites, resulting in complete degradation of its target proteins. Nevertheless, the protein shows a highly selective substrate profile. The presence of various potential cleavage sites in proteins that are not degraded by the bacterial protease indicates that additional characteristics like surface charge and/or the three dimensional structure of a protein determines the highly selective substrate profile of prtP-encoded lactocepin. A similar structure-dependent target recognition was proposed for the Group A streptococcal (GAS) protease SpyCEP/ScpC.[167]

To date proteolytic activity of bacteria has mostly been associated with disease initiation or progression e.g. by the inactivation of host derived defence structures or matrix decomposition and certain proteases are therefore discussed as virulence factors.[168, 169] Group A streptococcus causes life threatening diseases like necrotizing fasciitis. The SpyCEP/ScpC protease is a member of the *PrtS*-encoded lactocepin family and degrades IL-8, KC and MIP-2. Furthermore the protease was shown to promote the pathogenesis of invasive skin and soft tissue infections in mice by decreasing PMN recruitment to the site of infection, facilitating GAS survival and systemic spread.[170, 171] The commensal and opportunistic pathogen *Finegoldia magna* expresses the subtilisin-like protease SufA, cleaving and inactivating the antimicrobial proteins midkine, BRAK/CXCL14, LL-37 and

MIG/CXCL9 in order to either facilitate an objectionable spread under pathological circumstances or to enhance survival during epithelial inflammation.[172-174] In addition, the highly unspecific matrix-metalloprotease GelE from *Enterococcus faecalis* OG1RF was found to exacerbate intestinal inflammation in a genetically susceptible host by impairing the barrier function of IEC.[146] In sharp contrast to these findings, our results raise the hypothesis, that selective degradation of pro-inflammatory chemokines by a bacterial protease can be a protective probiotic trait. In fact, lactocepin did not affct recombinant E-Cadherin *in vitro* and did not show any adverse effects on normal or compromised epithelial barrier function, neither in Ptk6 nor in T84 cells.[114, 138] Although lactocepin is highly expressed in L.p, none of the experimental studies using VSL#3 or L.p reported adverse effects of the probiotic mixture on intestinal barrier function.[102, 114, 175] VSL#3 was even shown to reduce bacterial translocation in a mouse model of sepsis.[176] Hence, prtP-encoded lactocepin, that derives from relatively harmless microbes such as L.p VSL#3[176] cannot be considered as a virulence factor but might be indeed a potent protective trait in the context of chronic inflammatory diseases, contributes for example to the observed anti-inflammatory effects of VSL#3 in IBD.[69-71] This assumption is supported by our observation that the direct application of prtP-encoded lactocepin by i.p injection in $TNF^{\Delta ARE/+}$ mice did also not result in any adverse effects.

As the Th1 cell driven immunopathology in the murine $TNF^{\Delta ARE/+}$ model is characterized by by highly elevated chemokine levels (as TNF is an inducer of chemokines such as IP-10)[111], $TNF^{\Delta ARE/+}$ mouse model was an adequate model to investigate the physiological effects of lactocepin on chemokines like IP-10 and the ileal inflammation. This proof of concept study revealed that the i.p application of lactocepin indeed reduced ileal IP-10 levels, resulting in diminished recruitment of CD3+ effector cells and MPO-positive immune cells. The reduction of the immune cell infiltration was most prominent in the muscularis, which might be due to the fact that lactocepin was applied i.p, which matches most a serosal application. In addition, activation of pro-inflammatory signaling pathways that play a role in chronic intestinal inflammation, such as NFκB,[177, 178] p38 MAPK[178, 179] and PI3P/Akt[180, 181] pathways

was reduced. In this context it has already been shown *in vitro*, that supernatants of TNF-activated IEC show reduced ability to recruit T cells after co-incubation with fixed L.p.[111, 114] It might well be that the observed reduction of immune cell infiltration is not exclusively due to IP-10 degradation but also due to the degradation of several specific pro-inflammatory chemokines. Interestingly, the absence of side effects during injection of the probiotic supernatants also reveals that filtrated CM L.p does not trigger systemic side effects like sepsis. This goes along with the finding that i.p injection of CM of *Feacalibacterium prausnitzii* in a TNBS-mouse model led to reduced inflammation and was not accompanied by any systemic negative impact.[51] Nevertheless, the i.p injection of bacterial supernatants remains an artificial scenario whereas oral uptake of lactocepin might be indeed considered as a clinically relevant therapeutic scenario.

Contrary to the direct application of active prtP-encoded lactocepin, the potential of orally applied lactocepin-expressing probiotics is strongly dependent on the location and level of lactocepin secretion in the intestine. Previous studies reported that neither feeding of VSL#3 nor feeding of the single strain L.p resulted in reduction of ileal inflammation in $TNF^{\Delta are/+}$ mice.[111, 182] These results may be due to the lack of colonization of the administered strains in the ileum. The ileum seems to be a less favourable bacterial reservoir compared to the colon, as the ileum habours considerably fewer bacteria than the cecum or the colon.[43] On the other hand, feeding of VSL#3 was reported to reduce cecal inflammation in $IL\text{-}10^{-/-}$ mice, without ameliorating colonic inflammation.[111] In accordance with this finding, oral uptake of prtP-encoding L.c resulted in a potent, but intestinal site-specific, anti-inflammatory effect in T cell transferred $Rag2^{-/-}$ mice. This was demonstrated by strongly reduced levels of IP-10, significantly reduced T cell infiltration, and significantly reduced inflammation in the cecal tissue compared to mice that were fed lactocepin-deficient L.c $prtP^{dis}$. Colonic inflammation was not affected by oral feeding of lactocepin. Mono-association studies with L.c and L.c $prtP^{dis}$ proved that L.c does express functional prtP-encoded lactocepin in the cecum of mono-associated mice. As colonic and ileal inflammation was not ameliorated by feeding prtP-encoding bacterial strains, intestinal site specific expression studies of prtP-encoded

lactocepin would be of great interest. Furthermore, site-specific expression levels of lactocepin by the intestinal microbiota or selective strains like probiotic L.p remain to be elucidated in health and disease in order to assess the anti-inflammatory impact of additional uptake of lactocepin expressing bacteria. Besides expression rates, activity and/or target site accessibility of prtP-encoded lactocepin may vary in the different luminal environments of the investigated intestinal compartments, resulting in segment specific effects of prtP-encoded lactocepin. Fluorescent labeling of *in vivo* expressed lactocepin would give new insights into the location and level of lactocepin expression and should be addressed in future experiments.

Oral application of isolated prtP-encoded lactocepin may overcome the intestinal site specific efficacy of the probiotic protease. However, stability studies revealed that lactocepin does presumabely not retain its proteolytic activity after the gastrointestinal passage. Nevertheless, oral application of CM L.p may be possible e.g. when using capsules that are resistant to low pH. Yan et al. encapsulated LGG-derived and purified p40 in hydrogel beads to protect the protein from degradation. Feeding these beads resulted in prevention as well as treatment of inflammation in DSS-induced colitis by maintaining barrier function in the colonic epithelium in an EGF receptor-dependent way.[105] Release of probiotic lactocepin in the small or large intestine may be sufficient to mediate anti-inflammatory effects, because explant culture experiments showed that L.p-produced lactocepin is able to access tissue-distributed IP-10. Translocation of secreted bacterial compounds has been already observed in literature.[183, 184] Vandenbrouken *et al.* gavaged anti-TNF nanobody-expressing *L. lactis* to mice that were suffering from DSS-induced colitis. Immunohistochemical stainings detected these nanobodies in the colonic lamina propria of these mice.[185] However, DSS-treatment is known to lead to massive destruction of the epithelial layer, resulting in a leaky barrier. An impaired barrier function may therefore also be a prerequisite for anti-inflammatory effects mediated by orally applied prtP-encoded lactocepin.

Access of prtP-encoded lactocepin to tissue-distributed IP-10 may also be promoted by mucosal attachment of the bacterium. In this context, the number of L.c attached to the cecal

mucosa of mono-associated mice was found to be significantly higher compared to L.c prtP^{dis}, although the number of luminal cecal bacteria was comparable between the groups. This finding is consistent with the study of Habimana et al. who reported that the non-proteolytic domain of prtP is involved in the adhesion of lactococci to solid surfaces and solvents.[186] In inflamed lesion of UC patients, the mucosal adherence of lactobacilli is reduced compared to non-inflamed tissue biopsies[187] and hence may result in a reduction of putative prtP-encoding strains. In this context, it would be interesting to discuss the question whether an additional supply of prtP-encoding L.p leads to a detectable rise of intrinsic luminal prtP-encoded lactocepin. *In vitro* we made first observations, that co-incubation of L.c with a in terms of IP-10 degradation inactive *Lactobacillus delbrueckii* results in an increased proteolytic activity than single strain L.c, suggesting a kind of competitive upregulation of prtP-encoded lactocepin expression in the presence of *Lactobacillus delbrueckii.*

Although many lactobacilli and lactococci are known to encode prtP, only few bacterial strains were found to exert analogous, but in most cases much weaker, anti-IP-10 activity compared to L.p. The underlying reason might be different regulation of prtP expression, high variations in prtP expression levels as well as strain-specific substrate profiles of the bacterial protease. Indeed, BL23, like the active fecal isolate L.c, encodes for a lactocepin that is 99% homologous to prtP of L.p. However, in contrast to L.c and L.p, BL23 does not express the protease in CM although it was found to express active prtP-encoded lactocepin in milk culture. Furthermore, CM PHa[130 135] does not show any anti-IP-10 activity despite the presence of functional lactocepin which is 98% homologous to prtP of L.p, demonstrating different substrate profiles of by Pha-produced versus by L.p-produced prtP-encoded lactocepin. These highly diverse expression profiles and substrate specificities of prtP-encoded lactocepins from different lactococci and lactobacilli are of great relevance for the potential anti-inflammatory effects of the respective strains but the underlying reasons for these differences remain to be determined.

In summary, probiotic prtP-encoded lactocepins were found to exert anti-inflammatory effects via selective degradation of an array of pro-inflammatory chemokines, notably IP-10

(Figure 5-1). The physiological relevance of this highly selective mechanism could be confirmed by means of a genetic loss-of-function approach with an isogenic prtP-disruption mutant in L.c. Our study characterizes the selective degradation of pro-inflammatory chemokines by probiotic prtP-encoded lactocepins as a protective microbial structure-function relationship in the context of chronic intestinal inflammation and enables a structure-function-based selection of putative probiotic strains. In addition, prtP-encoded lactocepin was found to selectively degrade IP-10 in inflamed intestinal tissue, showing that the protease remains its protective activity in a physiological context, suggesting further research of lactocepin-based therapies in chemokine-mediated diseases. Especially skin-related disease such as psoriasis or allergic eczema may be of interest, as direct application of lactocepin in close proximity to inflammation-driving chemokines could easily be achieved. In the context of intestinal inflammation, tagging and overexpression of prtP-encoded lactocepin as well as encapsulation of the purified protein would further contribute to the development of lactocepin-based therapies.

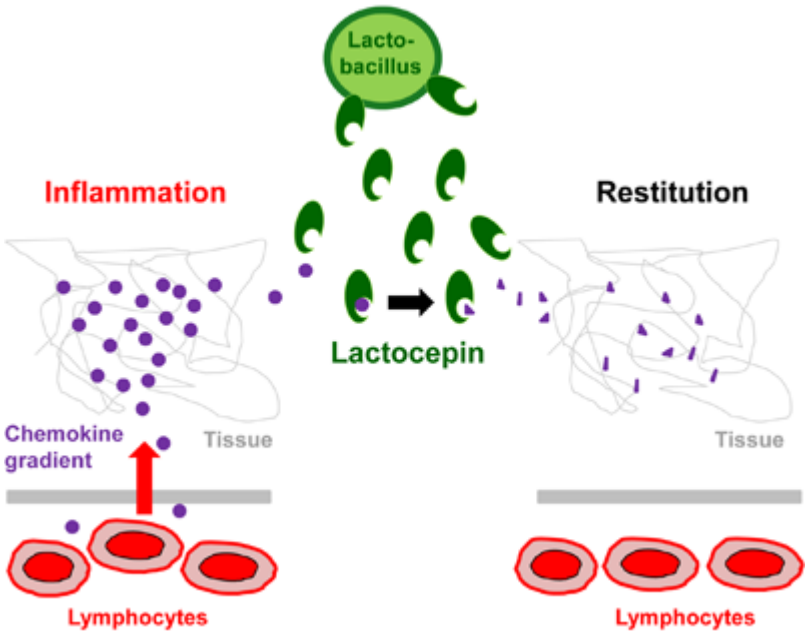

Figure 5-1: Proposed anti-inflammatory mechanism of probiotic prtP-encoded lactocepin in the context of intestinal inflammation.[138]

Probiotic prtP-encoded lactocepin selectively degrades IP-10 and other pro-inflammatory chemokines. The probiotic protease thereby reduces pro-inflammatory lymphocyte infiltration into the tissue, resulting in less inflammation.

6 APPENDIX

Table 6-1: Proteins identified in CM L.p via LC-MS/MS analysis

Molecular weight, MW; isoelectric point, pI; number of assigned peptides, n° peptides; sequence coverage, SC.

Protein name	MW	pI	N° peptides	SC (%)
Lactocepin precursor	200.1	4.8	20	15.5
L-lactate dehydrogenase	35.5	5.1	13	41.1
60 kDa chaperonin	57.4	4.7	12	24.8
Elongation factor Tu	43.4	4.8	7	25.3
Enolase 1	47.0	4.5	7	26.4
Phosphoglycerate kinase	42.8	4.9	7	17.8
GMP synthase	57.8	5.2	6	13.9
Enolase	46.5	4.6	5	16.7
ATP synthase subunit beta	51.4	4.5	4	15.5
D-2-hydroxyisocaproate dehydrogenase	36.9	5.1	4	13.5
ATP synthase subunit alpha	54.6	4.8	3	5.4
Chaperone protein dnaK	66.3	4.7	3	10.1
Glyceraldehyde-3-phosphate dehydrogenase	36.5	5.4	3	15.1
Glucose-6-phosphate isomerase	49.5	4.6	3	6.9
Inosine-5'-monophosphate dehydrogenase	52.8	5.7	3	7.9
Hypothetical protein lin2081	45.4	5.1	3	7.5
Bifunctional autolysin	136.7	-1.4	2	1.4
10 kDa chaperonin	10.0	4.8	2	24.7
Elongation factor G	76.9	4.6	2	8.1
Elongation factor Tu-A	44.0	5.2	2	11.5
Galactokinase	42.4	4.9	2	5.4
2,3-bisphosphoglycerate-dependent phosphoglycerate mutase 2	26.1	4.8	2	9.6
ATP-dependent hsl protease ATP-binding subunit hslU	53.2	5.4	2	4.3

Protein name	MW	pI	N° peptides	SC (%)
Phosphoenolpyruvate-protein phosphotransferase	63.0	4.5	2	3.5
DNA-directed RNA polymerase beta chain	132.2	4.8	2	1.8
DNA-directed RNA polymerase subunit beta'	135.7	6.2	2	1.6
Valyl-tRNA synthetase	100.9	5.8	2	2.3
Putative reductase CA_C0462	45.6	9.1	2	3.3
6-phosphogluconate dehydrogenase, decarboxylating 2	51.7	5.1	1	4.1
Fructose-bisphosphate aldolase	31.0	6.5	1	4.5
Bifunctional protein folD	30.6	5.3	1	3.9
DNA gyrase subunit A	100.1	4.7	1	2.1
1-(5-phosphoribosyl)-5-[(5-phosphoribosylamino)methylideneamino]	25.7	5.5	1	5.9
Pyruvate kinase	52.0	5.9	1	1.9
S-adenosylmethionine synthetase	43.4	5.3	1	3.8
Gamma-glutamyl phosphate reductase 1	45.4	5.3	1	4.8
dTDP-glucose 4,6-dehydratase	39.3	5.5	1	5.2
DNA-directed RNA polymerase alpha chain	34.7	4.7	1	5.8
30S ribosomal protein S2	30.2	5.1	1	7.1
30S ribosomal protein S3	24.6	10.3	1	6.9
30S ribosomal protein S7	17.8	10.6	1	9.0
Alanyl-tRNA synthetase	97.8	4.8	1	2.3
Glutamyl-tRNA synthetase	55.3	4.8	1	4.1
Threonyl-tRNA synthetase	73.4	4.9	1	1.6
Probable transaldolase	23.2	5.6	1	5.5
Hypothetical 30.6 kDa protein in fumA 3'region	30.6	7.8	1	5.3

Table 6-2: Proteins identified in CM BL23 via LC-MS/MS analysis

Molecular weight, MW; isoelectric point, pI; number of assigned peptides, n^{o} peptides; sequence coverage, SC.

Protein name	MW	pI	n^{o} peptides	SC (%)
60 kDa chaperonin	57.4	4.7	31	62.5
L-lactate dehydrogenase	35.5	5.1	15	41.1
Elongation factor Tu	43.4	4.8	13	33.7
Chaperone protein dnaK	66.3	4.7	7	14.0
Phosphoglycerate kinase	42.8	4.9	6	18.0
10 kDa chaperonin	10.0	4.8	5	71.0
Enolase 1	47.0	4.5	5	23.6
D-2-hydroxyisocaproate dehydrogenase	36.9	5.1	4	13.5
Elongation factor G	77.0	4.7	4	6.9
ATP synthase subunit beta	52.2	4.6	3	7.7
Probable ATP-dependent Clp protease ATP-binding subunit	77.4	5.5	3	5.0
Enolase	46.5	4.6	3	13.9
Glyceraldehyde-3-phosphate dehydrogenase	36.5	5.4	3	10.1
Glucose-6-phosphate isomerase	49.5	4.6	3	6.9
GMP synthase	57.8	5.2	3	8.7
30S ribosomal protein S2	30.2	5.1	3	20.2
Tryptophan synthase alpha chain	28.7	5.6	3	16.2
ATP synthase subunit alpha	56.1	4.8	2	3.5
Bacitracin synthetase 1	597.9	5.3	2	0.6
ATP-dependent Clp protease ATP-binding subunit clpL	77.8	4.7	2	3.6
Elongation factor Tu-A	44.0	5.2	2	14.0
2,3-bisphosphoglycerate-dependent phosphoglycerate mutase 2	26.1	4.8	2	9.6
Inosine-5'-monophosphate dehydrogenase	52.8	5.7	2	4.7
Protein recA	37.3	8.7	2	3.0

Protein name	MW	pI	n° peptides	SC (%)
50S ribosomal protein L2	30.2	11.0	2	14.0
DNA-directed RNA polymerase alpha chain	34.8	4.6	2	6.1
Threonyl-tRNA synthetase	73.7	5.0	2	3.2
Tryptophan synthase beta chain	43.6	5.0	2	10.8
Probable amino-acid ABC transporter extracellular-binding protein ytmK	30.2	9.4	2	13.3
Acetyl-coenzyme A carboxylase carboxyl transferase subunit alpha	36.5	5.7	1	4.9
Fructose-bisphosphate aldolase	31.0	6.5	1	4.5
ATP-dependent Clp protease ATP-binding subunit clpE	83.1	4.9	1	1.7
ATP-dependent Clp protease proteolytic subunit	21.6	4.8	1	6.1
GTP-dependent nucleic acid-binding protein engD	40.0	4.7	1	3.8
Glucosamine--fructose-6-phosphate aminotransferase	65.4	4.9	1	1.5
6-phosphofructokinase	34.0	5.0	1	5.6
Pyruvate kinase	52.0	5.9	1	1.9
S-adenosylmethionine synthetase	43.4	5.3	1	3.8
S-adenosyl-methyltransferase mraW	34.9	5.8	1	9.7
N-acetylmuramic acid 6-phosphate etherase 1	32.1	5.2	1	5.0
Adenylosuccinate lyase	49.3	5.4	1	4.2
30S ribosomal protein S10	11.7	10.1	1	13.7
30S ribosomal protein S4	23.7	10.8	1	3.4
30S ribosomal protein S7	17.8	10.6	1	9.0
30S ribosomal protein S9	14.1	11.8	1	7.7
Glutamyl-tRNA synthetase	56.1	5.2	1	6.3
Triosephosphate isomerase	27.0	4.5	1	10.3
UvrABC system protein B	76.9	5.4	1	2.5

Table 6-3: Sequence of prtP in Lactobacillus paracasei VSL#3

The nucleic acid sequence of L.p prtP has been deposited at GenBank database and can be retrieved by the following accession number: JQ625054.

```
0001    TGTCTTGGGT  AATGCTCCCT  TACCGGATGT  ATGTTGACCA  TTACGATCAG  TCGTATCATC
0061    CGTGCTCGTT  GACGTCGTTG  TGGCTGGCTT  TGTTGGGATA  TTACCGCCAG  AACTAGGCTG
0121    GCTGCCCTCA  TCGCCTTTGT  CCTTACCTGT  GTCGCCTGGA  GCGGGCGCCG  GGGTACCTTG
0181    ACCGCCGCCT  TTGTCGCTAG  TACCGGCTGT  ATCGTCGCCT  TTACCAGCTG  TCTTGGCCGC
0241    TTCAACGGCA  GCTGCCACCT  TCGTTTTCAG  ACTTTGCAAA  GCTTGTAAAT  GTGCAAGCTT
0301    GTCAGACGCA  TCAGCACTGG  CTTGACCAGA  CGTCAATGTG  TCAGCAATGT  CGGCATACCA
0361    AGTTTTGCCA  GTTGCAGCAT  CTTTAGCATT  GCCAACCTCA  GCCGGTGTTG  CCGCTTTAAT
0421    ACCCTCCGCA  AGTTTTGCTA  ATACTGCATC  AAGTACCTTG  GCAAGACTCG  CTTGAAGCTG
0481    GTCGGCCGTT  TGCGTGCCTG  CTTGAGCTTG  TGCCACTAGC  TCGTCTAACG  CTGCCGTAAA
0541    TGTTTTGCCA  GTGCTTGGAT  CAGTTTGATT  GCCCAAGTCC  GTTCCCAGCG  CAGCCTTCAC
0601    AGATTGCAAC  TGGTTAAGCA  GCGAAGTCTT  CTTATCTGCA  GGTAATTTGG  CAGCTAATTG
0661    ATTGATCGCA  GTTTGCAGGT  CTCGATTGGC  ACCAGTAAGT  GAATCAACTG  AGGCGTCCGT
0721    CTGATCAAGC  GCCGTTTGTG  CCTTCTGCGT  TGCTGCTGCT  AAAGCAATCG  TTGTGGCATC
0781    ATCATACTTA  CCGCTGGCAC  TTAGCGTTTT  GGCGGAAGCA  ATCAGATTAG  TCAGTGCCTG
0841    CTTAGCTGCC  TGCAATTGTG  CAGGATCATC  AGTCTTGATA  TTGGTCACAA  CATAGTCGAC
0901    GGCTGGTGAT  TCATTACCGT  ATAAATCAGT  CGACTTAAAC  TTGAAGGTGC  CATTTGCCGT
0961    GACCGTGACA  CCGGCTGCCG  GCACATCCTG  ATATGTCTTG  CCACCATCAG  CACTATACTG
1021    AACCGTTTCA  CCCGTTGCGG  CAGCGTTTGC  CGTCAGAGTC  ACCGTTTTGG  CTGGTTCAGT
1081    GGTACTTGGC  GTCACAGTTG  GTGCTGCCAG  TGTTTTCTTT  GGTTCGTAGT  AAACTGTGAT
1141    CTTCTTGGTC  GTGGTGTTGT  TTTCGCTATC  TGTAACAGCA  ACAGTCAGCA  CGTTTTTGCC
1201    TTCGAGCAAT  TTAACGGGCT  GATCAATAGC  CATATGACCC  GGTTTGCCGC  TATTGATGTT
1261    GATGTCTGCG  TATTGGCTGG  CGACATGACT  GCCGTTAATT  GCCAGACTCA  GATATTGTGC
1321    ATTGTCAGTG  GCCGTTCCAG  TAATCTGGAA  GTTCGGATCG  TTGGTATAAA  CCGGTGCATC
1381    TGTCGAGCTG  TCCAATGACA  ATGTTGGCGC  CACTGCATCC  AAAATGAAGG  TCAACGCTTC
1441    TTGGAAGGTC  TTGTTTTGAG  TGGTGTCACC  CACAACAACC  CCAAACGGTT  TTTGTCCAAG
1501    AGTACCTAAA  TCTAAAGTGA  AACTGAAAGT  CAGATCATCC  TTGATTGGAA  TTTGCTTACC
1561    ATCAACCTGC  AACGTTGTCG  TTGGGTGCTT  GACCTTACCA  GTAATCGTCG  CAATCCCGGT
1621    CTTAGGGTCA  TAGAACTTAG  CCGAGGTGGC  ATTGAATTCA  TTGGCACCAA  ATGTCACACC
1681    TTGATCGAAC  GTCACAGCAT  TCTTCAACAC  ATCAGGATCA  TAAGACGAGG  TAATCGTCTT
1741    TTGCTCCGTC  GTGGTGTTGC  CGTCTTCGTC  GGCGGCGGTC  ACCTTGATGG  TATTGCCACC
1801    ATAATTAACC  GGTACATCAA  CACTAAAGTG  ATGTTGTGCA  TCAAGTGCTG  CTACGGTGTC
1861    GCCAACATTA  ACTGTCTTGG  TGTCAGCACT  AACCGTCCCT  GTAACCTTGA  TGGTCGCTTC
1921    GGATGTCTGA  TCCGAGCCAT  TGTTGAATTT  CAAATCAGTA  AAGGTTGGCG  CCGTCAGCCG
1981    AACTTTGGTG  TCAAAATGTT  TAAGCAACTG  GGTGTGCGCC  TTATCGGCAT  ATAGATCCAC
2041    TTGCGCGGCG  TAATCAGCAT  TCGTGACCGC  CATTGAGGCA  GTGAAACTGT  TAGTCGCAGC
2101    ATCGTAGGTT  GTGTTCAAAT  CATGTTTCTT  TCCTTGTGCA  TCAGTGTAAG  TACCGTCAAC
2161    CGCTGCTGGA  TACGTTCCAC  TAAACGTATA  CGTCCCGCCA  CCTTGAGTAT  TGGCTTCGTA
2221    GCCAGTTGTG  TTACTTGAGA  TCTTGTCTGG  GATACCGCCG  CCGTTCACAA  TTAAATCAAA
2281    CGATGTAGAC  CCCGGCTTCT  GAACGCTGGC  ATCTTGATCA  GTGGCATTAG  ATGCATTATC
2341    AGTCAAGTAC  AGCTCAGCCG  AATTGTCGCC  ATTGCCAAGT  GCTTGGGCCT  GTTCATCAGA
2401    TAATGGCGTT  TCAATTTTGG  TGTACCCATC  AGCCGTTGTC  CCAGCATCGG  TAAAGGTAGC
2461    ATCAAGATTC  GTCACTTCAT  TAATTGCAGT  TTTAACGCTC  TTGGTGGCAT  CAAGACCACT
2521    CAAATCATCC  TTGGCTTCAG  CTGTCAAATA  ATACTGGGTT  TTCCCATTTT  CCGTTTTGGC
2581    TGACAAAGCG  ACATGACGAA  CTGTCGGCGC  CTTAGAGTCG  AGCTTGAAAG  GCACATCAAA
2641    CACTTGACGT  TTGTCGCCGC  CTTCCGGTAC  ACCGGAAATA  CGATAAGTAT  AACTGCCATC
2701    ATCAGCCGTC  TTGATGTTGC  CATCACGTTG  ATCATAATAG  GTGCCATCCC  ACGCTGGAGC
2761    ATTGTAGTAG  ATGTACTGCT  GCGAATGAGC  ATCATAATAG  GTCTTCGTCT  GATTGGTGGA
2821    ACTGCTGAGA  GTCGTAACTT  TATTGCCCTG  ACCATCAAGA  ATATCAACTT  GGACGTTGCT
2881    GATATTGCGC  AATAGATAAT  ACTGCATGCT  GATGTCATTA  TATAAGGCAT  TCTTGTCACT
2941    CGAAAAAGCA  ATCGCCTGAT  CATCAACTGT  CTGGTTGCCA  TCAGCGTCTG  TGACCATGCC
3001    GCCATAATAT  TGATTGCCTG  TATTTTTGTT  CGTCAATAGT  GGCACGGTGC  CATAATTACC
3061    ACCAGCAGGA  CTATAAGTGA  TCCCATTGAG  ACTATCGACA  ATCTTACCGT  CATTCCAGTC
3121    ACCAAAGAAG  CCCATGTATG  GCAAGTTCAA  GCGCGATCCA  TCGCTACCCT  TAAAGTTCAG
3181    AAAACCTTCA  ACAAATTGCT  GTTGGTCAAA  AGACTTCGGC  AAAGATAGTG  TGAATTCAAT
3241    CTGCGCCGTT  TTCCCAGCAG  GCACAGTTAT  GTCACTGCCG  GCTTTAATGG  CTGCTCCATC
3301    AATCTTCTTG  TCATACAAAA  CCCCAGAATT  AGGGTCAGTC  GCTGATGTAT  AAACGGCATT
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3361   AGTATCCGTA  TTACTGTCCA  TTTGATAGGT  TAGTTCATGG  GTCGTGCGGT  TCGTGAAGGT
3421   CAGTTTAAAG  GTCTTGTCCG  TACTCGTGAA  GTCTTTCAAT  TCAACTGCCG  GGTAGCCGTT
3481   TTCGGAGACA  ACCGTTGACG  GATTCTTTTC  TAATGCATCA  ATGGCTGCCT  TCACATCAAC
3541   AAGACCGGCA  CCTTGCCGCC  GCGGCGATAC  GATAACATTG  TTGTAGTTAA  TATCGTTGAT
3601   TGGCTGGGCA  GTATTCATCT  CAACTGTCTT  AAGAAAATCG  GTGAGCGCTG  TCCCTTTAAG
3661   TTGTTTGTAG  TCAGCATAAA  ATGGGTTGTT  TTTGTTATTC  AACGCTTGTT  TCAACAATGC
3721   TTGTGAACCG  GCAATAAATG  GCGAGGCCAT  TGACGTACCA  GACATATTTG  TGTAGCCATT
3781   GTTGTTTTGC  GTTGACCAGA  TGTTGCCGCC  TGGTGCGGTA  ATATCTGGTT  TGAAGGAAAG
3841   ATTGGAAACT  GGCCCATAGG  ATGTGAAGTC  AGACATCTTG  TCTTCAGTAT  ATTTCTGATT
3901   TGGTAACAGC  GTCAGGGCAA  TCTTGACACC  GAGACTATCA  TCCGGGTGTG  CTGTGACCCA
3961   GTCAACCAGC  TTTTGACCGG  TTACACTGGA  GAGCACAAAT  GTTGGGAAGG  TGGTGGTTAA
4021   CGCAATAGAA  GTCAGCGGTG  TTGCTGTGCC  ATCGTTGTTG  ACAATGATCA  AGCCAGCAGC
4081   ACCAGCGGCT  TGGGCGTATT  TTTGTTTGTC  AGCAAAGGTA  AGTTCGCCAC  GTTTAACGAT
4141   GGCAATTTTG  CCTTTAGCGT  CAGCAGTATA  GTCGGTTGCT  GCCCCTTTGC  TGAGGCTGCC
4201   ACTAGCATCT  TTGACAACAT  AAAACTTCTT  TTGGTCAAAG  CTACCAGTGA  AATCGTTGCT
4261   TGAAAGCTGA  ATGGTTTCCG  GTCCAAGCTG  TAAACCTGTA  CCATCTGTAA  TGGTCACTGC
4321   CTGACTGATG  ACATCCGTGT  TCTCAGCGGA  AGCAACTGTG  GTCGCTCCTC  GTGATGTCCC
4381   TGGCGTTCCC  ACCATTTCAT  TGTCTTGCAA  ACCGTAATAA  TCCTTGTTGA  CACCTTCAGT
4441   TGCTGAACCG  GATGTTCCTG  AGTTCCCAGC  AGAAATGACG  GCGGCTGTTC  CTGATTCGTT
4501   AGCATTTTGC  ACCGCAGCAA  TTTCTGGATC  CTCCAAGGTT  TGGTTGCCTG  AATCAGATCC
4561   TAAGGACATG  TTGAGGACAT  CGGCACCGAT  TTTTGCCGAG  TCTTCAATGG  CAGAAACCAA
4621   GGTAGCTGAC  CCGGTTGTTG  CAGAAGTGTC  AGAGTTGGTG  AAAACTTTCA  TTGCCAGTAG
4681   CTGTGCTTCT  GGCGCAACTC  CGACAACAGA  CTTGGTTGGA  TCGTCACCTG  TCCCGTTAGC
4741   ACCGATGATC  CCAGCAACAT  GCATGCCGTG  TTGTTCGTCA  ACCGTATCAT  CTGTAATGGT
4801   GTCGTTATTA  TCAGCGTAAT  TAAACCCATA  TGGCACTTTT  GAAGTAAAAT  AGCGGCCATG
4861   CTTGGCGGTA  TCAGTGAATC  TTTCAACATC  AGATTTGGTT  AATTTGACGT  CTTTATCATC
4921   GCTTAGCCGC  ATGTCTTTAT  GTGTTGGATC  AATGCCAGTG  TCAATAACCG  AGACAACTGT
4981   GCCTTCACCT  TTATATTTGT  AATTGGACCA  TACGGCCTGC  ACATTCGCCA  TCGAGTTAGC
5041   CTTAGCATCA  GTCGGATAGT  AAACTTTCGC  CAATGTGACT  GTTTTAACTC  CGGCAATTTG
5101   TTTCAGTTTA  GGGATATCAA  CTACCCTAAC  TTTAGTTGAA  AAGCCGTTAA  CGACATAGCC
5161   ATAACTTTCA  CCGGCAGTTT  GTTGGGTGAC  TTGTTCAACA  GCTGCCTTAA  CGCTTGCCTG
5221   AGCCGCGATC  ACTTTATTGG  TCTCCTGCTG  AATCTCCGCC  GTGCTGGAGT  AATCAGTTCT
5281   TATGCCGTTT  TCAGAGGCAG  GCGCTGCGCT  CATTTGAACT  ATGACGTCAA  CATAAATATC
5341   TTGCTGCTGA  ACTTTATTCA  GCTTATTGTA  ATCAATACCT  TTAGCCGCCA  ACTGTGTAGC
5401   AATCGCTTGA  TTCGTTGTCG  CTGCGGTTGT  GTCAGTGGCC  GCTTGCTTAG  CAGTCGCGGC
5461   CTTAACCGTA  TTTGCGAGTG  ATGATCCCTT  AGTTTGCTGC  GAGATAGC
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Table 6-4: Sequence of prtP in the human fecal isolate L.c BFLM 218.

The nucleic acid sequence of L.c prtP has been deposited at GenBank database and can be retrieved by the following accession number: JQ625055.

```
0001   TGTCTTGGGT AATGCTCCCT TACCGGATGT ATGTTGACCA TTACGATCAG TCGTATCATC
0061   CGTGCTCGTT GACGTCGTTG TGGCTGGCTT TGTTGGGATA TTACCGCCAG AACTAGGCTG
0121   GCTGCCCTCA TCGCCTTTGT TCTTACCTGT GTCGCCTGGA GCGGGCGCCG GGGTACCTTG
0181   ACCGCCGCCT TTGTCGCTAG TACCGCTTGT ATCGTCGCCT TTACCAGCTG TCTTGGCCGC
0241   TTCAACGGCA GCTGCCACCT TCGTTTTCAG ACTTTGCAAA GCTTGTAAAT GTGCAAGCTT
0301   GTCAGACGCA TCAGCACTGG CTTGACCAGA CGTCAATGTG TCAGCAATGT CGGCATACCA
0361   AGTTTTGCCA GTTGCAGCAT CTTTAGCATT GCCAACCTCA GCCGGTGTTG CCGCTTTAAT
0421   ACCCTCCGCA AGTTTTGCTA ATACTGCATC AAGTACCTTG GCAAGACTCG CTTGAAGCTG
0481   GTCGGCCGTT TGCGTGCCTG CTTGAGCTTG TGCCACTAGA TCGTCTAACG CTGCCATAAA
0541   TGTTTTGCCA GTGCTTGGAT CAGTTTGATT GCCCAAGTCC GTTCCCAGCG CAGCCTTCAC
0601   AGATTGCAAC TGGTTAAGCA GCGAAGTCTT CTTATCTGCA GGTAATTTGG CAGCTAATTG
0661   ATTGATCGCA GTTTGCAGGT CTCGATTGGC ACCAGTAAGT GAATCAACTG AGGCGTCCGT
0721   CTGATCAAGC GCCGTTTGTG CCTTCTGCGT TGCTGCTGCT AAAGCAGTCG TTGTGGCATC
0781   ATCATACTTA CCGCTGGCAC TTAGCGTTTT GGCGGAAGCA ATCAGATTAG TCAGTGCCTG
0841   CTTAGCTGCC TGCAATTGTG CAGGATCATC AGTCTTGATA TTGGTCACAA CATAGTCGAC
0901   GGCTGGTGAT TCATTACCGT ATAAATCAGT CGACTTAAAC TTGAAGGTGC CATTTGCCGT
0961   GACCGTGACA CCGGCTGCCG GCACATCCTG ATATGTCTTG CCACCATCAG CACTATACTG
1021   AACCGTTTCA CCCGTTGCGG CAGCGTTTGC CGTCAGAGTC ACCGTTTTGG CTGGTTCAGT
1081   GGTACTTGGC GTCACAGTTG GTGCTGCCAG TGTTTTCTTT GGTTCGTAGT AAACTGTGAT
1141   CTTCTTGGTC GTGGTGTTGT TTTCGCTATC TGTAACAGCA ACAGTCAGCA CGTTTTTGCC
1201   TTCGAGCAAT TTAACGGGCT GATCAATAGC CATATGACCC GGTTTGCCGC TATTGATGTT
1261   GATGTCTGCG TATTGGCTGG CGACATGACT GCCGTTAATT GCCAGACTCA GATATTGTGC
1321   ATTGTCAGTG GCCGTTCCGG TAATCTGGAA GTTCGGATCG TTGGTATAAA CCGGTGCATC
1381   TGTCGAGCTG TCCAATGACA ATGTTGGCGC CACTGCATCC AAAATGAAGG TCAACGCTTC
1441   TTGGAAGGTC TTGTTTTGAG TGGTGTCACC CACAACAACC CCAAACGGTT TTTGTCCAAG
1501   AGTACCTAAA TCTAAAGTGA AACTGAAAGT CAGATCATCC TTGATTGGAA TTTGCTTACC
1561   ATCAACCTGC AACGTTGTCG TTGGGTGCTT GACCTTACCA GTAATCGTCG CAATCCCGGT
1621   CTTAGGGTCA TAGAACTTAG CCGAGGTGGC ATTGAATTCA TTGGCACCAA ATGTCACACC
1681   TTGATCGAAC GTCACAGCAT TCTTCAACAC ATCAGGATCA TAAGACGAGG TAATCGTCTT
1741   TTGCTCCGTC GTGGTGTTGC CGTCTTCGTC GGTGGCGGTC ACCTTGATGG TATTGCCACC
1801   ATAATTAACC GGTACATCAA CACTAAAGTG ATGTTGTGCA TCAAGTGCTG CTACGGTGTC
1861   GCCAACATTA ACTGTCTTGG TGTCAGAACT AACCGTCCCT GTAACCTTGA TGGTCGCTTC
1921   GGAGGTCTGA TCCGAGCCAT TGTTGAATTT CAAATCAGTA AAGGTTGGCG CCGTCAGCCG
1981   AACTTTGGTG TCAAAATGTT TAAGCAACTG GGTGTGCGCC TTATCGGCAT ATAGATCCAC
2041   TTGCGCGGCG TAATCAGCAT TCGTGACCGC CATTGAGGCA GTGAAACTGT TAGTCGCAGC
2101   ATCGTAGGTT GTGTTCAAAT CATGTTTCTT TCCTTGTGCA TCAGTGTAAG TACCGTCAAC
2161   CGCTGCTGGA TACGTTCCAC TAAACGTATA CGTCCCGCCA CCTTGAGTAT TGGCTTCATA
2221   GCCAGTTGTG GTACTTGAGA TCTTGTCTGG GATACCGCCG CCGTTCACAA TTAAATCAAA
2281   CGATGTAGAC CCCGGCTTCT GAACGCTGGC ATCTTGATCA GTGGCATTGG ATGCATTATC
2341   AGTCAAGTAC AGCTCAGCCG AATTGTCGCC ATTGCCAAGT GCTTGGGCCT GTTCATCAGA
2401   TAATGGCGTT TCAATTTTGG TGTACCCATC AGCCGTTGTC CCAGCATCGG TAAAGGTAGC
2461   ATCAAGATTC GTCACTTCAT TAATTGCAGT TTTAACGCTC TTGGTGGCAT CAAGACCACT
2521   CAAATCATCC TTGGCTTCAG CTGTCAAATA ATACTGGGTT TTCCCATTTT CCGTTTTGGC
2581   TGACAAAGCG ACATGACGAA CTGTCGGCGC CTTAGAGTCG AGCTTGAAAG GCACATCAAA
2641   CACTTGACGT TTGTCGCCGC CTTCCGGTAC ACCGGAAATA CGATAAGTAT AACTGCCATC
2701   ATCAGCCGTC TTGATGTTGC CATCACGTTG ATCATAATAG GTGCCATCCC ACGCTGGAGC
2761   ATTGTAGTAG ATGTACTTCT GCGAATGAGC ATCATAATAG GTCTTCGTCT GATTGGTGGA
2821   ACTGCTGAGA GTCGTAACTT TATTGCCCTG ACCATCAAGA ATATCAACTT GGACGTTGCT
2881   GATATTGCGC AATAGATAAT ACTGCATGCT GATGTCATTA TATAAGGCAT TCTTGTCACT
2941   CGAAAAAGCA ATCGCCTGAT CGTCAACTGT CTGTTTGCCA TCAGCGTCTG TGACCATGCC
3001   GCCATAATAT TGATGACCTG TATTTTTGTT CGTCAATAGT GGCACGGTGC CATAATTACC
3061   ACCAGCAGGA CTATAAGTGA TCCCATTGAG ACTATCGACA ATCTTACCGT CATTCCAGTC
3121   ACCAAAAAAG CCCATGTATG GCAAGTTCAA GCGCGACCCA TCACTCCCCT TAAAGTTCAG
3181   AAAACCTTCA ACAAATTGCT GTTGGTCAAA AGACTTCGGC AAAGATAGTG TGAATTCAAT
3241   CTGCGCCGTT TTCCCAGCAG GCACAGTTAT GTCACTGCCG GCTTTAATGG CTGCTCCATC
3301   AATCTTCTTG TCATACAAAA CCCCAGAATT AGGGTCAGTC GCTGATGTAT AAACGGCATT
3361   AGTATCCGTA TTACTGTCCA TTTGATAGGT TAGTTCATGG GTCGTGCGGT TCGTGAAGGT
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3421   CAGTTTAAAG GTCTTGTCCG TACTCGTGAA GTCTTTCAAT TCAACTGCCG GGTAGCCGTT
3481   TTCGGCGACA ACCGTTGACG GATTCTTTTC TAATGCATCA ATGGCTGCCT TCACATCAAC
3541   CAGACCGGCA CCTTGCCGCC GCGGCGATAC GATAACATTG TTGTAGTTAA TATCGTTGAT
3601   TGGCTGGGCA GTATTCATCT CAACTGTCTT AAGAAAATCG GTGAGCGCTG TCCCTTTAAG
3661   TTGTTTGTAG TCAGCATAAA ATGGGTTGTT TTTGTTATTC AACGCTTGTT TCAACAATGC
3721   TTGTGAACCG GCAATAAATG GCGAGGCCAT TGACGTACCA GACATATTTG TGTAGCCATT
3781   GTTGTTTTGC GTTGACCAGA TGTTGCCGCC TGGTGCGGTA ATATCTGGTT TGAAGGAAAG
3841   ATTGGAAACT GGCCCATAGG ATGTGAAGTC AGACATCTTG TCTTCAGTAT ATTTCTGATT
3901   TGGTAACAGC GTCAGGGCAA TCTTGACACC GAGACTATCA TCCGGGTGTG CTGTGACCCA
3961   GTCAACCAGC TTTTGACCGG TTTTACTGGA GAGCCCAAAT GTTGGGAAGG TGGTGGTTAA
4021   CGTAATAGAA GTCAACGGTG TTGCTGTGCC ATCGTTGTTG ACAATGATCA AGCCAGCAGC
4081   ACCAGCGGCT TGGGCGTATT TTTGTTTGTC AGCAAAGGTA AGTTCGCCAC GTTTAACGAT
4141   GGCAATTTTG CCTTTAGCGT CAGCAGTATA GTCGGCTGCT GCCCCTTTGC TGAGGTCGCC
4201   ACTAGCATCT TTGACAACAT AAAACTTCTT TTGGTCAAAG CTACCAGTGA AATCGTTGCT
4261   TGAAAGCTGA ATGGTTTCCG GTCCAAGCTG TAAACCTGTA CCATCTGTAA TGGTCACTGC
4321   CTGACTGATG ACATCCGTGT TCTCAGCGGA AGCAACTGTG GTCGCTCCTC GTGATGTCCC
4381   TGGCGTTCCC ACCATTTCAT TGTCTTGCAA ACCGTAATAA TCTTTGTTGA CACCTTGAGT
4441   TGCTGAACCG GATGTTCCTG AGTTCCCAGC AGAAATGACG GCGGCTGTTC CTGATTCGTT
4501   AGCATTTTGC ACCGCAGCAA TTTCTGGATC CTCCAAGGTT TGGTTACCTG AATCAGATCC
4561   TAAGGACATG TTGAGGACAT CGGCACCGAT TTTTGCCGAG TCTTCAATGG CAGAAACCAA
4621   GGTAGCTGAC CCGGTTGTTG CAGAAGTGTC AGAGTTGGTG AAAACTTTCA TTGCCAGTAG
4681   CTGTGCTTCT GGCGCAACTC CGACAACAGA CTTGGTTGGA TCGTCACCTG TCCCGTTAGC
4741   ACCGATGATC CCAGCAACAT GCATGCCGTG TTGTTCGTCA ACCGTATCAT CTGTAATGGT
4801   GTCGTTATTA TCAGCGTAAT TAAACCCATA TGGCACTTTT GAAGTAAAAT AGCGGCCATG
4861   CTTGGCGGTA TCAGTGAATC TTTCAACATC AGATTTGGTT AGTTTGACGT CTTTATCATC
4921   GCTTAGCCGC ATGTCTTTAT GTGTTGGGTC AATGCCAGTG TCAATAACCG AGACAACTGT
4981   GCCTTCACCT TTATATTTGT AATTGGACCA TACGGCTTGC ACATTCGCCA TCGAGTTGGC
5041   CTTAGCATCA GTCGGATAAT AAACTTTCGC CAATGTGACT GTTTTAACTC CGGCAATTTG
5101   TTTCAGTTTA GGGATATCAA CTACCCTAAC TTTAGTTGAA AAGCCGTTAA CGACATAGCC
5161   ATAACTTTCA CCGGCAGTTT GTTGGGTGAC TTGTTCAACA GCTGCCTTAA CGCTTGCCTG
5221   AGCCGCGATC ACTTTATTGG TTTCCTGCTG AATCTCCGCC GTGCTGGAGT AATCAGTTCT
5281   TAAAGTGCCG TTTTCAGAGG CAGGCGCTGC GCTCATTTGA ACTATGACGT CAACATAAGT
5341   ATCTTGCTGC TGAACTTTAT TCAGCTTATT GTAATCAATA CCTTTAGCCG CCAACTGTGT
5401   GGCAATCGCT TGATTCGTTG TCGCTGCGGT TGTGTCAGTG GCCGCTTGCT TAGCAGTCGT
5461   GGCCTTAACC GTATTTGCGA GTGATGATCC TTTAGTTTGC TGCGAGATAG C
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7 LISTS OF FIGURES & TABLES

7.1 List of Figures

7.2 List of Tables

8 ABBREVIATIONS

5-ASA	5-aminosalicylic acid
Active	IP-10 degrading strain
AKT	protein kinase B
APC	antigen presenting cell
ARE	adenosine uracil rich region
B….	*Bifidobacterium …..*
BRAK	breast and kidney-expressed chemokine
dH_2O	deionized water
CARD	caspase activating receptor domain
CEP	cell envelope protease
CD	Crohn´s Disease
CD4	cluster of differentiation 4
CD3	cluster of differentiation 3
Cfu	colony forming units
CM	conditioned media
Cse L.p	cell surface extracts of *Lactobacillus paracasei* (VSL#3)
DC	dendritic cell
DSS	dextran sodium sulphate
DAMP	damage associated microbial patter
E.coli	*Escherichia coli*
ELISA	enzyme linked immunosorbent assy
GALT	gut associated lymphoid tissue
GAS	group A streptococcus
H&E	hematoxylin&eosin
i.p	intraperitoneal/ly
IBD	inflammatory bowel disease
IBS	inflammatory bowel syndrome
IEC	intestinal epithelial cells
IEL	intraepithelial lymphocytes
IFNγ	interferon γ
Ig	immunglobulin
IκB	inhibitor κB
IKK	inhibitor κB kinase complex
$IL10^{-/-}$	interleukin 10 knockout
Inactive strain	strain that fails to degrade IP-10
IP-10	interferon inducible protein 10
IL	interleukin
I-TAC	interferon-inducible T cell alpha chemoattractant

LC-MS/MS	liquid chromatography mass spectrometry mass spectrometry
L. …	*Lactobacillus* ….
L.p	*L. paracasei* (VSL#3)
L.c	*L. casei* BFLM 218
L.c prtP^{dis}	*L. casei* BLFM 218 isogenic prtP disruption mutant
LGG	*Lactobacillus rhamnosus* GG
L. lactis	*Lactococcus lactis*
MAMP	microbe associated molecular pattern
MAPK	mitogen activated protein kinase
Marim.	Marimastat
MHC	major histocompatibility complex
MIG	monokine induced by interferon □
MIP	macrophage inflammatory protein 2
NaF	sodium fluorescein
n.d.	not detectable
NFκB	nuclear factor κB
NOD	nucleotide binding oligomerisation domain
o.n.	over night
PAMP	pathogen associated molecular pattern
PI3P/Akt	phosphatidylinositol 3-kinase
PCR	polymerase chain reaction
PPARγ	peroxisome proliferator activated receptor γ
PRR	pattern recognition receptor
RT	room temperature
SAA	serum amyloid A
SC	sequence coverage
SCID	severe combined immunodeficiency
SDF-1	stromal derived factor 1
SDS	sodium dodecyl sulfate
SPF	specific pathogen free
STAT	signal transducer and activator of transcription
S.…	*Salmonella*
TBST	Tris-buffered saline tween
TCR	T cell receptor
TEAB	Tetraethylammonium bromide
TECK	thymocyte expressed chemokine
TER	transepithelial electrical resistance
TGFβ	transforming growth factor β
Th	T helper cell
TNBS	trinitrobenzene sulfonic acid
TNF	tumor necrosis factor

TLR	toll like receptor
Treg	T regulatory cell
UC	ulcerative colitis
WB	Western Blot
wt	wildtype
ZO	zonula occludin

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PUBLICATIONS & PRESENTATIONS

International peer-reviewed original manuscript and national publication

Lactocepin Secreted by Lactobacillus Exerts Anti-Inflammatory Effects By Selectively Degrading Pro-inflammatory Chemokines
von Schillde MA*, Hörmannsperger G*, Weiher M, Alpert CA, Hahne H, Bäuerl C, van Huynegem K, Steidler L, Hrncir T, Pérez-Martínez G, Kuster B and Haller D
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*These authors contributed equally to the work.

Probiotika: Quo vadis…?
von Schillde MA and Haller D
Pharmazie in Unserer Zeit 2012;41:149-153.

Published Abstracts

Probiotic-Derived Lactocepin Degrades the Pro-Inflammatory Chemokine IP-10: Impact on Chronic Intestinal Inflammation
von Schillde MA, Hörmannsperger G, Weiher M, Alpert CA, Hahne H, Bäuerl C, Blaut M and Haller D.
Gastroenterology, Volume 138, Issue 5, Supplement 1, S-615-S-616, May 2010

Structure Function Analysis of the Bacterial Protease Lactocepin in the Context of Probiotic Mechanisms
von Schillde MA, Hörmannsperger G, Weiher M, Alpert CA, Hahne H, Bäuerl C, Monedero V, Pérez-Martínez and Haller D.
Int J Med Microbiol 2011; Volume: 301, p. 1-126
Volume 301, Supplement 1, pp. 1-126, September 2011

Oral presentations

Probiotic Structure Function Analysis Reveals Prtp-Encoded Lactocepin to Mediate Anti-Inflammatory Effects via Selective Degradation of Pro-Inflammatory Chemokines
von Schillde MA, Hörmannsperger G, Weiher M, Alpert CA, Hahne H, Bäuerl C, van Huynegem K, Steidler L, Pérez-Martínez G, Kuster B and Haller D.
Accepted for oral presentation at the upcoming Digestive Disease Week 2012 (DDW), May 18-22 2012, San Diego, USA.

Structure Function Analysis of the Bacterial Protease Lactocepin in the Context of Probiotic Mechanisms
von Schillde MA, Hörmannsperger G, Weiher M, Alpert CA, Hahne H, Bäuerl C, Monedero V, Pérez-Martínez and Haller D.
63rd Annual meeting of the German society of Hygiene and Microbiology (DGHM), September 25-28, Essen, Germany

Probiotic-derived Protease Lactocepin Degrades the Pro-inflammatory Chemokine IP-10: Impact on Chronic Intestinal Inflammation
von Schillde MA, Hörmannsperger G, Weiher M, Alpert CA, Hahne H, Bäuerl C, Monedero V, Pérez-Martínez and Haller D.
15th International Congress of Mucosal Immunology (ICMI), July 5-9, 2011, Paris, France

Probiotic intervention in IBD: Probiotic function of the protease lactocepin
von Schillde MA, Hörmannsperger G, Weiher M, Alpert CA, Hahne H, Bäuerl C, Monedero V, Pérez-Martínez and Haller D.
3rd Annual meeting of the research project IPODD (intestinal proteases: opportunity for drug discovery), May 12-15 2011, Rome, Italy

Probiotic-derived Lactocepin degrades the pro-inflammatory chemokine IP-10: impact on chronic intestinal inflammation
von Schillde MA, Hörmannsperger G, Weiher M, Alpert CA, Hahne H, Bäuerl C, Monedero V, Pérez-Martínez and Haller D.
EMIG, September 29 – October 2 2010, Amsterdam, The Netherlands

Probiotic-derived Protease Lactocepin Degrades the Pro-Inflammatory Chemokine IP-10: Impact on Chronic Intestinal Inflammation
von Schillde MA, Hörmannsperger G, Weiher M, Alpert CA, Hahne H, Bäuerl C and Haller D.
2nd Annual meeting of the research project IPODD (intestinal proteases: opportunity for drug discovery), July 6-9 2010, Tübingen, Germany

Bacteria-derived protease and protease-inhibitor activity under chronic intestinal inflammation: two probiotic approaches
von Schillde MA, Hörmannsperger G, Weiher M, Alpert CA, Bäuerl C, Blaut M and Haller D.
1st Annual meeting of the research project IPODD (intestinal proteases: opportunity for drug discovery), July 15-18 2009, Prague, Czech Republic

Poster presentations

Probiotic Structure Function Analysis Reveals PrtP-Encoded Lactocepin to Mediate Anti-Inflammatory Effects via Selective Degradation of Pro-inflammatory Chemokines
von Schillde MA, Hörmannsperger G, Alpert CA, Hahne H, Bäuerl C, and Haller D.
49. Wissenschaftlicher Kongress der Deutschen Gesellschaft für Ernährung (German Nutrition Society), March 14-16, 2012, Freising-Weihenstephan, Germany

Probiotic-Derived Protease Lactocepin Degrades the Pro-Inflammatory Chemokine IP-10: Impact on Chronic Intestinal Inflammation
von Schillde MA, Hörmannsperger G, Weiher M, Alpert CA, Hahne H, Bäuerl C, Monedero V, Pérez-Martínez G and Haller D.
4th Seeon Conference of the section “Microbiota, Probiota and Host“ (German Society for Hygiene and Microbiology, DGHM), April 15-17 2011, Seeon, Germany

Probiotic-Derived Lactocepin Degrades the Pro-Inflammatory Chemokine IP-10: Impact on Chronic Intestinal Inflammation
von Schillde MA, Hörmannsperger G, Alpert CA, Hahne H, Bäuerl C, Blaut M and Haller D.
Digestive Disease Week (DDW) May 1-5, 2010, New Orleans, USA

VSL#3-derived *L. casei* induces post-translational degradation of IP-10 Protein in Intestinal Epithelial Cells: Impact on Chronic Inflammation
Hörmannsperger G, **von Schillde MA**, Clavel T, Hoffmann M, Reiff C, Kelly D, Loh G, Blaut M, Hölzlwimmer G, Laschinger M and Haller D
2nd Seeon Conference (German Society for Hygiene and Mikrobiology, DGHM), April 23-25, 2009, Seeon, Germany

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ERKLÄRUNG

Hiermit erkläre ich, dass ich die vorliegende Doktorarbeit selbständig angefertigt habe. Es wurden nur die in der Arbeit ausdrücklich benannten Quellen und Hilfsmittel benutzt. Wörtlich oder sinngemäß übernommenes Gedankengut habe ich als solches kenntlich gemacht.

Freising, den 17.4.2012