

Inflamed gut mucosa: downstream of interleukin-10

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ABSTRACT

Background Interleukin-10 is a pleiotropic cytokine, whose main function is limitation and ultimately termination of immune responses. This is especially true for environmental interfaces such as the gastrointestinal tract. IL-10 acts as a key mediator for maintaining gut homeostasis. IL-10 knockout mice are well established as a genetic model for inflammatory bowel disease (IBD), and sequence variants in the IL-10 locus contribute to ulcerative colitis (UC).

Design This review covers the significance of IL-10 signalling in the intestinal immune response both in health and disease. It explains the biological role of IL-10, its deregulation in IBD and its contribution to intestinal inflammation via endoplasmic reticulum stress response.

Results Many IBD susceptibility genes have been discovered in the past years, linking fundamental biological systems, like innate and adaptive immunity, stress responses, autophagy and mucosal barrier to the pathogenesis of Crohn's disease (CD) and UC. IL-10 has long been known for its substantial role in regulating gut immunity, but its contribution to IBD was somewhat elusive. A recent study identified mutations in either IL-10 receptor subunits that are associated with early-onset enterocolitis, a severe phenotype of IBD. Other than genetic variants of IL-10 receptors, IL-10 and STAT3 genes are also associated with IBD, emphasizing the involvement of the IL-10 signalling cascade in the pathogenesis of CD and UC.

Conclusions The discovery of inherited deregulations in the IL-10 signalling cascade is not only considered the missing link between IL-10 and intestinal homeostasis, but also demonstrates how findings made in animal models help explaining human disease.

Keywords Inflammatory bowel disease, interleukin-10, interleukin-10 receptor.

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Inflammatory bowel disease

Inflammatory bowel diseases (IBD) are chronic immune-mediated intestinal disorders. Both subtypes, Crohn's disease (CD) and ulcerative colitis (UC), are considered to arise as a consequence of an aberrant intestinal immune response in genetically predisposed individuals [1]. UC is strictly mucosal, less penetrative and spreads continuously from the rectum to more proximal regions of the colon. UC carries features of autoimmunity against colonic epithelial cells or the commensal microbiota. In contrast, CD is more complicated with transmural and discontinuous inflammation that can virtually affect any part of the gastrointestinal tract, but is most often found in the ileocecal segment [2]. CD is rather considered as barrier dysfunction with enhanced intestinal permeability and bacterial translocation. Approximately, one-third of all patients with IBD show

extraintestinal manifestations that may affect the skin (erythema nodosum, pyoderma gangrenosum), joints (peripheral arthritis, ankylosing spondylitis), eyes (uveitis), hepatobiliary tract (hepatic steatosis, primary sclerosing cholangitis), bones (osteoporosis), and patients are at enhanced risk for arterial and venous thrombosis [3]. IBD is a multifactorial disorder, and both genetic as well as environmental factors trigger alterations in intestinal microbiota and drive the host immune response.

Genetic aspects of IBD

A well-known effect indicating genetic influence is familial clustering. IBD occurs more frequently among members of the same family, and the concordance rate between identical twins is 50% for CD and 15–20% for UC. Compared with CD, the inheritable component is apparently weaker for UC, but both

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disease phenotypes seem to share a group of susceptibility genes [4]. Several genome-wide association studies have been performed to date and have identified risk loci for IBD as seen in Table 1. The risk loci involved in sensing and handling microbiota in the gut (NOD2) and genes involved in autophagy (ATG16L1 and IRGM) are only associated with CD [5]. On the other hand, extracellular matrix protein 1 (ECM1) and other genes involved in mucosal barrier function (e.g. HNF4 α , CDH1, LAMB1), as well as IL-10, are risk factors solely found in patients with UC [6]. Deregulations in the adaptive immune response, especially in the IL-23/Th17 axis (IL23R, IL12B, STAT3), confer a common risk for IBD [6].

Environmental and lifestyle aspects of IBD

There is strong evidence linking modern Western lifestyle to the epidemiology of CD and UC. Cigarette smoking provokes

Table 1 Genetic mutations in inflammatory bowel disease

Gene	Locus	Function	CD	UC
IL23R	1p31	IL-23 mediates the formation of Th17 cells	✓	✓
IL12B	5q33	Subunit of IL-12 and IL-23, Th1 polarization	✓	✓
MHC	6p21	Strongest association with HLA DRB1*0103 allele	✓	✓
JAK2	9p24	Tyrosine kinase involved in IL-23 signalling pathway	✓	✓
ORMDL3	17q21	Part of the unfolded protein response	✓	✓
NOD2	16q21	Intracellular pattern recognition receptor	✓	
ATG16L1	2q37	Required for autophagy as part of a protein complex	✓	
IRGM	5q33	Control of intracellular pathogens via autophagy	✓	
PTGER4	5p13	Receptor for prostaglandin E2	✓	
IL10	1q32	Anti-inflammatory cytokine		✓
ECM1	1q21	Involved in the intestinal barrier		✓
HNF4A	20q13	Increased epithelial permeability in knockout mice		✓
CDH1	16q22	E-cadherin part of adherens junction and Wnt-signalling		✓
LAMB1	7q31	Part of the basement membrane, cell adhesion		✓

CD, Crohn's disease; UC, ulcerative colitis.

divergent effects in patients with CD or UC. The percentage of actively smoking patients with UC (10–15%) is significantly lower than in the general population (25–40%), whereas a larger proportion of patients with CD are active smokers (45–55%) [7]. In fact, smoking may protect against UC but aggravates CD. The worldwide incidence of IBD is steadily on the incline and reaches areas where those diseases were considered extremely rare (such as the Asia-Pacific countries). Adaptation of Western lifestyle including nutritional changes and more frequent use of antibiotics is likely responsible for transformations within the gut microbiome. Also, modern dietary habits are variably ignored within the IBD pathogenesis though excluding the intestine from nutritional contact or switching the diet to medieval food quality improved mucosal healing in CD [8,9].

Gut microbiota in IBD

Evidence for alterations of the gut microbiota in IBD exists. It is currently unclear whether such alterations are cause or consequence of chronic intestinal inflammation. Dietary compounds and their products, the biggest source of intraluminal antigens in the gastrointestinal system, affect commensal bacteria. As shown in Table 1, multiple-risk loci for IBD play a role in bacterial sensing and thereby modulate the host immune response. Gut microbes are an essential trigger for inflammation in genetically predisposed individuals. Also, animal models of IBD do not develop enterocolitis when raised under germ-free conditions [10,11]. In line with these observations, certain patients benefit from antibiotic therapy against various anaerobic microorganisms (such as bacteroides).

Gut immune response

The molecular mechanisms underlying these distinct clinical and pathological characteristics of CD and UC are only partially understood. The gut as an environmental interface plays an important role in maintaining a delicate immune homeostasis. Food antigens, commensal bacteria, enterocytes, Paneth cells, goblet cells, immune cells and their products like cytokines and antibodies are all in close proximity at the mucosal barrier. An inadequate immune response (when encountering potential pathogens), or an overreacting immune system (against food antigens or parts of the commensal flora), poses potential threat to this homeostasis, resulting in disease manifestation. Therefore, multiple mechanisms evolved to co-ordinate homeostatic responses, and one such mechanism is cytokine signalling. Cytokines as polypeptide mediators permit communication between hematopoietic and nonhematopoietic cells and orchestrate immune responses to various stimuli. On the other hand, cytokines also play a substantial role in the pathogenesis of autoimmune diseases. The healthy

intestinal mucosa contains an array of cytokines secreted by immune cells – such as neutrophils, macrophages, dendritic cells (DCs), lymphocytes or mast cells – but also enterocytes. A balanced interplay between proinflammatory [e.g. tumour necrosis factor α (TNF- α), interleukin-1 β , 6, 12, 23] and anti-inflammatory mediators [e.g. transforming growth factor β (TGF- β), interleukin-10 (IL-10)] is important to prevent disease. Antibodies that prevent TNF- α from binding to its receptor (e.g. Infliximab) are one of the most effective drugs in IBD that may cause mucosal healing. Mice deficient in IL-10 production spontaneously develop enterocolitis, and the very same has been observed for infants with homozygous mutations in the IL-10 receptor genes [12,13]. Knowledge about the importance of IL-10 in the pathogenesis of CD and UC is continuously growing, and its appeal was resurrected with the discovery of disease causing mutations that disrupt IL-10 signalling. Hence, this review reflects on recent progress in our understanding of IL-10 signalling in IBD.

Interleukin-10

IL-10 was first identified as a cytokine, secreted by CD4⁺ Th2-cells, that inhibits cytokine production in antigen-presenting cells [14]. Owing to this observation, Fiorentino *et al.* proposed the name cytokine synthesis inhibitory factor. In the following years, a range of cytokines related to IL-10 were discovered, making IL-10 the founding member of the type II cytokine family that includes IL-19, IL-20, IL-22, IL-24, IL-26, IL-28 and IL-29 [15]. IL-10 has important effects on the intestinal immune system, depending on cell type, time and location of release, strength of signal and interaction with other cytokines. It modulates both innate and adaptive immune responses. The complexity of the immune response is well represented in the gastrointestinal tract, which is exposed to various microorganisms and xenobiotics.

IL-10 in intestinal innate immunity

IL-10 is expressed by many cells of the innate and adaptive immune system. Innate immunity triggers IL-10 expression in a toll-like receptor (TLR)-dependent and (TLR)-independent fashion. TLRs are a type of pattern recognition receptors (PRRs) that recognize particular molecular patterns from pathogens. Following TLR binding, the signal is transmitted via the activation of the extracellular signal-regulated protein kinase (ERK), p38-mitogen-activated protein kinase (p38-MAPK), phosphoinositide 3-kinase (PI3K) and nuclear factor- κ B (NF κ B). PI3K as well as p38-MAPK pathways also acts downstream of IL-10 receptor signalling [16,17]. All of these proteins mediate the expression of IL-10 in macrophages and DCs, which are a major source of IL-10.

Dendritic cells

Dendritic cells act at the interface of the innate and adaptive immune system by extending their processes into the intestinal lumen, sampling antigens and presenting them to T cells for their activation. This task is mainly performed by immature DCs, which show a high capacity for endocytosis, but are not well equipped for antigen presentation by major histocompatibility complex II (MHC II). Sensing of bacterial and viral products via PRRs or activation with inflammatory cytokines such as IL-1, TNF- α and interferons leads to maturation of DCs. IL-10 interferes in the maturation process of DCs by suppressing Akt activity and downstream inhibitor of kappaB kinase (IKK). It thereby prevents the nuclear translocation of NF κ B [18].

IL-10 also blocks the function of mature myeloid DCs through several mechanisms. It prevents antigen presentation by downregulation of MHC class II molecules and the costimulatory molecules CD80 and CD86, hence collectively impairing the capacity for T-cell activation. Moreover, the secretion of various proinflammatory cytokines, like IL-1, IL-6, TNF- α and IL-12, is downregulated by IL-10 [19]. Contrariwise, IL-10 upregulates its own expression in an autocrine fashion, although mature DCs lose sensitivity over time because of IL-10R1 downregulation [20].

Macrophages

Proinflammatory stimuli activate macrophages, an event that is terminated by IL-10. Interestingly, in macrophages, the activation of the IL-10 signalling pathway leads to downregulation of its own expression [21]. Lamina propria mononuclear cells from patients with IBD generally produce less IL-10 and are less responsive to IL-10 [22]. Similar to DCs, the expression of MHC class II, costimulatory molecules, proinflammatory cytokines and the generation of NO in macrophages are reduced in response to IL-10. Proliferation of LPS-treated macrophages is reduced in a Signal Transducer and Activator of Transcription 3- (STAT3)-dependent manner, whereas the reduction in cytokine secretion has not shown to be dependent on the JAK/STAT signalling cascade [23]. Also, it has been shown that microRNAs are involved in the inhibitory effect of IL-10. microRNAs can influence post-transcriptional gene expression leading to degradation of the target mRNA and translational repression. IL-10 inhibits the transcription of miR-155 that has shown to be induced by TLR4 signalling in macrophages [24]. This leads to an increase in the expression of SHIP1, a negative regulator of TLR4 signalling [25].

IL-10 also inhibits autophagy induction in murine macrophages through the PI3K pathway [26]. Autophagy, a process important for cellular homeostasis under stress conditions, is also associated with protective innate and adaptive immune response [27]. Moreover, induction of autophagy in

macrophages is important for removing intracellular pathogens and antigen presentation. Recent studies have implicated autophagy genes like *ATG16L1* and *IRGM* in the pathogenesis of IBD (Table 1).

Neutrophils

Neutrophils act as the first line defence against intruding bacteria and play an important role during early infection. The expression of IL-10 in neutrophils depends on the colligation of TLR2 and C-type lectin receptors, like Clec5A and Clec7A [28]. Only a simultaneous activation of the downstream molecules, MyD88 (myeloid differentiation primary response gene 88) and spleen tyrosine kinase, leads to a substantial release of IL-10.

Freshly isolated neutrophils from healthy donors are unresponsive to IL-10 [29]. The reason for this lies in the inducible nature of IL-10R1. This receptor subunit is neither present on the cell surface nor in the cytoplasm, but is synthesized *de novo* in an inflammatory environment. Therefore, neutrophils stimulated with LPS or IL-4 or isolated from septic patients do express IL-10R1, which renders such cells responsive to IL-10 [30].

Natural killer cells

The effect of IL-10 on NK cells is more of a stimulatory nature. While IL-10 neither increases proliferation nor cell migration by itself, it might act as a costimulatory factor in combination with IL-18 [31]. Nevertheless, IL-10 upregulates genes involved in NK cell cytotoxicity with somewhat different expression patterns compared with IL-2 [32]. This suggests that IL-10 and IL-2 involve distinct pathways for the activation of NK cells.

Mast cells

Mast cells as effectors of innate and Th2-mediated immunity are present throughout all layers of the gastrointestinal wall and are also regulated by IL-10. Induction of apoptosis in mast cell progenitors and in differentiated mast cells (in combination with IL-4) is mediated by IL-10 [33,34]. The expression and function of the high-affinity receptor for immunoglobulin E, Fc receptor for IgE and c-Kit, are inhibited by IL-10 in a PI3K-dependent way [35,36]. Besides other effects, the inhibitory qualities of IL-10 on mast cells explain why this cytokine is also investigated in the context of allergic diseases. Although mast cells from IL10^{-/-} mice produce significantly more proinflammatory cytokines *in vitro*, depletion of such cells had no effect on disease severity in this mouse model [37].

Intestinal epithelial cells

Intestinal epithelial cells (IEC), like enterocytes, goblet cells and Paneth cells are targets of immune-regulatory effects of IL-10. As outlined later, IL-10 also controls pathways that enable cells to respond to an accumulation of unfolded proteins within the

lumen of the endoplasmic reticulum (ER), called ER stress. The proteome of IEC from IL10^{-/-} mice suggests an inadequate response to oxidative and ER stress, as well as defects in energy metabolism and regulation of apoptosis [38]. Deregulated ER stress responses have been linked to IBD, through XBP1 and ORMDL3. Deletion of XBP1 in IECs leads to the formation of enteritis in mice associated with Paneth cell dysfunction and depletion [39]. Whereas many genetic pathways in CD (e.g. NOD2, ER stress, autophagy) could all be traced back to dysfunction of Paneth cell or enterocytes, the contributing cell type and pathogenetic mechanism of IL-10 receptor mutations remain elusive [40].

IL-10 in intestinal adaptive immune response

Th1, Th2 and Th17 cells

T-helper 1 cells (Th1) involved in cell-mediated immunity as well as Th2 cells that primarily promote humoral immunity express IL-10. The effect of IL-10 on T cells is mediated indirectly. Altered antigen presentation by DCs as well as down regulation of IL-12, IL-18 and IFN- γ leads to the deprivation of appropriate stimuli for naïve T cells to enter the Th1 path. Th1 cells require the activation of STAT4 and ERK signalling, resulting from the fact that IL-10 production in these cells is highly dependent on IL-12 [41]. Th2 cells rely on IL-4 (an important cytokine for Th2 polarization) and the activation of STAT6 and GATA-3 for their IL-10 production [42]. Naïve CD4⁺ T cells are capable of differentiation into another subtype of T cells that produces IL-10, as well as IL-17, and are therefore called Th17 cells. Differentiation into the Th17 cell lineage involves IL-6, IL-21, IL-1 β , IL-23 and TGF- β [43–46]. Several genes involved in Th17 signalling are associated with CD or UC, like IL23R, IL-12B, CCR6 and STAT3 (Table 1) [2]. Although TGF- β and IL-6 have been shown to increase IL-10 secretion by such cells, IL-23 had no effect on IL-10 expression [47]. The transcription factor responsible for relaying the signal from the receptor to the IL-10 promoter is c-Maf and also depends on STAT3 [48].

Effects of IL-10 on Th2 cells are ambiguous. IL-10 readily suppresses the expression of IL-4 and IL-5 [49] arresting Th2 growth [50]. On the other hand, an abundance of data highlights the function of IL-10 to channel the immune response from Th1 towards the Th2 pathway. IL-10 secreted by Th2 cells controls the generation of Th1 cells. In fact, IL-10 is a strong inhibitor of IL-12, which induces Th1 polarization.

Tregs and other T-cell populations

Regulatory T cells (Treg cells) are pivotal for the modulation of intestinal immune responses. Intestinal autoimmunity found in IL10^{-/-} mice can be virtually replicated by conditional deletions in CD4⁺ or FOXP3⁺ cells, although not as pronounced in the

Treg cell-specific deletion [51,52]. IL-10 is a central cytokine produced by Treg cells [53]. IL-10 and TGF β secretion by those cells is essential for their suppressive function. The pathways leading to IL-10 expression in CD4⁺CD25⁺ Tregs and inducible Treg cells are incompletely understood, but may involve IL-2 and IL-4. However, IL-2 is certainly not the main cytokine driving IL-10 expression, as Treg cells of IL-2^{-/-} mice still produce considerable amounts of IL-10 [54]. Interestingly, IL-10 did not reduce IFN- γ and TNF- α expression in these mice. It is quite surprising that IL-10 itself cannot trigger its own secretion in an autocrine manner in FOXP3⁺ and FOXP3⁻ Tregs, but TGF- β seems to be mediating this process [55].

Differentiation of CD4⁺Foxp3⁺ IL-10-producing cells in the colon can be induced by microbial products. Colonization of germ-free mice with *Bacteroides fragilis* induces the formation of IL-10-producing Foxp3⁺ Tregs via polysaccharide A and active TLR2 signalling [56]. Also, the expression of the chemokine receptor 6 (CCR6) seems to determine the fate of Treg cells, as IL-10-producing Treg cells in the colon [57].

Naïve T cells are characterized by high expression of CD45Rb, a receptor tyrosine phosphatase important for antigen-induced T-cell proliferation, whereas regulatory T cells (CD4⁺CD25⁺ Tregs) express low levels of CD45Rb [58]. Transfer of CD4⁺CD45RB^{hi} T cells into immunodeficient mice leads to formation of a colitis-like phenotype that can be ameliorated by cotransfer of CD4⁺CD45RB^{low} cells, in a TGF- β - and IL-10-dependent manner [59]. In a recent study, it was found that IL-10 secretion by CD11b⁺ myeloid cells (not by Tregs) of the lamina propria is vital for the prevention of colitis in this T-cell transfer model [60]. IL-10 secretion by such cells has shown to be of importance for maintaining the expression of Foxp3 in Treg cells and thereby regulates their function. Treg cells without a functional IL-10R2 receptor subunit lost Foxp3 expression under inflammatory conditions and as a consequence their ability for suppression. Active Foxp3 expression by Tregs is crucial for the inhibition of activated effector T cells and its mutation leads to severe autoimmune diseases in mice and humans [61].

Double negative T cells (CD4⁻CD8⁻) also respond to IL-10 exposure, but undergo apoptosis [62]. This immunosuppressive cell type represents around 1% of all peripheral T-lymphocytes. They inhibit a variety of immune responses, via direct killing of effector T cells in an antigen-specific manner [63,64]. They were first described by Strober *et al.* for their natural suppressor functions and have shown to be of importance in a series of transplantation experiments in mice, because of their ability to inhibit graft rejection and graft-versus-host disease [65–67]. Double negative T cells have shown in the past to be active producers of IL-10 [68], but Hillhouse *et al.* [62] demonstrated that double negative T cells from an autoimmune-prone mouse strain show a 10-fold higher IL-10 secretion than autoimmune-resistant strains and their overall number is markedly reduced.

The role of double negative T-cell in the pathogenesis of IBD has yet to be elucidated.

B cells

B cells respond differently to IL-10. Here IL-10 provides a proliferation stimulus [69], induces the secretion of IgM, IgA and IgG [70] and the immunoglobulin class-switch to IgA, IgE, IgG1 and IgG3 [71]. B cells are also an important source of IL-10 and thereby regulate autoimmunity in a variety of animal models, especially in mice with experimental autoimmune encephalomyelitis [72]. Moreover IL-10-producing B-cell subsets have been identified in models of experimental colitis, but are not required to induce enterocolitis in IL10^{-/-} mice [73,74].

IL-10 receptor signalling in IBD

The IL-10 homodimer executes its signalling through a receptor complex comprised of two chains each of IL-10R1 and IL-10R2. Ligand binding to IL-10R1 forms a receptor complex with IL-10R2 and activates intracellular receptor-associated kinases JAK1 and TYK2. Phosphorylation of tyrosine residues on the intracellular portion of IL-10R1 leads to the recruitment of STAT3 via its SH2 domain to the complex. Again phosphorylation of tyrosine residues results in activation, dimerization and nuclear translocation, where STAT3 binds to promoters with STAT-binding elements. Although anti-inflammatory properties of IL-10 depend on activation of STAT3, it simultaneously activates STAT1 and STAT5 (the latter only in nonmacrophage cells). IL-10 has been shown to activate PI3K/Akt pathway to promote proliferation in selected cells, as well as the p38/MAPK pathway, to inhibit TNF- α translation and induction of hemeoxygenase-1 in macrophages [75–77].

IL-10 receptor signalling mediated by IL-10R1 and IL-10R2 is affected by single nucleotide polymorphism (SNP) in the encoding genes [12]. Two common variants of the IL-10R1 (S138G; rs3135932) and G330R; rs2229113) are associated with different disease phenotypes [78,79]. The allelic frequency in a Caucasian cohort is relatively high (around 16% for the S138G and 33% for the G330R), and S138G was found to be in strong linkage disequilibrium with G330R. Our functional studies on IL-10R1 variants indicate that IL-10R1 S138G variant is a loss-of-function allele for IL-10-induced STAT1 and STAT3 activation that may protect from UC [78]. The S138G variant has more pronounced loss-of function effects on viral cytomegalovirus (CMV) IL-10 signalling [80]. On the other hand, IL10R1-G330R alters the kinetics of STAT phosphorylation indicating that the position of G330 is important in stabilizing the STAT signal and links to progression of hepatic fibrosis in hepatitis C [81,82].

While searching for mutations in IL-10R1 our laboratory also identified a truncated IL-10R1 allele that was missing a 179-basepair fragment in a heterozygous otherwise healthy

semite [83] (Fig. 1). In a recent report from Glocker *et al.* [12] similar IL-10R1 and IL-10R2 mutations were detected in homozygous mutants from inbred families. G141R mutation in the *IL10RA* gene, which codes for the IL-10R1 subunit, is a missense mutation localized in the IL-10-binding region, while the T84I mutation resides in a highly conserved sequence. The W159X variant in the *IL10RB* gene, which encodes the IL-10R2 subunit, represents a nonsense mutation. All three mutations have shown to disrupt IL-10 signalling and thereby their observation linked early-onset IBD to IL-10R mutations, both in IL-10R1 and IL-10R2. It is likely that several loss-of-function mutations exist in IL-10Rs that are still undetected. Individuals with heterozygous mutations in IL-10R are carriers only. Remarkably, some artificially introduced mutations in the cytoplasmic domain of murine *IL10RA* did not halt IL-10 signalling, but promoted it [84]. This mutation did not interfere with receptor number or binding affinity. To what extent those findings can be transferred to humans is unknown, but they provide an example for the possibility of nondeleterious mutations in IL-10R genes.

Not only genetic variants of the IL-10 receptors, but also of the IL-10 and STAT3 genes, are associated with IBD, which underscores the involvement of the IL-10 signalling cascade in the pathogenesis of CD and UC [85,86]. A SNP (rs3024505) located directly adjacent to the IL-10 gene has shown to

constitute a risk allele for UC [85]. Risk alleles have also been identified for STAT3, the key signal transduction molecule for the anti-inflammatory properties of IL-10 [87]. Table 2 provides a summary of IL-10 pathway genes that were genetically linked to CD or UC (Table 2).

Pathogenesis of colitis under defective IL-10 signalling

IL10^{-/-} and IL10Rb^{-/-} mice develop spontaneous enterocolitis [13,88]. The exact mechanism by which defective IL-10 signalling leads to intestinal inflammation is unknown, but most likely is a result of the breakdown of the symbiotic relationship between the indigenous microflora and their host. Activation of TLRs by commensals is vital for gut homeostasis [89], although it results in inflammation when deprived of essential immunoregulatory cytokines like IL-10.

Bacterial sensing

One of the most important susceptibility genes for CD is the nucleotide-binding oligomerization domain 2 (NOD2) [90]. NOD2 is an intracellular PRR that recognizes muramyl dipeptide structures from bacterial cell walls and is constitutively expressed in IECs, macrophages and DCs. CD-associated NOD2 mutations are located in the 3' end of the receptor,

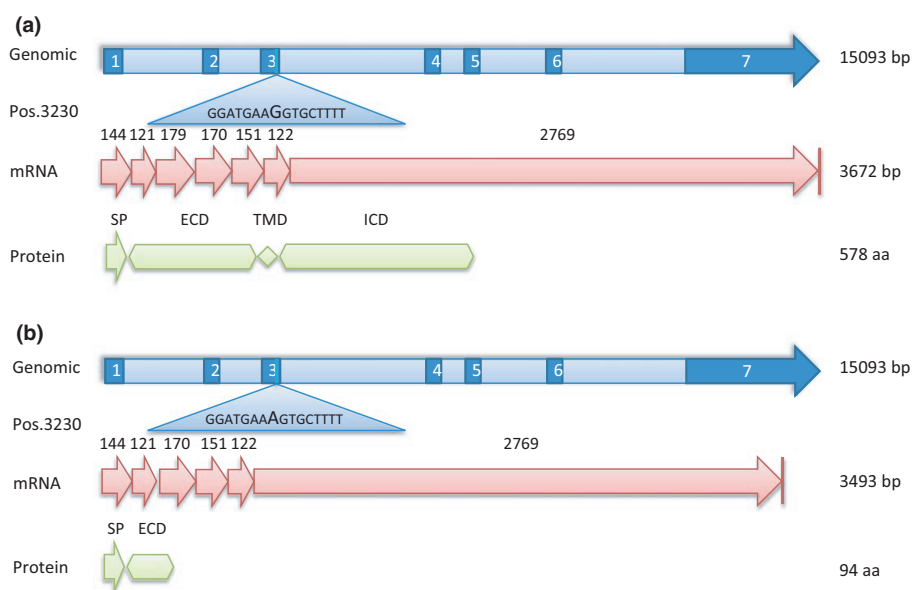


Figure 1 IL-10 receptor mutation in inflammatory bowel disease. (a) Genomic localization of EX3del mutation on *IL10RA* in a heterozygote healthy individual. (b) The G-to-A point mutation at the splice acceptor site of exon 3 was identified at position 3230 (g. 3230G>A; NC_000011) leading to the loss of a 179 bp fragment (exon 3) and an associated frameshift. The predicted 94-aa protein lacks both transmembrane and intracytoplasmic domains. SP, signalling peptide; ECD, extracellular domain; TMD, transmembrane domain; ICD, intracytoplasmic domain.

Table 2 Mutations of IL-10 pathway genes and association with inflammatory bowel disease phenotype

Gene	Locus	Amino acid substitution	Disease	References
IL10	1q32	F129Y	UC	[85]
IL10RA	11q23	T84I	Early-onset enterocolitis	[12]
	11q23	G141R	Early-onset enterocolitis	[12]
IL10RB	21q22	W159X	Early-onset enterocolitis	[12]
STAT3	17q21	Intronic	CD, UC	[86]

CD, Crohn's disease; UC, ulcerative colitis.

the so called leucine-rich repeats and likely interfere with ligand binding [91]. A functional NOD2 signalling cascade contributes to intestinal homeostasis via the secretion of IL-10, TGF- β and IL-1Ra and by inducing autophagy in macrophages [92,93]. Furthermore the production of defensins in Paneth cells is mediated by NOD2 and NOD2 mutations lead to Paneth cell dysfunction [94].

Despite the involvement of NOD2 in a plethora of signalling pathways, neither NOD2^{-/-} mice nor mice that carry the homologue to the most common NOD2 variant, display spontaneous intestinal inflammation [95,96]. Mutations in leucine-rich repeat region of NOD2 lead to a reduction of IL-10 secretion, because of disrupted complex formation of NOD2 with phosphorylated p38 and the transcription factor hnRNP-A1 (a mechanism that is important for steady state, as well as microbe-induced IL-10 expression) [97]. This strengthens the concept of microbiota-dependent triggering of intestinal inflammation in genetically susceptible hosts.

MyD88 is a cytosolic adaptor protein used by nearly all TLRs for signalling, culminating in activation of AP-1 and NF- κ B [98]. TLR-induced NF- κ B activation in mucosal immune cells is a substantial event for colitis formation in IL10^{-/-} mice [99]. IL10^{-/-}; MyD88^{-/-} double knockout mice do not develop spontaneous enterocolitis, further highlighting the importance of bacterial sensing by immune cells [100]. Interestingly, IL2^{-/-} mice develop a colitis phenotype very similar to IL10^{-/-} mice, also with a predominant Th1 cytokine profile. IL2^{-/-}; MyD88^{-/-} double knockout mice on the other hand still develop intestinal inflammation, in opposition to IL10^{-/-}; MyD88^{-/-} mice, suggesting different pathogenetic mechanisms, but a comparable disease phenotype [89].

Under physiological conditions, TLR-mediated MyD88-dependent signalling is negatively regulated by IL-10. As depicted in Fig. 2, IL-10 induces ubiquitination and proteasomal degradation of the essential signalling molecules IRAK4, IRAK1 and TRAF6 [101]. This seems to happen in an autocrine loop, because IL-10 expression is a result of active TLR/MyD88

signalling in some cells [102]. TLR/MyD88 signalling is also crucial for the pronounced Th1/Th17 immune response in IL-10-deficient conditions which resembles the immunological scenario seen in CD. For instance activation of TLR4 by LPS or TLR9 by CpG, DNA promotes a Th1 response by release of IL-12p70 and IL-23 by DCs [103].

Th1 response

A wide variety of data highlights the crucial role of the IL-12/IL-23 axis in IBD. IL-12 mediates Th1 polarization, while IL-23 leads to the formation of Th17 cells. Genetic variations in genes of the IL-12/IL-23 axis (IL12B, IL-23R, JAK2) are associated with both subtypes of IBD, and monoclonal antibodies (Ustekinumab) targeting the shared p40 subunit of IL-12 and IL-23 are under clinical investigation [6,104]. Diverse approaches to block the function of either IL-12 or IL-23, be it monoclonal antibodies or double knockouts, led to amelioration or abrogation of disease in IL10^{-/-} mice [105,106]. *In vivo* presumably IL-10 from CD4⁺ T cells is performing this task, targeting the transcription of the p40 subunit among other mechanisms [107]. T cells extracted from draining mesenteric lymph nodes of IL-10^{-/-} mice primarily produce IFN- γ , further corroborating the hypothesis of a Th1-mediated disease. Although blockage of IFN- γ with antibodies only leads to amelioration of disease, antibodies against IL-12 administered to young knockout mice prevent it [108].

Th2 response

Also, Th2 cytokines can reduce the Th1/Th17-driven inflammation in IL-10^{-/-} mice. Excessive Th2 responses are usually linked to UC, where high levels of IL-13 are detected. IL-13 decoy receptor (IL-13R α 2) scavenges the Th2 cytokine IL-13. IL-10^{-/-} mice have higher levels of IL-13R α 2, fuelling Th1/Th17-mediated responses. On the contrary, IL-10^{-/-}; IL-13R α 2^{-/-} double knockout mice have a markedly reduced disease severity and were unresponsive to aggravation of disease through piroxicam treatment and the consecutive IFN- γ - and IL-17A-driven intestinal inflammation [109]. In general, IL-10^{-/-} mice display normal Th2 responses.

Spontaneous enterocolitis in humans

The enterocolitis seen in patients with mutations in the IL-10 receptor subunits most likely constitutes a distinct entity that cannot be lumped together with UC or CD. Nevertheless, clinical features like perianal abscesses and enterocutaneous fistulas are present that resemble CD and the underlying excessive Th1/Th17 cell response [12]. Patients with mutations in IL10RB present in addition with folliculitis, which is likely the result of abrogated signalling of cytokines that are dependent on IL-10R2 as a cosignalling subunit (e.g. IL-22, IL-26, IL-28A,

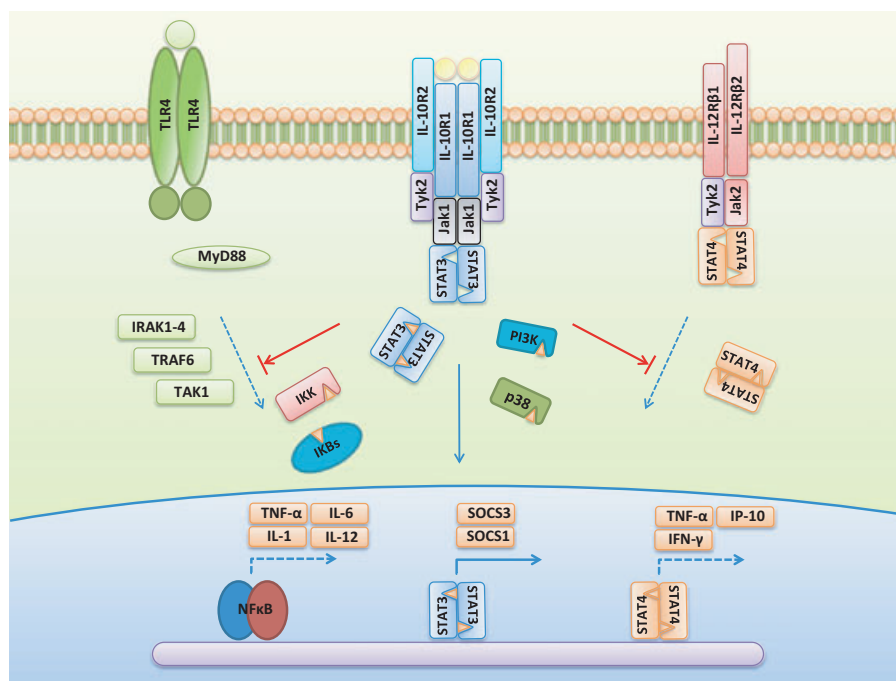


Figure 2 Interleukin-10 signalling in inflammatory bowel disease (IBD). IL-10 signals through a complex, consisting of two copies each of IL-10R1 and IL-10R2. Ligand binding to the receptor upregulates the activity of the Janus kinases Jak1 and Tyk2. Phosphorylation of the receptor subunits as well as of the kinases (not shown) leads to the binding and phosphorylation of STAT molecules. STAT3 molecules dimerise and translocate to the nucleus, where they dock to STAT-binding elements on the DNA and upregulate IL-10-responsive genes like SOCS3 or SOCS1. Other cytoplasmic molecules are also capable of binding to the activated receptor complex. This event results in the activation of the PI3K and MAP kinase pathway. Phosphorylation of STAT3 promotes the anti-inflammatory properties of IL-10. IL-10-mediated signalling inhibits MyD88-dependent TLR signalling by inducing proteasomal degradation of essential signalling molecules, like IRAK1, IRAK4 or TRAF6. Thereby, IL-10 inhibits NF- κ B and the expression of inflammatory cytokines, such as TNF- α , IL-1 β or IL-6. Disease-promoting effects of IL-12 in IBD are also antagonized by IL-10. IL-12 signals through a heterodimeric receptor formed by IL-12 β 1 and IL-12 β 2. As a consequence of ligand binding, Jak2 and Tyk2 mediate the activation of STAT4, the critical component of the IL-12 signalling cascade. IL-12 thereby activates Th1 cells and natural killer cells and leads to the secretion of IFN- γ , TNF- α and IP-10. IL-10 readily suppresses IL-12 production that is regulated by NF- κ B, by inhibiting the transcription of both subunits p35 and p40 of IL-12. Hence, IL-12 signalling is inhibited by withdrawing the production of the corresponding ligand. STAT, Signal Transducer and Activator of Transcription; SH2, Src Homology 2; SOCS, suppressor of cytokine signalling; PI3K, Phosphoinositide 3-kinase; MAPK, Mitogen-activated protein kinase; MyD88, myeloid differentiation primary response gene (88); TLR, toll-like receptor; IRAK, interleukin-1 receptor-associated kinase; TRAF, TNF receptor-associated factor; NF- κ B, nuclear factor-kappa B; IFN- γ , interferon-gamma; TNF- α , tumour necrosis factor-alpha; IP-10, inducible protein 10.

IL-28B and IL-29). Further investigations can explain whether these rare genetic mutations in the IL-10 signalling cascade exclusively account for the dramatic disease phenotype in paediatric patients or are also relevant to the moderate intestinal inflammation seen in everyday clinical practice.

The present model of the pathogenesis of enterocolitis under defective IL-10 signalling is far from being complete, but is suggestive of several ways through which this cytokine operates and manipulates to maintain gut homeostasis. However, one must not forget that there are many genetic models of colitis such as knockouts of IL-2, STAT3, TCR- α , TGF- β , overexpression of IL-7, TNF- α , HLA-B27, transfer of

CD4⁺CD45Rb^{high} into lymphocyte deficient hosts, just to mention a few [110]. The future task will be to dissect the involvement of each and every pathway, which result in the loss of tolerance to commensals in the gut.

Viral IL-10 signalling and IBD

Epstein-Barr virus (EBV), CMV and other members of the Herpes virus family acquired structural homologues to human IL-10, called viral IL-10 (vIL-10). Interestingly, superimposed CMV infections in patients with IBD are associated with re-activation and aggravation of disease, seen in a higher prevalence

of toxic megacolon and need of surgical intervention [111]. The pathomechanism explaining the association between CMV infections and IBD flares is still elusive. Immunosuppressive therapies, a common concept in IBD, with associated compromise of host's T-cell response are known to re-activate CMV from latent infection sites, like the bowel. As a matter of fact, CMV re-activation almost always occurs in patients with UC under corticosteroid therapy. The role of cmvIL-10 in this scenario is unknown. Responsible for keeping CMV virus in latency are CMV-specific CD8⁺ memory T cells [112]. In immunocompetent individuals, populations of stable memory T cells exist at low levels in parallel with T cells that expand to high frequencies in the course of CMV re-activation [113]. This phenomenon called 'memory inflation' is inhibited by IL-10, thus facilitates viral expansion [114]. This might explain the expression of IL-10 homologues by members of the Herpes virus family. Sequence identity between hIL-10 and cmvIL-10 lies only around 27%, compared with 83% homology between hIL-10 and ebvIL-10 from EBV [115,116]. While ebvIL-10 has a 1000-fold decrease in binding affinity for IL-10R1 when compared with hIL-10, cmvIL-10 retained a high IL-10R1-binding affinity and displays even more efficient binding to IL-10R2 than hIL-10 [117]. The biological consequence resulting from this is that cmvIL10 leads to a stronger phosphorylation of STAT3 in immature DCs than ebvIL-10 or hIL-10 [118]. Similar to ebvIL-10 homodimers, heterodimers consisting of hIL-10 and vIL-10 also lead to an enhanced STAT3 phosphorylation [119]. We were able to demonstrate that signalling through common variants of IL-10R1 (S138G, G330R) reduces phosphorylation of STAT3 and hence should render individuals carrying those allelic variants more resistant to immune-evasion strategies by CMV [80]. In the latent phase of CMV infections, a different IL-10 homologue is expressed, called LA-cmvIL-10. LA-cmvIL-10 shows similar signalling qualities as cmvIL-10, although not as pronounced [119]. Its binding affinity to variants of IL-10RA is unknown.

IL-10 and ER stress response in intestinal inflammation

Impairment of ER stress response has recently become a novel concept of IBD pathogenesis [39]. ER stress occurs under circumstances that lead to the accumulation of misfolded proteins in the ER. Unfolded protein response (UPR) is a cellular stress response to cope with ER stress, which either is a consequence or a cause of intestinal inflammation [120,121]. In the ER, glucose-regulated protein 78 (grp78) plays an essential role in folding and assembly of proteins and acts as a sensor for misfolded proteins to induce UPR [122]. The ultimate aim of this pathway is to prevent the secretion of misfolded proteins, halt cell cycle for removal of such proteins and thereby promote cellular sur-

vival. In case of a nonreversible damage, the same pathway is able to commit cells to apoptosis [123]. IL-10 has been involved in modulating ER stress response. Characterization of the proteome in *Enterococcus faecalis*-monoassociated IL10^{-/-} mice revealed ER stress as epithelial response to intestinal inflammation [38]. Grp78 levels are elevated in IEC from IL10^{-/-} mice as well as in patients with IBD [124]. *Vice versa*, reconstitution of a functional IL-10 signalling cascade led to a decrease in grp78 levels, an observation that can be attributed to the phosphorylation of p38-MAPK by IL-10 [124]. As shown in Fig. 3, IL-10 signalling in unstressed cells blocks grp78 transcription via phosphorylated p38-MAPK by inhibiting nuclear translocation of transcription factor ATF-6 and hence, its binding to the grp78 promoter. (Fig. 3) The proinflammatory activity of grp78 is ascribed to the activation of NF-κB pathway [125]. TNF-induced inflammatory response alters the cellular localization of grp78 from ER to cytoplasm. Cytoplasmic localization of grp78 and its consecutive recruitment to the IKK complex have shown to be crucial for TNF-induced phosphorylation of RelA and thereby enable NF-κB-mediated transcription. IL-10 was found to prevent grp78 association with IKK complex and completely blocks TNF-induced phosphorylation of RelA with unclear functional consequences [124]. IL-10 also inhibits NF-κB activity either through the suppression of IKK activity or by interfering with the DNA-binding activity of NF-κB [126]. It has also been shown that the p38 pathway induced by IL-10 affects TNF-α mRNA translation [75] and also anti-inflammatory response of macrophages through heme oxygenase-mediated pathway [127]. Nevertheless, such findings suggest that IL-10 mediates ER protective effects through induction of p38-MAPK pathway. Regulation of ER stress response by IL-10 is another potential mechanism of its action against inflammation. Contrariwise, IL-10 transcription has shown to be upregulated by active ER stress in Treg cells, an effect mediated by phosphorylation of the translation initiation factor eIF2α [128].

Therapeutic implications of IL-10 in IBD

The potent anti-inflammatory properties of IL-10 made it appealing to study its therapeutic implications in various human diseases. These include the following: psoriasis [129], wound healing [130], rheumatoid arthritis [131], sepsis [132,133], organ transplantation [134,135], visceral and limb ischaemia [136], atherosclerosis [137], chronic Hepatitis C [138,139], HIV infection [140], post-ERCP pancreatitis [141], coeliac disease [142] and IBDs [143], especially CD. The first therapeutic administration of recombinant human IL-10 (rhIL-10) back in the mid-1990s raised hope for a potential treatment option in CD [144]. Indeed, IL-10 treatment led to the induction of remission in patients that were otherwise refractory to treatment. Two large, multi-centred follow-up studies using

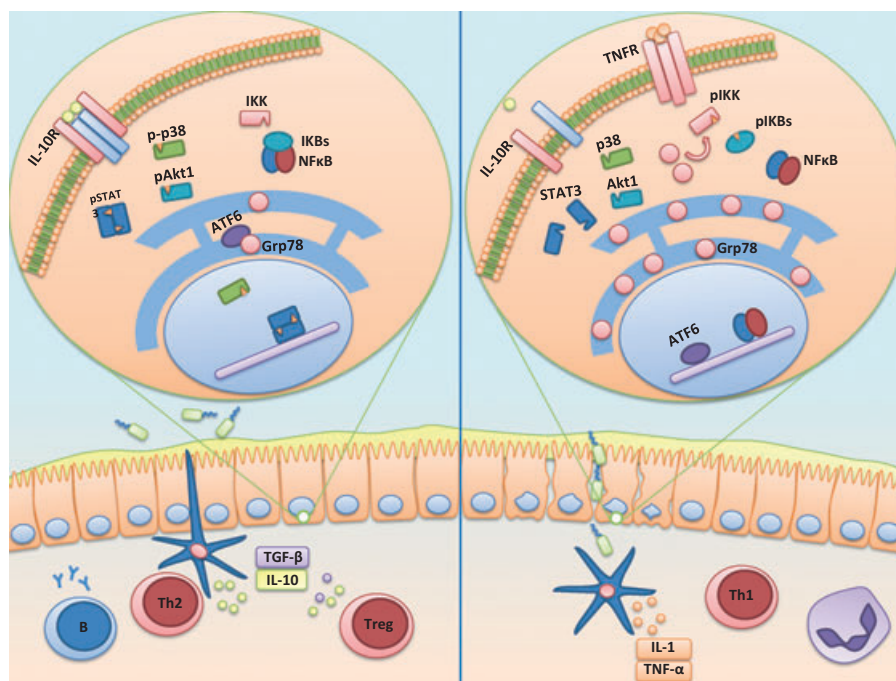


Figure 3 IL-10 regulates ER stress response in intestinal inflammation. Left: Under normal conditions, both innate and adaptive immune arms maintain gut homeostasis. Immunoregulatory cytokines like IL-10 and TGF- β are critical in controlling inflammation. In the absence of ER stress, UPR proteins like ATF-6 remain in an inhibitory state and are not translocated to nucleus. Right: Intestinal translocation of gut bacteria can induce ER stress and inflammation. Inflammation-induced ER stress triggers UPR via upregulation of grp78. grp78 plays a central role in resolving ER stress. TNF- α activates NF κ B and leads to relocalization of grp78 from ER to the IKK complex. In the absence of IL-10-mediated activation of p38 signalling pathway, TNF induces recruitment of ATF-6 to the grp78 promoter. ATF-6, Activating transcription factor; grp78, 78 kDa glucose-regulated protein; NF- κ B, Nuclear factor κ of activated B cells; TNF, Tumour necrosis factor; IKK, Inhibitor of kappaB kinase; ER, endoplasmic reticulum; UPR, unfolded protein response.

subcutaneous dosing were unable to confirm the results from the intravenous pilot trial [145,146]. Although an improvement in clinical response was observed, no difference in the remission rate was detectable. Out of several hypotheses explaining the disappointing results, short half-life of IL-10 (2–3 h) in the systemic circulation is likely a contributing factor. The benefit of IL-10 in the treatment of various human diseases has yet to be evaluated. High levels of IL-10 in the bloodstream are associated with an increased level of IFN- γ and neopterin, the latter a product secreted by macrophages upon stimulus of IFN- γ and thereby indicating a proinflammatory status. This might explain certain adverse events in patients, such as anaemia of chronic disease, thrombocytopenia, fever and headache [147,148]. Presumably, IL-10 might act differently *in vivo* in a complex network of cytokines, antigens and cell types than assayed *in vitro*. This is for instance seen in the upregulation of CD64 (Fc-gamma RI) on neutrophils and monocytes, an event that is usually triggered by proinflammatory cytokines like IFN- γ and proved useful for the differentiation between infectious enterocolitis and IBD [149,150]. In addition, insufficient

local tissue concentrations of IL-10 might account for the negative results. Most studies used the subcutaneous route of administration. Different approaches have been tested that might yield to a more specific delivery of IL-10 to the intestinal mucosa. Another route of raising local cytokine concentrations for the treatment of regional disorders in the gut is mucosa-targeted gene delivery with polymer-based microparticles. Those particles encapsulate gelatine nanoparticles containing plasmid DNA-expressing murine IL-10 [151]. As the polymer-based microparticles are selectively degraded by lipases in the small and large intestine, the gelatine nanoparticles are released at the desired site of action. The IL-10 gene containing particles successfully reduced the levels of inflammatory cytokines as well as disease activity scores in the trinitrobenzene sulfonate (TNBS)-induced model of colitis.

Administration of oral inoculation of genetically modified *Lactococcus lactis*, (a noninvasive, gram-positive bacteria) which expresses murine IL-10, decreased experimental colitis in the DSS model and IL-10^{-/-} mice [152]. Another study in which CD patients were treated in a phase I clinical trial with genetically

engineered *Lactococcus lactis*-producing human IL-10 (LL-Thy12) was considered safe and led to the improvement of disease [153].

Another option tested was the systemic or rectal application of adenoviral vectors encoding IL-10 [154,155]. Systemic injection of adenoviruses to DSS mice as well as rectal delivery to IL-10^{-/-} mice led to significant improvement of colonic inflammation in these mouse models of IBD. Major drawback of systemic administration is the high-degree infection of hepatocytes by adenoviruses, as well as unwanted systemic immunosuppression. Rectal delivery through enemas to IL-10^{-/-} mice has shown to reduce inflammation, while sparing systemic side effects.

The immunomodulatory factor low-calcium response V is a protein usually secreted by enteropathogenic *Yersinia* to evade the immune system, by stimulating the production of IL-10. It was found that *L. lactis* strain expressing low-calcium response V ameliorated disease activity in TNBS- and DSS-treated mice, but not IL10^{-/-} mice, suggesting that IL-10 is required for this effect [156]. The exact mechanism by which these genetically modified bacteria exhibit their anti-inflammatory properties is not clear.

Conclusions

Out of several pathways contributing to the pathomechanism of IBD, impaired IL-10 signalling has proven to be one of them. Rare IL-10R mutations link the importance of IL-10 as a key mediator of gut immune homeostasis with the pathogenesis of IBD. However, even after more than 20 years of research, several questions remain unsolved including which cell type (enterocytes, Paneth cells or immune cells) is most dependent on the presence of functional IL-10. Nevertheless, our current knowledge credits IL-10 a significant role in orchestrating intestinal immune homeostasis, although the exact function remains elusive.

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