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Functional epithelial cell proteomics under conditions of chronic intestinal inflammation

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3. Shkoda A, Ruiz PA, Kim SC, Sartor RB, Daniel H, Haller D. Identification of dynamic proteome changes in primary intestinal epithelial cells after the colonization of germ-free wild type and IL-10 gene deficient mice with colitogenic *Enterococcus faecalis*. Innate Immunity and IBD Stuttgart 4-5 November, 2005.

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Content

List of abbreviations

2D	Two-dimensional
AP-1	Adapter-related protein complex-1
Apaf-1	Apoptotic protease-activating factor 1
APC	Antigen-presenting cell
BCR	B-cell receptor
CARD	Caspase recruitment domain-containing protein
CARMA-1	CARD-containing maguc protein 3
CD	Crohn's disease
cDNA	complementary DNA
CFU	Colony forming unit
cIAP1	cellular inhibitor of apoptosis protein 1
CK	Creatine kinase
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Desoxyribonucleic acid
ECL kit	Enhanced chemiluminescence light-detecting kit
EDGF	Epithelial-derived growth factor
ER	Endoplasmatic reticulum
ERSE	ER-stress response element
ESI	Electrospray ionisation
FBS	Fetal bowine serum
GF	Germ-free
HCCA	Hydroxy-ciano-cynamic acid
HLA	Human leukocyte antigen
IAP2	Inhibitor of apoptosis protein 2
IBD	Inflammatory bowel disease
IEC	Intestinal epithelial cell
IEF	Isoelectric focusing

IFNγ	Interferon gamma
IL	Interleukine
IP-10	Interferon-gamma-inducible protein-10
IPG	Immobilized pH gradient
IRAK	Interleukin-1 receptor-associated kinase
IRF3	Interferon regulatory factor 3
ITAM	Immunoreceptor tyrosine-based activation motif
LCM	Laser capture microdissection
LLRs	Leucine-rich repeats
LPS	Lipopolysaccharide
LTA	Lipoteichioic acid
MALDI-TOF-MS	Matrix assisted laser disorption ionisation time-of-flight mass spectrometry
MARCO	Macrophage receptor with collagenous structure
MAPK	Mitogen-activated protein kinase
MC	Morbus Crohn
MHC	Major histocompartibility complex
Mn-SOD	Magnesium superoxiddismutase
MSR	Macrophage scavenger receptor
NES	Nuclear export sequences
NF-κB	Nuclear factor kappa B
NLS	Nuclear localization sequences
NOS	Nitric oxide synthase
PAMPs	Pathogen associated molecular patterns
PCR	Polymerase chain reaction
PGN	Peptidoglycan
PKCθ	Protein kinase C theta type
RHD	Rel homology domain
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SAP	Serum amyloid protein
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SMAD	Mothers against decapentaplegic homolog
SOCS	Supressor of cytokine signalling
SODD	Silencer of death domains
STAT	Signal transducer and activator of transcription
TAB	TGF-beta-activated kinase 1-binding protein 1
TAK	TGF-beta-activated kinase 1
TCR	T-cell receptor
TFA	Triflouracetic acid
TGF-β1	Tumor growth factor beta 1
TIR	Toll-interleukin-1 receptor domain
TLR	Toll-like receptor
TRADD	Tumor necrosis factor receptor type 1-associated DEATH domain protein
TRAF 6	TNF receptor-associated factor 6
TNF	Tumor necrosis factor
TRAM	Translocating chain-associating membrane protein
TRIF	TIR-domain-containing adaptor inducing IFN-beta
UC	Ulcerative Colitis
WT	Wild type
XIAP	X-linked inhibitor of apoptosis protein
ZAP70	70 kDa zeta-associated protein
ZO-1	Zonular occludens protein 1

1 Introduction

1.1 Immune response in the gut

The immune system is one of the evolutionary consequences of the continuous interaction between infectious micro-organisms and multicellular organisms developed to protect the host. The immune system consists of two main components: innate and adaptive immunity. The innate immune response provides a first line of defense against invading microbes [1, 2]. From the first minutes after birth animals and human are surrounded by millions of bacteria, viruses and antigens which can potentially cause a disease. It would be reasonable for the host to develop identification systems that help to recognize and distinguish the commensal microbiota from pathogens [3, 4]. This recognition is mainly made by a genome-encoded system of receptors and it is crucial for the outcome of bacteria-host interactions. Innate immunity provides to the host an ability for specific recognition of proteins, lipids, carbohydrates, nucleic acids and other complex bacterial antigens. Due to the mechanisms of adaptive immunity the host is able to remember an infectious agent and to induce a prompt immune response by the clonal expansion and activation of antigen-specific cells [5, 6, 7]. Taking into consideration that microbes or viruses are also changing and mutating due to their own adaptive mechanisms, it seems optimal for the host to recognize archaic conservative bacterial patterns and to induce an individual response on highly variable antigens.

The gastrointestinal tract presents a system that is extremely open for a tremendous number of microbial species. These bacterial species enter the gut and establish a symbiotic community of microbiota individual for each host. Normally, the immune system of the gut is remaining relatively unresponsive to food antigens and to the commensal microbiota. Spontaneous or induced disbalance between the commensal microbiota and genetically susceptible host leads to the inadequate reaction of mucosal immune system resulting in chronic intestinal inflammation [8, 9, 10].

Intestinal inflammation can be characterized by cell activation and by production of biologically active mediators like cytokines and chemokines. These mediators cause chemotaxis and infiltration of the mucosal tissue by lymphocytes, granulocytes, macrophages and eosinophils secreting bactericidal and pro-inflammatory peptides. Excessive secretion of pro-inflammatory cytokines can cause oxidative stress, tissue injury and leakage of the intestinal epithelial barrier allowing massive invasion of microorganisms into intestinal tissue [11, 12]. The initial contact of the host with commensal non-pathogenic microbiota drives the induction of “physiologic” pro-inflammatory

response followed by its resolution and establishment of ignorance to the constant presence of commensal bacteria. To some extent pro-inflammatory reaction is greatly positive for the host that remains in pre-activated status. The microbiota-induced pro-inflammatory response will be terminated or neutralized by inherited host-derived inhibitory mechanisms [13]. However, under conditions of chronic intestinal inflammation the enteric microbiota appears to act as a surrogate bacterial pathogen.

1.1.1 Commensal microbiota of the gut

The commensal microbiota consists of multiple bacterial resident species that initially colonize the intestine, propagate and establish a complex ecosystem. About 10^{14} microbes of more than 1000 species inhabit the human gastrointestinal tract [14]. The most abundant microbiota is present in the distal parts of the gut such as the colon and ileum. The stomach and proximal gut regions prevent the continuous access of new bacteria from the environment due to the presence of proteolytic enzymes and acidified milieu. More than 90 % of the microbial population in the large intestine are obligate anaerobes. The most common inhabitants are represented by species such as *Bacteriodes, Eubacterium, Bifidobacterium, Fusobacterium*, less frequent there could be found *Lactobacillus, Enterobacter and E.coli* [15, 16]. Predominant species form an appropriate surrounding and a metabolic "niche" for the other less abundant and minor species. The commensal bacteria play an important role in various metabolic processes of the host. They participate in the fermentation of dietary compounds, synthesis of essential vitamins and short-chain fatty acids, degradation and conversion of xenobiotics and toxic metabolites. Enteric bacteria have an effect on functional and enzymatic activity of the intestinal epithelium. In addition, the commensal bacteria restrict the invasion of pathogens and their attachment to the mucosal surface [17]. On the other hand, resident bacterial species produce a number of immunogenic substances that could trigger immune response of the host. The propagation of the microorganisms is accompanied by the constant destruction of bacteria and by the release of a wide range of innate ligands such as fragments of the cell walls, DNA, surface and membrane glycoproteins or flagella. Some of these components have a strong immunomodulatory effects. For example, lipopolysaccharides (LPS) from the membranes of the Gram-negative bacteria induce activation of enterocytes, macrophages and lymphocytes followed by secretion of pro-inflammatory cytokines like IL-1 and TNF. Peptidoglycan from the cell wall of the Gram-positive bacteria can also activate immune competent cells upon its recognition by TLR2 and intracellular Nod proteins. Flagellin, bacterial heat shock proteins and DNA fragments with unmethylated CpG-motif also stimulate macrophages and dendritic cells to secrete pro-

inflammatory cytokines [18, 19, 20]. It seems obvious, that due to the host-derived protective mechanisms the mucosal tissue is able to resist the constant pro-inflammatory response on various immunogenic determinants of bacteria. Disruption of these mechanisms leads to the impaired immunologic homeostasis and to the development of chronic intestinal inflammation.

1.1.2 Role of the intestinal epithelium in the host protection

The epithelial cells are located in multiple organs and tissues of the body. They are responsible for important physiologic functions such as absorption, secretion, transport, defence and metabolism. The typical intestinal epithelial cell (IEC) has microscopically recognized structures such as microvilli, basal bodies and desmosomes that are not equally distributed on the cell surface. By forming millions of finger-like villi and crypts, the epithelial surface covers about 400 m^2 in the human gut [21]. In the small intestine the main antigenic load comes from dietary components. The large intestine is additionally in a constant contact with commensal microbiota and its metabolic products [22, 23]. Since intestinal epithelial cells are polarized, they have a strong physiological and functional connection to the extracellular matrix of the underlying tissue. Each epithelial cell maintains an intimate association with neighbouring cells sealing the surface of the gut with tight junctions (Figure 1). Due to the tight junction structures the epithelial monolayer creates a protective barrier. The intestinal epithelium plays an important role in the processes of inflammation, tissue injury and regeneration [24]. The intestinal epithelium presents a complex structure that possess barrier and immune surveillance functions. In addition, it was recently shown that the epithelium is a very dynamic tissue responding to internal and external stimuli. Activated epithelial cells can produce a broad spectrum of pro-inflammatory cytokines and growth factors, they can constitutively or inducibly express different co-stimulatory molecules, receptors and communicate with regulatory and effector cells of the immune system [25, 26]. Nevertheless, the role of the epithelium in the development of inflammatory bowel disease is still not completely understood. Functional integration of the epithelium with lymphoid tissue is presented by Peyer's patches, mesenteric lymph nodes, dendritic cells and T-regulatory cells of the lamina propria. It provides the basis for the homeostasis between the gut antigens and host immunity [27]. There are several epithelial cell types involved in the maintenance of the intestinal functions. About 80 % of IEC are represented by enterocytes that perform secretory and barrier functions. Goblet cells produce mucus and peptides for the growth and repair. Paneth cells secret antimicrobial defensins. Myofibroblasts secret proteins of extracellular matrix and basal membrane. M-cells are known as specialized epithelial cells of the follicle-associated epithelium of the gastrointestinal tract. In

addition, the intestinal epithelium contains neuroendocrine cells that produce serotonin, vasoactive intestinal peptide and other hormones [37].

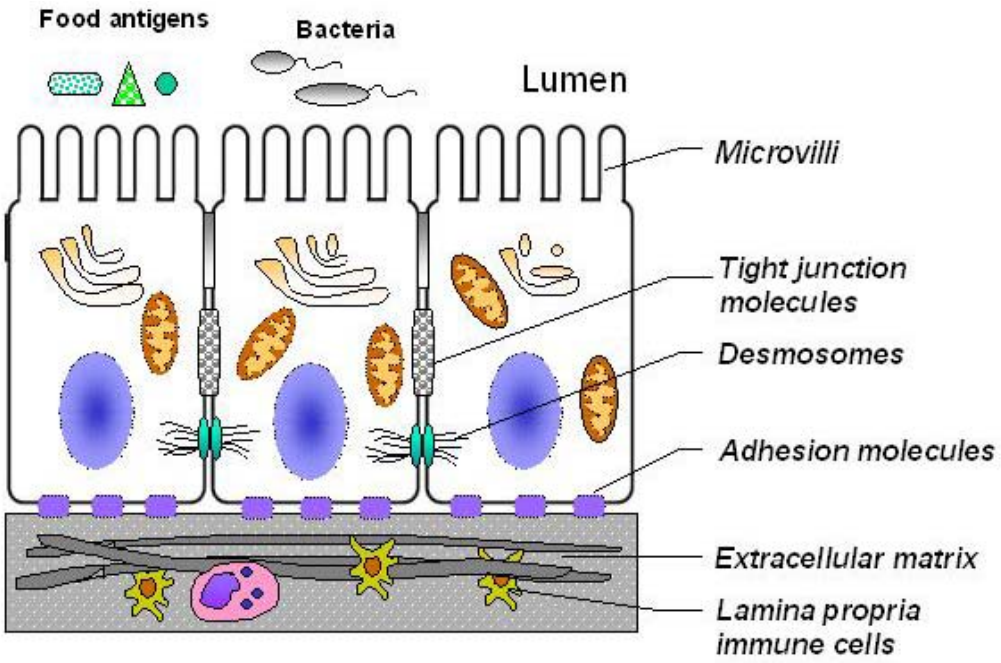

Figure 1. Morphologic structure of the intestinal epithelial cell.

It is supposed that one of the preconditions for the development of the intestinal inflammation is loss of ability of the epithelium to maintain the integrity and selective permeability [28, 29]. The regulation of expression of tight junctions and adhesion molecules is considered as one of the mechanisms of resistance to bacterial invasion in lamina propria and blood capillaries. Under conditions of chronic inflammation the integrity of the intestinal epithelium is lost or disturbed, accompanied by the increased apoptosis rates and poor regeneration [30, 31]. It is known that many junction proteins are bound structurally and functionally to the cytoskeletal components like F-actin or myosin [32]. Some pathogens like enteropathogenic *E.coli,* which causes diarrhoea upon infection in patients or in animal models, can increase tight junction permeability and reorganization of actin cytoskeleton upon phosphorylation of myosin light chains by specific kinase [33]. The increased epithelial permeability can be explored also under the influence of IFNγ and TNF found at the high levels in the intestinal mucosa under inflammatory conditions. The incubation of cultured IEC with TNF and IFNγ led to the redistribution of ZO-1, occludin, claudin-1,-4 and to phosphorylation of myosin light chain. The inhibition of specific phosphorylating kinase reversed the lost of the

epithelial permeability. These findings indicated that TNF and IFN utilize novel signalling pathways involving tight junction and cytoskeletal proteins [34]. It is also known that secretion of pro-inflammatory cytokine TNF can induce apoptotic pathway. Although apoptosis is a natural mechanism in the regulation of the cellular homeostasis, it also functions as a defensive mechanism by eliminating infected, mutated and transformed cells [35, 36]. Under normal conditions the cellular population or tissue establishes an optimal balance between growth/proliferation and apoptosis. Changing of this ratio towards the massive apoptotic cell death reflects an abnormal pathologic process. The disbalance between the cell death and regeneration leads to the destruction of tight junctions and to the loss of epithelium integrity.

1.1.3 Role of the gut-associated lymphatic tissue in mucosal homeostasis

Although the intestinal epithelium is considered as a major structural and functional component in defence of the mucosal surface, it presents an open system for environmental factors and different cells from the underlying tissues and organs. The gut-associated immune system fences off potentially harmful intestinal antigens from the systemic circulation and induces tolerance to luminal antigens. The mucosa-associated lymphatic system includes multiple lymphatic follicles (Peyer's patches) containing T- and B-cells, and free lamina propria lymphocytes [38]. B-cells of lamina propria produce large amounts of IgA directed against the mucosal microbiota. Secretory IgA prevents the adherence of bacteria to the mucosal surface [39]. Intraepithelial T-lymphocytes comprise effector and regulatory cells responsible for the cytotoxic functions and cell activation. The migration and homing of epithelial lymphocytes plays an important role in the recirculation of activated cells between the epithelium, organized lymphatic tissue and bloodstream [40, 41]. Immunologically active cells of the mucosal tissue are represented not only by T- and B-cells, but also by macrophages, eosinophils, mast cells and neutrophils. Schematically the integration of the intestinal epithelium with gut-associated lymphatic system is presented in Figure 2. Under conditions of chronic intestinal inflammation the mucosal tissue is infiltrated by granulocytes, macrophages and activated lymphocytes. The degree of infiltration is one of the important criteria to diagnose the severity and type of intestinal inflammation. Dendritic cells and regulatory T-lymphocyte also produce immunosuppressive cytokines TGFβ and IL-10 that act to inhibit pro-inflammatory cytokine secretion leading to the resolution of inflammation [42]. IEC can be either directly activated by bacteria to produce pro-inflammatory cytokines, or they can be additionally activated by regulatory cells from lamina propria. Since IEC express receptors for inhibitory

cytokines, the communication between immunosuppressive T-lymphocytes and enterocytes is possible even without their direct contact.

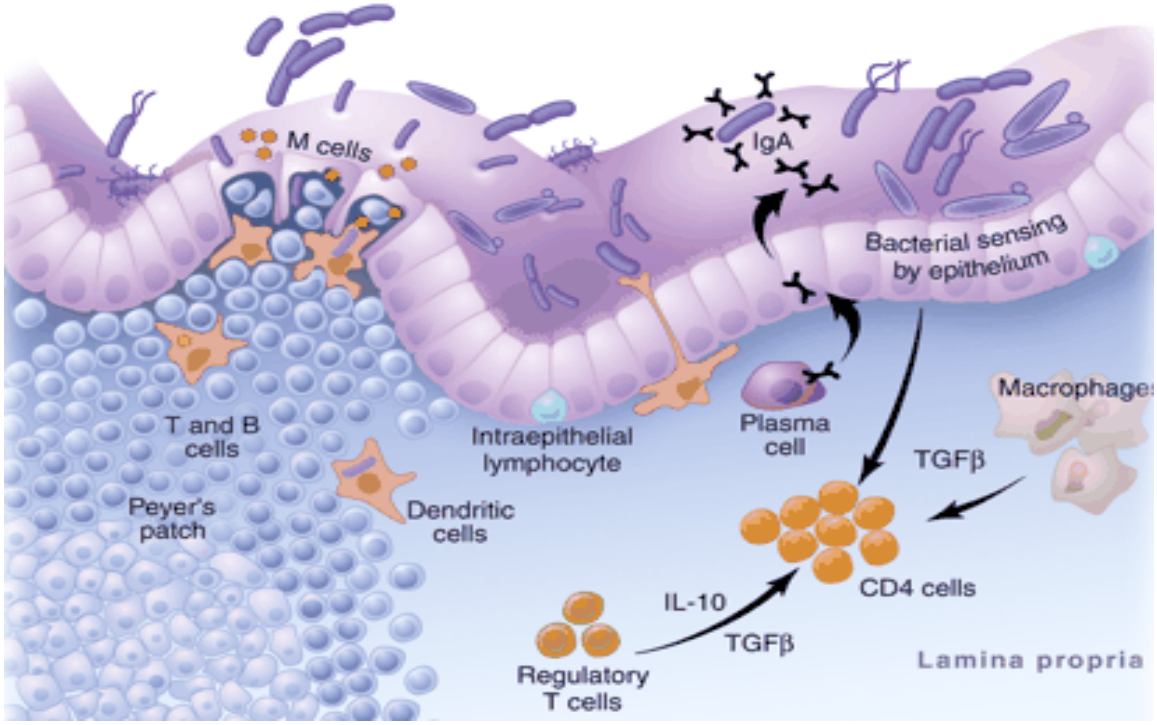

Figure 2. Structure of the gut-associated lymphatic tissue.
From *Science* 2005; 307: 1920-25.

1.2 Innate immune recognition

It was absolutely necessary for the host to develop specific identification mechanisms that would allow to recognize invariant molecular structures shared by many microorganisms. These molecular determinants are called pathogen-associated molecular patterns (PAMPs) [43]. The innate immune system utilizes a variety of pattern recognition receptors that are expressed on the surfaces of the cells or secreted to the extracellular space and bloodstream. Pattern recognition receptors are expressed by enterocytes, activated macrophages and other immune cells of the lamina propria. These receptors initiate and mediate important cellular processes such as opsonization, phagocytosis and presentation of antigens, activation of pro-inflammatory signalling pathways and induction of apoptosis [44]. Toll-like receptors (TLRs) are expressed by a variety of cells and play a crucial role in innate immunity and defense against microbial infection. Macrophages express scavenger receptor (MSR) with a broad binding affinity to LPS, LTA and double-stranded RNA.

MSR is functionally close to another protein MARCO binding the cell wall components of bacteria and mediating phagocytosis [45]. Some pattern recognition molecules are intracellular and interact with pathogens or viral particles that entered the host cell. This interaction prevents either the synthesis of virus-specific proteins and replication of viral genome or it activates signalling pathways and expression of the host genes, responsible for anti-viral (IFN) defence [46]. The cytoplasmic NOD1 and NOD2 proteins are also reported to be involved in activation of the NF-κB/MAPK pathways in response to components of the cell wall of Gram-positive bacteria. *Nod2* gene mutations lead to the increased predisposition to inflammatory bowel disorder and considered as one of the possible factors of the genetic susceptibility to Crohn's Disease in human [47].

1.2.1 Toll receptor signalling

Toll-like receptor was originally discovered in 1985 as a product of the gene coding a developmental regulator essential for the differentiation of *Drosophila* embryos [48]. Only later, in 1996 Toll-1 was described as an essential protein for antifungal response, but the mechanism of this action was still unknown [49]. The first mammalian Toll-like receptor (TLR-4) was identified in 1997 and its function was connected to the binding of the cell wall components of Gram-negative bacteria [50, 51]. Independently it was shown that mutation in one of the coding exons of TLR-4 gene correlated with hyporesponsiveness of C3H/HeJ mice to lipopolysaccharide [52]. Beginning from that moment several other mammalian TLRs were identified on the basis of their sequence homology. The specificities of all 13 members of TLR-family described to the present time were determined by systematically generated knock-out models and by the subsequent test of TLR-deficient cells for their response to isolated microbial components. It was found that TLR-2 appeared to bind peptidoglycans from Gram-positive bacteria, zymosan and bacterial lipoproteins. TLR-5 is able to bind flagellin. TLR-9 interacts with unmethylated CpG DNA that is present only in bacterial genomes [53, 54]. Schematically different Toll-like receptors and their binding ligands are presented in Figure 3.

Although TLR was initially described in *Drosophila* together with corresponding ligand Spätzle, which was not identified in mammalian, the final products of Toll-like activation are rather different: mainly antimicrobial peptides in insect and pro-inflammatory mediators (cytokines and chemokines) in mammalia. This functional diversity reflects the development of novel properties of the innate immune system during the evolution [55]. TLR-mediated cell signalling results in the activation of NF-κB and the expression of pro-inflammatory cytokines [56]. The members of TLR-

family have a similar molecular organisation. In addition, they partially share common intracellular signalling structures with IL-1 receptor like TIR domain and adaptor protein MyD88. The members of IRAK kinases family are connected to TRAF6 downstream MyD88 protein. Two different adaptors, connecting TRAF6 and IKK complex were proposed: TAK1 kinase with TAB1 and TAB2 members and ECSIT [57, 58]. The activation of adaptor protein TRAF-6 results in activation of MAPK kinases and IκB kinase followed by the dissociation and proteasomal degradation of phosphorylated IκB and nuclear translocation of NF-κB. Gene targeting of several adaptor proteins in mice abrogated NF-κB activation and led to the early embryonic lethality. MyD88 deficient mice did not respond to IL-1 and several Toll-ligands by the activation of NF-κB, MAPK, co-stimulatory and MHC molecules [59].

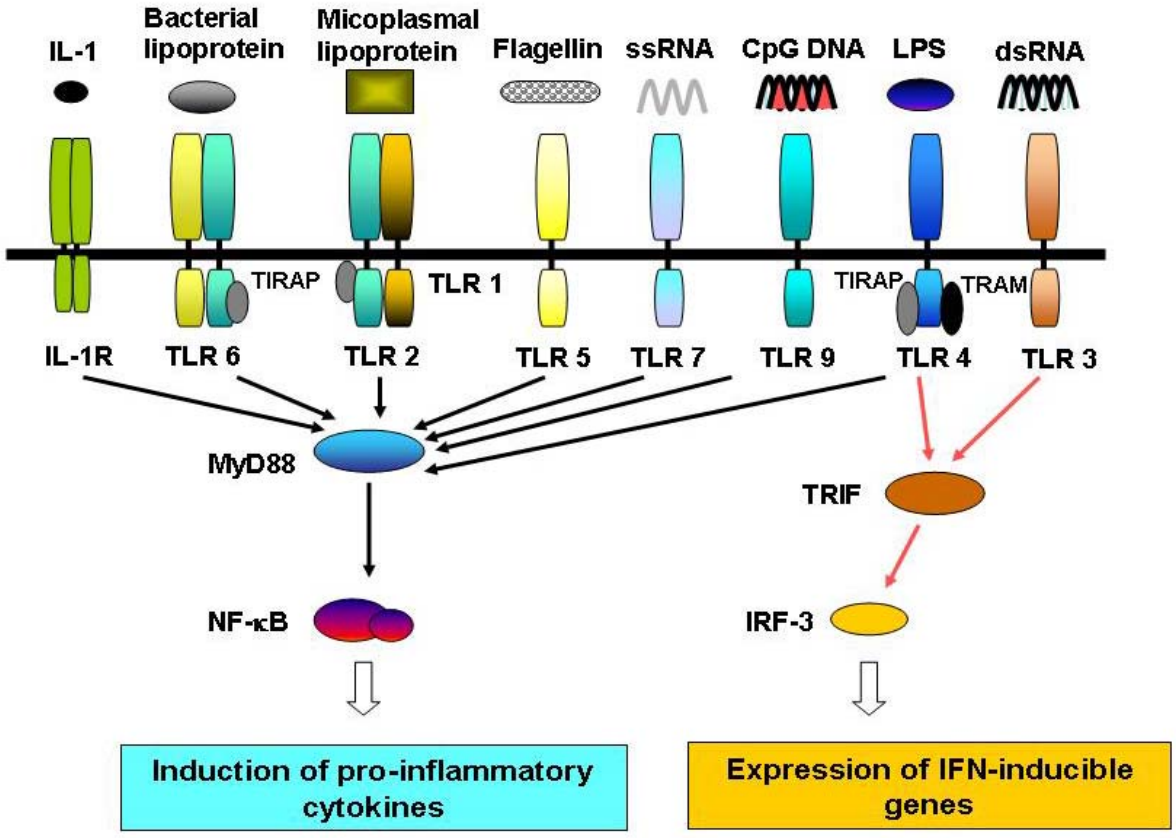

Figure 3. Mammalian Toll-like receptors and their ligands.

Later a MyD88-independent mechanism was described. It is supposed to be mediated by novel adaptor molecules TRIF and TRAM, participating in in IRF-3 activation and expression of IFN-

inducible genes [60]. Toll-receptor signalling results also in the expression of negative regulators for this pathway such as Tollip, IRAK-M and SIGRR, creating a feedback regulatory loop that is important for the prevention of inflammatory response to the commensal microbiota [61]. Toll-receptor recognition triggers multiple functions in epithelial cells. For example, recognition of TLR2 or TLR9 ligands increased the gut barrier functions and epithelium integrity. Also, the normal microbiota was able to induce cytoprotective proteins hsp25 and hsp72 in colonic epithelial cell lines [62, 63]. It is not excluded that the activation of intracellular signalling cascades takes place not only in the host cells, but also in bacterial cells of commensals after the contact with mucosal surface. Due to such bacteria-host interactions commensal microorganisms could be induced to produce mediators that prevent pro-inflammatory response in the intestinal epithelium. Another possibility for IEC to ignore the constant signalling from PAMPs would be to down-regulate expression levels for pattern recognition molecules by their compartmentalization or degradation [64]. One of the important roles in gut hyporesponsiveness to commensal microbiota belongs to the regulatory cells of lamina propria and gut-associated lymphatic tissue that produce inhibitory cytokines TGFβ and IL-10. Mice, deficient to produce IL-10 or TGFβ develop severe intestinal inflammation after bacterial colonization [65]. However, the molecular mechanisms of TGF/IL-10-mediated control of inflammation are not completely understood. It is also not clear how exactly potential pathogens and non-pathogenic commensal bacteria are initially distinguished by the epithelial cells.

1.3 Signalling to NF-κB: importance for innate immunity and inflammation

Nuclear factor kappa B (NF-κB) belongs to the family of transcription factors playing extremely important role in the regulation of a wide range of biologic processes such as cell differentiation, embryonic development, pathogen-recognition and inflammation [66, 67]. Upon activation in a complex signalling cascade this transcription factor translocates from the cytoplasm to the nucleus and regulates expression of NF-κB dependent genes. There are about 200 genes that can be induced or regulated by NF-κB, including genes of cytokines, growth factors, acute phase response proteins, cell-adhesion and apoptosis molecules. In turn, NF-κB family members can be activated by cytokines, mitogens, intracellular stress, bacterial or viral products [68, 69]. At the moment five members of the NF- κB family are identified and cloned: RelA (p65), RelB, c-Rel, NF-κB2 (p52/p100) and NF-κB1 (p50/p105) [70]. In unstimulated cells these proteins exist as a homo- or heterodimers, bound to the inhibitory IκB proteins or p100/p105. The common feature in the

structure of NF-κB transcription factors is conserved Rel homology domain (RHD), responsible for the dimerisation, interaction to IκB and binding to κB-elements on the promoter sequences of the genes [71]. The binding to IκB prevents the translocation of the complex to the nucleus. NF-κB can be activated by the classical and alternative pathways, depending on the input signal from an appropriate receptor-ligand complex [72]. Phosphorylated IκB is recognized by ubiquitin ligase machinery, leading to its poly-ubiquitination and subsequent degradation. The dissociation of IκB from NF-κB dimers makes nuclear export sequences (NES) and nuclear localization sequences (NLS) on p65/p50 accessible for the transport to the nucleus. IκB kinase (IKK) is 700-900 kDa complex containing two kinase subunits (IKKα and IKKβ) and one regulatory subunit NEMO (IKKγ). IKKβ is mainly involved in the classical activation of NF-κB. IKKα phosphorylates p100 followed by its processing to p52 and it is more essential for the alternative pathway [73]. Upstream of IKK complex there are different adaptor proteins that are functionally bound to the transmembrane domains of various specific receptors for cytokines, growth factors, bacterial and viral ligands. There are numerous signalling pathways in the cell that lead to NF-κB activation via IKK complex, IκB phosphorylation and degradation. Tumor necrosis factor (TNF) is one of the 19 described members that signals to NF-κB via TNF-R superfamily. Interestingly, TNF is able to induce both, cell death and survival pathways. Through the induction of the TNF-mediated survival pathway this pro-inflammatory cytokine regulates the expression of anti-apoptotic genes such as cIAP1/2, Bcl-xl and secretion of pro-inflammatory cytokines [74, 75, 76]. On the other hand, if NF-κB signalling is blocked, TNF induces rapid apoptosis in most cell types. Since the family of TNF receptor lacks an intrinsic enzymatic activity, intracellular adaptor molecules bound to the cytoplasmic tail of the receptor are recruited. Different intracellular adaptors like RIP family, TRAF, TRADD and SODD proteins mediate TNF-induced activation of IKK, p-38 MAPK, AP-1, JNK and caspases [77, 78, 79].

Toll/IL-1 is another important signalling pathway for innate and adaptive immune responses leading to the expression of NF-κB dependent genes. Corresponding signalling mechanisms mediated by TNF-R and TLR2 are depicted in Figure 4. TCR and BCR-mediated NF-κB activation is critical for the antigen-specific proliferation and maturation of lymphocytes into effector cells. Although this pathway is still not fully investigated, several novel members and adaptor proteins like ZAP70, ITAM, PKCθ, and CARMA-1 were recently reported to signal upstream of IKK/NEMO [80, 81].

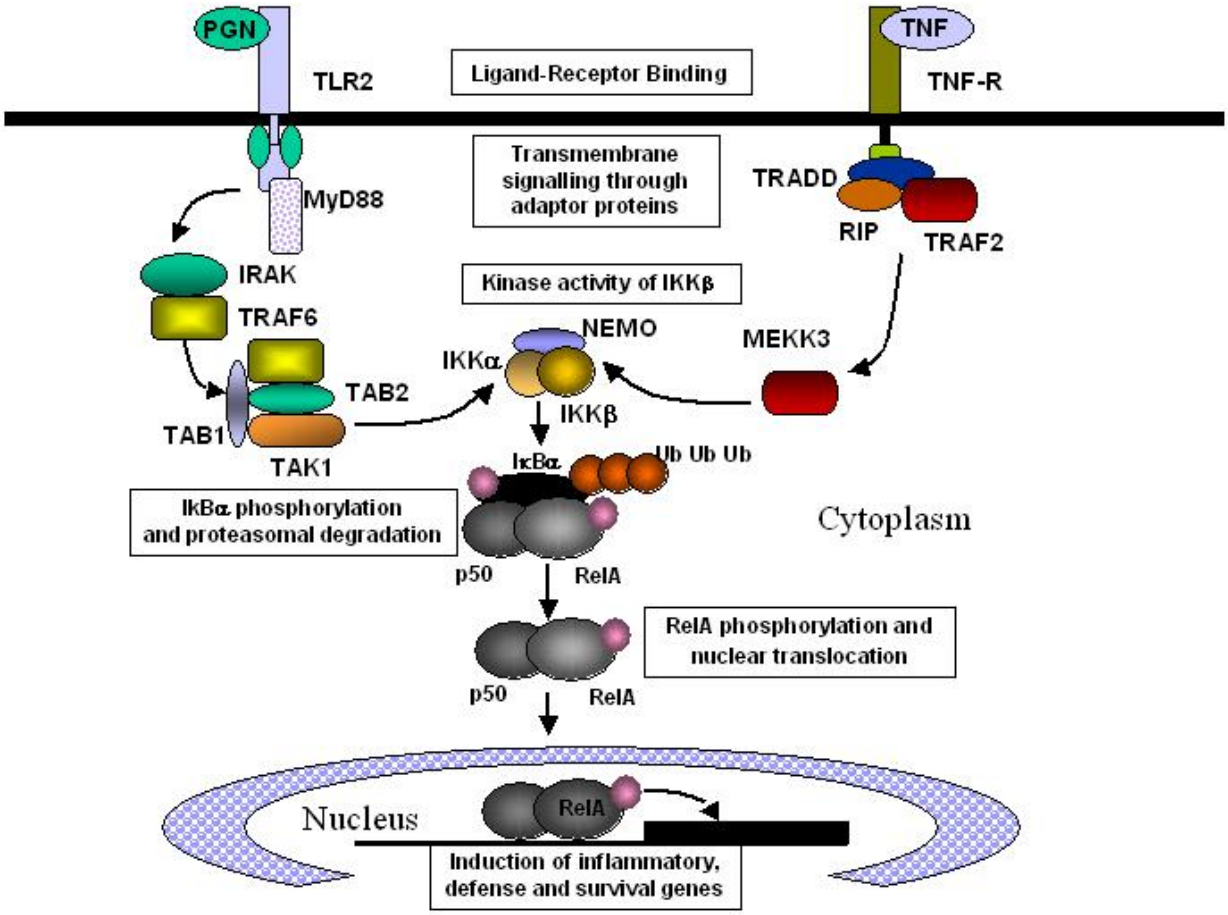

Figure 4. Mechanisms of TNF-R and TLR2-mediated NF-κB activation.

The transcription factor NF-κB is one of the basic regulators for the establishment of primary pro-inflammatory immune response, leading to the secretion of various cytokines including TNF, IP-10, IL-6, IL-1 and IL-8 [82, 83, 84]. These pro-inflammatory mediators attract and activate immun-competent cells. Multiple animal studies demonstrated that the deficiency of proteins involved in the NF-κB signalling cascade leads to the early embryonic lethality ($p65^{-/-}$), massive apoptosis ($IKK\beta^{-/-}$), inadequate humoral response ($p50^{-/-}$, $p52^{-/-}$) and other serious dysfunctions of the immune system [85]. These facts support the idea that although persistent NF-κB activation and pro-inflammatory gene expression can be harmful for the host, nevertheless, inability to signal to NF-κB due to genetical defects makes the host unresponsive to the uncontrolled invasion of virus or pathogens. It is crucial for the host to induce and inhibit pro-inflammatory response avoiding non-specific tissue damage by pro-inflammatory cytokines and chemokines. One of the negative regulators of NF-κB is TGFβ/Smad-mediated pathway that is important for the growth,

differentiation and immunosuppression. Multiorgan inflammation in TGFβ-null mice is thought to occur due to the loss of T-regulatory mechanisms, normally mediated by activated macrophages that produce TGFβ [84]. Host-derived protective mechanisms are aimed to control microbiota-induced "physiologic" inflammation by the regulation of TLR-mediated NF-κB signalling.

1.4 NF-κB activation and apoptosis

Apoptosis is an essential mechanism of multicellular organisms to regulate important cellular processes such as excessive cell proliferation, elimination of infected and malignant cells [87]. The cells undergoing apoptosis display typical morphological alterations such as membrane blebbing, cell shrinking, hypercondensation of chromatin and fragmentation of chromosomal DNA. All these alterations are driven by a complex network of intracellular signalling mechanisms, leading to the activation of caspases, proteins from cysteine proteases family [88]. The extrinsic apoptotic pathway is triggered by the binding of ligands to the extracellular death receptors as Fas, CD40 or TNF-R. The intrinsic apoptotic pathway involves mitochondria, releasing apoptosis-promoting factors like cytochrom c, Apaf-1 and others [89]. Two pathways act via different initiator caspases, but they both lead to the activation of two major executive caspase-3 and 7 (Figure 5). In contrast to the proliferation, growth or activation, apoptotic cells generally down-regulate their gene and protein expression. The essential metabolic pathways are suppressed in the apoptotic cell due to the failure to maintain oxidative respiration and energy homeostasis. It could be suggested that two distinct mechanisms such as apoptosis and NF-κB activation are mutually excluded and have no common crossing points. NF-κB was initially considered as an anti-apoptotic factor [90, 91]. Indeed, the direct evidence was provided by the target gene-disruption of NF-κB family members in animal models. RelA deficient mice died at the early embryonic stage due to the massive apoptosis of hepatocytes [92]. The same consequence was caused by the deletion of IKKβ/IKKα and NEMO genes in mice [93]. It was also reported that expression of anti-apoptotic genes such as cyclin D, mitochondrial Blf-1, Bcl-xl, TRAF1 and TRAF2 is also mediated by NF-κB [94, 95]. In addition, there is an evidence that apoptotic molecules can negatively regulate NF-κB. The cleavage of p65 by caspase-3 leads to the loss of trans-activation domain. Caspase-3-induced cleavage of IκBα makes this protein resistant to the proteasomal degradation in response to NF-κB inducers [96]. The evidence of the shared signalling between apoptosis and NF-κB is TNF-R-mediated NF-κB activation with subsequent induction of caspase inhibitors cIAP1, IAP2, XIAP and mitochondria stabilizers Bcl-xl and Bfl-1 [97].

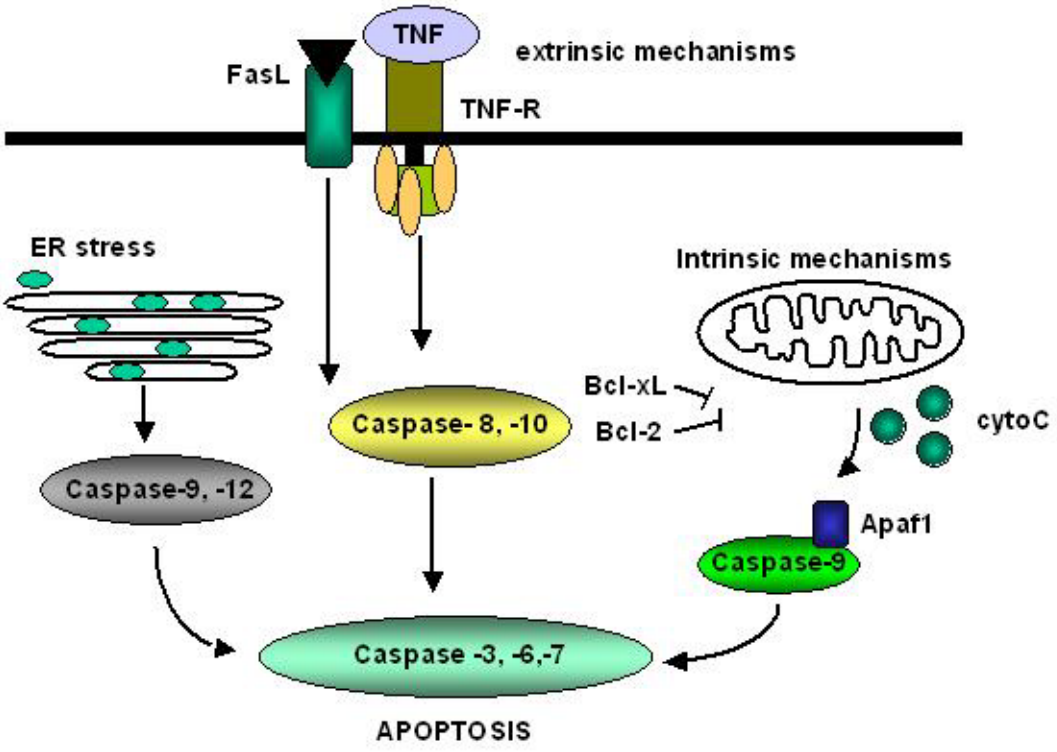

Figure 5. Apoptosis induction mediated by intrinsic and extrinsic mechanisms.

The regulation of apoptosis is especially relevant for the gastrointestinal tract, since the intestinal mucosa undergoes a process of continual cell turnover essential for the maintenance of its normal functions [98]. Pro-inflammatory cytokines are known to drive inflammation, but emerging data suggest additional important roles for TNF by influencing mucosal barrier functions, adhesion/junction molecule expression and epithelial apoptosis [99]. The introduction of anti-TNF antibodies for the therapeutic purposes suppress the severity of intestinal inflammation by the induction of T-cell apoptosis. However, the increased rates of apoptosis in intestinal epithelium would result in disruption of integrity and tissue injury [100, 101]. The reduction of the excessive epithelial apoptosis and simultaneous inhibition of pro-inflammatory cytokine secretion is considered as one of the therapeutic strategies for the treatment of IBD.

1.5 Animal models of chronic intestinal inflammation

Despite the great progress in understanding of the phenomenon of the gut hyporesponsiveness to the commensal microbiota, the contribution of the intestinal epithelium to the host protective mechanisms still remains unclear. There is no doubt that bacteria have a profound effect on the

intestinal epithelium after the first days of colonization. The positive outcome of this bacteria-epithelial cross-talk is a basis for the interaction between innate and adaptive immune system directed to distinguish pathogens from commensals [102]. These molecular mechanisms present a complex system of signalling pathways. They start from extracellular ligand-receptor interactions through diverse adaptor proteins leading to the activation of corresponding transcription factors. There are many experimental animal models with gene deletions and mutations that result in impaired homeostasis to the intestinal microbiota leading to the intestinal inflammation [103, 104]. The generation of these animal models displays a simplified way to understand bacteria-host interactions. For most of the animal models the absence of commensal bacteria (germ-free conditions) does not lead to the intestinal inflammation.

One model of spontaneous intestinal inflammation similar to Crohn's disease is the SAMP1/Yit (Fc) mouse model of ileitis. The inflammation is microbiota-driven and absent in germ-free mice [105]. Interestingly, no inborn mutations were found in these mice, but various alterations in the epithelial structure and T-regulatory populations were observed. In addition, it was reported that NOD2 expression was increased as well as the expression of chemotactic and adhesion molecules for neutrophil trafficking [106, 107]. Another model that is susceptible for the development of spontaneous colitis is C3H/HeJBir mice. They develop focal colitis localized to the cecum and accompanied by the induction of Th1-response towards some bacterial antigens [108].

Transgenic animals that are deficient in production of cytokines or growth factors (TGFβ, IL-10, IL-2) develop the intestinal inflammation of different severity after the colonization with non-pathogenic microorganisms.

TGFβ is an autocrine pleiotropic regulator of activated macrophages and other immune cells, involved in the inhibition of NF-κB signalling by induction of TGF/smad2 signalling. Mice lacking TGFβ or TGFβII receptor develop multiorgan inflammation, fibrosis and T-regulatory immunosupression and die at the age of 5 weeks [109]. TGFβ-null mice are hypersensitive to endotoxin due to the increased expression of TLR4. It is supposed, that TGFβ-deficiency provokes abnormal T-regulatory response. Dominant-negative mutations in TGFβII receptor lead to the development of severe colitis, wasting and diarrhoea in mice [110, 111]. One of the major regulators of TGFβ-signalling is Smad3. Smad3-deficient mice live 1-3 months and die due to the multifocal pyogenic abscesses of the intestinal tissue [112].

IL-10 is another anti-inflammatory cytokine that is produced by macrophages, dendritic cells, activated Th1 cells and NK-cells. IL-$10^{-/-}$ mice do not develop intestinal inflammation under germ-

free conditions; if colonized they develop severe colitis, anaemia and growth retardation [113, 114, 115]. The pathogenesis of this colitis model is not fully understood. It is suggested that enhanced Th1 responses and subsequent immune activation of macrophages and dendritic cells result in production of high levels of pro-inflammatory IL-1, IP-10, IL-6, TNF and IFNγ. The administration of anti-IFNγ antibodies to IL-10 deficient mice abrogated the development of colitis similarly with delivery of IL-10 to the gut [116]. It is still not much known about altered signalling in native epithelial cells in the absence of IL-10. STAT-3 is transcription factor important for IL-10-mediated signalling. STAT-3 deficiency is embryonic lethal. Conditional gene targeting of STAT-3 in isolated cells stimulated with LPS led to the increased production of pro-inflammatory cytokines, resistant to the treatment with IL-10. Conditional STAT-3 knockout mice develop severe colitis with granulocyte infiltration of large intestine and die after 2 months [117].

One of the experimental models of IBD is A20-deficient mice. A20 is a negative regulator of NF-κB pathway involved in the proteosomal degradation and de-ubiquitination mechanisms. *A20*$^{-/-}$ mice die shortly after birth with multiple signs of severe gut inflammation. LPS-stimulated macrophages of these animals produced *ex vivo* much higher levels of TNF, than *A20*$^{+/-}$ or wild type animals [118].

P-glycoprotein is a member of ATP-binding transmembrane transporters family, expressed on the apical surface of the epithelial cells. This protein was initially described as a product of multiple drug-resistance gene *mdr1a,* known to be associated with resistance of the malignant cells to chemotherapeutic agents. *Mdr1a* $^{-/-}$ mice develop transmural colitis accompanied by Th1-cytokine profile and disruption of barrier functions of the epithelium [119]. Mice deficient in α-subunit of Gi2 protein, one of the adenylate-cyclase-mediated signal transducers, also suffer from severe pancolitis [120]. The defects of the epithelial barrier function and infiltration of the lamina propria with activated T-cells could be already detected at early stages of the inflammation. Dominant negative mutation of N-cadherin also results in the development of intestinal inflammation in mice due to the disruption of intra-epithelial interaction and tight junctions. This animal model suggest an important role of adhesion/junction proteins in the maintenance of the epithelial integrity [121].

IL-2 plays an important regulatory role in the establishment of natural immunotolerance to self-antigens. IL-2 knockout mice develop multiple pathologies with severe autoimmune component. One of the pathologic signs of IL-2 deficiency is pancolitis with massive hyperplasia and lymphocyte infiltration of the colonic epithelium. Mice lacking IL-2 receptor demonstrate similar pathology and decreased $CD4^+CD25^+$ subpopulation of the regulatory T-cells [122]. Mice lacking T-

cell receptor (α-chain) suffer from chronic diarrhoea, rectal prolapse and acute inflammation of the colon, mediated by Th2 response. The colitis appeared only after bacterial administration and was characterized by the autoimmune response of the mice to multiple host-autoantigens due to the activation and expansion of B-cells [123].

Not only deletion, but also the overexpression of certain molecules in experimental models can cause inflammatory bowel disorders. The genetic modification of the untranslated region of TNF gene increases mRNA stability and expression levels of TNF in response to the normal microbiota. The severity of bacteria-induced ileocolitis can be attenuated or eliminated on the TNF-receptor deficient background [124]. In STAT-4 transgenic mice, immunized with synthetic antigens, unremitting colitis, diarrhoea and weight loss were accompanied by massive infiltration of the inflamed intestine with T-cells and excessive secretion of IFNγ and TNF [125]. The increased amounts of macrophages-secreted TNF, IL-8, IL-1 and IL-1 were observed in transgenic CD40-overexpressing mice developing multiorgan inflammation and colitis [126].

To summarize, these selected examples demonstrate the importance of essential cytokines, growth factors and other regulatory molecules of the immune system for the establishment of the complex mechanism of the gut hyporesponsiveness to the commensal enteric microbiota. In each case the bacterial colonization is one of the most critical moments for the host to ensure the induction of appropriate protective mechanisms directed to tolerate enteric non-pathogenic microorganisms.

1.6 Human Inflammatory Bowel Disease

Inflammatory bowel disease (IBD) is a chronic relapsing idiopathic inflammation defeating the gastrointestinal tract. Two distinct pathologies are recognized in human IBD: Crohn's disease (CD) and ulcerative colitis (UC). Both disorders affect mostly people in early adult life in approximately equal female/male proportion with combined frequency 150-200 cases on 100 000 European population [127]. Symptomatically Crohn's disease is more pleomorphic and can affect any part of the gastrointestinal tract from the oral cavity to the rectum. More often CD involves ileal regions with discontinuous segments of transmural inflammation. The clinical features of CD depend on the localization and typically include obstructive symptoms of abdominal pain, vomiting, weight loss and bloody diarrhoea. Ulcerative colitis affect the rectum and proximal colon and has similar symptoms. In addition, for CD and UC the development of fistulas between the gut epithelial tissue and other organs like bladder or skin can generalize inflammation due to the infection of these organs with unusual microbial environment [128, 129]. The symptoms of IBD can be partially treated by

the use of powerful drugs like prednisolone, azulfidine or its chemical analogs. However, drug-therapy often produces multiple side effects like diabetes, osteoporosis and depression. In some cases debilitating surgery and even the entire colon resection can only be curable for prevention of the death in IBD patients [130, 131]. The development of effective therapy specifically attenuating the intestinal inflammation is not possible without complete understanding of the disease etiology and detailed molecular mechanisms of pathogenesis.

Although many aspects of the etiology and pathogenesis of IBD are still unknown, it is believed that CD and UC are immune-mediated diseases in the genetically susceptible host, resulting in aberrant immune response, loss of tolerance to the normal intestinal microbiota and chronic gut inflammation [132, 133]. Much effort has been recently made for the identification of the predisposition or susceptibility markers of IBD. Nevertheless, no single gene locus was found to be completely responsible for the initiation of the disease. Broad genetical studies including allele polymorphism in human population, family studies of siblings and twins and large-scaled introduction of genomic approach allowed to extend the knowledge about genetic aspects of IBD. Thus, the population studies showed that there are ethnic groups with increased rates of IBD like Ashkenazi Jews, in contrast to the population of Afro-Caribbean's with a very low rate of disease in comparison to the average frequency [134, 135]. The family studies demonstrated significantly higher rates of the disease concordance in monozygotic twins, particularly for Crohn's disease [136]. Multiply affected families and siblings-pairs were investigated word-wide by full genome screening in order to look for the potential chromosomal loci with hypothetical markers of predisposition. Several chromosomal regions with strong reproducible linkage to IBD were localized. Later, positional cloning and sequencing technologies were involved for the fine mapping of these identified loci [137, 138]. The localization of *Nod2* gene on the chromosome 16 and investigation of its possible functions in human and animal models significantly improved the hypothesis about genetic predetermination of the increased risk to IBD. Mutations of this gene were found in patients with Crohn's disease, but not in patients with ulcerative colitis, increasing up to 40 times the relative risk for CD [139]. Mice, mutated or deficient in *nod2* gene exhibited enhanced NF-κB activation in response to bacteria-derived muramyl-dipeptide and increased secretion of IL-1β [140]. NOD1 and NOD2 proteins that are expressed by the antigen-presenting and epithelial cells can recognize peptidoglycan of the bacterial cell wall. In comparison to TLRs that have extracellular and transmembrane domains, NOD proteins are localized in the cytosol and can bind only processed or transported bacterial products. This interaction could be achieved by phagocytosis in antigen-presenting cells and by the apical

peptide transporters in epithelium [141]. One of the main outcomes of NOD1 and NOD2 activation is NF-κB signalling. In case of NOD2, the effector molecule RICK mediates K63-linked poly-ubiquitination of IKKγ and phosphorylation of IKKβ, resulting in IκB degradation and NF-κB translocation to the nucleus. In addition, NOD1 and NOD2 are involved in the activation of JNK, ERK and p-38 MAPK pathways by unknown mechanism [142, 143].

Searching for the genetic predisposition to IBD still leaves many open questions: to what extend two similar disorders (CD and UC) share common susceptibility genes; whether the true gene mutations or just certain polymorphic alleles are associated with disease; how close are the animal models of intestinal inflammation to human IBD.

The genetic background of the host is only one of the possibilities to provoke disease development. The environmental factors like smoking, synthetic food ingredients or drugs with side effects to the gut are also reported to influence IBD [144]. The commensal microbiota is considered as one of the most important factors that could initiate uncontrolled immune response in the absence of adequate regulatory mechanisms [145, 146]. However, the animal models show that intestinal inflammation often develops only after the bacterial colonization of the germ-free host. For example, in IL-10 knock-out mice colonized with *Enterococcus faecalis* the initial early response to these commensal bacteria occurs within 1-2 weeks, but histologically diagnosed intestinal inflammation develops then after 2-3 month [115]. Since in human the disease can affect the adult population, such a long delay between early and late responses seems unrealistic. One of the possibilities to explain IBD etiology would be to suppose that intrinsic defects in innate immunity become visible during the interaction with adaptive immune mechanisms involving the balance between T-regulatory and T-effector functions. The excessive effector T-cell activation or deficient T-regulatory function can lead to the loss of mucosal homeostasis by the induction of Th1 or Th2 pathways, characterized by certain cytokine-expression profiles. Th1-mediated response with secretion of IFNγ, IL-12 and TNF was reported for CD patients. In contrast, for UC patients Th2-mediated response with secretion of IL-4, IL-5 and IL-13 was mainly observed [147, 148]. The population of the regulatory $CD25^+$ T-cells is thought to have suppressive function by production of TGFβ and inhibition of NF-κB mediated expression of pro-inflammatory genes [149]. T-regulatory cells, producing the inhibitory cytokine IL-10 can be induced by dendritic cells in Peyer's patches. There is experimental evidence that IL-10 is required for TGFβ secretion and signalling [150].

The therapeutic approaches of IBD treatment are aimed to suppress the excessive effector T-cell functions by diminishing of the cytokine secretion and correction of Th1/Th2 balance. Currently,

the use of azathioprine and anti-TNF or anti-IL-12 antibodies was successfully applied for the treatment of Crohn's disease. This treatment is aimed to induce apoptosis of Th1-cells rather than to block TNF-activity [151, 152, 153]. The delivery of TGFβ and IL-10 could be potentially applied directly to the inflamed mucosa by genetically modified microorganisms, as it was previously done in animal models [154, 155]. Recent studies indicated that the regeneration of the damaged intestinal mucosa can be accelerated by epithelial-derived growth factor (EDGF) which could be also considered as a candidate for the delivery into affected gut regions [156].

Other therapeutic strategy for IBD is based on the ability of some probiotic microorganisms to restore the bowel and immune functions by unknown mechanisms. It should be further investigated how these microorganisms can be beneficial to the host. The treatment with probiotic microorganisms can be achieved through the supplements or appropriate diet, containing probiotic species [157, 158]. The identification of novel diagnostic markers together with understanding of etio-pathogenesis of IBD will help to work out such prophylactic and therapeutic strategies that contribute to the resolution of intestinal inflammation by affection of specific cell populations and intracellular pathways with minimal side effects.

1.7 Proteomics: identification of novel targets and molecular mechanisms

1.7.1 Proteome analysis: principles and main strategy

Proteome analysis is the investigation of all proteins present in a cell, tissue or organism at any one time. Since cells are constantly responding to the environmental stimuli, their protein expression profiles are persistently changing. In a wide sense modern proteomics includes a conglomerate of advanced analytical methods and knowledge directed to assess protein expression status of the cells in its full complexity, taking into account not only relative quantification, but very important, metabolic activity and turnover (metabolomics), modifications and interaction of the proteins in complexes and signalling cascades (interactomics) and functional analysis [159]. By use of carefully controlled growth conditions and well-established experimental design it is possible to obtain detailed information about the physiological status of the living cells during the disease development and to investigate the influence of certain bioactive substances or drugs on the cell systems.

The development of DNA microarray technology allowed analysing gene expression at the level of transcriptome. The mapping of new gene loci and prediction of corresponding protein sequences in

mammalian species contributed to the creation of the broad gene and protein databases for many taxonomic groups including rat, mouse and human [160]. Although proteomics would be not possible without previous achievements in the genomics, measuring of the gene expression at the protein level is potentially more informative for a better understanding of physiologic and pathologic state of the organism. Normally, every cell of the organism contains an identical copy of the genome. However, the information stored in the genome is used differentially in various cell types due to the tissue-specific protein expression [161]. In many human diseases namely an incorrect modification of a normal protein leads to the development of pathology. Finally, proteins are always useful for the diagnostic of disease. In addition, they are the main drug-targets during the treatment.

Generally, for successful conduction of proteome analysis it was important to develop all key technologies, including improved two-dimensional protein separation with a high resolution, creation of computer programmes for analysis of 2D-gel images, introduction of high-sensitivity mass-spectrometers and availability of genome and protein databases. The separation of the complex protein mixtures by two-dimensional gel electrophoresis into reproducible patterns gives an opportunity to detect quantitative and qualitative differences between several samples. 2D-SDS-PAGE, initially described by O'Farrell in 1975 is based on the separation of the proteins according to isoelectric point and molecular weight [162]. Isoelectric focusing, improved by the application of the immobilized pH gradient (IPG) strips for the first dimension significantly increased the quality and reproducibility of the method [163]. The second dimension, followed by the staining of the gels for the analytical or micro-preparative purposes allowed the separation of thousands of proteins extracted from the cells, tissues and biological fluids. One of the limitations of this method is the loading capacity of IPG strips. Even using of the overlapping IPG stripes with narrow pH gradients not always allows to visualize low-abundant proteins together with high-abundant [164]. Another key moment in proteomics is a sample preparation that should be done as simply as possible in order to avoid the protein degradation and get all proteins solubilized. In order to achieve a true representation and a deep coverage of proteins, several strategies can be undertaken during the protein separation. Recently applied fractional proteome analysis allows significantly increase the solubility of the cellular organelles in specific detergents. In addition, it is an initial step in order to elucidate cellular functions, mechanisms of intracellular transport and compartmentalization [165, 166]. Proteome analysis of the isolated functional units inside of the given cellular compartment demonstrates the analytical power of this approach for the future. The proteome analysis of the mammalian spliceosom [167], analysis of interchromatin granule clusters in mice [168], proteomics of

the nuclear envelope in murine cells [169] or spindle pole by yeast [170] are the examples of unique exemplary studies.

Unfortunately, 2-D SDS-PAGE by itself would be still only a descriptive technique without the availability of the reliable tools for the identification of proteins in complex mixtures. The revolutionary techniques such as matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS), electro-spray ionization (ESI) and tandem mass spectrometry replaced less sensitive slow protein degradation methods [171]. The fundamental principle of MS analysis involves the conversion of the subject molecules to either cations or anions in the ion source, followed by their separation according to the mass/charge (m/z) ratio in the mass analyser and subsequent detection. In MALDI the sample is embedded in a large excess of organic matrix that has a strong absorption at the laser wavelength. Following the laser irradiation of the sample surface, the matrix accumulates energy, initiating the proton transfer between the matrix and analyte and formation of singly-charged ions. In simple terms, gas-phase ions, produced in the ion source are introduced into the mass analyser and differentiated according to their mass/charge ratio on the basis of their motion in a vacuum under the influence of electric or magnetic fields. ESI produces ions directly from solution where the sample passes under the pressure through a capillary. The strong electric field induces the charge accumulation at the liquid surface and leads to the formation of the highly charged droplets [172]. There are several different types of mass-spectrometers that combine ESI or MALDI with a variety of mass analysers for the detection of ions from high-molecular-mass proteins and polypeptides with extremely low concentrations.

The development of the computer algorithms for the interpretation of mass-spectrometry data with information in databases allows the identification of the proteins by searching for all peptide fragments with the same mass as a selected ion and by prediction of expected unique amino acid sequence [173]. The existing databases are constantly growing being completed by novel proteins with unknown or hypothetically predicted functions, leading to the accumulation and systematization of the proteins within every taxonomic group. The more genes and corresponding proteins are described, cloned and documented, the higher probability for their identification by proteome analysis. Like in genomics, Human Proteome Organisation (HUPO) coordinates the proteomic projects worldwide, creating proteomic databanks obtained from different cell, tissue and organ-types [174].

1.7.2 Proteomics for disease: importance of clinical studies

Despite tremendous advance in understanding of molecular mechanisms of the human diseases, pathogenesis and etiology of many disorders are still completely unknown even if the symptoms can be already successfully treated. Most of such diseases have multifactorial ethiology and can not be explained by mutation or dysfunction of any single genetic locus. These diseases should be investigated from the different aspects, using a broad spectrum of clinical and molecular biology methods in order to estimate the interaction between the genes, their products and environment. Knowledge of the sequence of the human genes has provided a framework for genomic approach to search disease-responsible genes. A similar knowledge of the human proteome is currently lacking. Ideally, such database would include information about the sequences of all human proteins, their post-translation modifications, tissue/organ distribution and functions [175].

Of course, proteome analysis in the human system is much more complex and sophisticated in comparison to the cell culture or isolated primary cells from the animal models. First, the human population is extremely polymorphic and heterogeneous. Even basic structural or metabolic proteins can have multiple isoforms and allelic variants. As a consequence, the same disease can have completely individual properties in every single person and affect different organs or system with different severity. Second, since proteomics is an approach that mainly deals with relative quantification, it requires a reference group for comparison. It would be extremely important to have control groups consisting of healthy individuals within a “normal” phenotype. However, due to the heterogeneity of the human population, this “reference” group alone can be as a subject for the study of individual variations between healthy persons. Third, there are technical and ethical problems of availability of the biological material from human. If the clinical material can be often taken from patients during biopsy or surgical operation, it is very difficult to obtain biomaterial of certain tissue/organs from the healthy individual. It is necessary to perform proteome analysis using 2D-patterns generated from every single patient and control individuals with subsequent cross-comparison and appropriate statistic. The selection of significant reproducibly-regulated proteins and their further verification by other molecular methods would give an opportunity to estimate variations and relative risk within the group of the patients.

The main directions in human proteomics include the conduction of clinical studies in order to find new disease markers, potential drug targets and to investigate host-pathogen interactions. In addition, it is necessary to improve and standardize the procedure of tissue/organ profiling in order to accumulate specific databases that are open for clinicians.

1.7.3 Epithelial protein expression profiling

Proteome analysis in primary IEC and different epithelial cell lines is aimed to study epithelial cell biology in health and disease. This would allow to identify novel prognostic, diagnostic and therapeutic markers. It seems attractive to link disease biomarkers through specific mechanisms of pathology to potential drug targets. Epithelial cell proteomics may bridge an existing gap between acute and chronic inflammation, tissue injury, multiple organ dysfunction and normal functions [176]. Since epithelial cells share many common structural, morphological and functional properties there are some general aspects important for the development of methodological strategies for the proteomic approach. One of the important aspects is sample preparation and purity, especially with respect to isolation of primary epithelial cells. In addition, it is still a great need to separate cellular proteins with high resolution, taking into consideration the limitations of currently used two-dimensional gel electrophoresis and multidimensional chromatography. Thus, a quite good resolution can separate and detect about 1000-3000 proteins, but it is still far from the estimated minimal number of 50 000 peptides produced by the average cell during the life cycle [177, 178]. Sensitivity limits of 2D-gel staining can also significantly influence the protein visualization. Therefore, it is necessary to improve the resolution, detection and identification of low-abundant proteins, achieved by different methods of fractionation, extraction and concentration. Of course, the interaction of the epithelium with other cell types is functionally important and leads to the introduction of new methods for highly selective isolation of the cells during sample preparation. The epithelial cells form complex architectural structures, tightly connected with tissue specificity and function, such as crypts in gastrointestinal tract, alveoli in respiratory system or renal capillary. In these systems not all cells are equally involved in the intracellular communication, cell signalling, interaction with potential pathogens and external stimuli. Laser Capture Microdissection (LCM) is a novel technique that allows to isolate selected tissue regions and even single cells [179]. For example, it is possible to obtain separately inflamed and non-inflamed regions of the intestine. By using of specific fluorescent markers it is also possible to distinguish activated cells from non-activated. This is an exiting and attractive start to link epithelial cell proteomics with histology of clinical samples, but there is a need in good established histopathology expertise. Although the amounts of protein from the captured cells may be limited and not sufficiently accurate for the preparative purposes, combination of LCM with fluorescent staining, followed by the sensitive analytic staining techniques allows to identify protein expression changes between selected populations containing about 25000 cells [180]. Another possibility to increase protein resolution

would be the fractional isolation of sub-cellular components and organelles, which allows to concentrate the proteins with similar solubility and localization. Most differentiated epithelial cells are polarized, so that they display differential distribution of structural, adhesion/junction proteins, transporters, receptors and signalling molecules on their apical and basolateral surfaces. Thus, by Boniha V. et al apical microvilli from mouse retinal pigment epithelia were isolated and subjected to proteome analysis [181]. It is still difficult to investigate extracellular or secretory proteins by proteomic approach. However, combination of *in situ* labelling with isotope-coded affinity tags and mass-spectrometry gives a chance to follow many cellular metabolic pathways. Lee S. et al analyzed rat intestinal epithelial cells transformed with cyclooxygenase-2 in order to investigate synthesis and transformation of bioactive lipids [182]. Protein expression profiling in isolated primary cells gives much more biologically and functionally relevant information about the cell physiology than proteome analysis in epithelial cell lines. Most of the epithelial cell lines were originally generated on the base of malignant or viral transformation allowing their immortalization. To this reason cultured epithelial cells often change their "natural" protein expression profile. Cultured epithelial cells can be undifferentiated, they can be resistant to spontaneous or induced apoptosis or loose certain receptors and corresponding signalling pathways. Some of these effects can be detected by proteome analysis and more important, must be correctly and carefully interpreted and extrapolated to clinical-relevant studies. Therefore, it is always useful to combine proteomic techniques with the subsequent verification of the obtained data by other molecular biology methods in different cell systems and various organisms. This principle is a basis for functional proteomics that makes this approach not only descriptive, but also gives an opportunity to demonstrate the biological relevance of differential protein expression.

2 Aims of the present work

The main focus of the present work is to use functional proteome analysis for the characterization of molecular and cellular host-derived protective mechanisms in intestinal epithelium responsible for the establishment of hyporesponsiveness to the commensal microbiota. We use wild type and IL-10 gene deficient mice monoassociated with Gram-positive *Enterococcus faecalis* as a simplified model to study bacteria-epithelial interactions in the genetically susceptible host under conditions of chronic intestinal inflammation. Investigation of TLR2-mediated NF-κB activation and its regulation by TGFβ/Smad signalling together with epithelial protein expression profiling and functional analysis would allow to identify novel targets and mechanisms involved in control of inflammation in native IEC. Functional analysis of detected proteins by use of siRNA-mediated knock-down strategy would allow to estimate their contribution, biologic and physiologic relevance for the generation of pro-inflammatory and anti-inflammatory host immune responses.

In addition, we use primary IEC from patients with Crohn's disease and ulcerative colitis to reveal protein expression changes in inflamed mucosal tissue. This initial screening is aimed to introduce proteomic approach for the investigation of protein expression changes in human intestinal epithelium under pathologic conditions. This would allow to identify novel diagnostic protein markers and to explain unknown aspects in etiology and pathogenesis of human IBD in order to find effective therapeutic strategies in perspective.

3 Materials and Methods

3.1 Reagents and Chemicals

Table 1. Reagents and Chemicals.

Reagent	Supplier
30 % Acrylamid/ 0.8 % Bisacrylamid	Roth, Karlsruhe
Acetonitril	Roth, Karlsruhe
Agarose GTQ	Roth, Karlsruhe
Ammoniumpersulfat (APS)	Roth, Karlsruhe
Ammoniumhydrogencarbonat	Roth, Karlsruhe
Acetic acid	Roth, Karlsruhe
Bromphenol Blue Na-salt	Roth, Karlsruhe
Bio-Rad Protein assay Kit	Bio-Rad, Munich
CHAPS	Roth, Karlsruhe
Chloroform	Merck, Darmstadt
Coomassie brilliant Blue (G250)	Serva, Heidelberg
Dithiothreitol (DTT)	Roth, Karlsruhe
DEPC (diethylpyrocarbonat)	Roth, Karlsruhe
ECL Detection Kit	Amersham Bioscience, Freiburg
Ethanol absolute	Roth, Karlsruhe
Ethidiumbromid	Roth, Karlsruhe
Gentamycin	Gibco BRL, Karlsruhe
L-Glutamin	Gibco BRL, Karlsruhe
Glycerin	Roth, Karlsruhe
Glycin	Roth, Karlsruhe
2-Iodacetamid	Merck, Darmstadt
Isopropanol	Roth, Karlsruhe
Methanol	Roth, Karlsruhe
Milk powder, blotting grade	Roth, Karlsruhe
Natrium Carbonat	Roth, Karlsruhe
Ponseau S	Roth, Karlsruhe
Neomycinsulfat	Roth, Karlsruhe
Sodium chloride	Roth, Karlsruhe
Sodiumdodecylsulfat	Merk, Darmstadt
Pharmalyte pH 3-10	Amersham Bioscience, Freiburg
Potassium chloride	Merk, Darmstadt
Proteinaseinhibitor Mix Complete Mini	Roche Diagnostics, Mannheim
Sucrose D(+)	Roth, Karlsruhe
Silicon Oil	Serva, Heidelberg
Tetramethylenethylendiamine (TEMED)	Roth, Karlsruhe
Thiourrea	Roth, Karlsruhe

Trifluoracetic acid	Roth, Karlsruhe
Tris(hydroxymethyl)aminomethan	Roth, Karlsruhe
Thiourea	Roth, Karlsruhe
Trypsin	Promega, Madison, WI USA
Trypsin-EDTA	Gibco BRL, Karlsruhe
Trypan Blue Stain 0.4%	Gibco BRL, Karlsruhe
Urea	Roth, Karlsruhe

Table 2. Specific primers for Light Cycler Real-Time PCR.

Encoded protein	Forward Sequence	Reverse Sequence	Amplicone size (bp)
Grp-78	5`-tgaccaaaaccgcctg-3`	5`-caatgtccgcatcctg-3`	257
Creatine kinase	5`-gctctttcccccaagt-3`	5`-tgcccacagtcttaatg-3`	185
Galectin-3	5`-attgtgtgaacacgaag-3`	5`-tcatggcgtggttagc-3`	239
IL-6	5`-acaacgatgatgcactt-3`	5`-cttggtccttagccact-3`	334
IP-10	5`-tccctctcgcaaggac-3`	5`-ttggctaaacgctttcat-3`	209
TGF-β1	5`-cgccatctatgagaaaacc-3`	5`-gtaacgccaggaattgt-3`	190
TGF-βRII	5`-catttggttccaaggtgc-3`	5`-tggtagtgttcagcgag-3`	282
18 S	5`-ccaaagtctttgggttccgg-3`	5`-aacaactaagaacggccatg-3`	204
GAPDH	5`-atcccagagctgaacg-3`	5`-gaagtcgcaggagaca-3`	198

Table 3. Small interference RNA sequences.

Target gene	Target sequence	Double-stranded ribonucleotide sequence
Grp-78	5´-ccgataatcagccaactgtaa-3´	**sense:** r(gauaaucagccaacuguaa)dtdt **antisense** r(uuacaguuggcugauuauc)dgdg
Galectin-3	5´- agggaactatgtaattatcaa-3´	**sense:** r(uugauaauuacauaguucc)dcdt **antisense** r(ggaacuauguaauuaucaa)dtdt

Table 4. Cytokines and pharmacological inhibitors.

Substance	Supplier	Working concentration
rIL-10	R&D system, Heidelberg, Germany	20-50 ng/ml
rTNF	R&D system, Heidelberg, Germany	10 ng/ml
rTGFβ	R&D system, Heidelberg, Germany	20 ng/ml
MG132	BioMol, Plymouth meeting, PA	20 μM
SB203580	Calbiochem, Läufelfingen, Germany	20 μM

Table 5. Primary and secondary antibodies for Western Blot analysis.

Antibody	Supplier	Dilution	Size kDa	Origin
Actin β	MP Biomedicals Inc, OH	1:1000	42	mouse
IL-10 R	Santa Cruz Bio Inc, CA	1:1000	90-110	rabbit
STAT-3	Cell Signalling Tech Inc, MA	1:1000	79, 86	rabbit
IKKα	Santa Cruz Bio Inc, CA	1:1000	85	rabbit
Cleaved Caspase-3	Cell Signalling Tech Inc, MA	1:1000	17,19	rabbit
CD-3	Santa Cruz Bio Inc, CA	1:1000	23	mouse
E-cadherin	Santa Cruz Bio Inc, CA	1:1000	124	rabbit
Grp-78	Calbiochem, Läufelfingen	1:2000	78	rabbit
Galectin-3	Santa Cruz Bio Inc, CA	1:1000	31	rabbit
Creatine Kinase	Santa Cruz Bio Inc, CA	1:1000	47	goat
Rel-A (total)	Cell Signalling Tech Inc, MA	1:1000	65	rabbit
Phospho-Rel-A	Cell Signalling Tech Inc, MA	1:1000	65	rabbit
p-38 (total)	Cell Signalling Tech Inc, MA	1:1000	43	rabbit
Phospho-p38	Cell Signalling Tech Inc, MA	1:1000	43	rabbit
STAT-1	Cell Signalling Tech Inc, MA	1:1000	84, 91	rabbit
Annexin 2	Santa Cruz Bio Inc, CA	1:1000	36	goat
Rho GDI α	Cell Signalling Tech Inc, MA	1:1000	26	rabbit
AIF-1	Cell Signalling Tech Inc, MA	1:1000	57, 67	rabbit
Phospho-STAT-1	Cell Signalling Tech Inc, MA	1:1000	84, 91	rabbit
Smad-2	Cell Signalling Tech Inc, MA	1:1000	55	goat
Phospho-Smad-2	Cell Signalling Tech Inc, MA	1:1000	58	rabbit
Phospho-AMPKα	Cell Signalling Tech Inc, MA	1:1000	62	rabbit
TLR-2	eBioscience, CA	1:300	97	rabbit
Phospho-STAT-3	Cell Signalling Tech Inc, MA	1:1000	79, 86	rabbit
Smad7	Cell Signalling Tech Inc, MA	1:1000	51	rabbit
Grp-78 (anti-human)	Sigma Aldrich Gmbx, Munich	1:2000	78	rabbit
ATF-6α	Santa Cruz Bio Inc, CA	1:1000	86	rabbit
ECL Anti-mouse IgG-HRP	Amersham Bioscience, Freiburg	1:1000	-	sheep
ECL Anti-rabbit IgG-HRP	Amersham Bioscience, Freiburg	1:1000	-	donkey
ECL Anti-goat IgG-HRP	Santa Cruz Bio Inc, CA	1:1000	-	donkey
TRITC-anti-rabbit IgG	Dianova, Hamburg	1:100	-	rabbit

Table 6. Molecular biology and protein biochemistry reagents.

Reagent	Supplier
TransMessenger Transfection Reagent Kit EC-R Buffer Transfection Reagent siRNA Suspension Buffer	Qiagen GmbH, Hilden Germany
Trizol Reagent	Invitrogen GmbH, Karlsruhe, Germany
cDNA and RT-PCR reagents 5 x First Strand Buffer M-MLV Reverse Trancriptase DTT 0.1 M RNaseOUT Ribonuclease Inhibitor Random Primers dNTPs 100 mM	Invitrogen GmbH, Karlsruhe, Germany
Light Cycler FastStart DNA Master SYBR Green I kit	Roche Diagnostics GmbH, Mannheim, Germany
Protein A/G PLUS Agarose	Santa Cruz Biotechnology Inc., Santa Cruz, USA
ECL-Detection Kit	Amersham Biosciences Europe GmbH, Freiburg, Germany
Dual Color Protein Standart	Bio-Rad Laboratories GmbH, Munich, Germany
Bio-Rad Protein Assay Kit	Bio-Rad Laboratories GmbH, Munich, Germany
Qproteome protein fractionation kit	Qiagen GmbH, Hilden Germany

Table 7. Cell culture reagents.

Reagent	Supplier
DMEM	Invitrogen GmbH, Karlsruhe, Germany
FBS	Invitrogen GmbH, Karlsruhe, Germany
L-Glutamine	Invitrogen GmbH, Karlsruhe, Germany
Antibiotic/Antimycotic	Invitrogen GmbH, Karlsruhe, Germany
Trypsin-EDTA	Invitrogen GmbH, Karlsruhe, Germany
Neomycin sulfate	Invitrogen GmbH, Karlsruhe, Germany

3.2 Buffers and Solutions

- PBS (without Ca and Mg) pH 7.4

NaCl	137 mM
$Na_2HPO_4x2H_2O$	4.3 mM
KCl	2.6 mM
KH_2PO_4	1.5 mM

- Semi-Dry Blotting Buffer

Tris	25 mM
Glycine	192 mM
Methanol	20 % (v/v)

- 1xTBS-T

Tris	20 mM
NaCl	137 mM
Tween 20	1 % (v/v)

- Ponceau

Ponceau S	2.5 % (w/v)
Acetic acid	20 % (v/v)

- Laemmli Buffer

TrisCl 1.5 M pH 6.8	25 % (v/v)
SDS	1.6 % (w/v)
Glycerol	15.8 %
Bromphenol Blue	trace
DTT	1 mM

- Lysis Buffer

Urea	7 M
Thiourea	2 M
CHAPS	2 %
Pharmalyte pH 3-10	0.8 %
DTT	1 %
Protease inhibitor	40 µl/ml

- Rehydration Buffer

Urea	8 M
CHAPS	0.5 %
DTT	15 mM
Pharmalyte pH 3-10	0.5 %
Bromphenol Blue	trace

- SDS-equilibration Buffer

TrisCl pH 8.8	50 mM
Urea	6 M
Glycerol	30 % (v/v)
SDS	2 % (w/v)
Bromphenol blue	trace
DTT	0.01 g/ml
2-Iodacetamide	0.04 g/ml

- Displacing Solution

TrisCl 1.5 M pH 8.8	25 % (v/v)
Glycerol	50 % (v/v)
Bromphenol Blue	few grains

- Fixing Solution

Ethanol absolut	42 % (v/v)
Acetic acid	9.6 % (v/v)

- Sealing Solution

Agarose	0.5 % in 1xSDS-Running Buffer
Bromphenol Blue	traces

- Coomassie Staining Solution

Coomassie G-250	0.0625 % (w/v)
Methanol 100 %	25 % (v/v)
Ammonium Sulfate	10 % (w/v)
Phosphoric Acid	2 %

- DEPC-water

DEPC	1 % (v/v)

- SDS-Running Buffer

Tris	25 mM
Glycine	192 mM
SDS	0.1 % (w/v)

- SDS-polyacrylamide soltion (12.5 %)

For 12 Gels:	
Acrylamide stock	333 ml
TrisCl pH 8.8	200 ml
Bidestilled water	250 ml
SDS 20 %	4 ml
APS 10 %	8 ml
TEMED	500 µl

- 10xTAE Buffer

Tris	0.4 M
EDTA-Na_2-salt	0.01 M
Acetic acid	0.2 M

- 1.5 M TrisCl (pH 8.8)
- 0.5 M TrisCl (pH 6.8)
- 1 % TFA
- 0.1 % TFA
- 50 mM NH_4HCO_3
- 10 mM NH_4HCO_3

3.3 Technical equipment

Thermocycler	Thermo Electron Corporation (Waltham, USA)
pH Meter	Eutech Instruments Pte Ltd (Singapore)
Laboratory scales	Gottl. Kern & Sohn GmbH (Balingen, Germany)
Vortex	VELP Scientifica srl (Usmate, Italy)
Light microscope Leica DMIL	Leica Microsystems GmbH (Wetzlar, Germany)
Centrifuge	SIGMA Laborzentrifugen GmbH (Osterode, Germany)
Biophotometer	Eppendorf AG (Hamburg, Germany)
Spectrophotometer Nanodrop ND-1000	PEQLAB Biotechnologie GmbH (Erlangen, Germany)
Thermomixer	Thermo Electron Corporation (Waltham, USA)
Water bath	Thermo Electron Corporation (Waltham, USA)
IPGphor	Amersham Biosciences Europe GmbH (Freiburg, Germany)
Ettan Dalt II System	Amersham Biosciences Europe GmbH (Freiburg, Germany)
Laminar flow	Kojair Tech Oy (Vilppula, Finland)
CO_2-incubator	Binder GmbH (Tuttlingen, Germany)
MALDI-TOF-MS	Bruker Daltonik GmbH (Leipzig, Germany)
Semi-Dry Transfer chamber	Bio-Rad Laboratories GmbH (Munich, Germany)
Confocal microscope	Leica Microsystems GmbH (Wetzlar, Germany)
Image Scanner	Amersham Biosciences Europe GmbH (Freiburg, Germany)
Light Cycler	Roche Diagnostics GmbH (Mannheim, Germany)
Speed vacuum centrifuge RC 10.10	Jouan (Saint-Herblain, France)
Laborshake	C. Gerhardt GmbH & Co KG (Königswinter, Germany)

Electrophoresis Unit	PEQLAB Biotechnologie GmbH (Erlangen, Germany)
Electrophoresis vertical Unit	Bio-Rad Laboratories GmbH (Munich,Germany)
Power supply	Bio-Rad Laboratories GmbH (Munich,Germany)
Semi-DryTransfer Cell	Bio-Rad Laboratories GmbH (Munich,Germany)
Multi Doc-It Digital Imaging System	UVP Inc. (Upland, USA)

3.4 Methods and techniques

3.4.1 Isolation of primary intestinal epithelial cells from IBD patients

Patients. Tissue specimens were obtained from patients with CD and UC undergoing surgical resections. Ileal regions of inflamed mucosal tissue were taken from six patients with active CD, colonic mucosa was isolated from inflamed mucosal tissue of patients with UC. Histological analysis was performed in order to assess the severity of inflammation (Institute of Pathology, University of Regensburg, Germany). In addition, colonic mucosal tissue was obtained from six patients with colorectal carcinoma as non-inflammatory control. Intestinal epithelium from control patients was taken at least 5 cm distant from the tumor. The study was approved by the ethics committee of the University of Regensburg and performed in accordance with the declaration of Helsinki.

Isolation of intestinal epithelial cells. Primary human IEC from ileal and colonic tissue sections were isolated according to the protocol described by Grossmann et al [183]. Briefly, the mucosa was stripped from the submucosa short after the intestinal resection and washed with phosphate buffered saline (PBS). The mucus was removed by treatment for 15 min with 1 mM dithiothreitol. After the washing process the mucosa was incubated with 1.5 mM EDTA in Hanks balanced salt solution without calcium and magnesium and tumbled for 10 min at 37°C. The procedure was repeated several times to yield complete crypts. Supernatant containing debris and mainly villus cells was discarded. To separate IEC from contaminating non-epithelial cells, the suspension was allowed to sediment for 15 min. The cells containing mainly complete crypts were collected and washed twice with PBS. Fractions of primary IEC were combined and collected in sample buffer for subsequent proteome analysis and Western blot. Epithelial cell suspension was centrifuged at 1400 rpm for 5 min and IEC were lysed in 200-500 µl (depending on pellet size) of ice-cold Lysis Buffer. Protein extracts were homogenized by ultrasonication (amplitude 35, cycle 0.5 and 10 impulses) and centrifuged at 10000xg for 30 min at 4°C. Protein concentration in supernatants was determined by Bradford using Bio-Rad protein assay kit. The samples were aliquoted and stored at -80°C. The purity of native IEC was assessed by measuring of $CD3^+$-T-cell contamination using immunoreactive anti-CD3 antibody in Western blot analysis.

3.4.2 Animals and bacterial monoassociation

Germ-free IL-10 gene deficient mice 129 SvEv TAC (derived by Dr. Edward Balish, University of Wisconsin, Madison, WI) were monoassociated at 12-16 weeks of age with the colitogenic *Enterococcus faecalis* strain OG1RF (a generous gift from M. Huycki, University of Oklahoma, Oklahoma City, OK). The mice were maintained in the Gnotobiotic Animal Core at the College of Veterinary Medicine, North Carolina State University (Raleigh, NC). The purity of colonization and absence of contamination with other bacterial species were confirmed by culturing samples from the small and large intestine at necropsy and culturing serial faecal samples. Animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC), North Caroline State University. Wild type and IL-$10^{-/-}$ mice were killed after 3, 7, 14, 28 days and 14 weeks after initial bacterial colonization, germ-free animals of both experimental groups were used as controls. Sections of ileum, cecum, proximal and distal colon were fixed in 10% neutral buffered formalin and subsequently embedded in paraffin. Histology scoring scaled from 0 to 4 was applied by assessment the degree of lamina propria mononuclear cell infiltration, crypt hyperplasia, goblet cell depletion and architectural distortion of epithelial structure.

3.4.3 Isolation of primary mouse intestinal epithelial cells

Primary intestinal epithelial cells from colon and cecum of wild type and IL-10 $^{-/-}$ mice were isolated and purified as follows: the intestinal tissue was cut into pieces and incubated at 37 °C in DMEM, containing 5% FCS and 1 mM dithiothreitol (DTT) for 30 minutes. The remaining tissue was incubated in 30 ml of PBS containing 1.5 mM EDTA for additional 10 minutes. The supernatants were filtered, centrifuged at 400xg for 5 minutes and the pellet was resuspended in DMEM with 5% FCS. Finally, IEC suspension was purified by centrifugation through 25%/40% discontinuous Percoll gradient at 600xg for 30 minutes. Western blot analysis was performed using mouse anti-CD3-antibody to confirm the purity of epithelial cells and the absence of $CD3^{+}$-T-cell contamination. Freshly isolated murine splenocytes were used as a positive control. Primary epithelial cells were collected in appropriate sample buffers for mRNA isolation, Western Blot and proteome analysis.

3.4.4 Cell culture

Cell line origin. Mode-K cell line was initially generated from enterocytes of C3H/HeJ mice immortalized by SV40 large T gene transfer. C3H/HeJ mice have a missense mutation in one of the exons of TLR-4 gene [184, 185]. Mode-K are adherent, not differentiated cells that exhibit morphological and phenotypic characteristics of normal enterocytes.

Mode-K cell culture. Mode-K cells (a generous gift from Ingo Authenrieth, University of Tubingen, Germany) in passage 10-30 were cultured according to general cell culture aseptic protocol at humidified atmosphere with 5 % CO_2 at 37 °C in growth medium DMEM (without sodium pyruvate, high glucose, with pyridoxine chloride), supplemented with 10 % v/v FBS, 2mM L-Glutamine and Antibiotic/Antimycotic. Cells were grown in 25 and 75 cm^2 flasks, and 6-well plates; medium was changed by aspiration of spent culture supernatant followed by replacement with a fresh medium every 48 hours. The cells were passaged at sub-confluent density with Trypsin-EDTA: briefly, cell monolayer was washed twice with 4-5 ml of pre-warmed PBS, Trypsin-EDTA aliquots (2 and 4 ml for small and big flask respectively) were added and the cells were placed into CO_2-incubator for a few minutes. The cells were collected and trypsin action was stopped by adding 4 ml of complemented medium. The cells were then centrifuged at 1400 rpm for 5 minutes and the pellet was resuspended in a fresh culture medium at the cell concentration of approximately $2x10^5$ cell/ml.

Stable transfection of Mode-K cells with IL-10 receptor (IL-10R) was performed in our laboratory by Pedro Ruiz (TUM) and the cells were provided for further experiments. Mode-K IL-10R-transfected cells were grown in complete DMEM medium containing Neomycin.

3.4.5 Bacterial infection and cell stimulation

Enterococcus faecalis (OG1RF strain) was aerobically cultured at 37 °C in Luria Broth (LB) containing tryptone (1%), yeast extract (0.5%) and NaCl (0.5%). First, the bacteria were inoculated from the frozen stock culture (-80°C) into 5 ml of LB-medium and cultured overnight. Bacteria were then transferred from overnight culture into fresh LB-medium (1 % inocculate) and cultured until they reached stationary growth phase. Bacteria were harvested by centrifugation at 3000xg for 15 minutes, washed with PBS and diluted in DMEM. Mode-K cells were infected with *E. faecalis* at a moi (multiplicity of infection) =30-100 for different time points.

Cytokine stimulation. Mode-K cells were stimulated with following cytokines: TNF (10ng/ml), TGFβ1 (20 ng/ml), IL-10 (20-50 ng/ml) or their combinations. Where indicated the cells were pre-

incubated with proteasome inhibitor (MG132) and p-38 MAPK inhibitor SB203580 1 hour before cytokines or bacteria-stimulation.

3.4.6 Adenoviral infection

Mode-K cells were infected with Ad5Smad7 (a generous gift from D. Brenner, Columbia University, New York, NY) for 24 hours in serum-reduced (2%) culture medium as previously described [115]. Ad5GFP was used as a viral negative control. The spent culture supernatants were removed, cells were washed twice with PBS and fresh cell culture medium was added.

3.4.7 Small interference RNA cell transfections

Specific synthetic double-stranded ribonucleotides were generated for mouse galectin-3 and grp-78. All siRNA and tranfections reagents were supplied by QIAGEN. Fluorescent-labelled negative control siRNA (Alexa Fluor 546) with no homology to mammalian genes was used to optimize and assess the specificity of transfection. The content of each lyophilized tube with siRNA was dissolved in appropriate volume of Suspension Buffer to obtain 20 nM solution. The tubes were heated to 90°C for 1 min and incubated for 1 hour at 37 °C.

Transfection procedure. Mode K cells were seeded 24 hours before the day of transfection in 24-well plates at optimal initial concentration 80 000 cells/well in 0.5 ml of appropriate growth medium, containing serum and antibiotics. Cells were grown under normal conditions (37 °C and 5% CO_2) to 50-80% confluence. The maximal silencing effect was achieved after 72 hours using the ratio 0.5 μg siRNA : 4.5 μl of RNAiFect Transfection Reagent (1:9) per well for galectin-3 and the ratio 1.5 μg siRNA : 13.5 μl of RNAiFect Transfection Reagent (1:9) for grp-78. The calculated amounts of siRNA were diluted with EC-R Buffer and RNAiFect Transfection Reagent to give a final volume 100 μl. The transfection mixture was incubated 10-15 min at room temperature to allow complex formation. While complex formation was taking place, the growth medium was replaced with 300 μl of fresh complete medium. The transfection mixture was added to the cells (100 μl/well) and the plates were gently swirled to ensure equal distribution of complexes. The cells were incubated under the normal growth condition. The silencing effect was monitored by Western Blot analysis after 24, 48 and 72 hours.

3.4.8 Real-Time PCR (Light Cycler)

3.4.8.1 Isolation of total RNA

Total RNA from primary isolated IEC and Mode-K cells was extracted using Trizol Reagent according to the manufacturer's protocol. Briefly, the cells were lysed in 500-800 µl of Trizol Reagent and 160 µl of chloroform was added to each sample. After centrifuging of the mixture at 14000 rpm for 30 min at 4 °C the upper RNA containing phase was transferred into a new RNAse free tube and precipitated with isopropanol. After centrifugation at 12000 rpm for 30 min the pellet was washed by adding 1 ml of 75 % ethanol and the tubes were respun at 7500x*g* for 10 minutes at 4 °C. After removing ethanol the RNA-pellet was air-dried for 10-15 min at room temperature. Extracted RNA was dissolved in 20 µl of sterile DEPC-water and RNA concentration was calculated from the absorbance rate at 260 nm. RNA purity and integrity was assessed by verifying the absorption ratio (A_{260}/A_{280}) with optimal values in range between 1.6 and 1.9. Purified RNA was stored at –80 °C.

3.4.8.2 cDNA generation

Reverse transcription was performed from 1 µg of total RNA in a two-step reaction: 30 µl of "master-mix 1" containing 1µg RNA, 8 µl of 5x First-strand buffer, 4 µl of DTT (100 mM) and 6 µl of desoxyribonucleoside triphosphate mixture (300 mM) was incubated for 5 min at 65 °C; the second step was performed by adding to each probe 10 µl of "master-mix 2", containing 0.2 µg of random hexamers, 40 U of RNase Out and 200 U of MMLV-reverse transcriptase. The final mixture was incubated for 60 min at 37 °C followed by heating to 99 °C for 1 min.

3.4.8.3 Primer design and Real-Time PCR

Gene-specific nucleotide sequences were taken from the GenBank database (http://www.ncbi.nlm.nih.gov/BLAST/). Primer design was done using Light Cycler Probe Design software (Roche Diagnostics, Mannheim, Germany). The optimal primer pairs were selected with regard to primer dimers formation, self-priming and primer melting temperature.

One-step PCR was performed in glass capillaries at Light Cycler equipment according to the instruction to Light Cycler-Fast Start DNA Master SYBR Green I kit. The total probe volume of 10 µl, containing 1µl of reverse transcribed cDNA, 1.2 µl of MgCl2 (4 mM), 1 µl of LC-Fast Start DNA Master Mix and 0.2 µl of reverse and forward primers (20 mM) was denaturated at 95 °C for

10 min, followed by 50 cycles including heating 15 sec at 95 °C, annealing 10 sec at 60 °C and extension at 72 °C for 20 sec. Authenticity and relative amounts of the amplified products were estimated by the presence of SYBR green fluorescence and melting curve analysis. The specificity of amplified cDNA products was checked by gel electrophoresis in 2 % agarose. Calibration curves were generated by measuring serial dilutions of stock cDNA to calculate the amplification efficiency (E). The crossing point (Cp) of the log-linear portion of the amplification curve was determined. The relative induction of gene mRNA expression was calculated using the following equation: $E^{\Delta Cp \text{ (control samples – treated samples)}}$ and normalized to the expression of house-keeping genes GAPDH and 18S ribosomal mRNA.

3.4.9 Western Blot analysis

Mode-K cells grown in 6-well culture plates were lysed in 200 µl of Laemmli buffer. The samples were denaturated for 5-10 minutes at 95 °C and 20-50 µg of total protein was subjected to electrophoresis in 10 % polyacrylamide gels. For better resolution of proteins with relatively small or high molecular weight 15 % and 7 % gels were prepared. Proteins were transferred to PVDV membrane (equilibrated with 100% methanol for 1 min) in Semi-dry Transfer Cell for 45 min and 0.2 A/gel. The equal loading, quality of separation and transfer was controlled by adding Ponceau-dye to the membranes for 1 minute. Membranes were incubated for 1 hour in 0.5 % milk powder in TBS-T. After blocking the membranes were washed three times with TBS-T and incubated with primary antibodies from 1 hour to overnight at 4 °C (incubation time was optimized depending on the type of antibody) with agitation. The dilution of primary antibodies was optimized ranging from 1:200 to 1:2000. The membranes were washed 2-3 times with TBS-T and incubated with anti-mouse-, anti-rabbit- or anti-goat-HRP-conjugated detection antibodies for 1 hour. Detection was performed using an Enhanced Chemiluminescence Light-detection kit (ECL).
Primary IEC were lysed either directly in 1 x Laemmli buffer or in Proteome Lysis Buffer and then an appropriate amount of total protein was combined with Laemmli Buffer 1:1 and separated by SDS-PAGE.

3.4.10 Co-immunoprecipitation

Mode-K and Mode-K/IL-10R-transfected cells were cultured in 75-cm^2 flasks followed by stimulation with TNF for various time points. Where indicated the cells were pre-incubated with rIL-10 for 24 hours prior TNF-stimulation. Cells were rinsed once with ice-cold PBS, resuspended

in 1200 µl of ice-cold 1xLysis Buffer containing 1mM phenylmethylsulfonyl fluoride (PMSF) and scraped off the flask. The tubes were then centrifuged at 12000xg for 10 minutes at 4 °C and supernatants were pre-cleared for 3 hours with 100 µl of A/G agarose beads at 4 °C. Total protein concentration was normalized and immunoprecipitation was performed with 25 µl of rabbit anti-IKKα antibody overnight at 4 °C. The immune complexes were collected by co-incubation of cell lysates with 200 µl of protein A/G agarose beads for 30 minutes, washed twice with 1xLysis Buffer and resuspended in 3xLaemmli buffer. The samples were denaturated for 5 min at 95 °C and resoluted by SDS-PAGE.

3.4.11 Cellular fractionation

Qproteome mitochondria isolation kit was used for the separation of cytoplasmic, microsomal and mitochondrial protein fractions. ModeK/IL-10R cells were cultured in 75 cm^2 flasks. The cells were scraped, washed with 0.9 % sodium chloride and centrifuged at 500xg for 10 min at 4 °C. The cell pellet was resuspended in 1 ml of ice-cold lysis buffer containing protease inhibitor and incubated on ice for 10 minutes. The cell lysate was then cenrifugated at 1000xg for 10 min at 4 °C and supernatant containing cytosolic fraction was collected. The cell pellet was then resuspended in 1.5 ml of ice-cold Disruption Buffer and centrifuged at 1000xg for 10 min at 4 °C. The supernatant was transferred in a new tube and centrifuged at 6000xg for 10 min at 4 °C. The microsomal protein fraction was collected in separate tube. The pellet of mitochondria was resuspended in 100 µl of Mitochondria Storage Buffer. Proteins from the cytosolic and microsomal fractions were concentrated by acetone precipitation. Briefly, 4 volumes of ice-cold acetone was added to each fraction and incubated for 15 min on ice. The tubes were then centrifuged at 12000xg for 10 min and supernatants were discarded. Protein pellets were air-dried and then dissolved in 100 µl of lysis buffer. Protein concentration was determined by Bradford assay. The protein extracts were mixed with 3xLaemmli buffer, 50 µg of total protein was separated by SDS-PAGE and subjected to immunoblotting with specific antibodies.

3.4.12 Immunostaining and fluorescence microscopy

Mode K/IL10R cells were grown on sterile glass coverslips in 6-well culture plates to 80 % confluence. The cells were washed with 2 ml of PBS and fixed for 5-10 min by adding 500 µl of ice-cold methanol. The cells were washed and incubated with 250 µl of blocking reagent (10 % goat serum in PBS) for 30 min at room temperature. Then 400 µl of primary grp-78 antibody (dilution 1:

600) or ATF-6α antibody (dilution 1:250) was added and the coverslips were incubated for 30 min. After washing with 2 ml of PBS the coverslips were incubated with 400 µl of secondary antibody (anti-rabbit TRITC-conjugated IgG, dilution 1: 100) for 30 min protected from the direct light. The coverslips were washed three times with 2 ml of PBS and stained with 200 µl of DAPI (1 µg/ml) for 5 minutes. The glass coverslips were finally washed twice with PBS and placed up-side down on a microscope slide with a drop of 50 % glycerol in PBS. The slides were subjected to confocal immunofluorescent microscopy. The images were analyzed by Leica confocal software version 2.5.

3.4.13 Two-dimensional Gel-Electrophoresis and proteome analysis

3.4.13.1 Sample preparation for Isoelectric Focusing

Primary mouse intestinal epithelial cells were isolated and purified as described above. The pellet was lysed in 200-300 µl of ice-cold Lysis Buffer containing DTT and protease inhibitor. The cell lysates were homogenized by ultrasonication (amplitude 35, cycle 0.5 and 10 impulses) and left on ice for 15-20 min for complete lysis. The lysates were then centrifuged at 10000xg for 30 min at 4°C. The supernatants were collected and total protein concentration was determined by measuring of optical density (OD) at 600 nm using Bio-Rad protein assay kit. Cell extracts were aliquoted and frozen at -80°C for further applications.

3.4.13.2 Isoelectric Focusing (First Dimension)

Protein separation was performed by using the immobilized pH-gradient strips (IPG) with linear pH gradient from 3 to 10. The strips were rehydrated in Reswelling Tray overnight in 350 µl of Rehydration Buffer containing DTT and Bromphenol Blue at room temperature under the silicone oil to avoid evaporation and crystallization. 500 µg of total protein was applied by cup-loading, allowing maximal loading volume 100-120 µl of protein extract. If necessary, the concentrated samples were diluted in Lysis Buffer to avoid protein precipitation. Isoelectric focusing was performed on Amersham IPGphor Unit under the following running conditions: 20 °C, 50 mA per IPG-strip and current parameters, depicted in table 8:

Table 8. Running conditions for IEF.

Voltage	Voltage gradient type	Time h:min	Total Vhours
500	Gradient	0:01	
4000	Gradient	1:30	
8000	Step-n-hold		28 000

After IEF the stripes were stored at -80 °C or directly used for the second dimension (SDS-PAGE).

3.4.13.3 SDS-PAGE (Second dimension)

SDS-PAGE was performed on Amersham Bioscience EttanDalt II System. SDS-polyacrylamide gels (12.5 %) were casted 2 days before and stored at 4 °C. SDS-running buffer was always freshly prepared according to the protocol**.** First, IPG-stripes were incubated in DTT-containing Equilibration Buffer for 15 min on the shaking platform. Then the solution was replaced with Iodacetamide-containing Equilibration Buffer and incubated for additional 15 minutes. Finally, the stripes were briefly rinsed with bidestilled water and running SDS-buffer before placing on the gel-surface. The stripes were sealed with agarose and run for 1 hour at 4 mA/gel, followed by 12 mA/gel overnight.

3.4.13.4 Micropreparative staining of 2D-gels with Coomassie Blue

The gels were fixed in Fixing Solution at least 4 hours and then stained overnight in Coomassie Blue solution by slow shaking. Destaining was performed in bidestilled water until the protein spots were clearly visible on transparent background. The gels were scanned on Image Scanner and stored at 4 °C with 7 % acetic acid as a preservative.

3.4.13.5 Image analysis

ProteomWeaver 3.1 Software (Definiens, Munich) was used to perform analysis of the gel images, allowing both automatic and manual spot detection, quantification and spot matching, including background subtraction and volume normalization. Images of replicate gels were usually collected into the same group; control group usually served as a reference for treatment. Spot detection was performed automatically by Proteomweaver software. Depending on the resolution and staining quality additional manual adjustments were performed taking into consideration spot radius, intensity and contrast limit. The software allowed matching of every gel pair and matching of all gels across the whole experiment. The criteria for spot picking were as follows: regulation factor

(RF) for up-regulated spots RF>=2, for down-regulated RF<=0.5; quality and reproducibility of spots and T-test when applicable.

3.4.13.6 Spot preparation and MALDI-TOF-MS

The spots were picked using 2-3 mm sterile skin biopsy punch and collected into 200 µl eppendorf tubes. In order to destain the proteins from Coomassie Blue, gel cuts were washed first with 50 µl of 50 mM ammonium hydrocarbonate for 15 minutes. The supernatants were discarded and spots were washed with 50 µl of mixture of acetonitrile and ammonium hydrocarbonate (1:1) for additional 15 minutes. These steps were repeated twice followed by washing with 50 µl of pure acetonitrile. The spots were dried in Vacuum Centrifuge for 5 min and stored at -20 °C.

For MALDI-TOF-MS application the dried spots were rehydrated by adding 5-6 µl of 0.02 mg/ml trypsin in 10 mM ammonium hydrocarbonate and incubated for 1-2 hour at 4 °C. Then the probes were incubated for another 16-20 hours at 37 °C. The tryptic fragments were then extracted from the gel with 7 µl of 1 % trifluouracetic acid by ultrasonication for 15 min at room temperature. The tubes were briefly spinned and supernatants collected for MS-analysis. Mass-spectrometric analysis was performed using mass spectrometer from Brucker Daltonics and Autoflex Control Software (Leipzig, Germany) according to manufacturer's instructions. The protein extracts were spotted on the "Anchor-Chip Target 400" according to method described by Gobom et al [186]. Briefly, the matrix was prepared by saturation of hydroxy-cyano-cynnamic acid (HCCA) in mixture of acetone and 0.1 % TFA (97:3). 1 µl of saturated matrix was spotted on the target. 3-4 µl of trypsin-digested protein extracts were added to the matrix and incubated for 5 min to allow matrix-protein binding. Finally, 2 µl of 0.1 % TFA was added to every spotted sample and immediately removed in order to remove not-bound proteins, salts and other substances that can influence spectrometric measurements. For internal calibration after each 8 measurements 1 µl of Peptidstandartmix was spotted on the target. The measurements were performed both manual and using the option "Automatic run". Peptide fragments were analysed using Autoflex Analysis Software. The proteins were identified using Mascot Server 1.9 (Brucker Daltonics) and BioTool program. The search parameters within every taxonomic group (*Mus musculus, Homo sapiens*) allowed carboxyamidomethylation of cystein and one missing cleavage. Only proteins with significant scores and relative high percentage of the sequence coverage were selected as positively identified. In addition, the same protein was picked several times from different gels and analysed by MALDI-TOF. The observed and calculated molecular weight and pH values were taken into consideration.

4 Experimental results

4.1 Differential signal transduction in native IEC from WT and IL-10$^{-/-}$ mice in response to colonization with *E. faecalis*.

Germ-free WT and IL-10$^{-/-}$ mice at the age of 10 weeks were colonized with *E. faecalis.* Mice were sacrificed after 3, 7, 14, 28 days and after 14 weeks. Histopathological changes in the cecal and colonic mucosa were observed only in IL-10$^{-/-}$mice but not in wild type mice after 14 weeks of bacterial colonization. Primary IEC from the large intestine were obtained and purified from germ-free and colonized WT and IL-10$^{-/-}$ mice in order to monitor inflammatory status and to investigate signal transduction, gene expression and protein expression changes. Schematically, the experimental design and investigated parameters are depicted in Figure 6.

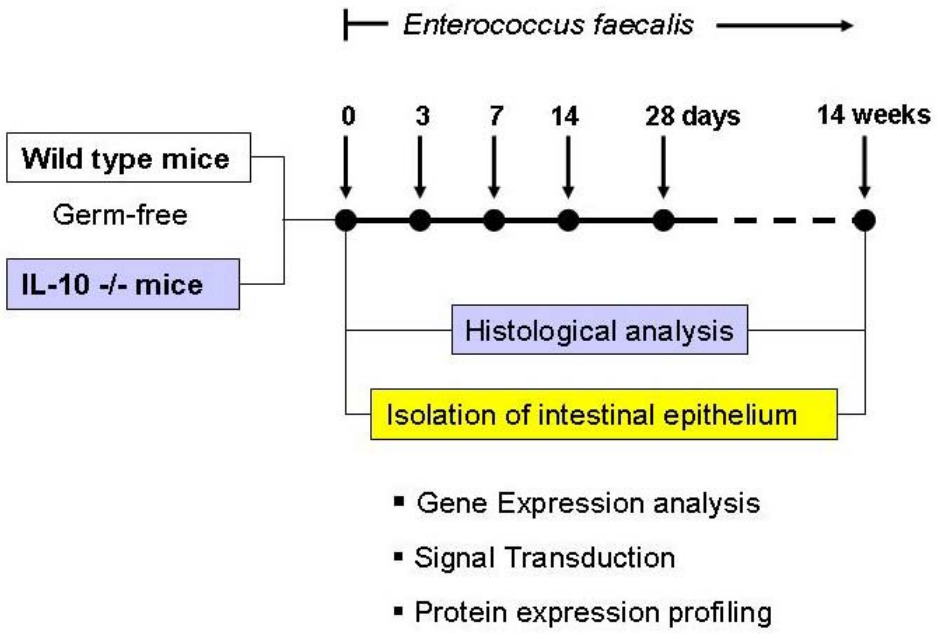

Figure 6. Experimental design and main approaches to study bacteria-epithelial interactions in WT and IL-10$^{-/-}$ mice, colonized with *E. faecalis*.

The purity of native IEC was confirmed by the absence of CD3^{+} T-cells by Western Blot analysis using anti-CD3 antibody. T-cells are the predominant cell type present in intraepithelial compartments and lamina propria especially under conditions of chronic intestinal inflammation (Figure 7).

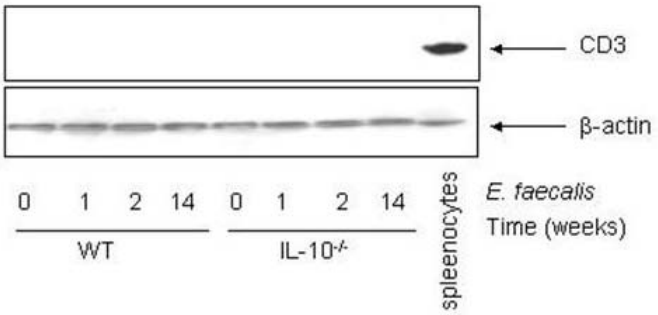

Figure 7. Purity control of native murine IEC.

Native IEC were isolated and purified from large intestine of WT and IL-10$^{-/-}$ mice after 1, 2 and 14 weeks of colonization with *E.faecalis*. 50 µg of total protein lysate containing equally pooled aliquots from each animal group was subjected to SDS-PAGE and immonoblotting with CD3-antibody. Freshly isolated murine splenocytes were used as a positive control; β-actin was used as loading control.

Histological analysis in paraffin-embedded mucosal tissue specimens was performed in WT and IL-10$^{-/-}$ mice after 1 and 14 weeks of *E.faecalis*-monoassociation. The severity of colitis was assessed by the degree of mononuclear infiltration in lamina propria, as well as by the degree of epithelial cells destruction and crypt hyperplasia. Histopathology was detected in distal colon of IL-10$^{-/-}$ mice after 14 weeks of colonization. No detectable inflammation was observed in WT animals at this time point. The corresponding scores according to the scale from 0 to 4 are presented in Table 9.

Table 9. Blinded histological analysis of paraffin-embedded tissue sections of cecum and distal colon.

Histology (Score ± SD)	Wild Type			IL-10 Knockout			Gut region
	GF	1	14	GF	1	14	
	0.2 (0.1)	0.6 (0.1)	0.5 (0.1)	0.3 (0.1)	0.8 (0.1)	3.6 (0.2)	Distal Colon
	0.3 (0.2)	0.4 (0.0)	0.6 (0.1)	0.2 (0.1)	0.6 (0.0)	1.3 (0.4)	Cecum

Since only IL-10$^{-/-}$ mice developed colitis after 14 weeks of monoassociation, it could be expected to observe some differences in expression of pro-inflammatory mediators in this group of animals. Interferon-gamma inducible protein-10 kDa (IP-10) is a CXC chemokine and a chemoattractant for CXCR3+ T cells. The expression of this cytokine is strongly associated with Th1-type of immune response and mediated by activation of NF-κB [187]. Light Cycler RT-PCR analysis of isolated

mRNA showed that expression of IP-10 was triggered by colonization of mice with *E.faecalis.* After the first week IEC from both WT and IL-10$^{-/-}$ mice showed increased IP-10 mRNA expression (7.3-fold and 11.1-fold respectively). However, after 14 weeks IP-10 mRNA expression significantly decreased (2.5 fold) in IEC of WT mice. In contrast, IP-10 mRNA expression in IEC of IL-10$^{-/-}$ mice was further increasing (18.9-fold) at this time point, correlating with histopathology in the colon (Figure 8). Of note, bacterial colonization of large intestine was comparably similar in WT and IL-10$^{-/-}$ mice (4-6 x10^9 CFU/g luminal content).

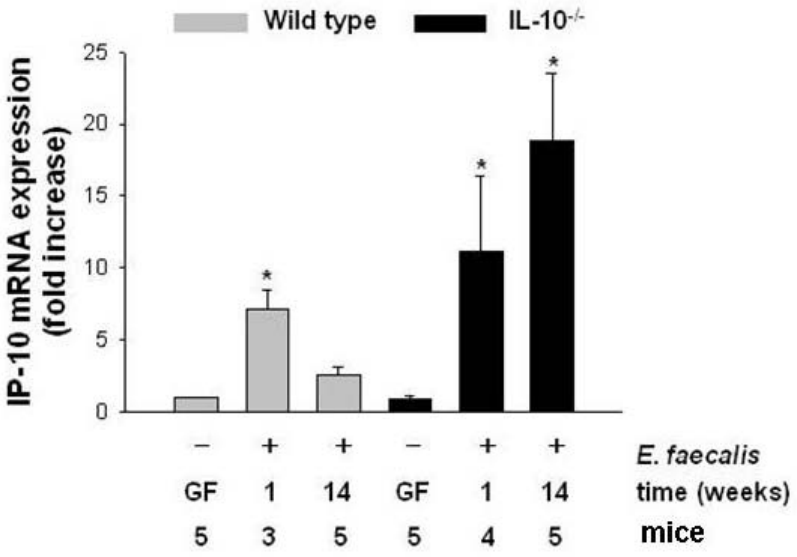

Figure 8. Differential IP-10 gene expression in primary IEC of WT and IL-10$^{-/-}$ mice colonized with *E.faecalis*.

Total RNA was isolated from large intestine of WT and IL-10$^{-/-}$germ-free and colonized animals (1 and 14 weeks), cDNA was generated and Light Cycler RT-PCR was performed using specific primers for murine IP-10 and GAPDH. Germ-free animals were taken as a control for appropriate calculations of fold-induction. Mean-fold increase and standard deviation are shown in every group.

It is known that Gram-positive bacteria can interact with TLR2 due to the presence of peptidoglycan and lipoteicheoic acid in their cell walls [53]. This binding induces activation of TLR2-mediated NF-κB signal transduction pathway that proceeds via MyD88-IRAK-TRAF-NIK-IKK and leads to the expression of NF-κB-dependent genes [188]. Therefore, we next measured TLR2 protein expression and the level of activated NF-κB signalling in native IEC. Primary IEC were isolated after 3, 7, 14 and 28 days of colonization of WT and IL-10$^{-/-}$ mice, phosphorylation of RelA (Ser536) and expression levels of TLR2 were measured by Western Blot analysis. Interestingly, IEC of WT mice revealed transient activation of RelA after one week of bacterial monoassociation but not after 14 and 28 days. In contrast to WT, persistent phosphorylation of RelA was detected in

IEC of IL-10$^{-/-}$ mice at all time points. In addition, phospho-RelA was already present in IEC of germ-free IL-10$^{-/-}$ mice. Total RelA was not changed in all groups and showed equal expression. TLR2 protein expression was differentially regulated in IEC of WT and IL-10$^{-/-}$ mice. TLR2 was initially expressed by IEC of germ-free WT mice and also after 3 and 7 days of colonization, but after day 14 and 28 TLR2 protein expression was significantly down-regulated. It correlates with the absence of RelA phosphorylation in IEC of WT mice after 14 and 28 days and could be explained by the termination of IEC stimulation by bacterial determinants due to down-regulation of TLR2. Consistent with the persistent induction of IP-10 gene expression and NF-κB activation in IEC of IL-10 deficient mice, TLR2 was persistently expressed in intestinal epithelium of these mice after 3, 7, 14 and 28 days after bacterial colonization (Figure 9).

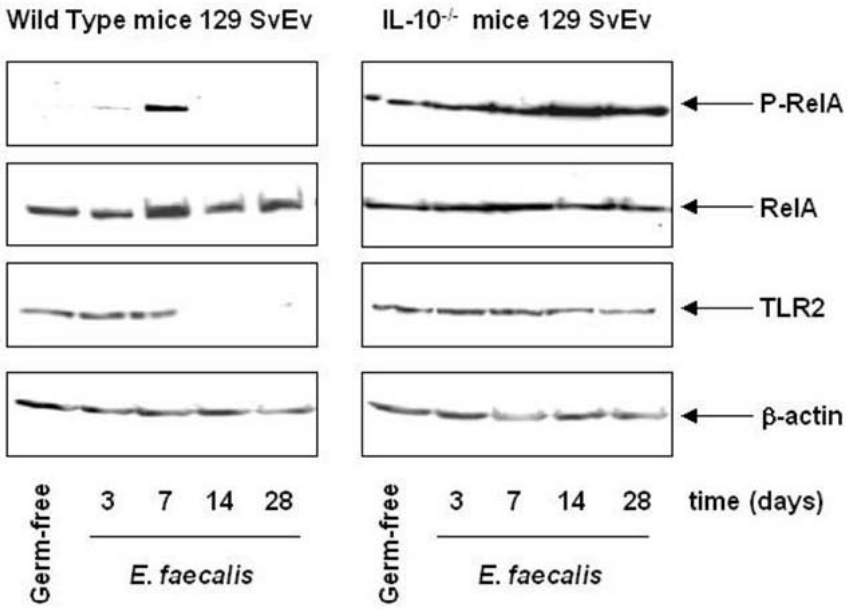

Figure 9. Differential NF-κB activation and TLR2 expression in isolated IEC from WT and IL-10$^{-/-}$ mice colonized with *E.faecalis*.

Murine IEC were isolated, purified and 50 μg of total protein extracts was subjected to SDS-PAGE separation with subsequent immunoblotting using specific monoclonal antibodies to phosho-RelA (serine 536), total RelA, TLR2 and β-actin. Samples were prepared by pooling of equal protein amounts from all animals in each group. (N=3-5).

Taking into consideration that prolonged NF-κB activation and high detectable levels of IP-10 gene expression were found in IEC of IL-10$^{-/-}$ mice after 28 days, it could be suggested that persistent NF-κB activation was still present after 14 weeks in inflamed mucosa. In addition, some other signalling pathways could be differentially involved in either regulation or suppression of

inflammation in WT, or in manifestation of colitis in IL-10$^{-/-}$ mice. Signal transducers and activators of transcription (STAT) factors function as modulators of cytokine signalling. IFN-gamma, produced by population of activated Th1-cells is a potent activator of STAT-1 [189]. Western Blot analysis performed in IEC of WT and IL-10$^{-/-}$ mice at early (1 week) and late (14 weeks) colonization time points demonstrated not only opposite NF-κB signalling but also differential involvement of STAT-1 and p-38 MAPK signalling pathways. Thus, phospho-RelA and phospho-STAT-1 were detected in IEC of IL-10$^{-/-}$ mice after 14 weeks and not detected in WT. Phosphorylated p-38 was not detected at the late disease stage in IL-10 deficient mice, whereas in WT mice the induction of phospho-p38 was observed after 1 and 14 weeks, suggesting protective role of p38 MAPK signalling for the prevention of the intestinal inflammation in WT mice (Figure 10).

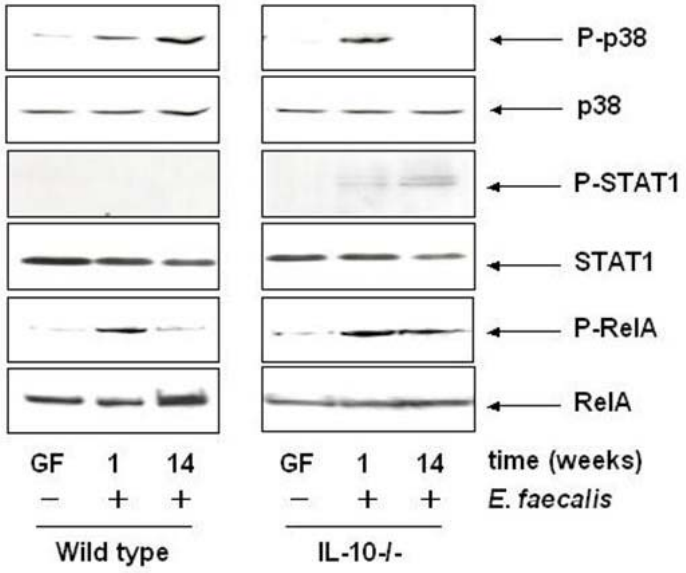

Figure 10. Differential involvement of NF-κB, p-38 MAPK and STAT-1 signalling pathways during early and late response of IEC from WT and IL-10$^{-/-}$ mice to *E.faecalis*-colonization.

Protein lysates from IEC of WT and IL-10$^{-/-}$ mice were resolved by SDS-PAGE; proteins were transferred onto membrane and probed with monoclonal antibodies for mouse phospho-p-38, total p-38, phospho-STAT-1, total STAT1, phospho-RelA and total RelA. Total amount of 50 μg of proteins was applied as pooled sample in every experimental group.

Mitogen-activated protein kinases (MAPKs) are activated by a variety of extracellular stimuli and have important functions as mediators of signal transduction. Cell signalling, mediated by p-38 MAPK activation is known to be involved in protection of several cell types from apoptosis [190]. In addition, this pathway is involved in IL-10 and TGFβ-mediated immunosuppressive mechanisms.

It was shown in intestinal epithelial cell line Mode-K that *E. faecalis* could directly induce RelA phosphorylation and activation followed by the subsequent induction and expression of pro-inflammatory cytokines like IL-6 and IP-10. Mode-K cells were stimulated with *E.faecalis* for 0-4 hours and phospho-RelA and total RelA were measured by Western Blot analysis. As shown in Figure 11A, during the first hours after stimulation, phospho-RelA was transiently detected in cultured IEC. Next, the induction of IL-6 and IP-10 gene expression was measured in Mode-K cell line after stimulation with *E.faecalis* for different time points (0-36 hours). Light Cycler RT-PCR analysis was performed using specific primers for IP-10 and IL-6. Transient induction of both genes in cultured IEC was observed after 12 and 24 hours, at expression maximum after 12 hours. Transcription levels of IP-10 and IL-6 genes decreased to normal values after 36 hours. (Figure 11B)

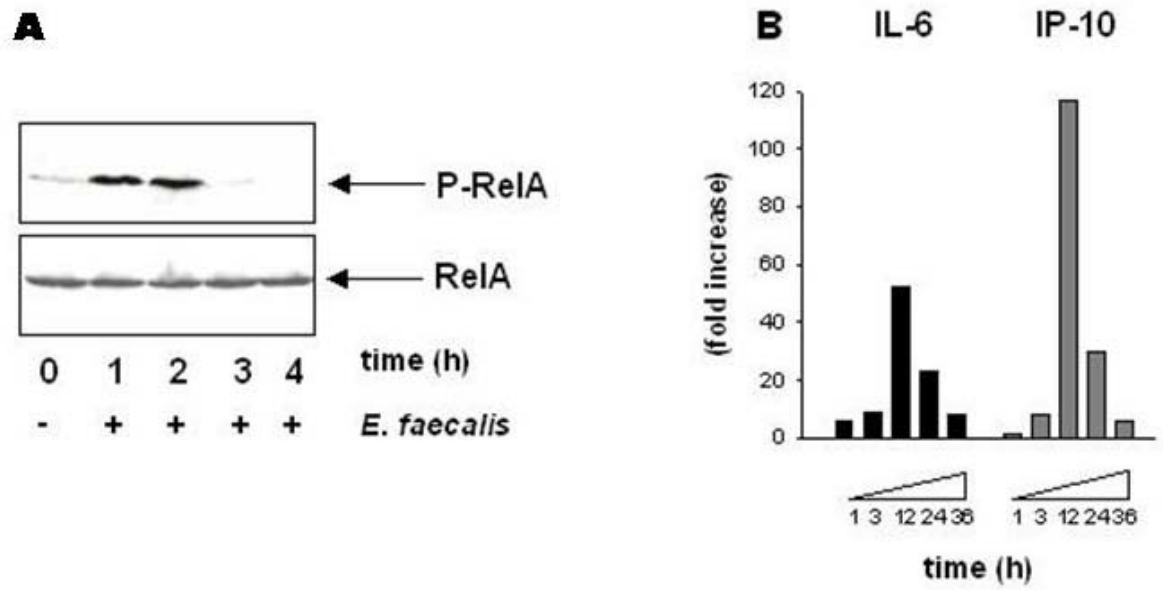

Figure 11. A and B. *E. faealis* triggers transient NF-κB activation and pro-inflammatory cytokine gene expression in Mode-K cell line.

A, Mode-K cells were stimulated with *E.faecalis* (moi 100) for 0-4 hours, cells were lysed and 20 μg of total protein extract was resolved by SDS-PAGE and analyzed by Western Blot using monoclonal antibodies to mouse phospho-RelA and total RelA. B, Mode-K cells were stimulated with *E.faecalis* for 0-36 hours, total RNA was extracted and reverse transcribed, Light Cycle RT-PCR was performed using specific primers to mouse IL-6, IP-10 and GAPDH, mean fold induction was calculated against non-stimulated cells as described in Materials and Methods.

4.1.1 Protective host-derived mechanisms mediated by TGFβ/Smad signalling

It is known that TGFβ is a pleiomorphic cytokine that can act as immunosuppressive modulator by activation of Smad signalling pathway in various cell types [42]. In addition, there is some evidence from animal models of experimental colitis about interrelated roles of TGFβ and IL-10 in immune response regulation [191, 192]. Since IEC of IL-10$^{-/-}$ mice lack IL-10-mediated protective response, it could be suggested that absence of IL-10 might be compensated by TGFβ/Smad-mediated mechanisms. First, we measured the expression levels of TGFβ1 and corresponding TGFβII receptor in isolated IEC of WT and IL-10$^{-/-}$ mice as well as in the total mucosal tissue of both animals groups. Since it is almost impossible to measure secreted levels of functional TGFβ *in vivo* and to distinguish whether TGFβ is produced by intestinal epithelial cells or by activated immune cells of lamina propria, the expression levels of TGFβ and TGFβRII receptor were assessed at the mRNA levels. As shown in Figure 8, IEC of WT and IL-10$^{-/-}$ mice were similarly induced to express TGFβ mRNA after 7 and 14 days of colonization. Expression of TGFβRII was also induced, particularly in IEC of WT mice. Expression levels of TGFβ and TGFβRII in total mucosal tissue were not changed in both groups (Figure 12).

Since the expression of TGFβ1 and TGFβRII mRNA was similar in IEC of WT and IL-10$^{-/-}$ mice, one could potentially expect similar TGFβ/Smad2 responses in the epithelium. However, Western Blot analysis using phospho-Smad2 antibodies detected activated TGFβ/Smad signalling only in IEC of WT mice, but not in IL-10$^{-/-}$ mice. As shown in Figure 13, phospho-smad2 was detected in IEC of WT mice at day 7 after colonization, followed by the induction of smad7, suppressor of TGFβ/smad2 after day 14 and 28. No phospho-smad2/smad7 was detected in IEC of IL-10$^{-/-}$ mice, despite the similar TGFβ/TGFβRII mRNA expression levels. Total smad2/3 was equally expressed in IEC of WT and IL-10$^{-/-}$ mice. Taken together, our results showed that IEC from IL-10$^{-/-}$ mice were able to induce TGFβ1 gene expression, but could not initiate the TGFβ/smad2 signalling, suggesting that some intrinsic defects in these cells prevented smad2 phosphorylation.

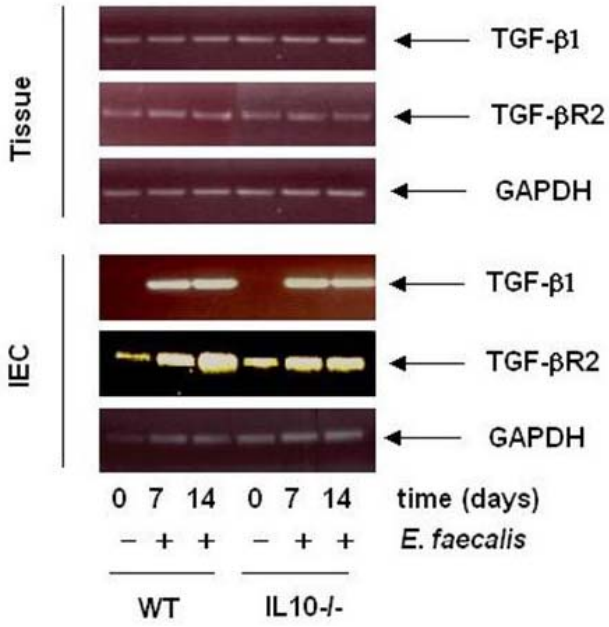

Figure 12. TGFβ1 and TGFβRII mRNA expression in native IEC and total mucosal tissue of WT and IL-10$^{-/-}$ mice.

Total RNA from murine native IEC and mucosal tissue was isolated, revere transcribed and used as a template in RT-PCR with specific primers for TGFβ1, TGFβRII and GAPDH. After Light Cycle RT-PCR amplification products were resolved on 2 % agarose gel.

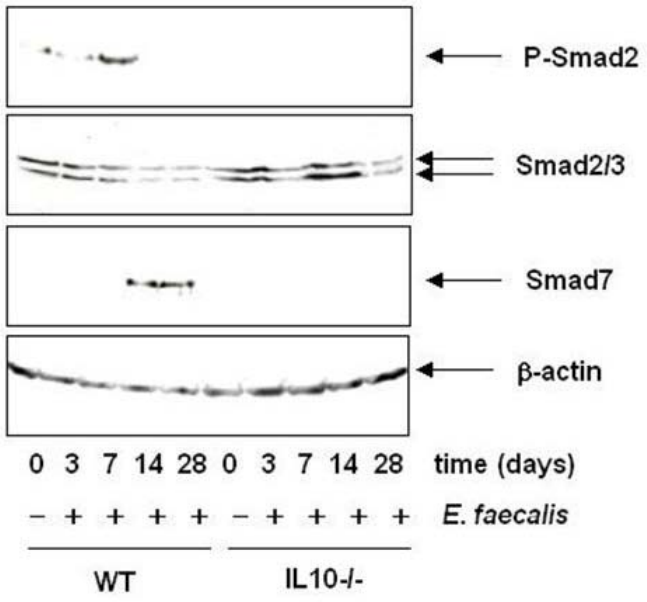

Figure 13. Differential TGFβ/Smad2 signalling in native IEC from WT and IL-10$^{-/-}$ mice monoassociated with *E.faecalis*.

Protein extracts from IEC of WT and IL-10$^{-/-}$ mice (3-28 days) were subjected to SDS-PAGE and ECL immunodetection using anti-phospho-smad2, smad2, smad7 antibodies was performed. β-actin was used as a loading control. Pooled samples containing 50 μg of total protein were generated for each time-point, including germ-free conditions.

Interestingly, *ex vivo* stimulation of freshly isolated native IEC from WT and IL-10$^{-/-}$ animals with TGFβ1 demonstrated that phosphorylation of smad2 could be induced in IEC of both types (Figure 14). It could be suggested that the absence of biologically active form of TGFβ in native IEC blocked smad2 phosphorylation.

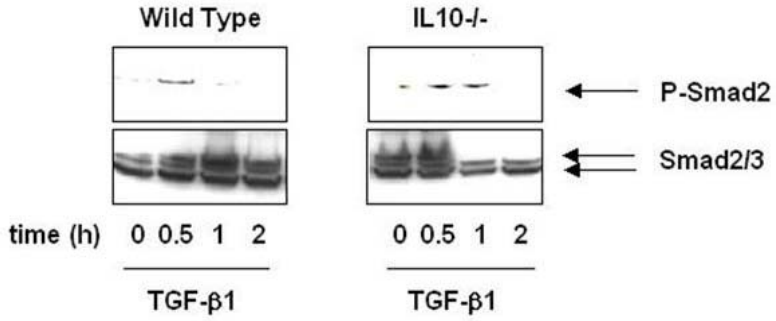

Figure 14. TGFβ1 triggers smad2 phosphorylation *ex vivo* in primary IEC of WT and IL-10$^{-/-}$ mice.

Native IEC were stimulated *ex vivo* with TGFβ1 (20 ng/ml) for 0-2 hours, cells were lysed in Laemmli buffer and 20 μg of total protein was subjected to SDS-PAGE and immunoblotting followed by ECL-immunodetection using mouse phospho-smad2 antibody. Smad2/3 was used as a control of equal expression.

4.1.2 Molecular mechanisms of TLR2 regulation by TGFβ/Smad2 signalling

Functional TGFβ/Smad2 signalling was detected in IEC of WT, but not in IL-10$^{-/-}$ mice after 7 days of bacterial colonization. Next, we thought to investigate, whether TGFβ/Smad-mediated signalling is involved in regulation of TLR2 expression in the intestinal epithelium. First, Mode-K cells were stimulated with exogenous TGFβ1 for 0-2 hours and phosphorylation of Smad2 was measured by Western Blot analysis. TGFβ triggered phosphorylation of Smad2 that could be inhibited by induction of Smad7 expression. The adenoviral infection of the cells with overexpressed smad7 molecule was shown to block smad2 phosphorylation in response to TGFβ1 (Figure 15). Of note, in IEC of WT mice smad2 phosphorylation followed by smad7 induction that is consistent with reverse regulation of TGFβ-mediated immunosuppressive effects. Since Smad2 phosphorylation in IEC of WT mice correlated with down-regulation of TLR2 expression, it could be suggested that TLR2 expression is regulated by TGFβ. Mode-K cells were stimulated with *E.faecalis* in the presence or absence of TGFβ for 0-2 hours. TGFβ stimulation did not lead to reduction of TLR2 expression in intact Mode-K cells. However, down-regulation of TLR2 expression was observed in bacteria-stimulated cells after addition of TGFβ (Figure 16A).

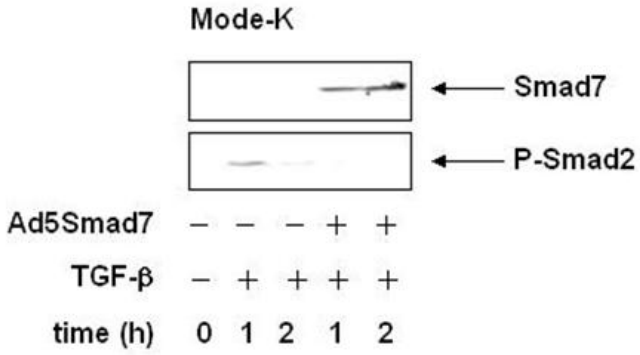

Figure 15. Adenoviral Smad7 overexpression prevents smad2 phosphorylation in TGFβ1-stimulated Mode-K cells.

Mode-K cells were stimulated with TGFβ1 for 0-2 hours, where indicated Mode-K cells were infected with adenovirus overexpressing smad7 during 24 hours in low serum culture medium. 20 μg of protein extracts were subjected to SDS-PAGE and immunoblotting with anti-phospho-smad2 and anti-smad7 antibodies.

As shown in Figure 16B, smad7 overexpression completely prevented the down-regulation of TLR2 in Mode-K cells, stimulated with *E.faecalis* and TGFβ1 due to the inhibition of smad2 phosphorylation. Of note, pre-treatment of the cells with proteasomal inhibitor MG132 did not influence down-regulation of TLR2 expression in Mode-K cells stimulated with *E.faecalis* and TGFβ. Perhaps, TLR2 down-regulation is mediated by mechanisms other than proteasomal degradation (Figure 16B).

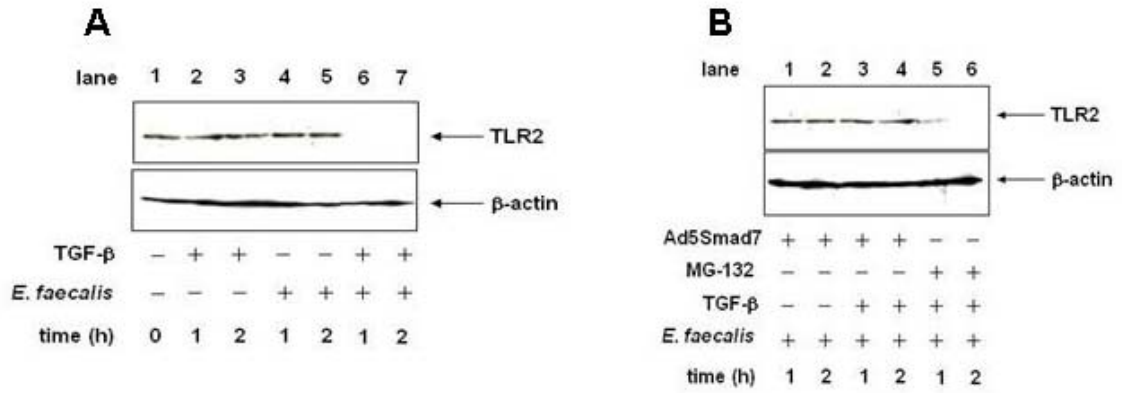

Figure 16. A and B. TGFβ/smad2 signalling triggers TLR2 expression in activated Mode-K cells.

A, Mode-K cells were stimulated with TGFβ1 alone, *E.faecalis*, and both TGFβ1 and *E.faecalis* for 0-2 hours. Westerm Blot analysis was performed using anti-TLR2 and β-actin specific antibodies. B, Mode-K cells were infected with smad7 overexpressing adenovirus or pre-incubated with proteasome inhibitor MG132 for 1 hour and stimulated with TGFβ1 and *E.faecalis* as described above. Western Blot analysis was performed using anti-TLR2 antibody and β-actin as a loading control.

Thus, investigation of cell signalling in native IEC demonstrated that in IEC of both WT and IL-10$^{-/-}$ mice the most decisive changes in signal transduction and gene expression occurred during the first two weeks after the initial contact of the animals with bacteria. Simultaneous down-regulation of TLR2- mediated NF-κB activation and induction of TGFβ/Smad2 signalling in IEC of WT mice and persistent TLR2-mediated NF-κB signalling in the absence of functional TGF/Smad signalling in IL-10$^{-/-}$ mice suggests that diverse cell signalling in murine IEC could lead to the different functional and physiological consequences that should be further characterized by other approaches.

4.2 Protein profiling in native intestinal epithelial cells from WT and IL-10$^{-/-}$ mice colonized with *E.faecalis*

Signal transduction and gene expression analysis, performed in native IEC of WT and IL-10$^{-/-}$ mice revealed the differential involvement of various signalling mechanisms, directed either to inhibit inflammatory response after bacterial colonization of animals, or oppositely, to keep IEC activated through the constant exposure to bacterial determinants due to the constant TLR2 expression. It seems obvious that each signalling cascade induces or regulates many genes and corresponding proteins that can be relevant for inflammation or defense. For this reason, protein expression profiling could help to monitor dynamic changes in the intestinal epithelium and identify novel molecular targets. Protein expression profiling of primary intestinal epithelial cell was performed in native IEC of WT and IL-10$^{-/-}$ mice colonized with *E.faecalis*, using 2D-SDS-PAGE and mass-spectrometric analysis. Two different time points were taken for the analysis. First, IEC from WT and IL-10$^{-/-}$ mice where isolated after 2 weeks of bacterial colonization. No inflammation could be detected histologically at this early time point, but the divergence in NF-κB and TGFβ/Smad signalling occurred between both groups. Second, IEC of WT and IL-10$^{-/-}$ mice were obtained after 14 weeks of monoassociation with *E.faecalis*. Only IL-10$^{-/-}$ but not WT mice developed severe intestinal inflammation and colitis. Two-dimensional gel electrophoresis and subsequent micro-preparative staining allowed to generate 2D-gel patterns from every single animal in each group and to conduct comparative image analysis in order to detect protein expression changes. Isoelectric focusing was performed by using IPG strips with broad pH gradient from 3 to 10, followed by SDS-PAGE separation of proteins in molecular weight range between 200 kDa and 5 kDa. Approximately 400-600 proteins could be detected by Proteomweaver software in each 2D-gel allowing the visualization of many abundant cellular proteins. The experimental design was planed in such way that all single 2D-patterns from each time point were run together in IEF and SDS-

PAGE, in order to reduce errors in technical reproducibility. Pooled samples from IEC of germ-free animals and separate samples for all colonized mice (N=5) were prepared. Gels that were obtained from germ-free WT and IL-10$^{-/-}$ mice were taken as a reference for comparative analysis. Protein spots differentially expressed in at least 3 of 5 animals with regulation factor higher than 2, were picked out of the gel, tryptic digested and subjected to MALDI-TOF-MS analysis. After 14 days of *E.faecalis*-monoassociation 14 and 17 differentially regulated proteins were identified in IEC of WT and IL-10$^{-/-}$ respectively. However, only 2 up-regulated protein in WT and 14 regulated in IL-10$^{-/-}$ were identified after 14 weeks of bacterial colonization (Table 10).

Table 10. Comparative number of differentially up- and down-regulated proteins identified by proteome analysis in IEC from WT and IL-10$^{-/-}$ mice after 2 and 14 weeks of monoassociation with *E.faecalis*.

Bacterial monoassociation	2 Weeks	14 weeks
WT (up-regulated)	3	2
WT (down-regulated)	11	0
WT (Total)	14	2
IL-10-/- (up-regulated)	10	5
IL-10-/- (down-regulated)	8	9
IL-10-/- (Total)	17	14

Protein expression profiling in IEC of WT and IL-10$^{-/-}$ mice revealed divergent regulation of protein expression after 2 weeks of bacterial colonization. The selected and identified regulated proteins in IEC of WT and IL-10$^{-/-}$ are shown in Figure 17 and 18 respectively. Except for one common protein, down-regulated in both groups (elongation factor TU), other identified proteins were distinct between WT and IL-10$^{-/-}$ and represented various functional categories. Table 11 and 12 compile the names of all proteins identified by mass-spectrometry including their calculated pI values, molecular weight and sequence coverage. For all proteins the regulation factor (mean fold change), standard deviation and frequency are shown. In most cases the calculated values of pI and molecular weight correlated well with the protein spots localization in the gel. Most of the up-

regulated proteins in both groups had regulation factors in the range between 2 and 3, except for oxoglutarate dehydrogenase with more than 5-fold induction in WT, and voltage dependent anion channel-2 with 3.7-fold induction in IL-10$^{-/-}$mice. Among the down-regulated proteins the regulation factor varied between 0.5 and 0.25 (2-4 fold reduction). Both groups of down-regulated proteins included a variety of metabolic enzymes, regulatory factors and other proteins, which could be potentially involved in pro- and anti-inflammatory response.

Proteome analysis was performed in IEC of WT and IL-10$^{-/-}$ mice after 14 weeks of monoassociation with *E.faecalis*. Interestingly, two proteins only (F-box protein 9 and DnaJ (Hsp40) homolog)) were up-regulated in IEC of WT mice at this time point compared to germ-free control (Figure 19, Table 13). In IEC of IL-10$^{-/-}$ mice 14 regulated (5 up-regulated, 9 down-regulated) proteins were identified. All identified proteins are presented in Figure 20 and listed in Table 14. Most of the differentially regulated proteins demonstrated fold induction or reduction ranging between 2 and 4. Of note, the expression of pyruvate kinase M2, hydroxymethylglutaryl-CoA-synthase and UDP-glucose dehydrogenase was down-regulated in IEC of IL-10$^{-/-}$ mice after 2 weeks of bacterial colonization. Protein expression of these enzymes was also decreased in IEC of IL-10$^{-/-}$ mice after 14 weeks of colonization under the inflammatory conditions. It could be suggested that inhibition of the corresponding metabolic pathways was involved in pathogenesis of chronic intestinal inflammation at early stage of bacterial administration. We next performed functional analysis of selected protein targets in order to explain how protein expression changes are associated with protective mechanisms in IEC.

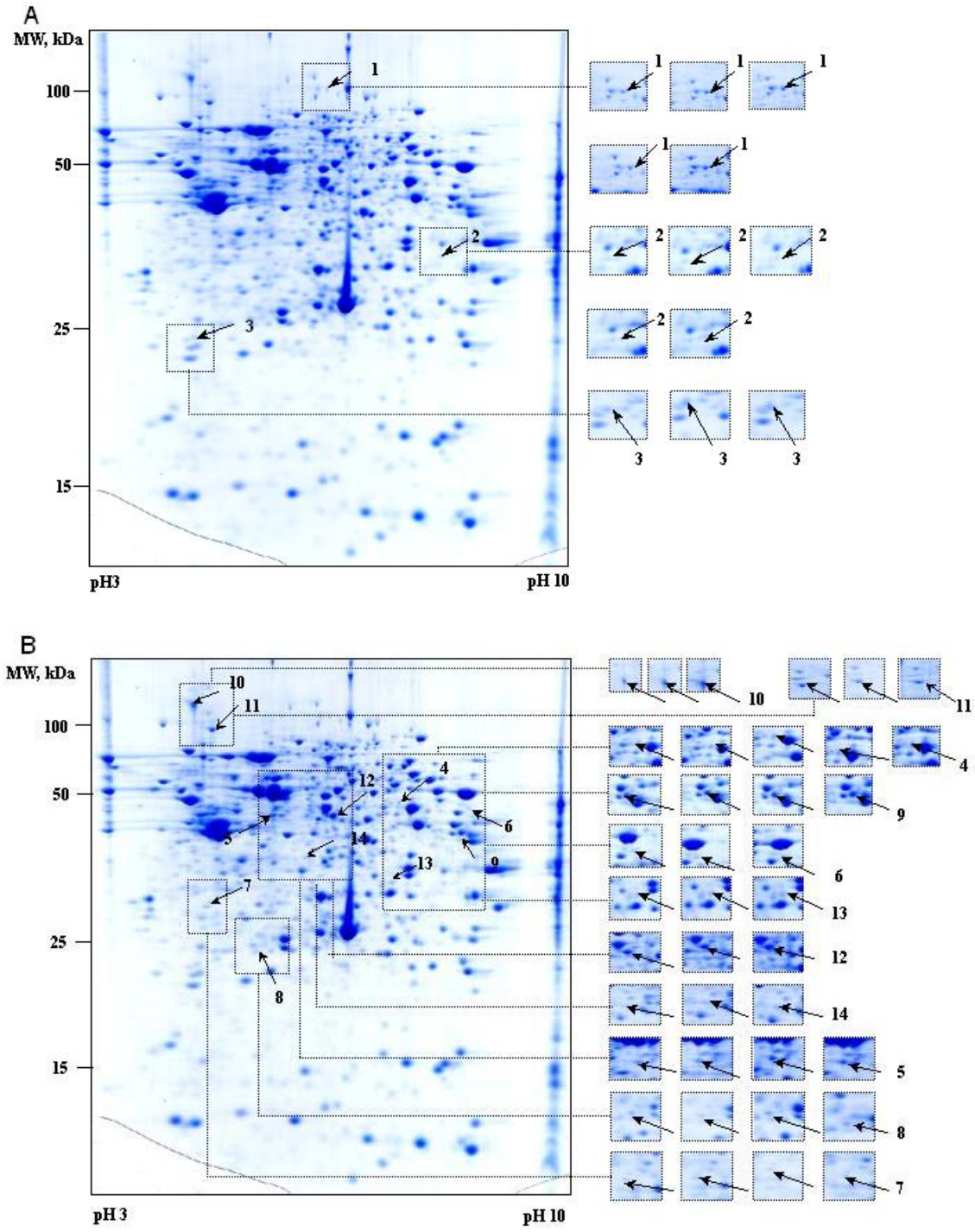

Figure 17. A and B. Coomassie stained 2D- gels with differentially regulated proteins in IEC from WT mice after 2 weeks of colonization with *E.faecalis*.

Native IEC were isolated and purified from large intestine of WT mice and 500 µg of total protein extract was subjected to IEF and 2D-SDS-PAGE. Gels were stained with Coomassie Blue and analyzed by Proteomweaver software. Indicated protein spots were picked and analyzed by MALDI-TOF-MS as described in Materials and Methods. Reference gel from germ-free mice and the corresponding regions from IEC of single colonized mice are shown. A, up-regulated proteins; B, down-regulated proteins.

Table 11. Differentially regulated proteins in IEC of WT mice after 2 weeks of colonization with *E. faecalis*.

N°	Accession Number	Protein	MW	pI	SC	RF	SD	F
1	AAH49104	Oxoglutarate dehydrogenase	117298	6.51	7	5.32	1.66	5/5
2	JC4398	Thiosulfate sulfotransferase	33673	7.71	18	2.61	0.13	3/5
3	BAB22060	Glyoxalase I	20967	5.24	45	2.52	0.52	3/5
4	AAC63098	Platelet-activating factor acetylhydratase 45 kDa subunit	44972	6.95	24	0.30	0.10	5/5
5	S29170	Annexin 7	50162	5.91	17	0.40	0.03	4/5
6	S60028	Ferredoxin NADP reductase precursor	54568	8.90	18	0.27	0.06	4/5
7	BAC401585	Chloride channel ABP	27338	5.09	38	0.42	0.10	4/5
8	AJMSRS	Argininosuccinate synthase	48840	8.36	15	0.32	0.03	4/5
9	BAC27697	Acetyl-CoA-acetyltransferase mitochondrial	45129	8.71	20	0.44	0.03	4/5
10	Q8K0P6	A430096B05 Rik fragment	133116	4.98	9	0.43	0.04	3/5
11	Q8BSR6	Valosin containing protein	89998	5.11	10	0.42	0.06	3/5
12	Q8BFR5	Elongation factor TU mitochondrial	49876	7.23	21	0.40	0.09	3/5
13	AAB03107	3-hydroxy-3-methylglutaryl-CoA lyase	34641	8.70	28	0.31	0.04	3/5
14	Q99K93	Elfin (PDZ and LIM domain 1)	36208	6.38	28	0.23	0.13	3/5

MW-molecular weight, Da; pI-isioelectric point; SC-sequence coverage, %; RF-regulation factor; SD-standard deviation; F-frequency

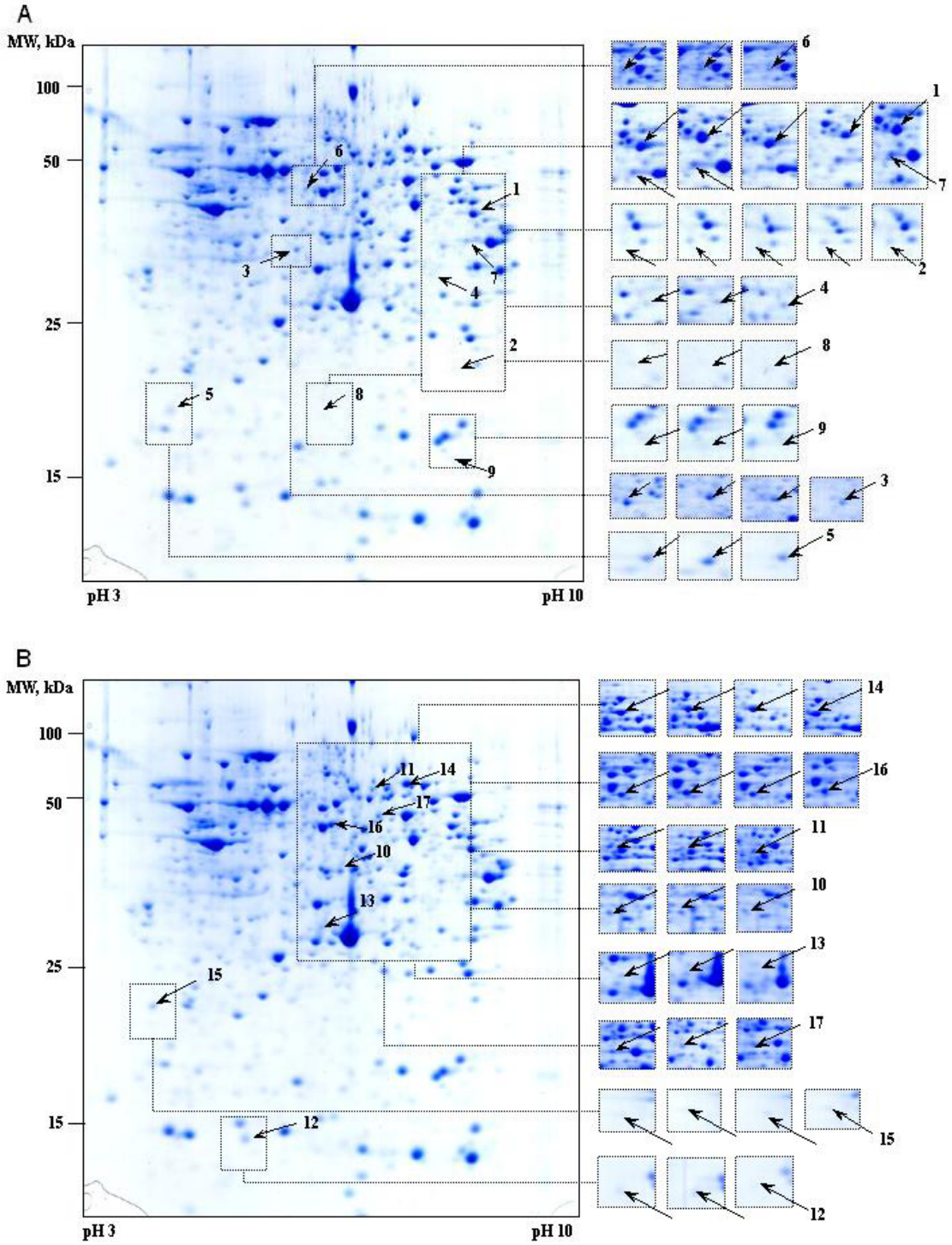

Figure 18. A and B. Coomassie stained 2D- gels with differentially regulated proteins in IEC from IL-10$^{-/-}$ mice after 2 weeks of colonization with *E. faecalis.*

Native IEC were isolated and purified from large intestine of IL-10$^{-/-}$ mice and 500 µg of total protein extract was subjected to IEF and 2D-SDS-PAGE. Gels were stained with Coomassie Blue and analyzed by Proteomweaver software. Indicated protein spots were picked and analyzed by MALDI-TOF-MS as described in Materials and Methods. Reference gel from germ-free mice and the corresponding regions from IEC of single colonized mice are shown. A, up-regulated proteins; B, down-regulated proteins.

Table 12. Differentially regulated proteins in IEC of IL-10$^{-/-}$ mice after 2 weeks of colonization with *E. faecalis*.

N°	Accession Number	Protein	MW	pI	SC	RF	SD	F
1	Q9Z2A3	Glial cell line derived neurotrophic factor family member receptor alpha 2b	41043	7.69	14	2.19	0.10	5/5
2	BAB22842	Succinate dehydrogenase complex, subunit B	32591	8.96	20	3.06	0.50	4/5
3	Q8CDP8	Ankyrin repeat domain-containing SOCS box protein Asb15	66440	5.67	10	2.97	0.34	4/5
4	Q99C98	Voltage dependent anion channel 2	32310	7.44	20	3.70	0.77	3/5
5	JQ0028	Cytokeratin 19, fragment	44515	5.28	26	2.81	0.74	3/5
6	T30173	Zink finger protein Pw1	158815	5.12	2	2.25	0.18	3/5
7	DEMSG	Glyceraldehydes-3-phosphate gehydrogenase, phosphorylated	36072	8.44	47	2.6	0.46	3/5
8	BAC37447	Destrin	18852	8.14	33	2.53	0.38	3/5
9	AAH08174	Peroxiredoxin 5	22226	9.10	24	2.96	0.74	3/5
10	CAD20433	Sequence 17 from patent WO0185986	30374	6.61	21	0.42	0.06	3/5
11	AAH06749	UDP-glucose dehydrogenase	55482	7.49	24	0.35	0.09	3/5
12	ILBP_ MOUSE	Gastrotropin Ileal lipid binding protein	14403	5.93	44	0.41	0.07	3/5
13	Q9CYW4	Hypothetical Haloacid dehalogenase	28237	6.31	27	0.41	0.05	3/5
14	S55921	Pyruvate kinase M2	58394	7.58	12	0.36	0.06	4/5
15	Q8CDX3	Hypothetical protein, fragment	29523	11.80	39	0.41	0.05	4/5
16	Q8BFR5	Elongation factor TU mitochondrial	49876	7.23	21	0.34	0.07	4/5
17	B55729	Hydroxymethylglutaryl-CoA-synthase, mitochondrial.	53115	7.46	11	0.39	0.06	3/5

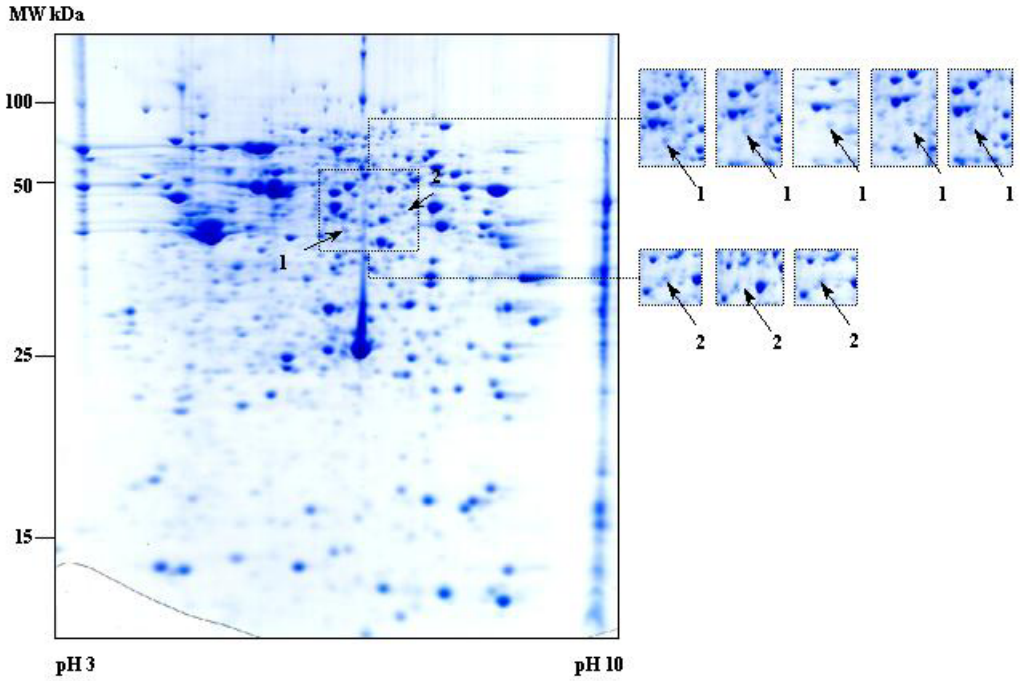

Figure 19. Coomassie stained 2-D gels with differentially up-regulated proteins in IEC from WT mice after 14 weeks of colonization with *E. faecalis*.

Native IEC were isolated and purified from large intestine of WT mice and 500 µg of total protein extract was subjected to IEF and 2D-SDS-PAGE. Gels were stained with Coomassie Blue and analyzed by Proteomweaver software. Indicated protein spots were picked and analyzed by MALDI-TOF-MS as described in Materials and Methods. Reference gel from germ-free mice and the corresponding regions from IEC of single colonized mice are shown. A, up-regulated proteins; B, down-regulated proteins.

Table 13. Differentially regulated proteins in IEC of WT mice after 14 weeks of colonization with *E. faecalis*.

N°	Accession Number	Protein	MW	pI	SC	RF	SD	F
1	Q8VDY6	F-box protein 9	51153	6.38	17	3.11	1.04	5/5
2	Q76G10	DnaJ (Hsp40) homolog, subfamily A member 1	45581	6.65	41	2.76	0.52	3/5

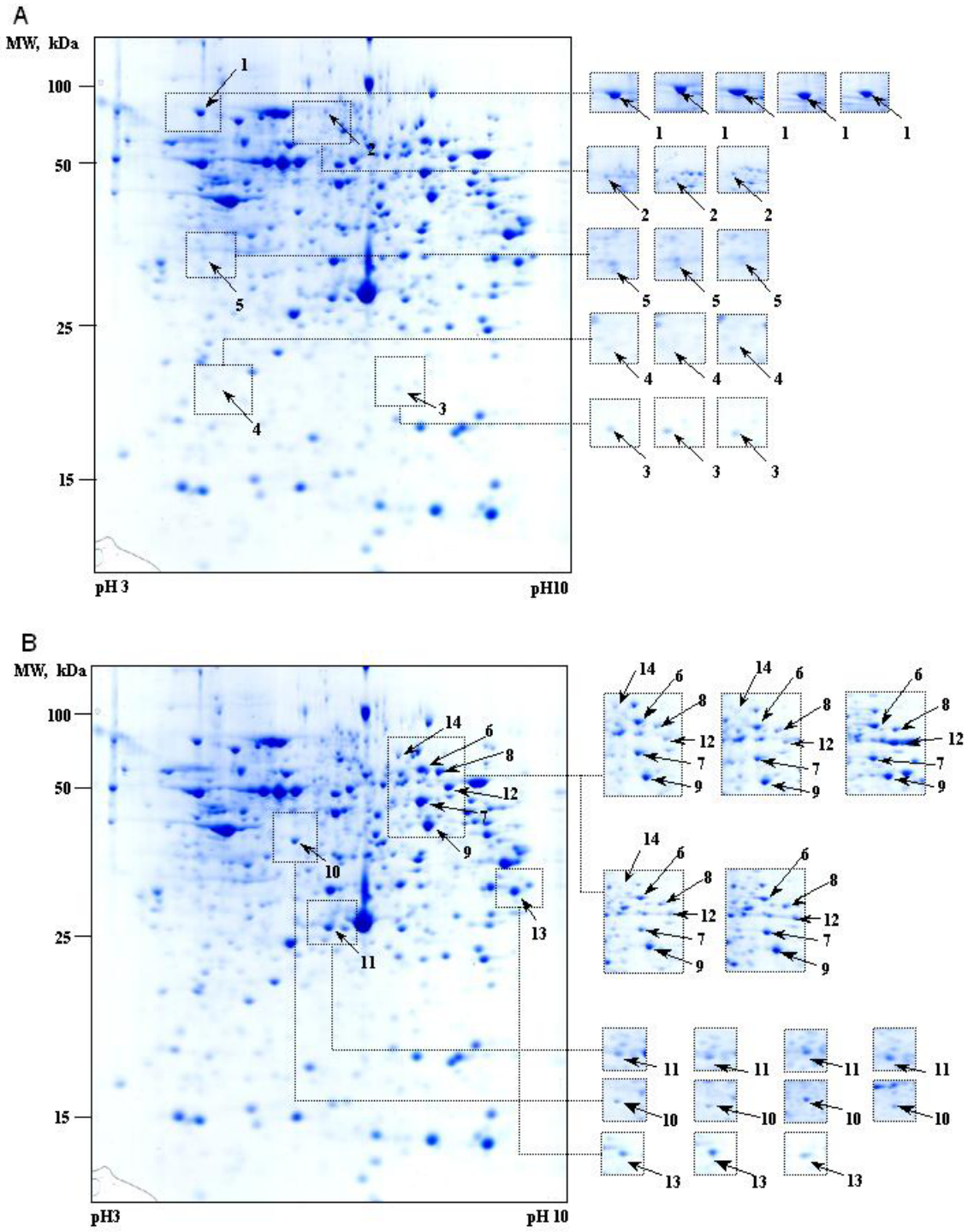

Figure 20. A and B. Coomassie stained 2-D gels with differentially regulated proteins in IEC from IL-10$^{-/-}$ mice after 2 weeks of colonization with *E. faecalis*.

Native IEC were isolated and purified from large intestine of IL-10$^{-/-}$ mice and 500 µg of total protein extract was subjected to IEF and 2D-SDS-PAGE. Gels were stained with Coomassie Blue and analyzed by Proteomweaver software. Indicated protein spots were picked and analyzed by MALDI-TOF-MS as described in Materials and Methods. Reference gel from germ-free mice and the corresponding regions from IEC of single colonized mice are shown. A, up-regulated proteins; B, down-regulated proteins.

Table 14. Differentially regulated proteins in IEC of IL-10$^{-/-}$ mice after 14 weeks of colonization with *E. faecalis*.

N°	Accession Number	Protein	MW	pI	SC	RF	SD	F
1	A37048	DnaK type molecular chaperone grp78	72491	5.12	27	2.42	0.50	5/5
2	Q922N3	Propionyl-CoA-carboxylase, alpha chain	80517	7.00	15	2.73	0.28	3/5
3	Q6ZQH1	MKIAA0186	19922	8.65	41	2.74	0.41	3/5
4	S31975	14-3-3 protein epsilon	29326	4.63	24	2.85	0.76	3/5
5	BAB27292	Tubulin beta 5	50064	4.78	28	3.43	0.50	3/5
6	CAA65761	Pyruvate kinase M2	58448	7.18	38	0.34	0.08	5/5
7	B55729	Hydroxymethyl-glutaryl-CoA–synthase mitochondrial	53115	7.46	14	0.28	0.08	5/5
8	AAH06749	UDP-glucose dehydrogenase	55482	7.49	37	0.35	0.06	5/5
9	S24612	Creatine kinase, mitochondrial	47373	8.39	43	0.40	0.06	5/5
10	Q9D154	Serin protease inhibitor clade B	42719	5.85	16	0.37	0.08	4/5
11	Q99J99	3-mercapto-pyruvate sulfotransferase	33231	6.11	21	0.40	0.08	4/5
12	JQ1004	Aldehyde dehydrogenase NAD precursor	55131	7.89	41	0.30	0.07	3/5
13	A45983	Lactose-binding lectin Mac-2, Galectin-3	27455	8.57	20	0.39	0.02	3/5
14	Q9QYS0	Sulfurylase/APS kinase isoform SK2	70991	7.31	13	0.39	0.01	3/5

4.3 Selective validation of protein expression in WT and IL-10$^{-/-}$ mice after 14 weeks of monoassociation with *E. faecalis*.

Three differentially regulated proteins in IEC of IL-10$^{-/-}$ mice were taken for the investigation of their possible functions under conditions of chronic intestinal inflammation. The selection criteria were based on importance of these proteins for multiple cellular processes and also on availability of appropriate antibodies and primers. Molecular chaperone grp-78 was up-regulated in IEC of all five IL-10$^{-/-}$ mice in comparison to germ-free conditions. The increased expression of grp-78 is a prototype marker of endoplasmic reticulum (ER) stress that could be induced after bacterial colonization and during the pro-inflammatory response [193]. The functions of grp-78 in the induction and regulation of ER-stress response under conditions of chronic intestinal inflammation are not yet investigated. The second protein, mitochondrial creatine kinase was identified to be down-regulated in IEC of IL-10$^{-/-}$ mice. Creatine kinase is one of the key enzymes involved in the maintenance of energy homeostasis in mitochondria and in the generation of phospho-creatine and ATP. One of the consequences of mitochondrial dysfunction is apoptotic cell death mediated by intrinsic mechanisms [194]. Galectin-3 was also found as a down-regulated protein in IL-10$^{-/-}$ mice. Galectin-3 is a protein with multiple cellular functions. It is involved in different signalling pathways from NF-κB activation to MAPK- and hypoxia-induced signalling [195]. The functional consequences of differential regulation of these selected proteins could be further investigated in cultured IEC. First, protein expression changes for three selected targets were additionally verified by Western Blot analysis and Real-Time PCR in pooled IEC samples from WT and IL-10$^{-/-}$ mice after 14 weeks of colonization compared to germ-free conditions. Western Blot analysis was performed using specific antibodies for grp-78, creatine kinase and galectin-3, followed by the densitometric measurement of the obtained bands. As shown in Figure 21A, grp-78 was induced in IEC of IL-10$^{-/-}$ mice and reduced in WT mice after 14 weeks. Oppositely, galectin-3 and mitochondrial creatine kinase protein expression levels were decreased in IEC of IL-10$^{-/-}$ mice but not in WT mice. Light Cycler RT-PCR was performed using specific primers for grp-78, galectin-3 and mitochondrial creatine kinase and mean-fold changes in mRNA expression were calculated as described in Materials and Methods. Grp-78 was up-regulated at mRNA expression level in IEC of IL-10$^{-/-}$ mice (4-fold induction); in IEC of WT mice grp-78 gene expression was decreased. Transcriptional levels of galectin-3 and mitochondrial creatine kinase revealed approximately 2-fold reduction in IEC of IL-10$^{-/-}$ mice and no significant changes in IEC of WT mice (Figure 21 B).

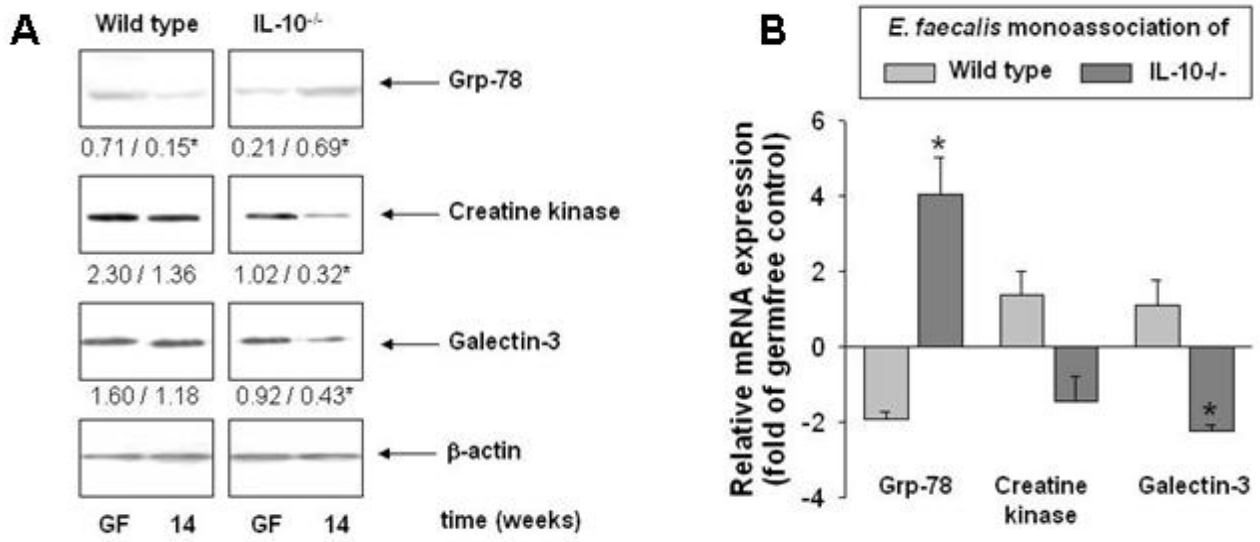

Figure 21. A and B. Differential protein and mRNA expression of grp-78, creatine kinase and galectin-3 in native IEC of WT and IL-10$^{-/-}$ mice.

A, Primary IEC were lysed, mixed 1:1 with Laemmli Buffer and 50 µg of total protein extract was subjected to SDS-PAGE. Immunoblotting analysis using specific antibodies to mouse grp-78, galectin-3, creatine kinase and actin was performed. Densitometric analysis was applied to quantify intensity of corresponding bands. B, Total RNA was extracted, purified and reverse transcribed from native IEC. Light Cycler RT-PCR with specific mouse primers to grp-78, galectin-3, creatine kinase and GAPDH was performed for all animals. Mean fold induction and standard deviation was calculated against GAPDH as a house-keeping gene, germ-free animals were taken as a control.

We next thought to investigate possible consequenses of regulation of grp-78, galectin-3 and creatine kinase under conditions of chronic inflammation. Of note, all selected proteins were expressed in Mode-K cell line that allowed to apply siRNA-mediated gene silencing technology for the characterization of grp-78 and galectin-3 functions and bacteria/cytokine stimulation for mitochondrial creatine kinase.

4.4 Functional characterization of selected protein targets

4.4.1 ER-stress response under conditions of chronic intestinal inflammation

4.4.1.1 Gene targeting of ER-chaperone grp-78 triggers NF-κB signalling in IEC.

Proteome analysis in IEC of WT and IL-10$^{-/-}$ mice after 14 weeks of monoassociation with colitogenic *E.faecalis* revealed increased expression of molecular chaperone grp-78 under conditions of chronic inflammation. In order to investigate the role of grp-78 in NF-κB-mediated signalling, grp-78 knock-down Mode-K cells were generated using siRNA-mediated specific gene-silencing. Significant reduction of grp-78 protein expression was achieved after 72 hours of transfection with specific grp-78 siRNA. No silencing effect was observed after transfection of the cells with control siRNA. Next, transfected Mode-K cells were stimulated with TNF for 1h and RelA phosphorylation was measured by immunoblotting. As shown in Figure 22A, almost no phosphorylation of RelA was observed in cells with grp-78 silencing effect. To the contrast, Mode-K cells transformed with control non-specific siRNA demonstrated transient NF-κB activation.

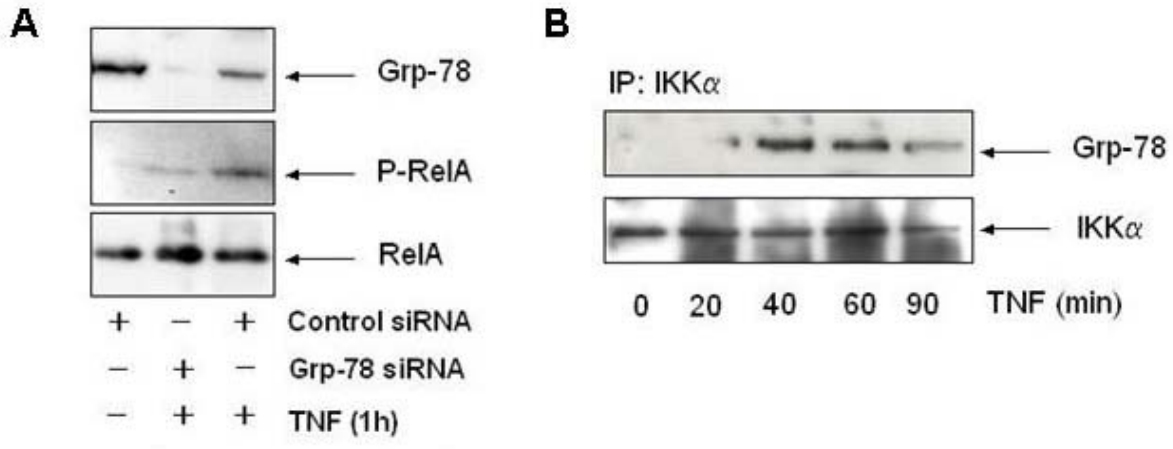

Figure 22. A and B. Grp-78 triggers NF-κB activation in TNF-stimulated cells.

A, Mode-K cells were transfected with specific siRNA for grp-78, after 72 hours cells were stimulated with TNF for 1h and Rel-A phosphorylation was measured by Western Blot analysis. Cell lysates were subjected to SDS-PAGE and probed with specific antibodies to grp-78, phospho-RelA and total RelA. B, Mode-K cells were stimulated with TNF for 0-90 min, cells lysates were co-immunoprecipitated with anti-IKKα monoclonal antibody and complexes were collected using agarose beads. Samples were denaturated, mixed with 1xLaemmli buffer and analyzed by Western Blot with specific antibodies to grp-78 and total IKKα to control equal binding.

It was reported recently that cytoplasmic heat shock proteins are involved in the IKK complex. They are supposed to play an important role in protein folding and stabilizing of IKK complex [196, 197]. Although grp-78 is one of the most abundant chaperones localized in endoplasmatic reticulum (ER), there is some evidence of redistribution of this protein between different cellular compartments [198]. We suggested that grp-78 could be translocated from ER to the cytoplasm for the regulation and stabilizing of IKK complex similar to other heat shock proteins. Indeed, grp-78 was co-immunoprecipitated with IKKα-subunit in Mode-K cells stimulated with TNF for 0-90 min. Grp-78 was recruited to the IKK complex after 40 min of TNF-stimulation (Figure 22B). Intracellular redistribution of grp-78 after stimulation of Mode-K cells with TNF was confirmed by the separation of total cell lysate in cytoplasmic, microsomal and mitochondrial protein fractions. Grp-78 was detected in the cytoplasmic and microsomal fractions of TNF-stimulated cells (Figure 23).

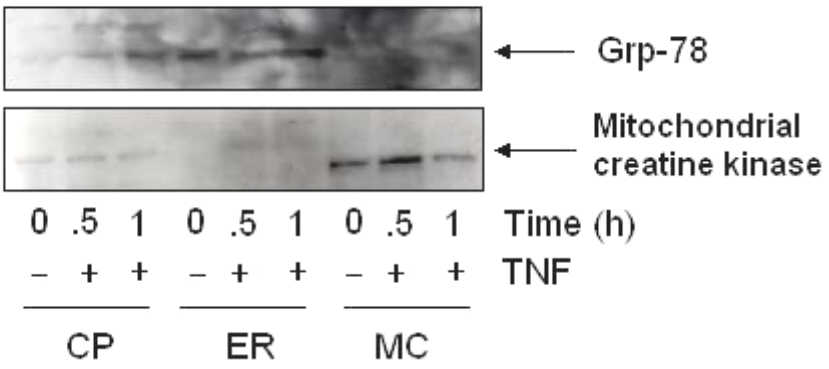

Figure 23. Fractional analysis of grp-78 in TNF-stimulated Mode-K cells.

Mode-K cells were grown in 75 cm^2 cultural flasks and stimulated with TNF for 0-60 min. The cells were harvested and total protein lysate was separated on cytosolic, microsomal and mitochondrial fractions using Qproteome kit. The proteins of different fractions were concentrated by acetone precipitation and dissolved in lysis buffer.50 μg of protein was mixed with 3xLaemli buffer and subjected to SDS-PAGE and immunoblotting using specific antibodies for grp-78 and mitochondrial creatine kinase was used to control the quality of fractionation.

In addition, immunostaining of grp-78 and fluorescence microscopy allowed to visualize grp-78 localization and intracellular trafficking upon TNF-stimulation. As shown in Figure 24, co-staining of the permeabilized cells with DAPI (blue) and specific anti-grp-78 antibody (TRITC-conjugated IgG was used as a secondary antibody, red) revealed grp-78 accumulation in perinuclear zone of unstimulated Mode-K cells. Stimulation of the cells with TNF for 1h induced redistribution of grp-

78 between perinuclear space of the ER compartment and the cytoplasm. Overlay images allow to vizualize partial relocation of grp-78 from perinuclear zone more to the periferic intracellular regions after TNF-stimulation.

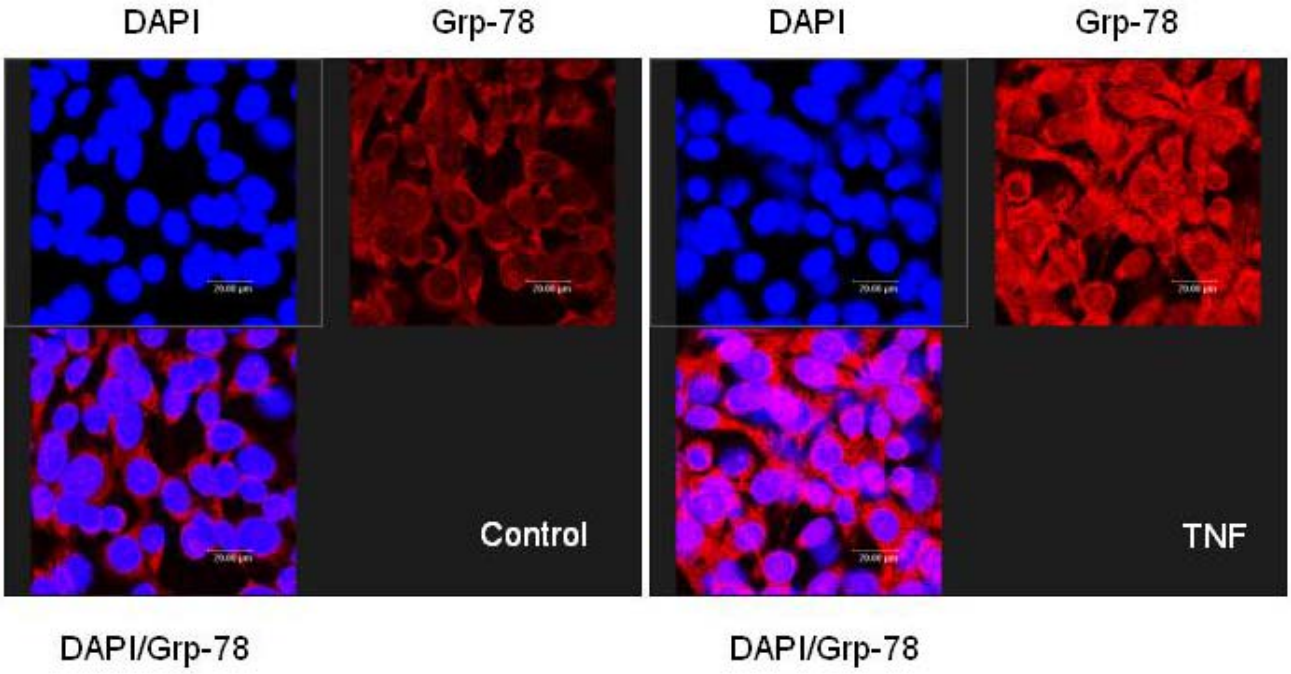

Figure 24. Immunostaining of grp-78 in intact and TNF-stimulated Mode-K cells.

Mode-K cells were grown on glass cover slips in 6-well plates. Confluent cells were stimulated with TNF for 1 h, fixed with methanol and incubated with primary rabbit anti-grp-78 antibody followed by incubation with anti-rabbit TRITC-conjugated IgG and DAPI. Stained cover slips were placed on microscopic slides and analyzed using inverted confocal microscope and Leica 2.5 software.

4.4.1.2 IL-10-mediated signalling in primary and cultured IEC.

IL-10 is known to be a pleiomorphic cytokine with immunosuppressive properties similar to TGFβ. TGFβ and IL-10 have tightly interrelated immunoregulatory functions and act as two synergistic cytokines in modulation of anti-inflammatory response [192]. However, the direct effects of IL-10 on IEC are not yet investigated. IL-$10^{-/-}$ mice developed severe colitis after bacterial colonization due to the absence of host-derived mechanisms regulating intestinal inflammation. In IEC of WT mice these protective mechanisms could be mediated not only by TGFβ/smad2, but also by p-38 MAPK signalling. Differential p-38 MAPK signalling was observed in IEC of WT and IL-$10^{-/-}$ mice after 14 weeks of colonization. IEC of WT mice expressed phospho-p-38 compared to IEC of IL-$10^{-/-}$ mice at the late stage of bacterial colonization. Of note, total p-38 and IL-10 receptor were constantly

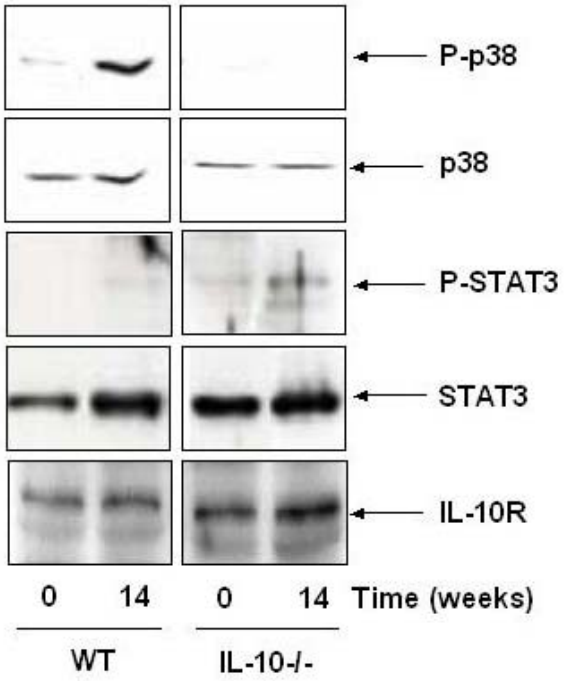

Figure 25. Differential p-38 MAPK signalling in IEC of WT and IL-10$^{-/-}$ mice after 14 weeks of monoassociation with *E. faecalis*.

IEC were isolated from germ-free and *E. faecalis*-colonized WT and IL-10$^{-/-}$ mice, 50 µg of total protein was separated by SDS-PAGE and analyzed by Western Blot using specific antibodies to mouse phospho-p38, p-38 and IL-10R.

and equally expressed in both IEC before and after bacterial monoassociation (Figure 25). To understand whether differential phosphorylation of p-38 may be the consequence of IL-10 signalling, the effects of IL-10 on p-38 MAPK pathways were investigated in Mode-K cells. IL-10 receptor reconstituted Mode-K cells were stimulated with recombinant IL-10. Non-transformed Mode-K cells were used to validate the specificity of IL-10 signalling via IL-10R. Phosphorylation of p-38 was detected only in IL-10R-transformed cells (Figure 26A). In addition, IL-10 was able to induce phosporylation of p-38 and STAT-3 in a time- and dose-dependent manner. In the absence of IL-10R no phospho-p-38 and phospho-STAT-3 was detected in Mode-K cells (Figure 26 B).

P-38 MAPK-mediated signalling comprises a multifunctional signalling network that influences cell growth, differentiation, apoptosis and cellular responses to stress [199]. Since this pathway represents a point of convergence for multiple signalling processes that are activated in inflammation, protective role of p-38-mediated IL-10 signalling on ER-stress responses was further investigated in IEC.

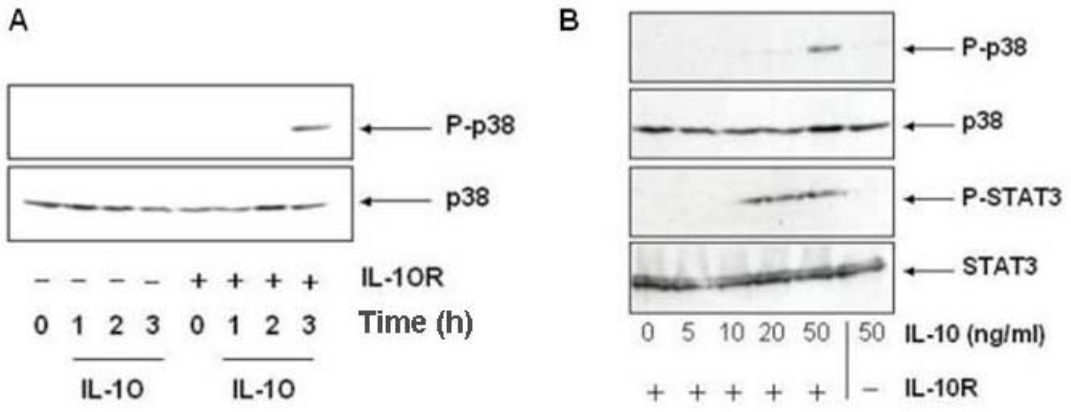

Figure 26. A and B. IL-10-mediated p-38 MAPK and STAT-3 signalling in Mode-K/IL-10R cells.

A, IL-10 reconstituted Mode-K cells were stimulated with rIL-10 for 0-3 h. Protein extracts were separated by SDS-PAGE and immunoblotted with specific antibodies to p-p38 and p-38. Non-transformed Mode-K cells were taken to demonstrate specificity of IL-10/MAPK signalling. B, Mode-K/IL-10R cells were stimulated with rIL-10 for 2 h in concentration range from 0 to 50 ng/ml. 20 μg of total protein extracts were subjected to SDS-PAGE and analyzed by immunoblotting using antibodies to p-p38, p-38, p-STAT-3 and STAT-3. Mode-K cells without IL-10R were used as a negative control.

4.4.1.3 IL-10 regulates expression of the molecular chaperone grp-78

Gene silencing of grp-78 suggested an essential role of this chaperone for TNF-induced NF-κB-activation. In addition, consistent with this finding it was shown in co-immunoprecipitation experiments that grp-78 was recruited to the IKK complex. Increased expression of grp-78 in IEC of IL-10$^{-/-}$ mice after 14 weeks of bacterial colonization correlated with the persistent NF-κB activation and intestinal inflammation in these mice. In contrast, IEC of WT mice revealed down-regulated grp-78 expression levels in the absence of NF-κB activation at this late colonization time point. It could be proposed that IL-10 prevented the induction of ER-stress in IEC of WT mice. Indeed, we were able to show in IL-10 receptor reconstituted Mode-K cells (Mode-K/IL-10R) that IL-10 treatment significantly reduced grp-78 protein expression already after 6 hours (Figure 27A). In contrast, TNF treatment did not inhibit grp-78 expression. This observation was confirmed by Light Cycler RT-PCR analysis. IL-10 treatment led to the reduction of grp-78 mRNA expression levels after 12 and 24 hours (Figure 27 B). Interestingly, pre-treatment of Mode-K/IL-10R cells with IL-10 followed by TNF-stimulation resulted in dramatic reduction of grp-78 mRNA levels after 24 hours. Of note, IL-10-mediated transcriptional down-regulation of grp-78 was observed only in IL-10 receptor reconstituted Mode-K cells (Figure 28)

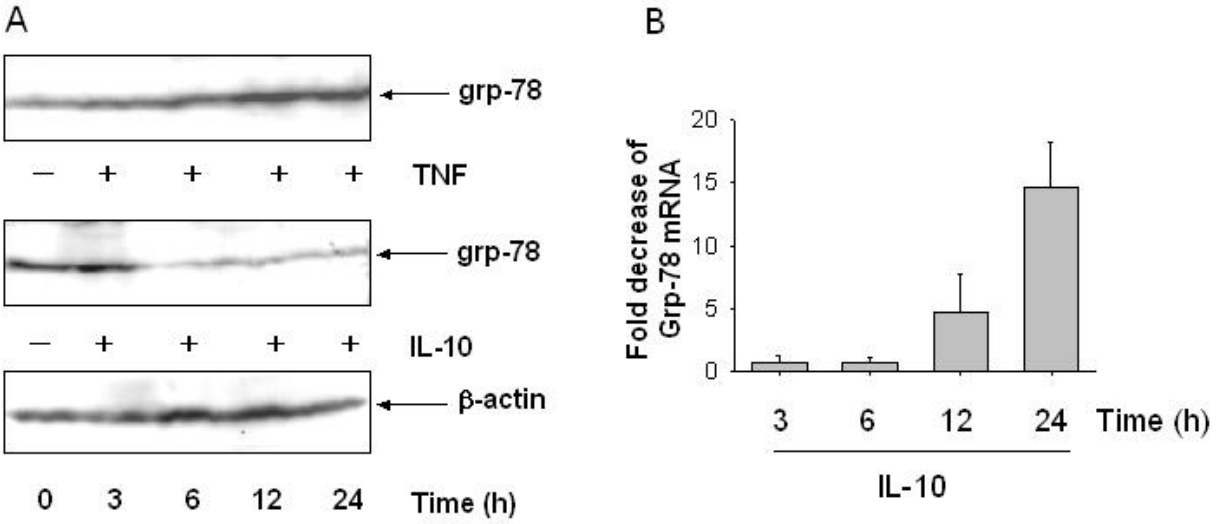

Figure 27. A and B. Down-regulation of grp-78 protein and mRNA expression by IL-10 in Mode-K/IL-10R cells.

A, Mode-K/IL-10 R cells were stimulated with rIL-10 and TNF for 0-24 h, 20 μg of total protein extract was analyzed by immunoblotting using specific antibodies to grp-78 and β-actin. B, total RNA was extracted and reverse transcribed, Light Cycle RT-PCR was performed with specific primers to grp-78, fold reduction was normalized against 18S gene and mean-fold changes and standard deviation were calculated for treatment triplicates.

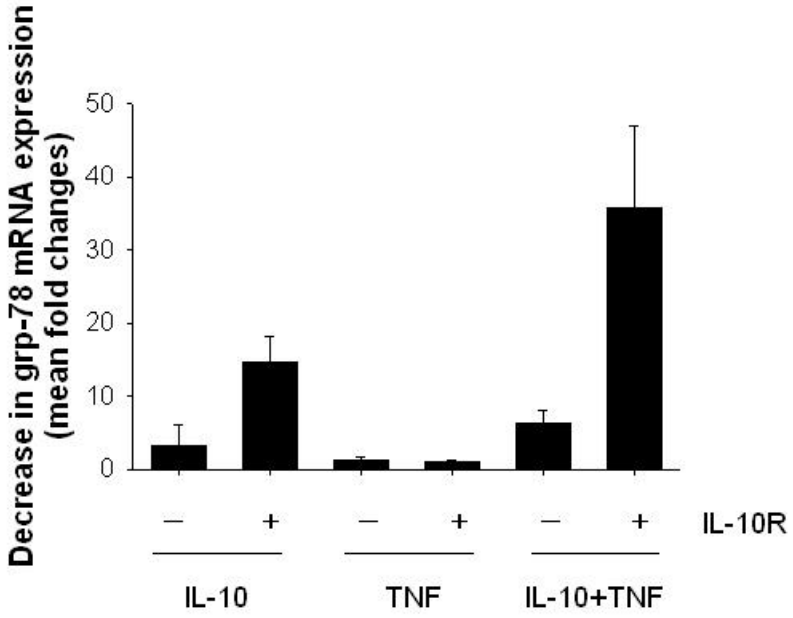

Figure 28. Inhibition of grp-78 mRNA expression by IL-10 in Mode-K/IL-10R cells.

Mode-K/IL-10R cells were stimulated with IL-10, TNF and combination of both cytokines for 24 h. Total RNA was purified and reverse transcribed. Light Cycler RT-PCR was performed with specific primers for grp-78 and normalized to 18S as a house-keeping gene. Mode-K cells not transformed with IL-10R were taken to assess specificity of IL-10 effect.

Since IL-10 was able to reduce grp-78 protein and mRNA expression, pre-treatment of the cells with IL-10 could affect grp-78 involvement to the IKK complex during the cell activation and modulate NF-κB activation. Indeed, the experimental data showed that pre-incubation of Mode-K/IL10R cells with rIL-10 for 24 h decreased grp-78 recruitment to the IKK complex and prevented phosphorylation of RelA after TNF-stimulation (Figure 29). This observation was consistent with our previous data where gene-specific siRNA silencing of grp-78 was able to decrease NF-κB activation in TNF-stimulated cells.

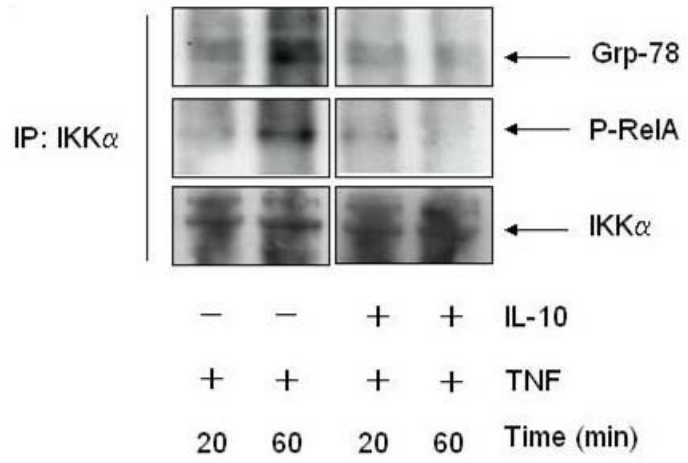

Figure 29. IL-10 triggers grp-78 involvement in IKK complex in TNF-stimulated cells.

Mode-K/IL-10R cells were pre-incubated with rIL-10 for 24h (where indicated), cells were stimulated with TNF for 20 and 60 min and total cell lysates were co-immunoprecipitated with anti-IKKα antibody and A/G agarose beads, complexes were dissociated by heating for 5 min, mixed with 3xLaemmli Buffer and subjected to Western Blot Analysis with specific antibodies to grp-78, phospho-RelA and IKKα.

4.4.1.4 Molecular mechanisms of IL-10-mediated regulation of ER-stress in IEC

We showed that IL-10 was able to regulate ER-stress response by inhibiting grp-78 at the levels of protein and mRNA expression. It is known that induction and expression of molecular chaperones during ER-stress is regulated primarily at the transcriptional level by binding of corresponding transcription factors to promoter-sequences. Promoter of grp-78 gene has a complex structure and contains multiple binding sites for transcription factors like ATF6, XBP1, TFII-I and others [200]. ATF6 is one of ER-membrane-anchored transcription factors activated by intramembrane proteolysis. Upon ER-stress, ATF6 is transported from the ER to the Golgi where it is processed to its active form followed by binding to grp-78 promoter in the nucleus [201]. We supposed that IL-10

could inhibit transcription of grp-78 gene by affecting binding of transcription factors to the promoter. Chromatin-immunoprecipitation (ChIP) was performed in our laboratory by Pedro Ruiz for joint publication and the data were kindly provided for the present manuscript. Briefly, Mode-K/IL-10R cells were stimulated with TNF in the presence or absence of IL-10. ChIP was performed using specific antibody to ATF6α, c-fos and Ac-P-H3. It was shown that IL-10 completely inhibited ATF6 binding to the grp-78 promoter sequences. In contrast, ATF6 recruitment to grp-78 promoter was clearly detected after TNF-stimulation. However, pretreatment of the cells with IL-10 prevented ATF6 binding to promoter sequences of grp-78 after TNF-stimulation (Figure 30A). In addition, it was demonstrated that this inhibitory effect of IL-10 on ATF6-binding was mediated by p-38 MAPK signalling pathway. Pre-treatment of the cells with p-38 MAPK inhibitor SB203580 reversed ATF6-binding to grp-78 promoter after the stimulation with IL-10 and TNF (Figure 30B)

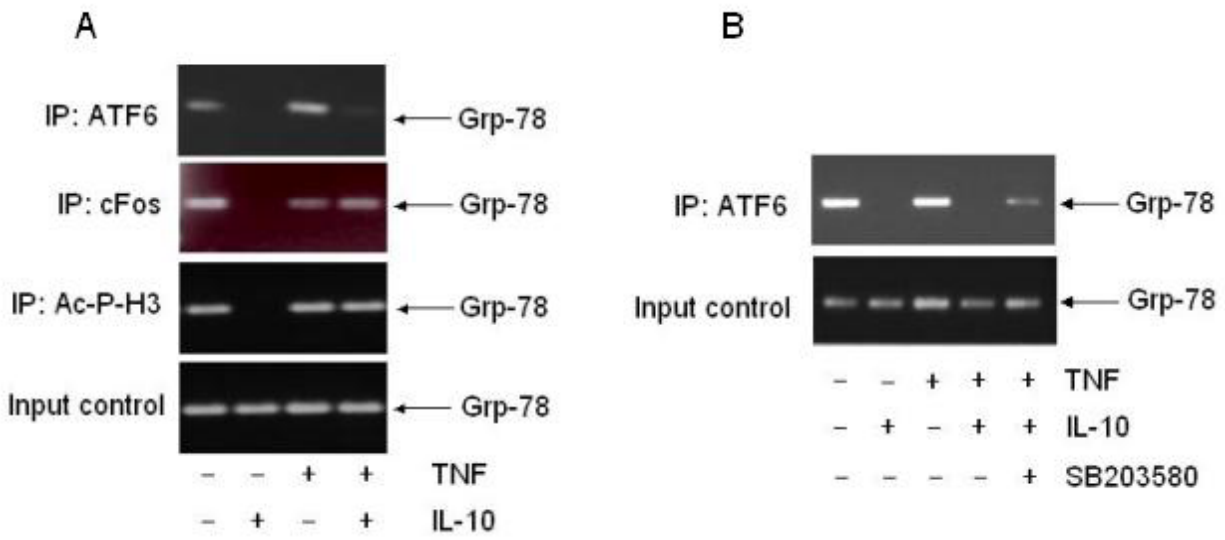

Figure 30. A and B. IL-10 mediated inhibition of ATF6α-binding to promoter of grp-78 gene.

A, Mode-K/IL-10R cells were pre-treated with IL-10 for 24 h (where indicated) and stimulated with TNF for 2 h. ATF6, c-fos and Ac-P-H3 DNA-binding to grp-78 gene promoter was measured using ChIP analysis. B, Mode-K/IL-10R cells were additionally pre-treated with SB203580, IL-10 and then stimulated with TNF. ATF6 binding to grp-78 promoter was measured using ChIP analysis. ChIP analysis was performed by P. Ruiz.

Immunostaining of ATF6 in Mode-K/IL-10R cells stimulated with TNF and IL-10 showed that TNF-stimulation led to the intracellular redistribution of ATF6α. In unstimulated cells ATF6 is bound to ER-membrane and could be detected in perinuclear space. Stimulation with TNF induced

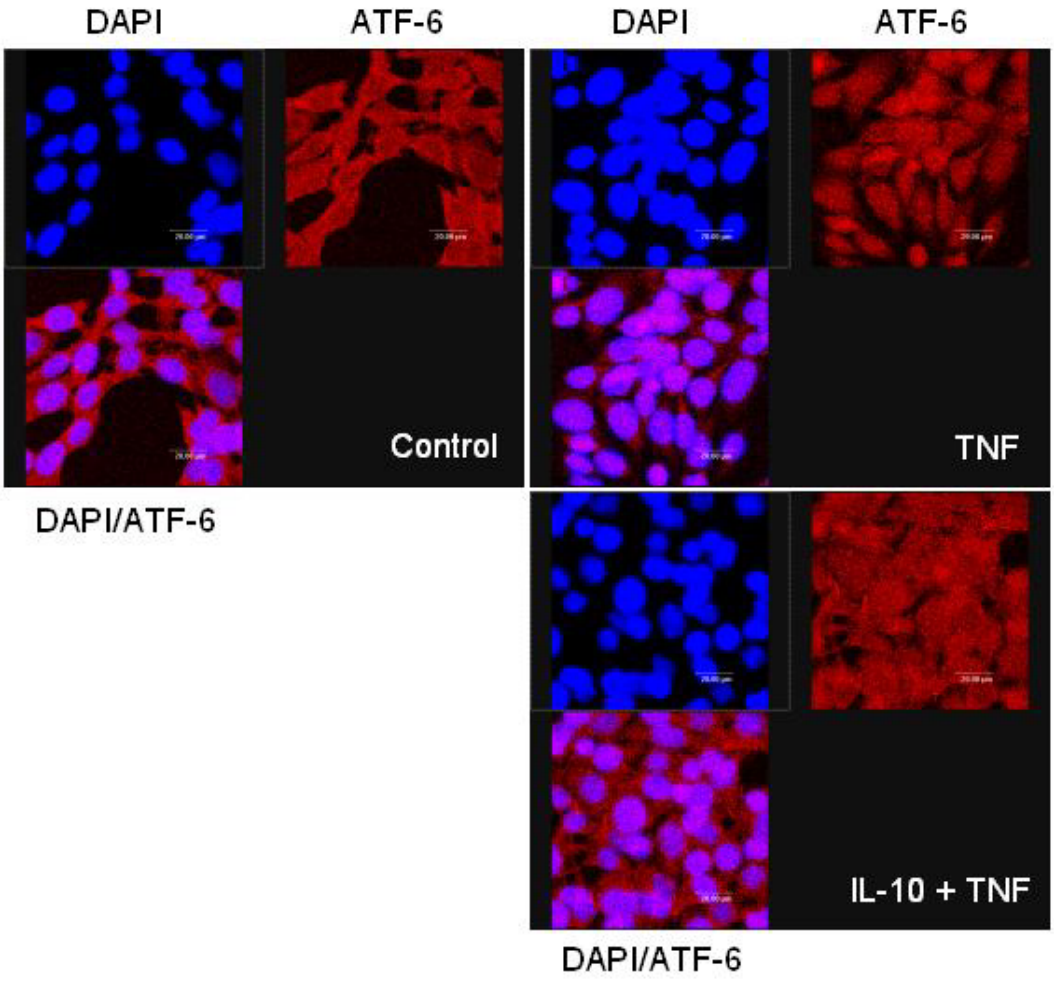

Figure 31. Immunostaining of ATF6 in Mode-K/IL-10R cells stimulated with IL-10 and TNF.

Mode-K cells were cultured on glass cover slips in 6-well plates. Confluent cells were stimulated with TNF for 30 min, where indicated the cells were pre-treated with IL-10 for 24 h prior to TNF-stimulation. The cells were fixed with methanol and incubated with primary rabbit anti-ATF6α antibody followed by incubation with anti-rabbit TRITC-conjugated IgG and DAPI. Stained cover slips were placed on microscopic slides and analyzed using inverted confocal microscope and Leica 2.5 software.

ATF6 activation and translocation to the nucleus. Pre-treatment of the cells with IL-10 for 24 hours prevented nuclear shuttling of ATF6 after TNF-stimulation and led to the accumulation of ATF6 in the cytoplasm (Figure 31). Thus, it was demonstrated that IL-10 inhibits ATF6 nuclear translocation and binding to the grp-78 promoter sequences. This inhibition is mediated by p-38 MAPK-dependent mechanism and could be prevented by specific p-38 MAPK inhibitor. Transcriptional regulation of grp-78 expression by IL-10 presents one of the novel protective mechanisms for cellular adaptation to ER-stress in intestinal epithelium.

4.4.2 Investigation of galectin-3 functions in IEC.

Galectin-3 is a multifunctional protein detected in the nucleus, cytoplasm and in extracellulal matrix of different tissues and cell types [195]. Since galectin-3 protein expression was down-regulated in IEC of bacteria-colonized IL-10$^{-/-}$ mice, the functional consequences of its down-regulation were investigated *in vitro* using small interference RNA (siRNA) technique. Mode-K cells were transfected with specific siRNA for galectin-3 and incubated for 0-72 hours. Significant reduction of galectin-3 was detected after 72 hours in comparison to control cells (no specific siRNA). Interestingly, that reduction of galectin-3 correlated with increased levels of cleaved caspase-3 (Figure 32A). To validate this finding we measured cleaved caspase-3 in IEC of WT and IL-10$^{-/-}$ mice after 14 weeks of colonization. Induction of cleaved caspase-3 was observed only in IEC of IL-10$^{-/-}$ but not WT mice after 14 weeks of colonization compared to germ-free conditions. It could be suggested that under conditions of chronic inflammation the increased rates of apoptosis could be observed in the intestinal epithelium. Massive apoptotic death of epithelial cells leads to the loss of protective barrier functions due to the disruption of adhesion/junction cellular structures. Indeed, the expression of E-cadherin, one of the important differentiation and adhesion/junction proteins in IEC, was significantly decreased in IEC of colonized IL-10$^{-/-}$ animals (Figure 32 B).

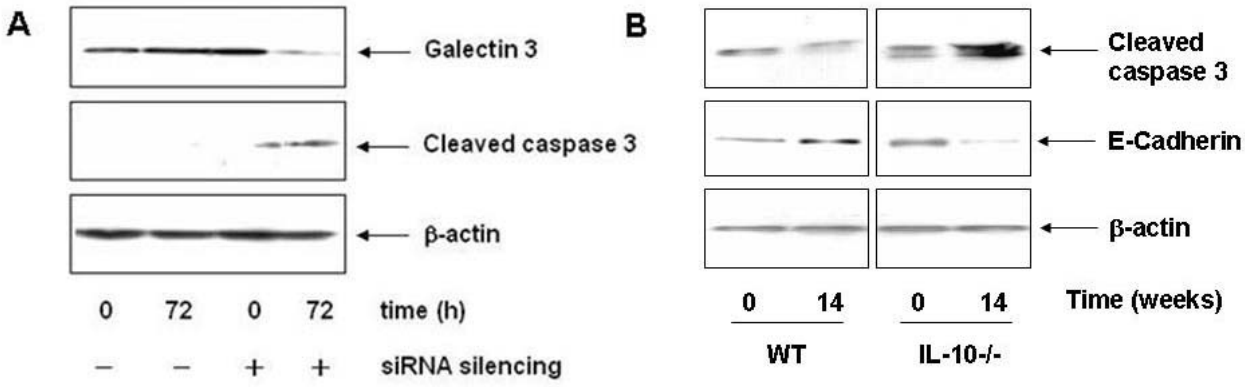

Figure 32. A and B. Gene targeting of galectin-3 and cleaved caspase-3 induction in Mode-K cell line and native IEC of WT and IL-10$^{-/-}$ mice.

A, Mode-K cells were transfected with control and galectin-3 specific siRNA for 0-72 h and 20 μg of total protein extract was subjected to SDS-PAGE followed by immunoblotting with specific antibodies to galectin-3, cleaved caspase-3 and actin. B, pooled samples containing 50 μg of protein lysate were generated using IEC of WT and IL-10$^{-/-}$ mice (germ-free and 14 weeks colonized) and subjected to Western Blot analysis using monoclonal antibodies to cleaved caspase-3, E-cadherin and β-actin.

4.4.3 TNF-mediated regulation of mitochondrial creatine kinase in Mode-K cell line.

Mitochondrial creatine kinase (mCK) is one of the central enzymes controlling cellular energy homeostasis. It participates in energy supply and protection of mitochondria from so-called permeability transition leading to necrosis or apoptosis. It is known that pathological conditions like oxidative stress, radical damage and calcium overload can alter mCK expression and activity. Since mCK is a prime target of oxidative and radical-induced damage, inactivation of this enzyme leads to the phosphocreatine-deficiency and to the perturbation of cellular pro-oxidant/anti-oxidant balance [194]. mCK was down-regulated in IEC of all IL-10$^{-/-}$ mice, but not in WT mice after 14 weeks of colonization with *E.faecalis.* In order to find out whether mCK expression is regulated by bacteria or by pro-inflammatory mediators, Mode-K cells were stimulated with *E.faecalis* and TNF for 0-24 hours. Interestingly, that bacterial stimulation of Mode-K did not affect mCK protein expression. However, TNF-stimulation of IEC led to the down-regulation of mCK after 12-24 hours (Figure 33). It could be suggested that under conditions of chronic intestinal inflammation in IL-10$^{-/-}$ mice the excessive secretion of pro-inflammatory cytokines influenced the expression levels and activity of mCK due to the high susceptibility of this enzyme to the oxidative stress. Perhaps, the down-regulation of mCK and affection of its activity could be also involved in the induction of apoptosis in native IEC of IL-10$^{-/-}$ mice due to the failure of mitochondrial homeostasis.

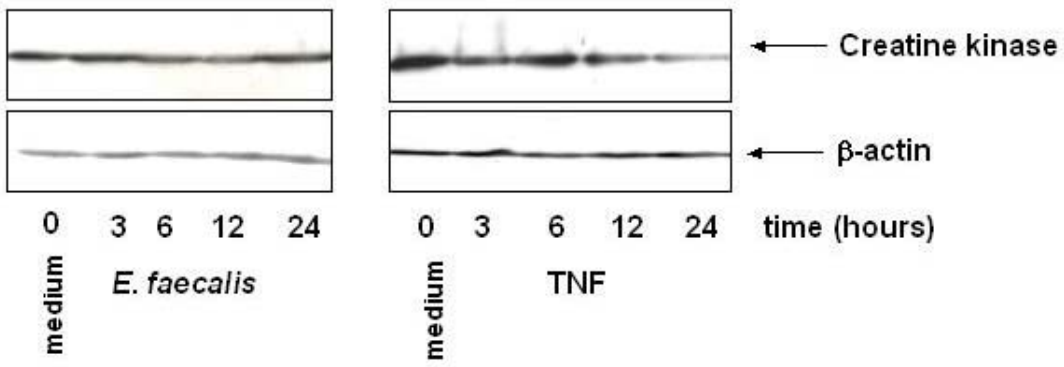

Figure 33. Differential regulation of creatine kinase by *E. faecalis* and TNF in Mode-K cell line.

Mode-K cells were stimulated with *E. faecalis* and TNF for 0-36 h. Cells were lysed in Laemmli buffer and 20 μg of total protein extract was subjected to SDS-PAGE and immunoblotting using specific antibody to mitochondrial creatine kinase. β-actin was used as a loading control.

4.5 Proteome analysis and intestinal epithelial cell profiling in IBD patients

4.5.1 Protein expression profiling in primary IEC from IBD patients and non-inflamed colorectal carcinoma patients

Comparative proteome analysis was performed using native purified IEC from the colon and ileum, taken from six patients with Crohn's disease (CD) and six patients with ulcerative colitis (UC). In all patients histological analysis revealed the presence of inflammation. Colonic epithelium with no histological signs of inflammation was obtained from six patients with colon carcinoma and used as a reference. The purity of isolated human IEC and the absence of T-cell contamination were assessed by immunoblotting using anti-CD3-antibody (Figure 34). In order to identify possible protein expression changes in patients with CD and UC in comparison to non-inflamed control samples, total protein extracts were separated by 2D-SDS-PAGE and obtained gels were subjected to proteome analysis and MALDI-TOF-MS. All gels were run and stained simultaneously to minimize qualitative differences through all 2D-SDS-PAGE procedures (Figure 35).

Protein spots that showed at least two-fold expression changes, consistently present in reference gels and in CD/UC gels were picked from all gels and subjected to MALDI-TOF-MS analysis. Totally, 25 proteins were identified in both groups of patients: 10 up-regulated and 7 down-regulated proteins in UC patients, 4 up- and 4 down-regulated proteins in CD patients respectively. All identified spots are listed in tables 15 and 16.

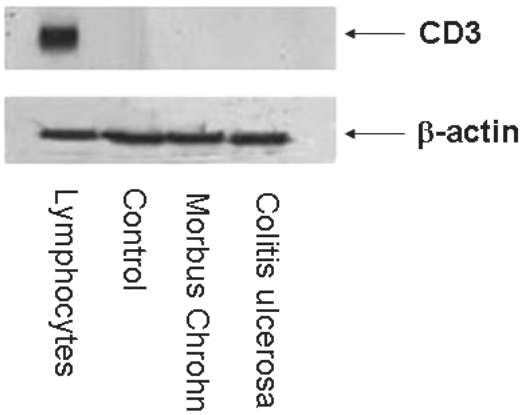

Figure 34. Purity control of human native IEC from IBD patients.

Native human IEC were isolated and purified, 50 µg of total protein lysate containing equally pooled aliquots from control, MC and UC group was subjected to SDS-PAGE and immonoblotting with CD3-antibody. Freshly isolated lymphocytes were used as a positive control.

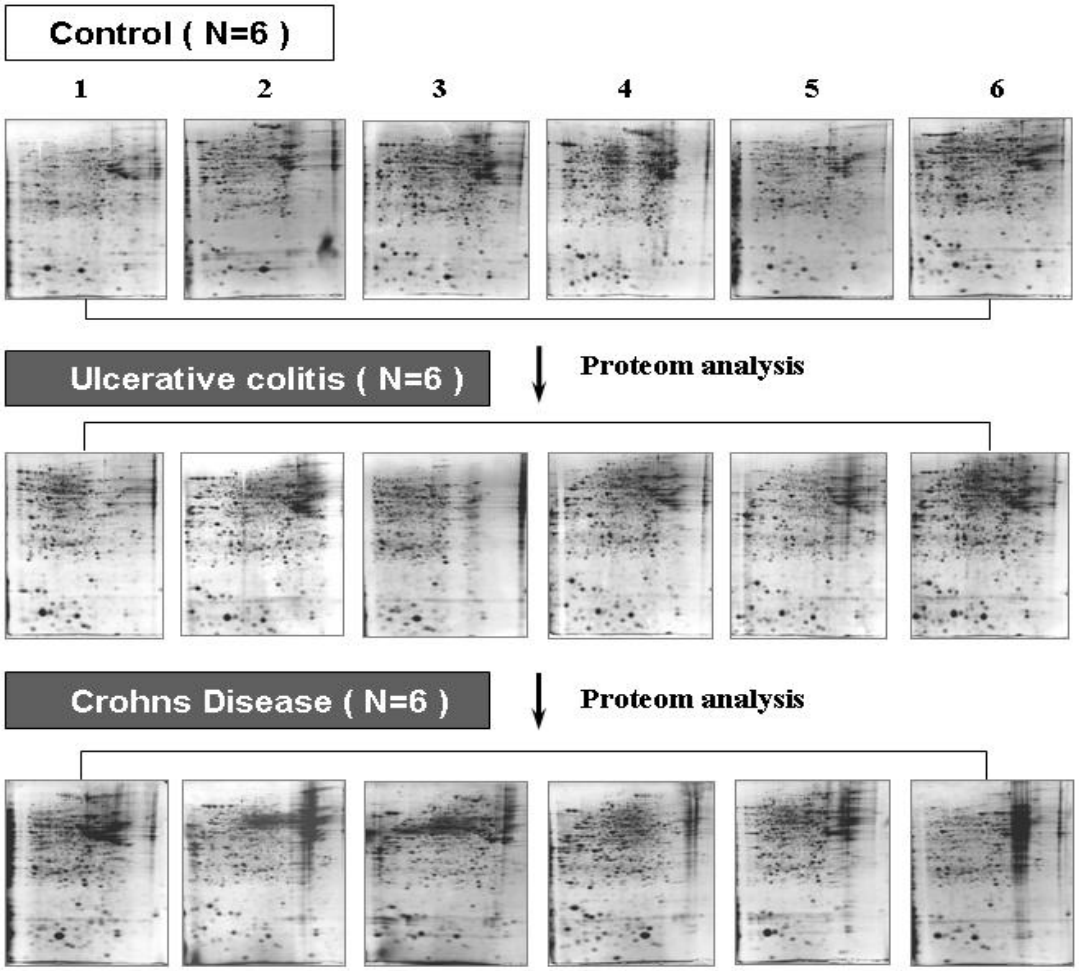

Figure 35. Proteome analysis in isolated IEC from patients with CD and UC: experimental design.

As proteome analysis showed, 3 proteins demonstrated similar dynamic changes in CD and UC in comparison to the reference group. Thus, Rho gdp dissociation inhibitor (GDIα) and L-lactate dehydrogenase were more than two-fold up-regulated, carbonyl reductase was down-regulated. Of note, RhoGDIα protein expression changes reached the criteria of statistical significance for both patient groups. In addition, four significantly regulated proteins were identified in CD patients: L-lactate dehydrogenase, villin 1, carbonyl reductase and 14-3-3 protein ε. Representative gels from each group of patients are shown in Figure 36A. Gel fragments with of RhoGDIα protein are separately shown for each patient with CD and UC. Western Blot analysis confirmed the increased expression of RhoGDIα in IEC from one of the patients with CD, UC and Sigmoid Diverticulits (SD) in comparison to one of the control patients. (Figure 36 B)

Table 15. Proteome changes in intestinal epithelial cells from patients with ulcerative colitis compared with surgical specimens from patients with colorectal cancer.

Spot Nr.	Access. Number	Protein	MW	pI	SC	RF	t-Test
1	BAA37169	IgG κ chain (fragment)	23690	6.92	51	2.07	0.1094
2	Q8WVXO_ HUMAN	Peroxisomale enoyl-coenzyme A hydratase-like protein	36078	8.47	24	2.09	0.1175
3	A55927	nicotinamide phosphor-ribosyltransferase	55772	6.69	47	2.13	0.1208
4	Q96AGO_ HUMAN	Staphylococcal nuclease domain containing 1	100294	6.52	22	2.12	0.1721
5	LEG-3_ HUMAN	Galectin-3	26098	8.61	34	2.66	0.0605
6	LDHA_ HUMAN	L-lactate dehydrogenase A chain	36819	8.46	53	2.04	0.0530
7	S65491	26S proteasome regulatory chain 12	36674	6.16	28	2.52	0.1431
8*	ACCOE	Rho gdp dissociation inhibitor (GDI) α chain ε	20571	6.73	40	2.20	0.0330
9	CRHU2	Carbonic Anhydrase II	29285	6.87	43	2.37	0.0920
10	MLRM_ HUMAN	Myosin regulatory light chain 2, nonsarcomeric	19707	4.67	47	2.33	0.0704
11	A48043	Ubiquinol-cytochrom c reductase core protein I	53270	5.94	26	0.45	0.2349
12	Q53G17_ HUMAN	NADH dehydrogenase (ubiquinone) Fe-S Protein 8	24232	5.53	36	0.43	0.144
13	DHCA_ HUMAN	Carbonyl Reductase (NADPH)	30510	8.55	47	0.49	0.1503
14#	KRHU9	Keratine 19, type I	44065	5.04	38	0.43	0.1163
15	AAA36425	Pancreatitis-associated protein	19654	8.16	40	0.25	0.2040
16	OWHU	Ornithine carbamoyl-transferase	40057	8.75	25	0.22	0.0701

17	1HJOA	heat shock 70kd protein 42kd ATPase n-terminal fragment	41973	6.69	37	0.34	0.2120

* Significantly regulated protein spots (p-values < 0.05)
# Spot location in the gel indicates differences to the hypothetical molecular mass

Table 16. Proteome changes in intestinal epithelial cells from patients with Crohn's disease compared with surgical speciments from patients with colorectal cancer.

Spot Nr.	Access. Number	Protein	MW	pI	SC	RF	t-Test
18	Q9BWC7_ HUMAN	Catechol O-methyl-transferase	20374	5.36	47	2.21	0.1156
19*	LDH_ HUMAN	L-lactate dehydrogenase A chain	36819	8.46	53	2.00	0.0385
20*	1CCOE	Rho gdp dissociation inhibitor (GDI) α chain E	20571	6.73	40	3.70	0.0041
21*	VILI_ HUMAN	Villin 1	92962	5.99	26	2.24	0.0106
22*	DHCA_ HUMAN	Carbonyl reductase	30510	8.55	47	0.47	0.0301
23*	Q4VJB6_ HUMAN	14-3-3 Protein ε isoform transcript variant 1	26658	4.76	39	0.39	0.0491
24#	KRHU9	Keratin 19 type I	44065	5.04	38	0.41	0.175
25	S55282	Isocitrate dehydrogenase (NAD) α-chain precursor	40022	6.47	22	0.36	0.0923

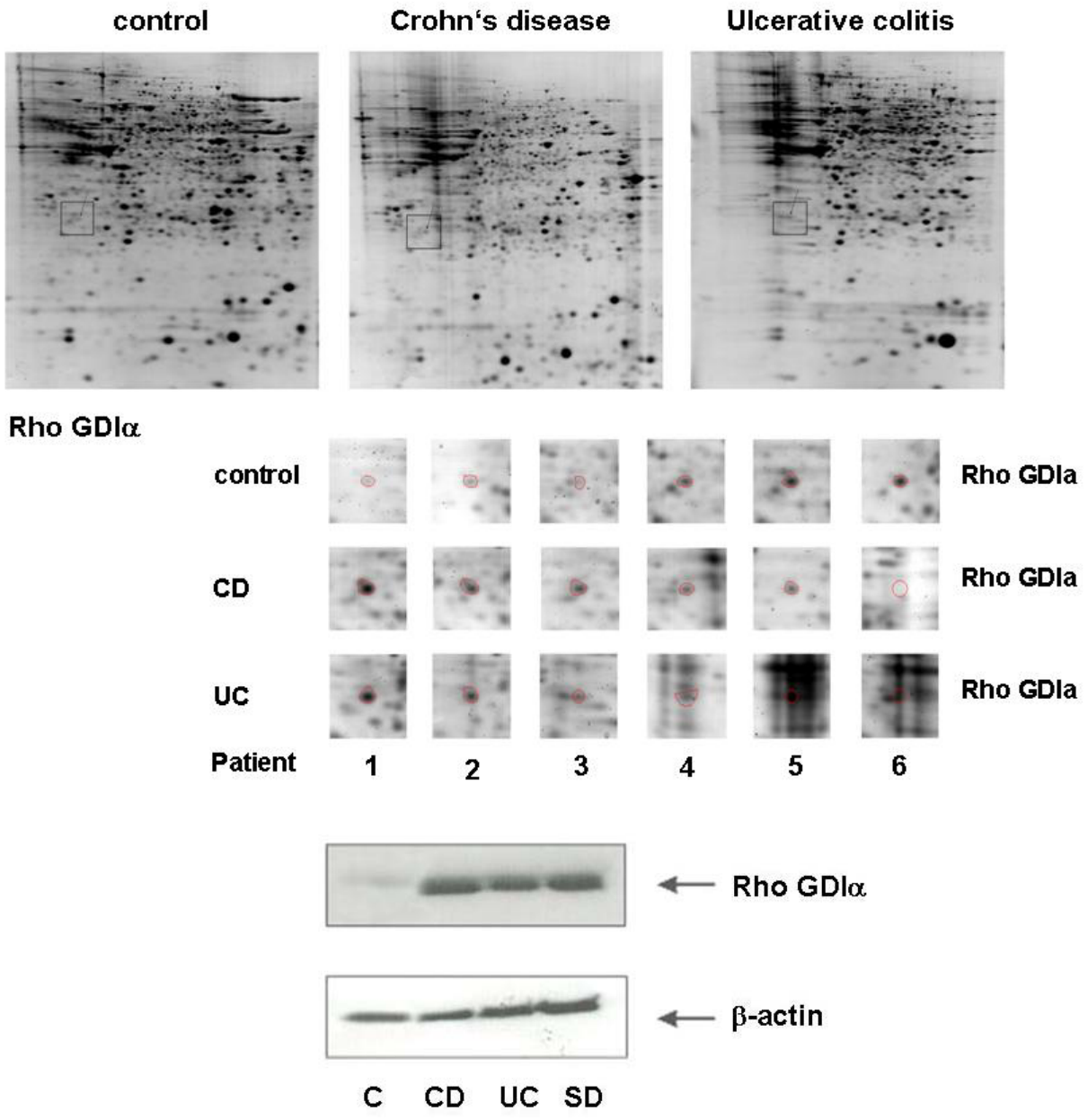

Figure 36. A and B. Differential expression of Rho GDIα protein in patients with CD and UC.

A, IEC were obtained from inflamed regions of ileum and colon of 6 patients with CD and 6 patients with UC respectively. Non-inflamed colonic epithelium was obtained from 6 patients with colon carcinoma. 2D-SDS-PAGE was performed, gels were stained with Coomassie and analyzed. Regulated spots were picked, tryptic digested and subjected to MALDI-TOF-MS. Representative 2D-gels from every group are shown; corresponding regions, depicting localization of Rho GDIα in analyzed gels are shown. B, verification of proteome results by Western Blot analysis: 50 μg of total protein obtained from one of the patients with CD, UC (column 1) and SD patient was subjected to SDS-PAGE and immunoblotting using specific antibodies to RhoGDIα and β-actin as a loading control.

4.5.2 Proteome analysis of human IEC from inflamed versus non-inflamed intestinal tissue of patients with Ulcerative Colitis and Crohn's Disease.

Initial proteome screening was performed in two UC patients and one patient with CD in order to identify possible dynamic changes in protein expression between IEC that were separately isolated from inflamed mucosal regions and non-inflamed regions of the same patient. This approach would give the possibility to identify and to compare inflammation markers in each individual of heterogeneous human population, avoiding the direct comparison to polymorphic "control" group. It is particularly important because of the absence of appropriate control non-inflamed mucosal tissue from healthy individuals. All 3 patients displayed relatively high variability in total number of regulated proteins. Specifically, 38 proteins (26 up- and 12 down-regulated) were significantly regulated in UC patient 1. Differentially regulated proteins identified in UC patient 1 are presented in Figure 37 and listed in Table 17. Interestingly, many proteins revealed relatively high regulation factors. For example, annexin 2 and programmed cell death inducing protein 8 (PDCD 8) displayed more than 7-fold induction in inflamed epithelial tissue (Figure 38A). For both proteins Western Blot analysis demonstrated strong induction in inflamed tissue in comparison to non-inflamed tissue of the same patient (Figure 38B). In addition, glutathione reductase, villin-2, proteasome activator PA28, NADH-ubiquinone oxidoreductase also revealed highly significant protein expression changes ranging between 3-4 fold. Proteome analysis performed in IEC from inflamed versus non-inflamed epithelium of the UC patient 2 allowed to identify only 3 significantly up-regulated proteins: splicing factor, serpinb1 and intestinal fatty acid binding protein. In a similar analysis using IEC of the patient with Crohn's disease 8 proteins (2 up- and 6 down-regulated) showed significant expression changes. Interestingly, selenium binding protein was down-regulated in CD patient, but up-regulated in inflamed tissue of the first UC patient. In contrast, the glycolitic enzyme fructose-bisphosphate-aldolase A was down-regulated in first patient with UC, but up-regulated in inflamed regions of CD patient. Similar proteins, found as regulated in the previous set of experiments, were also identified by proteome analysis within the same patients. Different chains of enzyme L-lactate dehydrogenase were detected as up-regulated in the analysis "inflamed versus non-inflamed" in UC patient 1 and in group "control-UC-CD". Two other similar proteins villin-2 and villin-1 were up-regulated in UC patient 1 and up-regulated in group of 6 CD patients respectively.

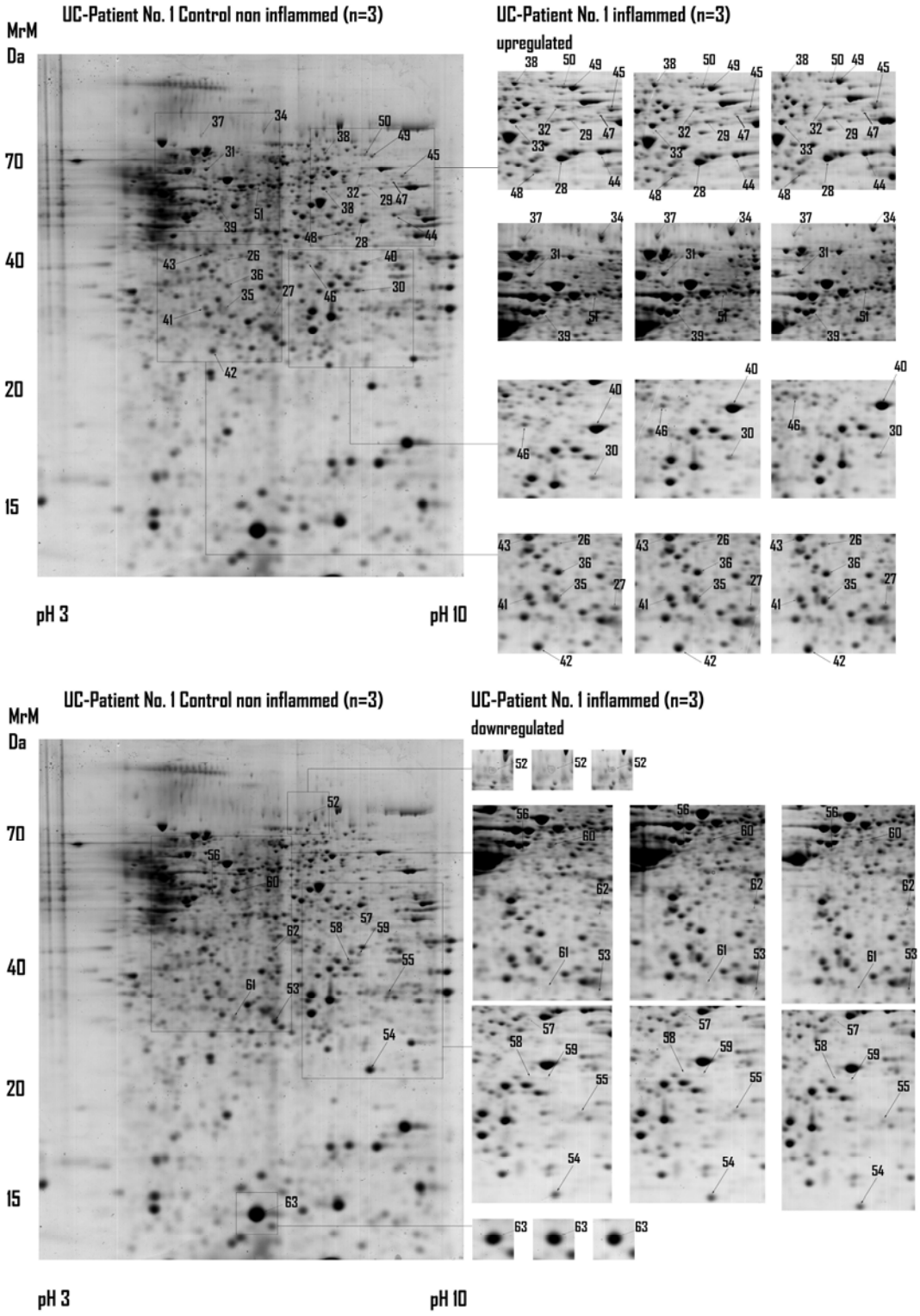

Figure 37. Coomassie stained 2-D gels with differentially regulated proteins in inflamed versus non-inflamed colonic epithelium of UC patient 1.

IEC were obtained from inflamed and inflammation-free regions of the colon of UC patient 1. 2D-SDS-PAGE was performed; gels were stained with Coomassie and analyzed. Regulated spots were picked, tryptic digested and subjected to MALDI-TOF-MS. Representative reference gel (big) and triplicates of gels from inflamed intestine region are shown.

Table 17. Proteome changes in intestinal epithelial cells from inflamed versus non-inflamed tissue regions in UC patient 1.

Spot Nr.	Access. Number	Protein	MW	pI	SC	RF	t-Test
26*	Q96GX7_ HUMAN	Selenium binding protein	52928	5.93	38	2.05	0.0191
27*	PGAM1_ HUMAN	Phosphoglycerate mutase 1	28769	6.75	64	3.41	0.0002
28*	A30789	Creatine kinase mitochondrial	47406	8.6	56	2.18	0.0123
29*	1BWCA	Glutathione reductase	50394	7.70	38	5.01	0.0001
30*	Q5J8M6_ HUMAN	Proliferating inducing gene 21	30237	8.02	57	2.41	0.0011
31*	Q9BU08_ HUMAN	Hypothetical protein (fragment)	59887	5.45	31	2.30	0.0114
32*	Q53HV2_ HUMAN	Chaperonin containing TCP1 subunit 7	59816	7.55	46	2.38	0.0277
33*	DEHUE	Glutamate dehydrogenase	61701	7.66	53	3.28	0.0010
34*	Q6NUR7_ HUMAN	Villin-2 (Ezrin)	69313	5.94	48	4.06	0.0001
35*	A54859	Proteasome activator PA 28	28876	5.78	65	5.75	0.0173
36*	ANXA4_ HUMAN	Annexin A4 (Lipocortin 4)	35957	5.85	73	2.81	0.0035
37*	NUAM_ HUMAN	NADH-ubiquinone oxidoreductase 75kDa subunit, mitochondrial precursor	80491	5.89	22	4.62	0.0093
38*	AAH01454	Phosphoenolpyruvat carboxykinase 2 mitochondrial	71452	7.57	48	4.87	0.0004
39*	AAH31559	BC031559 Keratin 20	48514	5.52	60	2.84	0.0022
40*	ANXA2_ HUMAN	Annexin A2 (Lipocortin 2)	38677	7.56	61	7.77	0.0091
41*	Q6PJ43_ HUMAN	Actin γ 1	29678	5.50	46	3.50	0.0203

42*	A37378	Glutathione transferase (GST–pi)	23569	5.43	63	2.31	0.0199
43*	Q96E67_ HUMAN	Actin β	40536	5.55	34	4.58	0.0219
44*	A32629	Ubiquinol-cytochrom c-reductase core protein II precursor	48611	8.74	35	2.92	0.0012
45*	CAP1_ HUMAN	Adenylyl cyclase-associated protein 1	51795	8.12	53	2.50	0.0002
46*	MDHC_ HUMAN	Malate dehydrogenase cytoplasmatic	36500	6.89	40	2.84	0.0001
47*	AAF04489	Methylmalonate semialdehyde dehydrogenase	58259	8.72	26	2.36	0.0004
48*	ADHX_ HUMAN	Alcohol dehydrogenase class III chi chain	40423	7.58	40	3.55	0.0012
49*	Q5JOZ7_ HUMAN	Programmed cell death 8 (Apoptosis inducing factor)	66539	9.03	30	7.49	0.0010
50*	A39018	Carnitine O-palmitoyltransferase II precursor	74244	8.38	44	3.58	0.0009
51*	LDHC_ HUMAN	L-lactate dehydrogenase C chain	36769	5.72	47	2.25	0.0017
52*	Q5U486_ HUMAN	KH-type splicing regulatory protein	73307	8.02	36	0.46	0.0475
53*#	A30789	Creatine kinase precursor, mitochondrial	47406	8.60	29	0.26	0.0040
54*	PEBP_ HUMAN	Raf-kinase inhibiting protein	21027	7.42	63	0.44	0.0170
55*	JC5893	Adenylate kinase 2B	25769	7.71	51	0.46	0.0119
56*#	Q597H_ HUMAN	Transformation-related protein 14	43248	5.49	47	0.41	0.0063
57*	Q53T69_ HUMAN	Trifunctional enzyme	59149	8.88	25	0.24	0.0015
58*	AAB60650	Heterogenous nuclear ribonucleoprotein A2	36041	8.67	38	0.25	0.0073
59*	ALDOA_ HUMAN	Fructose-bisphosphate-aldolase A	39720	8.37	31	0.42	0.0374
60*#	S24353	Proteasome 26S subunit MSS1	49002	5.71	52	0.25	0.0015

61*	AAH08250	Ethylmalonic encephalopathy 1	28368	6.35	55	0.47	0.0030
62*	AAH73991	Enolase 1 (α-Enolase)	37247	5.93	57	0.43	0.0206
63*	FZHUL	Fatty acid binding protein, intestinal	14256	6.60	80	0.27	0.0004

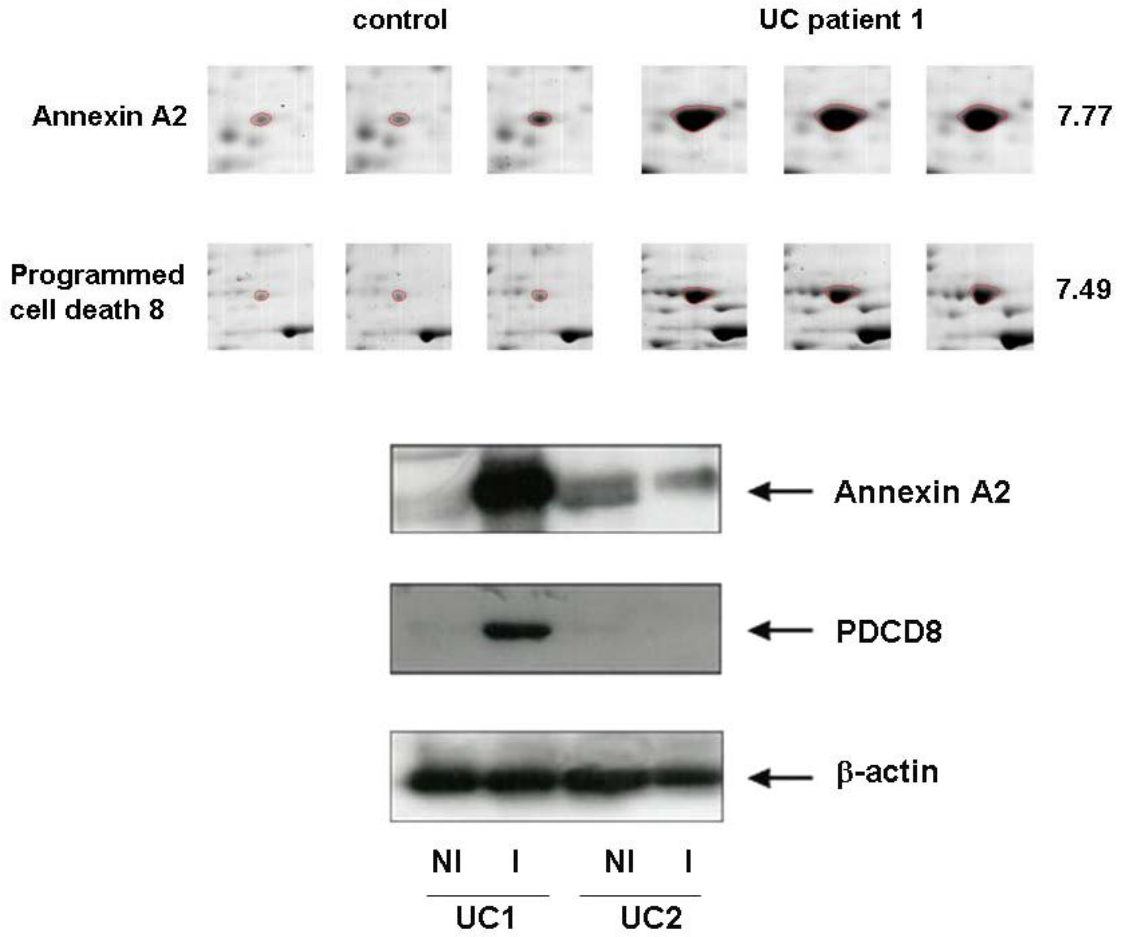

Figure 38. A and B. Induction of annexin-2 and PDCD 8 protein in inflamed versus non-inflamed regions of colonic intestine of UC patient 1.

A, 2D-SDS-PAGE gels were generated using IEC from inflamed and non-inflamed colon regions of UC patient 1. Triplicate gel fragments from non-inflamed and corresponding triplicates from inflamed epithelial tissue for annexin 2 and programmed cell death 8 protein are shown. B, verification of proteome analysis results by Western Blot analysis: total protein extracts (50 μg) were were subjected to SDS-PAGE and immunoblotting using specific antibodies to annexin-2, PDCD8, and β-actin as a loading control. NI-not inflamed tissue; I-inflamed tissue.

Table 18. Proteome changes in intestinal epithelial cells from inflamed versus non-inflamed colonic tissue regions in UC patient 2.

Spot Nr.	Access. Number	Protein	MW	pI	SC	RF	t-Test
64*	SFRS1_ HUMAN	Splicing factor	27711	10.37	45	2.36	0.0225
65*	S27383	Elastase Inhibitor (Serpin, clade B)	42829	5.90	36	2.22	0.0478
66*	FZHUL	Fatty acid binding protein, intestinal	14256	6.60	75	2.40	0.0363

Table 19. Proteome changes in intestinal epithelial cells from inflamed versus non-inflamed ileal tissue regions in CD patient 1.

Spot Nr.	Access. Number	Proteins	MrM	pI	SC	RF	t-Test
67*	CAA01217	Mature HSA	68425	5.67	70	4.80	0.0051
68*	ALDOA_ HUMAN	Fructose-biphosphate aldolase A	39720	8.39	33	4.80	0.0093
69*	Q96GX7_ HUMAN	Selenium binding protein	52928	5.93	79	0.36	0.0229
70*	S62767	Translation elongation factor (EF-Tu) precursor	49852	7.26	67	0.35	0.0134
71*	Q53RYI_ HUMAN	Aldolase 1 epimerase	37970	6.18	35	0.41	0.0154
72*	PPIA_ CERAE	Peptidyl-proplyl cis-trans isomerase A (cyclophilin A)	18098	7.82	60	0.48	0.0022
73*	1B56	Fatty acid binding protein, epidermal	15295	6.54	66	0.41	0.0300
74*#	CAF06488	AX961958 NID	30683	7.68	32	0.47	0.0103

4.5.3 Interpretation of proteome analysis in IBD patients using BiblioSphere Analysis Kit.

BiblioSphere Analysis Kit was developed to integrate literature and genomic network databases. The use of the Genomatix Software for analysis of protein expression changes allowed us to predict possible interactions between the genes and corresponding differentially regulated proteins.

All identified proteins were introduced to the software in form of corresponding gene IDs to generate a network, reflecting all possible functional inter-connections of the proteins on the base of literature co-citations in NCBI PubMed. In this analysis 62 members were accepted and clustered in 3 separated groups, depending on their co-citation levels (Figure 39). In the first cluster that was comprised of 38 tightly interrelated proteins, 19 proteins were involved in signal transduction mechanisms, 10 proteins in energy metabolism and other 11 proteins were associated with cytoskeletal or detoxification functions. Six linear co-cited proteins were included in the second cluster of the network. Additional 16 proteins revealed no known associations to each other and to the first two groups.

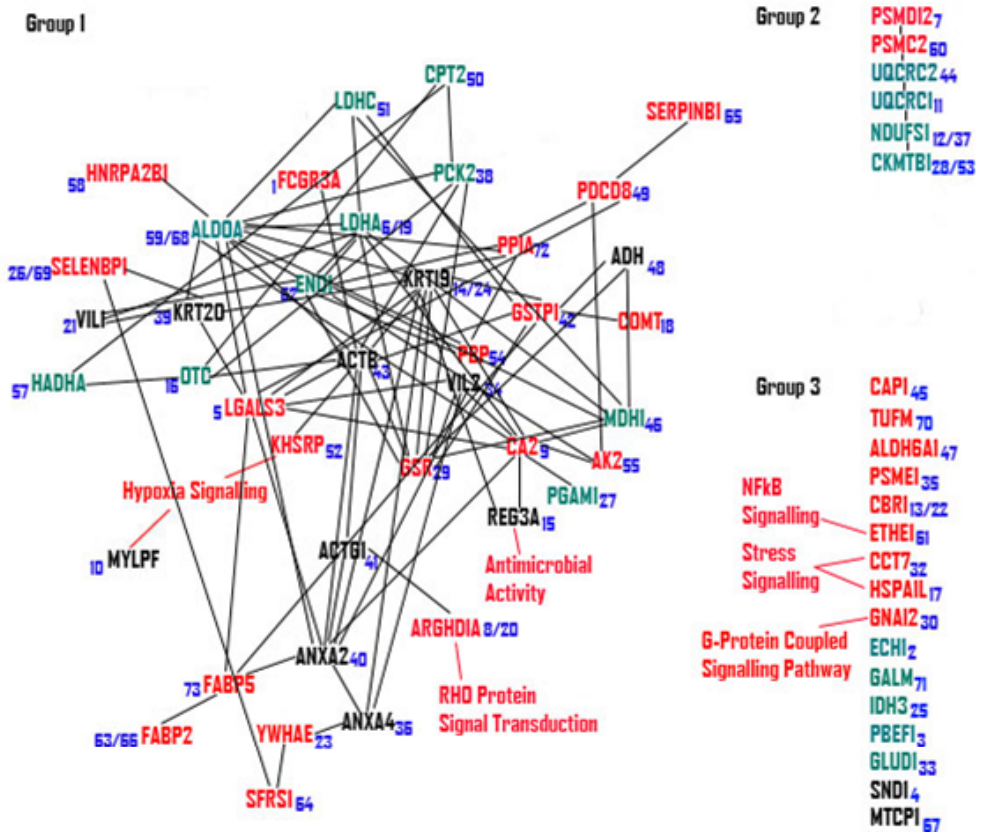

Figure 39. Computer analysis of protein-protein network in IBD patients using BiblioShere Analysis kit.

5 Discussion

5.1 Differential NF-κB and TGFβ/Smad signalling in IEC of WT and IL-10$^{-/-}$ mice colonized with *E. faecalis*

5.1.1 *E. faecalis* differentially triggers NF-κB activation and TLR2 expression in native IEC of WT and IL-10$^{-/-}$ mice

In this study we were able to show that TGFβ/Smad signalling is essential for the regulation of TLR2 protein expression in bacteria-activated intestinal epithelium. Induction of TGFβ/Smad signalling in native IEC at the early stages of colonization with *E. faecalis* presents one of the protective mechanisms to prevent pro-inflammatory response and intestinal inflammation. In IL-10$^{-/-}$ mice severe intestinal inflammation could be histologically detected after 14 weeks of bacterial monoassociation, and it was absent in germ-free animals. In contrast, WT animals did not develop colitis after 14 weeks of bacterial colonization. It is obvious, that IL-10 deficiency in mice is involved in pathogenesis of severe colitis [311]. Loss of epithelial cells homeostasis is an important early predictor of chronic intestinal inflammation [312]. Nevertheless, the molecular mechanisms of IL-10-mediated anti-inflammatory effects in native IEC are not enough investigated.

Colonization of germ-free WT and IL-10$^{-/-}$ mice with colitogenic *E. faecalis* differentially triggered signalling cascades in native IEC. NF-κB activation was observed already within the first week after the colonization. However, in comparison to the transient phosphorylation of RelA in IEC of WT mice, IL-10$^{-/-}$ mice revealed persistent NF-κB activation during the colonization time-course (3 days-14 weeks). As a consequence, the induction of NF-κB dependent pro-inflammatory cytokine IP-10 was persistently up-regulated in IEC of IL-10$^{-/-}$ mice. Of note, transient induction of IP-10 gene was also detected after 1 week of *E. faecalis*-monoassociation in IEC of WT mice. Nevertheless, this "physiologic" inflammation was controlled by host-derived immunosuppressive mechanisms. Increased NF-κB activity was previously observed in mucosal tissue in several animal models of intestinal inflammation and in IBD patients [202, 203]. Specific blockade of NF-κB activation with pharmacological inhibitors or antisense RelA oligonucleotides attenuated colitis severity in TNBS-treated mice [204]. Consistent with the fact that *E. faecalis* triggers NF-κB signalling via TLR2, we revealed differential TLR2 expression in IEC from WT and IL-10$^{-/-}$ mice. TLR2 was initially expressed by IEC of both germ-free groups of the mice. However, after 1 week of

colonization it was strongly down-regulated in IEC of WT mice and was constantly expressed in IEC of IL-10$^{-/-}$ mice. Down-regulation of TLR2 in IEC of WT mice correlated with the absence of RelA phosphorylation after 1 week of bacterial colonization. It seems reasonable to assume that the loss of TLR2 expression could be responsible for the termination of NF-κB signalling in these mice and appears to be as a protective mechanism to avoid excessive pro-inflammatory cytokine production. Although the modulation of the complex response to non-pathogenic bacteria in intestinal epithelial cell lines presents a simplified reductionistic model to study host-derived protective mechanisms, it helps to reproduce and characterize complex signalling networks in native IEC. In accordance with our results, stimulation of Mode-K cells with *E. faecalis* triggered NF-κB activation via TLR2 signalling cascade. Transient activation of NF-κB led to the transient induction of pro-inflammatory cytokines IP-10 and IL-6. The important question is how the host-derived mediators can regulate anti-inflammatory response in intestinal epithelium.

5.1.2 Host-derived mechanisms of TLR2 expression in IEC mediated by TGFβ/Smad2 signalling

TGFβ is one of the cytokines with pleiotropic properties involved in diverse cellular processes such as differentiation, proliferation, immunosuppression and inhibition of NF-κB-dependent pro-inflammatory response [205, 206]. In addition, the synergistic interaction between IL-10 and TGFβ was previously described [192, 207]. Investigation of TGFβ/Smad signalling in native IEC from WT and IL-10$^{-/-}$ mice showed that TGFβ-mediated phosphorylaton of Smad2 was transiently detected only in IEC of WT mice after 7 days of bacterial colonization, followed by the expression of the inhibitory Smad7 molecule. Despite the fact that inducible expression of TGFβ and TGFβRII mRNA were detected in IEC of both WT and IL-10$^{-/-}$ mice, Smad2 phosphorylation was absent in native IEC of IL-10$^{-/-}$ mice. Interestingly, TGFβ-mediated phosphorylation of Smad2 was observed *ex vivo* after the stimulation of primary IEC of IL-10$^{-/-}$ mice with exogenous TGFβ. It could be suggested that some intrinsic defects downstream of TGFβRII prevented the activation of TGFβ/smad2 cascade in IEC of IL-10$^{-/-}$ mice. Alternatively, the absence of functional TGFβ as well as bacteria strain-specific effects might also play a role in this phenomenon. We performed a series of experiments using cultured IEC in order to investigate the involvement of TGFβ-mediated signalling in the regulation of TLR2 expression and bacteria-induced NF-κB activation. It was shown, that addition of exogenous TGFβ down-regulated TLR2 protein expression in *E. faecalis*-stimulated Mode-K

cells. Of note, TGFβ alone was not able to decrease TLR2 expression. The inhibitory effect of TGFβ on TLR2 was Smad2-dependent, since overexpression of smad7 by adenoviral delivery abrogated TLR2 down-regulation in bacteria-stimulated Mode-K cells. Recent published results suggest an involvement of ubiquitination mechanism in the regulation of TLR2 [208]. By using proteasome inhibitor MG132 we showed that proteasomal degradation was not involved in TLR2 down-regulation. It was previously reported, that TGFβ down-regulated TLR2 expression in murine hepatocytes. Increased expression of TLR2 was observed after the treatment of hepatocytes with IL-1α. Pre-treatment with TGFβ strongly suppressed IL-1-mediated promoter activity of TLR2 and activation of NF-κB [209]. Similarly, in epidermal dendritic cells of the skin epithelium TLR2 levels were down-regulated after *in vitro* stimulation with different bacterial ligands, suggesting the low responsiveness of the host to the skin commensals [210]. In contrast, other reports described TGFβ-mediated Smad3/4 signalling pathway as a positive regulator of TLR2 induction in response to *Haemophilus influenzae* infection. This positive regulation of TLR2 was mediated by MAP kinase phosphatase 1 that inhibited p-38 MAPK, a known negative regulator for TLR2 induction [211, 212]. Perhaps due to the infectious nature of the pathogen, the host triggers defence mechanisms by cytokine and chemokine secretion, phagocytosis and chemoattraction. Nevertheless, it still remains unclear how the host distinguishes pathogen- and commensal-driven TLR2-mediated signalling. Investigation of TGFβ/Smad functions in cell culture allowed to simplify the sequence of signalling events in native IEC after the bacterial colonization. Initial contact of Gram-positive bacteria with IEC induces TLR2-mediated NF-κB activation. The expression of pro-inflammatory cytokines leads to the induction of protective immunosuppressive mechanisms *in vivo*. TGFβ/Smad2 signalling plays a pivotal role in the regulation of TLR2 expression. The reduction of TLR2 on the surface of IEC results in the termination of NF-κB activation and resolution of pro-inflammatory response in the absence of pathological process. This feed-back regulation explains the transient character of pro-inflammatory gene expression in the normal host. In contrast, in genetically susceptible host due to the failure of immunosuppressive mechanisms the colonization of the gut with commensal bacteria leads to the development of severe intestinal inflammation. This process is characterized by the massive cytokine overproduction, tissue damage and loss of epithelial integrity. Our data on differential cell signalling in IEC of WT and IL-$10^{-/-}$ mice support the hypothesis for interrelated synergistic functions of IL-10 and TGFβ.

5.2 Intestinal epithelial protein expression profiling at the conditions of chronic inflammation.

Signal transduction analysis in IEC of WT and IL-10$^{-/-}$ mice revealed similar NF-κB activation in the first week of colonization with *E. faecalis*, followed by the clear divergence at the later time points. Obviously, transient and persistent NF-κB activities could cause distinct effects on protein expression profiles in intestinal epithelium. Proteome analysis performed in IEC of WT and IL-10$^{-/-}$ mice was aimed to characterize protein expression changes at early (2 weeks) and late (14 weeks) colonization time-points. It could be expected that IEC of WT mice express more "protective" anti-inflammatory molecules than IL-10$^{-/-}$ mice. Indeed, proteome analysis performed after 2 weeks of *E. faecalis*-monoassociation demonstrated completely different up- and down-regulated proteins in IEC of WT and IL-10$^{-/-}$ mice in comparison to the equal germ-free conditions. Further, after 14 weeks, the differences in protein expression became more visible. Perhaps, excessive secretion of pro-inflammatory mediators in the absence of two interrelated immunosuppressive cytokines TGFβ and IL-10 induced strong differences in protein expression in IEC of IL-10$^{-/-}$ mice.
Differentially regulated proteins identified by proteome analysis in IEC of WT and IL-10$^{-/-}$ mice at early and late colonization time points will be selectively discussed below on the base of different experimental data present in the literature with respect to their possible functions under conditions of chronic inflammation

5.2.1 Differentially regulated proteins in IEC of WT and IL-10$^{-/-}$ mice after 14 days of monoassociation with *E. faecalis*

Wild type mice. Proteome analysis revealed 14 differentially regulated proteins in IEC of WT mice. Valosin-containing protein (VCP) was found to be down-regulated. It was previously shown that VCP could be co-precipitated within IκBα immune complexes [213]. The inactivation of the NF-κB inhibitor IκBα occurs through a series of ordered processes including phosphorylation, ubiquitin conjugation and proteasome-mediated degradation. VCP provides a physical and functional link between IκBα and the 26 S proteasome and plays an important role in the proteasome-mediated degradation of IκBα [214]. Similar evidence was provided by Asai et al, demonstrating that VCP influences NF-κB activation in VCP-transfected Dunn cells. TNF-stimulation of VCP-overexpressing cells triggered constant activation of NF-κB [215, 216]. In accordance with these

findings we showed that down-regulation of VCP correlated with inhibition of phospho-RelA in IEC of WT mice after 7 days of bacterial colonization. We also identified ferredoxin to be down-regulated in WT mice. Down-regulation of ferredoxin has probably protective anti-apoptotic effect, since up-regulation of this flavoprotein was reported to be involved in p53-induced apoptosis in several cell types [217]. Ferredoxin reductase transfers electrons from NADPH to cytochrome P450 via ferredoxin in mitochondria. Ferredoxin gene was found as a target for p53 family proteins that can be induced by DNA damage in apoptotic cells. Multiple cellular stresses, including production of reactive oxygen species (ROS) can activate p53 that induces the expression of ferredoxin and sensitizes cells to ROS-mediated apoptosis [218].

Interestingly, IEC of all WT mice revealed significant up-regulation (more than 5-fold) of oxoglutharate dehydrogenase. This enzyme is a key enzyme in the Krebs cycle and it is a crucial early target of oxidative stress, tightly connected with mitochondrial functions. Altered energy metabolism, including reduction in activities of the key mitochondrial enzyme of alpha-ketoglutarate dehydrogenase complex (KGDHC) is a characteristic for many neurodegenerative disorders [219]. Inhibition of this mitochondrial enzyme in PC12 cells led to the release of cytochrome c and caspase-3 activation [220, 221]. It was shown in other studies that inhibition of KGDHC led not only to the metabolic alterations and mitochondrial dysfunction promoting the cell death, but also affected Ca^{2+}-homeostasis between the cytosol and endoplasmatic reticulum [222, 223]. Probably, up-regulation of oxoglutarate-dehydrogenase in WT is one of the protective mechanisms of IEC to resist oxidative stress, caused by bacteria and cytokines. Detoxification proteins, such as thiosulfate sulfurtransferase (converts cyanides, sulphites, organic sulfinates, and dithiols to less toxic sulphur compounds) and glyoxalase I (glycation prevention) were also up-regulated in IEC of WT mice. Glycation of the proteins, nucleotides and basic phospholipids can be toxic for the cell, leading to the tissue damage. The enzymatic defence against glycation influences cellular activity and viability in different cell types. Decreased glyoxalase I activity during the aging process and oxidative stress results in increased glycation and tissue damage. Inhibition of glyoxalase I with specific pharmacological inhibitors leads to the accumulation of alpha-oxoaldehydes to cytotoxic levels [224, 225]. More than 3-fold reduction of platelet-activating factor acetylhydratase (PAF-AH) was observed in all WT mice after two weeks of colonization. Platelet-activating factor (PAF) is an important pro-inflammatory mediator of septic shock, produced by activated macrophages. It acts to perpetuate the response to endotoxin and metabolized by an endogenous PAF-AH. It was reported that PAF-AH significantly inhibits macrophage activation by lipopolysaccharide (LPS*) ex vivo*, decreasing

the expression of TNF, IL-1 and IL-6. This functional inhibition was associated with inhibition of ERK 1/2 kinase and NF-κB activation but not p-38 MAPK signalling [226]. The levels of circulating PAF-AH correlate with several disease syndromes, including alveolar inflammation, acute pancreatitis, and atherosclerosis [227, 228, 229]. The use of platelet-activating factor antagonists together with administration of epidermal growth factor are currently considered as effective therapeutic strategies for the treatment of necrotizing enterocolitis [230].

Reduction of argininosuccinate synthase (AS) in IEC of WT could correlate with resolution of pro-inflammatory response after 2 weeks, since induction of AS expression was previously reported in cytokine-stimulated cells (TNF and IFNγ) together with induction of nitric oxide synthase (NOS). NO is produced from arginine by NOS and citrullin as a by-product is recycled by AS in arginine [231]. In transgenic mice expressing human TNF increased levels of iNOS and AS were tightly associated with inflammatory polyarthritis and joint destruction [232]. Two important metabolic proteins, acetyl-CoA-acetyltransferase and 3-hydroxy-3-methylglutaryl-CoA lyase, were also found as down-regulated. These enzymes are localized in the mitochondrial matrix and catalyse the first and the last steps in the biosynthesis of ketone bodies respectively [233, 234]. Probably, down-regulation of both proteins could be a consequence of transient pro-inflammatory response in IEC of WT mice after bacterial colonization. Several other proteins, such as annexin 7, chloride channel ABP and elfin were down-regulated in IEC of WT mice, but their role under inflammatory conditions is not yet investigated. Thus, protein expression profiling in IEC of WT revealed differential regulation of proteins involved in maintenance of energy homeostasis in mitochondria, increased cell resistance to apoptosis and oxidative stress.

IL-10 deficient mice. IL-$10^{-/-}$ mice that were colonized for 2 weeks with *E.faecalis* revealed persistent activation of NF-κB resulting in protein expression changes different from WT mice. 9 up-regulated and 8 down-regulated proteins were identified at this time point. All mice demonstrated increased expression of glial-derived neurotrophic factor (GDNF). Up-regulation of GDNF has been already observed in human IBD and in the colonic tissue in experimental colitis of rats. In addition, GDNF induction has been studied in the colonic epithelial cell line HT-29. It was shown, that GDNF potently activates Akt and MAPK protecting the cells against apoptosis *in vitro* [235]. Interestingly, expression of zinc finger protein Pw1, up-regulated in IL-$10^{-/-}$ mice, could be interpreted divergently, as apoptosis-induced and NF-κB-activating mechanism. On the one side, Pw1 expression activates NF-κB via IκB dissociation and acts synergistically with TRAF2 in TNF response [236]. On the other side, several studies reported about direct involvement of Pw1 in p53-

mediated apoptosis. Mutant and anti-sense Peg3/Pw1 were able to prevent hypoxia-induced cell death [237, 238, 239]. Proteome analysis identified succinate dehydrogenase (SDHC) B-subunit to be up-regulated in IEC of IL-10$^{-/-}$ mice. Increased rates of apoptosis and oxidative stress were previously observed in a transgenic mouse cell line SDHC E69 with mutated SDHC gene. Apoptotic cell death was predicted by the increase in caspase-3 activity, decrease in mitochondrial transmembrane potential and structural changes in mitochondria [240]. SDHD knockout mice represented the first mammalian model lacking a protein of the electron transport chain and died at early embryonic stages [241]. It is known, that some pro-inflammatory cytokines induce production of ROS causing oxidative stress and tissue damage. Peroxiredoxins, proteins with strong anti-oxidative properties, protect the cells from overoxidation. Peroxiredoxin 5, the last discovered mammalian member, was detected in IL-10$^{-/-}$ mice as up-regulated after 2 weeks of colonization. Overexpression of peroxiredoxin 5 in either cytosolic or mitochondrial compartments significantly reduced cell death in various cell types. More effective protection was observed after the overexpression of peroxiredoxin 5 in mitochondria, the major target of peroxides [242, 243, 244]. Cytosolic peroxiredoxins are important regulators of TNF-signalling pathways mediated by MAPK (JNK, ERK, and p38) and SAPK [245]. Interestingly, after 2 weeks of bacterial colonization, IEC of IL-10-/- mice expressed simultaneously pro- and anti-apoptotic proteins. For example, voltage dependent anion channel 2 (VDAC2) revealed more than 3.5-fold induction in IL-10$^{-/-}$ mice in comparison to germ-free animals. In one report VDAC-2 was shown to act as a critical inhibitor of Bak-mediated apoptotic response [246]. In other studies this anion channel localized in the pore complex of the mitochondrial membrane, was associated with mitochondrial dysfunction, release of cytochrom C and apoptosis [247, 248]. Mitochondrial dysfunction could already start at this early stage of bacteria-epithelial contact in IL-10$^{-/-}$ mice even without any visible pathological signs. In addition, the expression of important metabolic enzymes such as pyruvate kinase, UDP-glucose dehydrogenase and mitochondrial hydroxylmethylglutaryl-CoA-synthase (HMGCoAS) was down-regulated after 2 weeks of colonization with *E.faecalis.* The expression levels of these enzymes were further reduced in IEC of IL-10$^{-/-}$ mice with colitis after 14 weeks. It seems possible that some hidden metabolic defects started during the first days of monoassociation of IL-10$^{-/-}$ mice with bacteria. Pyruvate kinase is a key glycolytic enzyme regulating cellular ADP/ATP traffic and synthesis of pyruvate. Together with the main ketogenesis enzyme HMGCoAS, pyruvate kinase provides energy-containing substances to the cell. Down-regulation of these important enzymes could have profound effects on metabolic processes in IEC. As a consequence, other cellular energy-consuming processes such as

selective permeability and transport could be affected in the intestinal epithelium. For example, down-regulation of the intestine-specific protein gastrotropin, involved in gastric secretion, transport and metabolisms of steroid hormones could also be explained by inhibition of metabolic processes and energy deficiency in the gut.

5.2.2 Differentially regulated proteins in IEC of WT and IL-10$^{-/-}$ mice after 14 weeks of monoassociation with *E. faecalis*

Intestinal epithelial protein expression profiling after 14 weeks of bacterial colonization revealed significant differences between WT and IL-10$^{-/-}$ mice. Only two up-regulated proteins were identified in IEC of WT. Totally 14 regulated proteins were identified in IEC of IL-10$^{-/-}$ mice. Several important proteins involved in energy metabolism, detoxification and stress-response were regulated in IEC of IL-10$^{-/-}$ mice in comparison to germ-free animals. Induction of molecular chaperone grp-78 indicated the presence of ER-stress in IEC. Regulation of ER-stress response in IEC under conditions of intestinal inflammation will be discussed later. Increased expression of tubulin beta can lead to the disruption of microtubules and inhibition of cell proliferation [249]. Propionyl-CoA-carboxylase is one of the enzymes catalyzing key reactions in gluconeogenesis, fatty acid metabolism and amino acid catabolism. This enzyme utilizes biotin as a co-factor and therefore plays an essential role in maintenance of metabolic homeostasis. Biotin deficiency is able to induce remodelling of chromatin by histones biotinylation and activation of NF-κB [250, 251]. Overexpression of 14-3-3 epsilon protein in IEC of IL-10$^{-/-}$ mice is a pro-apoptotic sign. Cleavage of 14-3-3 by caspase-3 facilitates interaction of Bcl-xL and Bad in mitochondria, promoting cell death [252]. In addition, 14-3-3-proteins are involved in Ras, Akt and TGFβ-mediated signalling [253, 254, 255]. Interestingly, the expression of detoxification enzymes 3-mercaptopyruvate sulfotransferase and aldehyde dehydrogenase (ALDH) was down-regulated in IEC of IL-10$^{-/-}$ mice. Accumulation of toxic metabolites during endobiotic and xenobiotic processing leads to the oxidative stress and cytotoxicity. Deficiency in ALDH gene function contributes the accumulation of excessive lipid peroxidation products, aldehydes, H_2O_2 and ROS. ALDH1A1 gene-targeting by specific antisense RNA was associated with increased susceptibility of the lens epithelial cells to oxidative damage and apoptosis [256, 257]. Oxidative stress-induced cytotoxicity in PC12 cells transfected with a mutant mitochondrial form of ALDH caused the excessive accumulation of toxic aldehyde derivatives [258]. In contrast, the overexpression of ALDH in transgenic system prevented acetaldehyde-induced cell injury and activation of stress signalling. These protective mechanisms were tightly associated with

ERK1/2 and p38 MAPK activation and could be abrogated by using specific MAPK inhibitors [259]. UDP-glucose dehydrogenase (UGDH) is a key enzyme of the unique pathway for the synthesis of UDP-glucouronate, the substrate for the numerous glucouronosyl transferases. Down-regulation of UDP-glucose dehydrogenase was observed in IL-10$^{-/-}$ mice after 14 days and after 14 weeks of *E.faecalis*-colonization. Other studies demonstrated that UGDH synthesis is modulated by the presence of TGFβ, pro-inflammatory cytokines and hypoxia-conditions. It was shown that IL-1α treatment reduced synthesis of chondroitin sulphate generated by UGDH. Oppositely, TGFβ was able to restore the normal activity of UGDH [260]. Another report showed that Specific protein-1 (Sp-1) could directly induce mRNA transcription of the UGDH gene by binding to its promoter. TGFβ specifically induced Sp-1 expression and promoter-binding, whereas hypoxia conditions inhibited these processes [261]. TGFβ/Smad signalling was absent in IEC of IL-10$^{-/-}$ mice during the early and late colonization stages in comparison to WT mice. It could be suggested that deficient TGFβ/Smad2 signalling together with high levels of pro-inflammatory cytokines led to UGDH down-regulation. Serine protease inhibitor b1 revealed almost 3-fold reduction in IEC from 4 of 5 IL-10$^{-/-}$ mice. Serpins are the family of proteins that inhibit proteases by specific irreversible mechanism. At the moment several serpin clades are described depending on their tissue distribution, secretion and cellular functions. Serpins of clade B (ov-serpins) function as intracellular mediators since they lack signal peptide necessary for secretion. The preliminary studies connected mammalian serpins with important cellular processes such as inflammation, apoptosis, microbial and viral infection, tumour invasion and hormone transport [262]. Due to the important biologic role of serpins, down-regulation of serpinb1 in IL-10$^{-/-}$ mice could be connected to the loss of protective mechanisms in IEC. Several cytoplasmic serpins have been shown to be anti-apoptotic molecules inhibiting proteolytic cascades of caspases activation. Serpins also protect the cells from their own proteases. For example, serpin-mediated inhibition of granzym B and cathepsin G was observed in granules of neutrophils, cytotoxic and NK-cells [263]. This mechanism allows degradation and digestion of bacteria by macrophages without proteolytic destruction of the host cell during degranulation. Interestingly, bacterial products and cytokines were shown to activate serpin gene-expression. In addition, NF-κB-binding sites and predicted STAT-binding sequences were found in the promoter structure of ov-serpis [264].

Mitochondrial creatine kinase. Mitochondrial creatine kinase (mCK) was down-regulated in IEC of all IL-10$^{-/-}$ mice after 14 weeks of *E.faecalis*-colonization. mCK presents a complex system for the generation of constant ATP levels in the cell. Under situations of compromised cellular energy

state, which are often linked to ischemia, oxidative stress and calcium overload, two characteristics of mitochondrial creatine kinase are particularly relevant: exquisite susceptibility to oxidative modifications and the compensatory regulation of its gene expression [194]. Reduction of mCK is implicated in mitochondrial dysfunction. mCK is a prime target for free radical damage during the oxidative stress. The inability to generate transmembrane potential and electron-transfer in respiratory chain, ATP depletion and release of cytochrom C are the typical symptoms of the apoptotic cell [265]. We were able to show that expression of mCK was not affected by bacteria-stimulation, but was negatively regulated by TNF in Mode-K cell line. These findings are consistent with similar observations of negative IFNγ-effect on mCK activity in cultured human myocytes. The cells were treated with various concentrations of recombinant human IFN-gamma and muscle cell proliferation, creatine kinase synthesis and muscle cell cytotoxicity were analyzed. Dose-dependent inhibitory effect of IFNγ on mCK synthesis was detected [266, 267]. An important role of creatine kinase was also demonstrated in transgenic mice, overexpressing mCK in the liver. TNF-mediated hepatic apoptosis in normally fed and creatin-fed CK transgenic mice was assessed. TNF-induced apoptosis was only inhibited in livers of CK-transgenic mice due to the maintenance of the mitochondrial functions [268]. The reduction of enzymes with respect to energy homeostasis indicates that IEC of IL-10$^{-/-}$ mice might not induce compensatory mechanisms in order to overcome energy deficiency and displayed the increased predisposition to apoptosis.

5.2.3 Galectin-3 is involved in anti-apoptotic protective functions in intestinal epithelium

Down-regulation of galectin-3 protein expression in IEC of IL-10$^{-/-}$ mice was detected after 14 weeks of bacterial administration. Galectins have been shown to play an important role in diverse biological processes, such as adhesion, proliferation, apoptosis, mRNA splicing, bacterial colonization and modulation of the immune response. Moreover, galectins play a key role in various pathological states, including autoimmune diseases, allergic reactions, inflammation, tumour spreading, atherosclerosis and diabetic complications [269, 270]. Targeting of galectin-3 by siRNA in Mode-K cell line demonstrated that gradual reduction of galectin-3 expression led to the induction of cleaved caspase-3, a major executive caspase of apoptosis. Down-regulation of galectin-3 in intestinal mucosa of colonized IL-10$^{-/-}$ mice correlated with increased cleaved caspase-3 levels in comparison to germ-free WT and IL-10$^{-/-}$ animals. It could be suggested that galectin-3 deficiency induces pro-apoptotic mechanisms in IEC under conditions of chronic inflammation. Reduction of E-cadherin expression indicates that barrier functions and integrity of the epithelium

are disturbed. The anti-apoptotic effect of intracellular galectin-3 was previously described in other cell systems [271, 272, 273]. Nevertheless, the molecular mechanisms of this protective anti-apoptotic effect are not enough investigated. There is a structural similarity between galectin-3 and inhibitor of apoptosis Bcl-2. Galectin-3 could replace Bcl-2 located on the outer mitochondrial membrane blocking the release of cytochrom C and preventing mitochondrial damage. Another explanation of anti-apoptotic functions of galectin-3 is the modulation of cyclins expression during the cell cycle [274]. In most reports galectin-3 appears to be an important molecule for cell survival, associated with beneficial effects for the host. Constitutive expression of galectin-3 was down-regulated in the intestinal epithelium of Crohn's disease patients [275]. In addition, galectin-3 gene therapy of bronchalveolar inflammation in rats inhibited IL-5 production by T-cells and eosinophils and normalized alveolar functions [276]. Interestingly, the addition of recombinant galectin-3 to activated human neutrophils led to p-38 phosphorylation. Disruption of this signalling pathway abrogated galectin-3-mediated modulation of neutrophil degranulation, phagocytosis and apoptosis [277]. Similar findings for the involvement of p-38 MAPK signalling in galectin-3 induction were observed by using specific p-38 MAPK inhibitor in activated macrophages [278]. We showed that phosphorylation of p-38 was present after 14 weeks of bacterial colonization in IEC of WT but not IL-$10^{-/-}$ mice. There is a single report describing cooperative interactions between galectin-3 and IL-10 in protection of breast carcinoma cells against ischemia-induced injury and oxidative stress. In this study the authors used co-cultures of carcinoma cells, overexpressing galectin-3 with liver fragments from wild type and IL-$10^{-/-}$ mice after liver ischemia. It was shown, that only galectin-3-overexpressing carcinoma cells remained viable after incubation with IL-$10^{+/+}$ liver fragments, but not with liver fragments of IL-$10^{-/-}$ phenotype. Investigation of this cooperative interaction between galectin-3 and IL-10 allowed hypothesizing, that IL-10 can suppress NO production by inhibition of iNOS and galectin-3 protects cells from NO-induced cytotoxicity [279]. The present functional study revealed anti-apoptotic role of galectin-3 in IEC. Down-regulation of galectin-3 in IEC promotes apoptosis-induced epithelium damage, leading to the disruption of mitochondrial homeostasis and to down-regulation of important metabolic pathways.

5.3 IL-10 mediated mechanisms for the regulation of ER-stress response in IEC under conditions of chronic intestinal inflammation

5.3.1 Molecular chaperone grp-78 and ER-stress

Induction of molecular chaperone grp-78 in native IEC from IL-$10^{-/-}$ mice after 14 weeks of monoassociation with *E. faecalis* indicated the presence of ER-stress in epithelium under conditions of chronic inflammation. Different physical, chemical and environmental factors induce ER-stress. Bacterial and viral products, glucose deprivation, hypoxia, oxidative stress and pro-inflammatory mediators were reported to be inducers of ER-chaperones and heat shock proteins [280, 281, 282]. ER-stress is often caused by the accumulation of misfolded or aberrant proteins that can not perform their normal functions in the absence of proper folding or post-translational modifications. These polypeptides will be either modified or removed by proteasomal degradation. Proteasomal degradation is one of the mechanisms of protein turnover during activation and induction of signalling cascades. Excessive accumulation of aberrant peptides leads to the formation of insoluble toxic complexes lethal for the cell. Grp-78 is an ATP-ase containing chaperone responsible for the stabilizing and folding of newly synthesized proteins. ER-chaperones and other related heat shock proteins work in multimeric complexes called “foldosomes”. They are structurally and functionally bound to kinases, membrane transporters and to energy-supplying machinery, since refolding of proteins always requires energy from ATP-hydrolysis. In addition, ATP-cleavage induces conformational changes of chaperone structure, allowing binding of target proteins [283]. Molecular chaperone grp-78 is reported to be broadly involved in multiple signalling cascades not only within ER, but also between different cellular compartments from complex Golgi to mitochondria and nucleus [284]. The network of main functional connections of grp-78 (Hspa5 gene) to other proteins, transcription factors and signalling pathways is depicted in Figure 40. This scheme is generated by singe Hspa5 gene analysis based on the literature co-citations integrated by BiblioSphere Software. Interestingly, grp-78 participates in distinct cellular mechanisms such as apoptosis, NF-κB activation, hypoxia, MAPK signalling and interaction with other related chaperones. Obviously, the biologic role of grp-78 depends on the cell types, their physiological status and ER-stress-inducing stimuli. However, the functions of grp-78 under conditions of chronic intestinal inflammation are not yet characterized. IEC of IL-$10^{-/-}$ mice revealed up-regulation of grp-78 after 14 weeks of bacterial colonization. Colonization of the gut with bacteria and bacterial products induces pro-

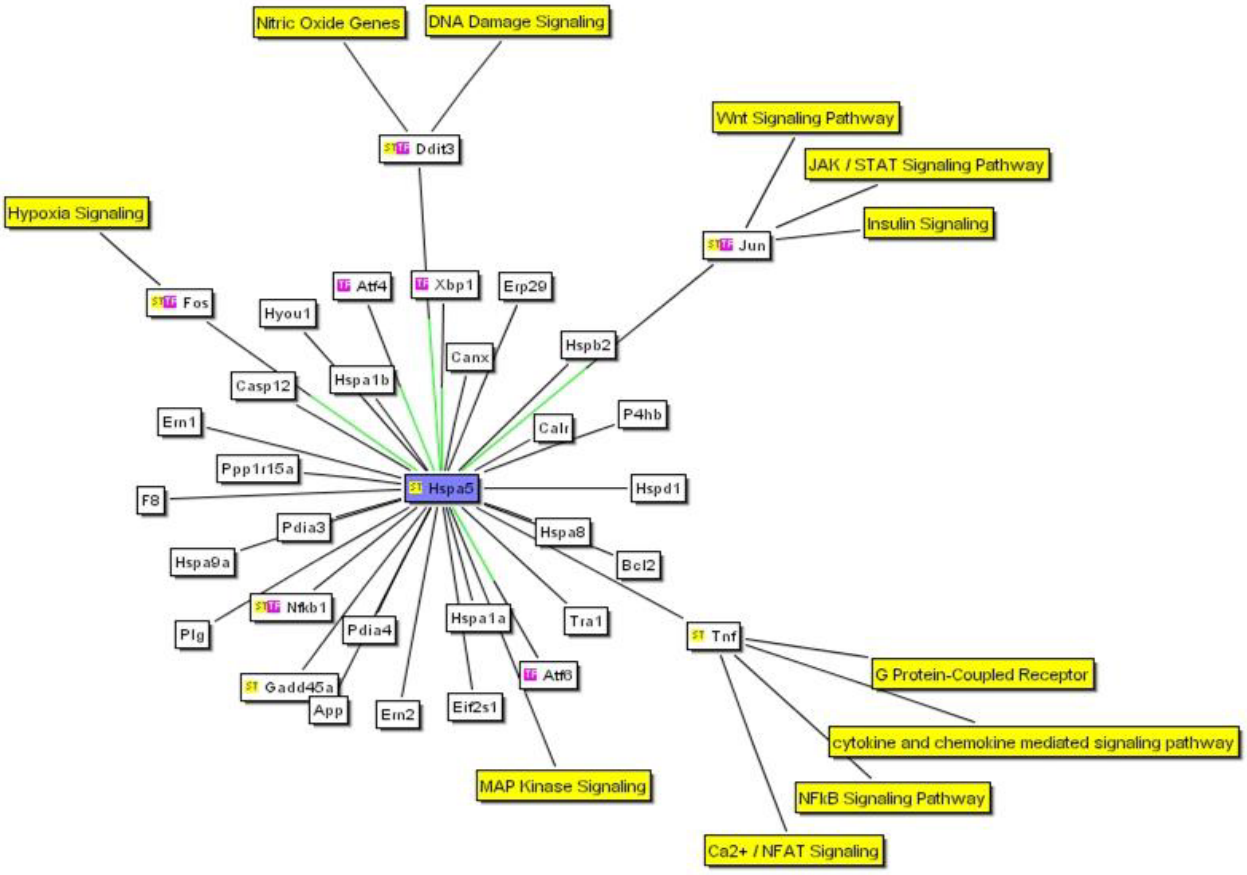

Figure 40. Important functional connections of molecular chaperone grp-78. Single gene analysis by BiblioSphere analysis kit.

inflammatory response and secretion of a broad cytokine spectrum. Cytokine overproduction can lead to the tissue damage and to the oxidative stress due to the production of ROS and NO. Pro-inflammatory and oxidative stress-response in the absence of inhibitory mechanisms induces ER-stress and increased expression of grp-78. Two distinct outcomes of ER-stress and unfolded stress response are described at the moment. The survival pathway is accompanied by increased expression of grp-78, correction of misfolded protein and degradation of unmodified peptides. Apoptotic death is induced due to the ER-overload and inhibition of proteasomal degradation and could be depicted as a maladaptation of the cell to ER-stress (Figure 41). Signal transduction studies and protein expression profiling showed that in IEC of IL-10$^{-/-}$ mice persistent NF-κB activation and pro-inflammatory cytokine secretion were associated with increased levels of cleaved caspase-3 and down-regulation of energy-generating enzymes. Increased expression of molecular chaperone grp-78 is simultaneously a ER-stress marker and an adaptive mechanism for cellular

survival. The question is whether grp-78 up-regulation in in IEC of IL-10$^{-/-}$ mice presents a consequence of inflammation or it is a compensatory mechanism to overcome ER-stress under conditions of chronic inflammation.

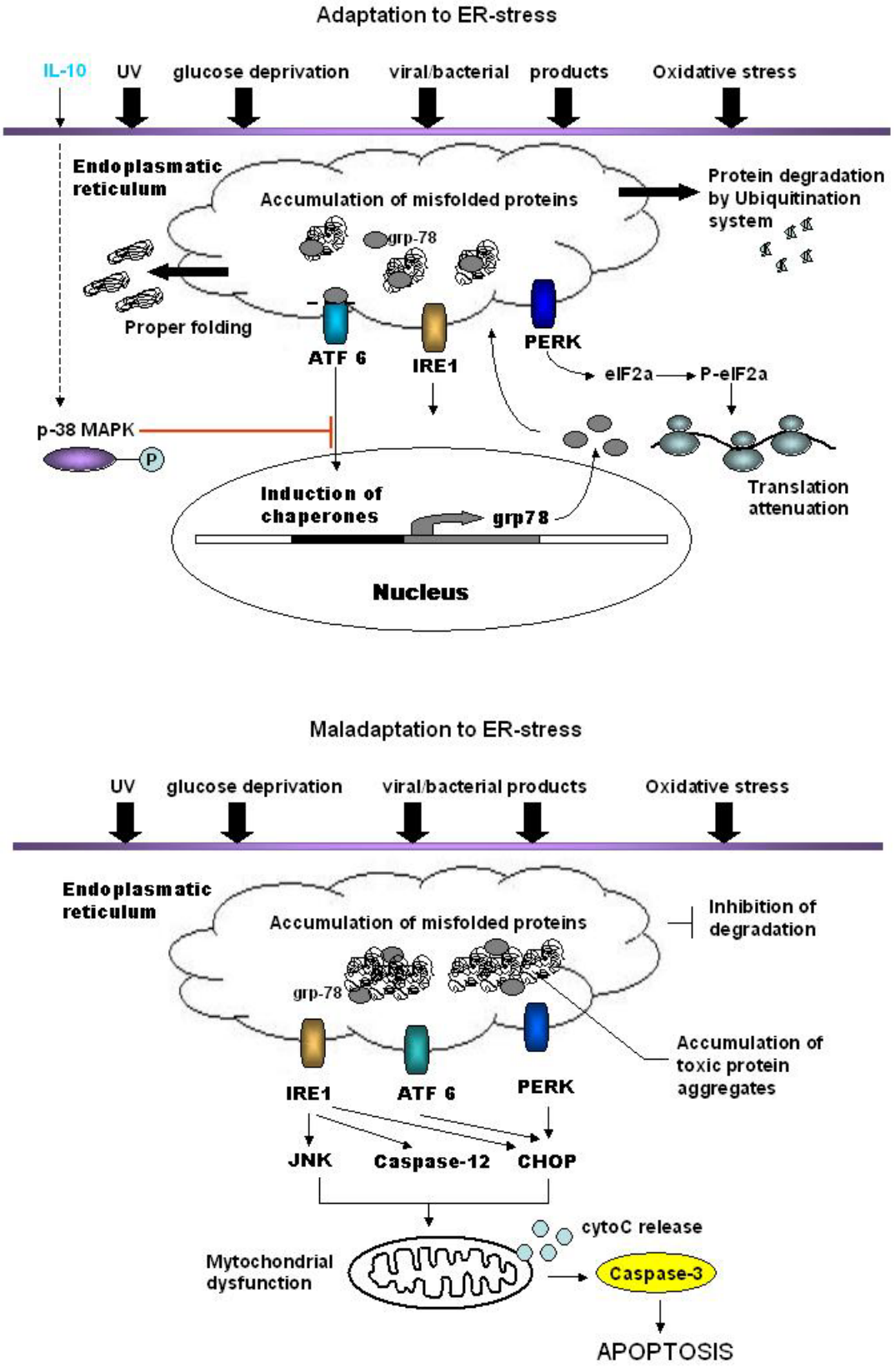

Figure 41. Mechanisms of cellular adaptation and maladaptation to ER-stress.

5.3.2 Molecular chaperone grp-78 is recruited to IKK complex and triggers RelA phosphorylation

The present work demonstrates an essential role of molecular chaperone grp-78 for NF-κB signalling. Our data showed that specific siRNA-mediated grp-78 gene silencing with subsequent TNF-stimulation of Mode-K cells led to the significant decrease in RelA phosphorylation in comparison to non-silencing conditions. We were able to show in immunoprecipitation experiments that grp-78 is recruited to the IKK complex shortly after the stimulation of Mode-K cells with TNF. TNF induced relocation of grp-78 from ER to the cytosoplasm for the association with the IKK complex. Immunostaining allowed to visualize grp-78 in the perinuclear space of unstimulated Mode-K cells. Stimulation with TNF induced the partial redistribution of grp-78 outside of ER-lumen. In addition, the presence of grp-78 in the cytosolic cellular fraction after TNF-stimulation was confirmed by fractionation analysis.

Simultaneous induction of grp-78 expression and NF-κB activation was already reported after challenging of the cells with various stimulators. It was shown that nicotine induced oxidative stress, induction of grp-78 and NF-κB activation in human gastrointestinal tract [285]. Nuclear translocation of NF-κB and activation of p-38 MAPK were observed during ER stress in MCF-7 cells [286]. In addition, NF-κB was reported to participate in a novel ER-nuclear signal transduction pathway distinct from the unfolded-protein-response [287]. Previous studies showed that grp-78/BiP is required for cell proliferation protecting the cell mass from apoptosis during early mouse embryonic development. Grp-78$^{-/-}$ embryos exhibited proliferation defects, massive increase in apoptosis and peri-implantation lethality [288]. Other studies suggested anti-apoptotic functions of several heat shock proteins by their involvement to the IKK complex. It was reported that binding of HSP90 to IKKγ and IKKα was absolutely essential for TNF-induced NF-κB activation. Inhibition of Hsp90 with geldanamycin enhanced TNF-induced cell death in HeLa cell line [196, 197]. Other data implicated Hsp70 and Hsp27 in suppression of TNF-induced inflammation by interaction with IKK subunits [289]. It was also shown that Hsp27 mediates cell survival by promoting proteasomal degradation of IκB and enhancing of NF-κB-activation [290]. It is still not completely clear how the binding of chaperones influences the activity of IKK complex. It is supposed that these proteins ensure stability and proper conformation of the IKK complex for further interaction with IkBα. Interestingly, inhibition of the heat shock proteins that were found within IKK complex with specific inhibitors led to the irreversible insolubility of IKK complex and NF-κB inhibition [294,

[295, 296]. Similar functions could be expected for grp-78 and ER-chaperones that transiently leave ER-lumen to interact with the IKK complex. We were able to show grp-78 relocation from ER to the cytoplasm upon TNF-stimulation of Mode-K cells. There is a limited number of reports demonstrating the redistribution of chaperones between cellular compartments under ER-stress conditions [291, 292, 293].

5.3.3 IL-10 regulates ER-stress response by inhibition of ATF-6 nuclear translocation to grp-78 promoter

A novel molecular mechanism was described for IL-10-mediated regulation of ER-stress in IEC under conditions of chronic intestinal inflammation. The investigation of signal transduction pathways that were differentially involved in the generation of early and late response after bacterial colonization in WT and IL-$10^{-/-}$ mice suggested p-38 MAPK signalling as one of the possible protective mechanisms in IEC of WT mice. Immunosuppressive properties of IL-10 mediated by p-38 MAPK and STAT-3 signalling mechanisms were previously described in regulatory and other immune system cells [297]. In addition IL-10 was shown to induce expression of heme oxygenase 1, one of the stress-response proteins with anti-inflammatory activity [288]. Our attempt to study IL-10-mediated effects in cultured IEC led to the generation of IL-10 receptor reconstituted Mode-K cells. Stimulation of these cells with rIL-10 revealed involvement of p-38 MAPK and STAT-3 signalling pathways in a time- and dose-dependent manner. In addition, we were able to show in Mode-K/IL-10R cells that IL-10 treatment significantly reduced expression of grp-78 at protein and mRNA levels, particularly after TNF-stimulation. Moreover, we found that IL-10 pre-treatment reduced recruitment of grp-78 to the IKK complex after TNF-stimulation. It has been previously shown that induction and expression of grp-78 gene can be regulated at the transcriptional level due to the complex organisation of its promoter. The promoter of grp-78 has several binding sites for transcription factors such as ATF-6, XBP-1 and TFII-I. The activated transcription factors interact with ERSE-elements of the promoter to induce grp-78 gene transcription [299, 300]. ATF6 is one of the transcription factors that translocates from ER to the nucleus after its specific proteolytic cleavage in Golgi complex [201]. Chromatin immunoprecipitation experiments suggested a novel mechanism for IL-10-mediated transcriptional regulation of grp-78 gene. We showed that IL-10 treatment inhibited binding of transcription factor ATF6α to grp-78 promoter. ATF6-binding to grp-78 promoter sequences was observed after the stimulation of the cells with TNF. Immunostaining and fluorescent microscopy allowed to visualize ATF6 in perinuclear space corresponding to ER. After

TNF-stimulation ATF6 was clearly detected in the nuclei of IEC. Pre-treatment of IEC with IL-10 completely prevented interaction of ATF6α with grp-78 promoter after TNF-stimulation. Immunostaining showed that IL-10 was able to block ATF6 shuttling between ER and nucleus resulting in diffuse accumulation of ATF6 in the cytoplasm. Specific p-38 MAPK inhibitor SB203580 was able to restore ATF6α-binding to grp-78 promoter after TNF-stimulation in IL-10 pre-treated Mode-K/IL-10R cells. Our findings were consistent with the previous studies that showed that induction of grp-78 mRNA requires the integrity of a signal transduction pathways mediated by p-38 mitogen-activated protein kinase [301, 302]. Interestingly, IL-10 specifically affected ATF6 binding, but did not affect histone acetylation and binding of another transcription factor c-fos in TNF-stimulated cells. Prolonged ER-stress in IEC of IL-$10^{-/-}$ mice is one of the consequences of bacteria-induced pro-inflammatory response in the absence of adequate immunoregulatory mechanisms. Pro-inflammatory cytokines including TNF were shown to induce ROS production [307]. ER is a primary target of toxic metabolites and oxygen radicals that affect protein synthesis and turnover. Cellular adaptation to ER-stress includes the induction of ER-chaperones, correction and proper folding of the proteins and destruction of misfolded peptides by proteasomal degradation. Maladaptation of the cell to ER-stress induces pro-apoptotic mechanisms due to the ER-overload and decrease in proteasomal degradation. It could be hypothesized that in WT mice colonized with *E.faecalis,* IEC escaped ER-stress due to the protective anti-inflammatory mechanisms mediated by IL-10. IL-10 was able to inhibit grp-78 transcriptional activity and to reduce its involvement to the IKK complex. In contrast, under pathological conditions in IEC of IL-$10^{-/-}$ mice ER-stress response could not be terminated because of several reasons. First, the excessive secretion of pro-inflammatory cytokines due to the persistent activation of NF-κB might cause an oxidative stress. As a compensatory mechanism grp-78 gene expression was induced. Nevertheless, ER-functions could not be restored in inflamed epithelium by up-regulation of grp-78. It seems possible that mitochondrial dysfunction and down-regulation of essential metabolic pathways led to the energy deficiency in IEC. Since ATP-derived energy is essential for the normal functions of ER-chaperones, the depletion of ATP contributes ER-stress response. As a consequence, ER-stress could cause cellular maladaptation and apoptotic death in IEC under conditions of energy deficiency. Investigation of grp-78 functions in IEC revealed novel properties of immunosuppressive cytokine IL-10. We were able to show that p-38 MAPK-mediated IL-10 signalling modulates ER-stress response by the regulation of transcriptional activity of grp-78 via ATF6-promoter binding.

5.4 Intestinal epithelial profiling in patients with Inflammatory Bowel Disease.

Intestinal epithelial profiling was performed in ileal and colonic tissue samples obtained from surgical resection of inflamed intestinal regions of patients with Crohn's disease and ulcerative colitis. Inflammation-free mucosal tissue was taken from patients with colon carcinoma and used as a reference. In the first set of experiments proteome analysis was performed in order to compare 2D-patterns from 6 CD patients and 6 UC patients and to reveal the proteins, differentially expressed in inflamed epithelium in comparison to non-inflamed mucosal tissue. Despite expected high variability and heterogeneity of human samples, as well as limited number of patients available, the initial proteome screening revealed several proteins significantly regulated within both groups of patients. From 25 identified proteins with at least 2-fold changes, one protein (RhoGDIα) was detected as significantly regulated for CD and UC. Only in group of CD patients villin-1, 14-3-3-ε protein, carbonyl reductase and L-lactate dehydrogenase A were detected as significantly regulated proteins. The latter two proteins were also detected in UC patients with more than 2-fold reduction and induction respectively, but they did not reach criteria of statistical significance.

Significant up-regulation of Rho GDIα protein was observed in group of patients with CD, UC and in patient with sigmoid diverticulitis, suggesting its important role in the modulation of inflammatory response. Rho-GDIα is a cytoplasmic protein that regulates the GDP/GTP exchange reaction of Rho proteins including Rac1 and Cdc42 by inhibiting dissociation of GDP and the subsequent binding of GTP. Initially, RhoGDIα was described as a polypeptide with pro-apoptotic and anti-proliferatory properties, involved in the regulation of cell cycle [303, 304]. Despite the initial negative functions attributed to RhoGDI, it is suggested that it may also act as a positive regulator necessary for the correct targeting and regulation of Rho activities. Disruption of the Rho GDIα gene in mice resulted in age-dependent degeneration of kidney functions leading to the renal failure and death with additional defects in male reproductive systems and in post-implantation embryos development [305]. Since members of the Rho family mainly regulate reorganization of the actin cytoskeleton, intracellular vesicle trafficking and cell motility, RhoGDI is mainly involved in transmembrane signalling cascades during actin filaments rearrangements [306]. The role of RhoGDIα in generating of pro- or anti-inflammatory response in the intestinal epithelium was not yet investigated. We may hypothesize that its induction is associated with pro-apoptotic functions followed by the destruction of the epithelial integrity and increased permeability. This idea could be

supported by the observation, that 14-3-3 ε protein was significantly up-regulated in CD patients. It was shown that 14-3-3 protein was overexpressed in apoptotic cells acting as one of the substrates for caspase-3 [252]. Of note, this protein was also up-regulated in IEC of IL-10$^{-/-}$ mice after 14 weeks of bacterial colonization.

In addition, 2D-gel patterns were generated using purified IEC that were separately obtained from inflamed and non-inflamed colonic regions of 2 UC patients, and corresponding ileal specimens from 1 CD patient. IEC of non-inflamed regions were used for the generation of the reference gels. Proteome analysis revealed 48 additionally regulated proteins (38 proteins for UC1 patient, 3 proteins for UC2 and 7 proteins for CD1). Most of these proteins displayed statistically significant changes within each analyzed group. Of course, the total number of regulated proteins varied between the patients due to heterogeneity of the human population, disease severity and genetical susceptibility. Certainly, more patients are required to get more objective information about inflammation markers for their classification according to criteria of relative risk in human population. Totally 62 differentially regulated proteins were taken for the generation of protein-protein network using BiblioSphere Analysis. Since gene and protein expression changes are the basic principles in the subsequent regulation of signalling cascades and metabolic pathways, integral analysis of these changes can potentially bring new information about the pathophysiological status of the IEC under inflammatory conditions. We classified all identified proteins in 3 clusters according to their co-citation levels. Most proteins were involved in signal transduction pathways (30 of 62 members), including hypoxia and stress response signalling (MYLPF, KHSRP, CCT7, and HSPAIL), NF-κB nuclear export signalling (ETHE1), surface-binding proteins (SELENBP1, FABP2 and YWHAE), red/ox signalling (GSR, GSTP1) and apoptosis (PDCD8).

In addition, 19 of 62 proteins within the network could be assigned to the proteins of energy metabolism and cellular homeostasis. Several key enzymes of glycolysis and Krebs cycle (LDHA, ENO1, MDH1, ALDOA and IDH3) as well as mitochondrial enzymes of respiration and electron transport were found. Recently, mitochondrial dysfunction and cellular oxidative stress are suggested to play an important role in the pathogenesis of inflammatory bowel disease [202]. Total ATP levels were decreased in the intestinal mucosal tissue of patients with ulcerative colitis [203]. The important functions of the intestinal epithelium such as absorption, epithelial transport of nutrients and selective permeability are highly energy-dependent processes. Destruction of epithelial integrity, increased gastrointestinal permeability and loss of barrier functions may occur in

conditions of ATP-depletion, stimulation of IEC with pro-inflammatory cytokines and disturbances of the normal gastrointestinal microflora. Up-regulation of L-Lactate dehydrogenase in CD and UC patients could suggest the induction of the compensatory cellular mechanism for anaerobic type of glycolisis for ATP-generation. Hypoxic conditions and oxidative stress that can be often observed in inflamed tissue lead to the mobilization of alternative pathways to restore energy homeostasis [202]. However, persistent inflammation can also induce cell damage and apoptotic death. Destruction of the epithelial structure and tight junctions is one of the first signs of increased apoptosis. Indeed, some of the regulated proteins identified in patients with UC and CD could be associated with mitochondrial dysfunction and oxidative stress. For example, the programmed cell death protein 8 (PDCD8) showed more than 7-fold induction in IEC from inflamed gut sections of UC patient 1 in comparison to non-inflamed colonic regions. PDCD8, also known as apoptosis-inducing factor (AIF) is localized in mitochondria in unstimulated cells. Following PARP-1 activation, AIF translocates to the nucleus triggering chromatin condensation, DNA fragmentation and nuclear shrinkage [308, 309]. AIF is supposed to be a major factor determining caspase-independent cell death, emphasizing the central role of mitochondria in the control of physiological and pathological state of the living cell.

Strong induction of annexin 2 was observed in inflamed versus non-inflamed mucosal tissue of the same UC patient. Annexins are Ca^{2+}-dependent phospholipid-binding proteins with anti-inflammatory properties present on the surfaces of secretory epithelium. It seems that annexin 2 induction is necessary for cell proliferation and prevention of apoptosis [310]. Overexpression of annexin 2 could appear as one of the protective mechanisms to protect epithelium from disruption due to apoptosis induction. Consistent with these finding IEC of bacteria-colonized IL-$10^{-/-}$ mice revealed persistent NF-κB activation and increased expression of pro-apoptotic cleaved caspase-3.

This pilot study is aimed to characterize protein expression changes in primary IEC of IBD patients. The preliminary results showed that despite the high heterogeneity of human samples the intestinal epithelial protein profiling can give the unique possibility for the direct investigation of molecular mechanisms of IBD ethiopathogenesis.

6 Summary

In this study we used IL-10 gene deficient mice monoassociated with Gram-positive *Enterococcus faecalis* in order to characterize bacteria-epithelial interactions and host-derived mechanisms involved in the regulation of chronic intestinal inflammation in genetically susceptible host. We showed that intestinal epithelial cells (IEC) of IL-$10^{-/-}$ mice lack protective TGFβ/Smad signalling and fail to inhibit expression of NF-κB-dependent genes of pro-inflammatory cytokines. In addition, the molecular mechanism for negative regulation of TLR2 expression by TGFβ/Smad signalling was described in IEC.

Protein expression profiling was performed in native IEC isolated from colonic tissue of wild type and IL-$10^{-/-}$ mice after 2 and 14 weeks of monoassociation with *E. faecalis*. Consistent with the presence of histopathology and persistent TLR2-mediated NF-κB activation in IEC of IL-$10^{-/-}$ mice, proteome analysis revealed divergent protein expression changes between WT and IL-$10^{-/-}$ mice at early and late colonization time points. Down-regulation of important metabolic, detoxification and immunregulatory proteins was associated with induction of endoplasmic reticulum stress (ER) in IEC of IL-$10^{-/-}$ mice under pathologic conditions.

We performed functional analysis of galectin-3 and molecular chaperone grp-78 regulated in IL-$10^{-/-}$ mice after 14 weeks of bacterial colonization. Using specific siRNA knock-down in intestinal epithelial cell line Mode-K we investigated functions of galectin-3 and molecular chaperone grp-78 in order to explain their contribution and physiologic relevance for disease development. We described a novel molecular mechanism of ER-stress response regulation mediated by IL-10 and grp-78. Increased expression of grp-78 was found in IEC of IL-$10^{-/-}$ mice after 14 weeks of bacterial colonization. Grp-78 gene targeting in Mode-K cell line showed that the absence of grp-78 modulated NF-κB signalling. We presented an evidence that grp-78 is recruited to the IKK complex upon TNF-stimulation. In addition, we found that IL-10 was able to down-regulate transcriptional activity of grp-78 via the inhibition of ATF6 nuclear translocation and binding to the grp-78 promoter. Pharmacological p-38 MAPK inhibitor blocked IL-10-mediated inhibition of ATF6 recruitment to grp-78 promoter in TNF-stimulated IL-10-receptor reconstituted Mode-K cells suggesting the involvement of p-38 MAPK signalling in regulation of ER-stress response. Specific gene targeting of another protein galectin-3 in Mode-K cell line resulted in the induction of cleaved caspase-3, suggesting anti-apoptotic functions of this protein in IEC. Consistent with this finding galectin-3 down-regulation in native IEC of IL-$10^{-/-}$mice correlated

with increased caspase-3 expression and down-regulation of E-cadherin. Thus, protein expression profiling in native IEC revealed involvement of ER-stress, energy depletion and disturbance of mitochondrial homeostasis in the pathogenesis of experimental colitis in IL-$10^{-/-}$ mice.

Finally, we performed a pilot study with a limited number of IBD patients to apply proteome analysis in human disease. We used native IEC isolated from inflamed mucosal tissue of patients with Crohn's disease (CD) and ulcerative colitis (UC). We identified Rho GDIα protein to be significantly up-regulated in inflamed mucosa of CD and UC patients in comparison to control non-inflamed samples from colon carcinoma patients. We also conducted proteome analysis in inflamed versus non-inflamed mucosal tissue separately obtained from CD and UC patients. This study is aimed to identify potential protein markers involved in etiopathogenesis of human IBD.

7 Zusammenfassung

In dieser Arbeit wurden Interleukin-10 (IL-10$^{-/-}$) gendefiziente Mäuse als Modell für chronische Entzündungen im Darm verwendet. Wildtyp und IL-10$^{-/-}$ Mäuse wurden mit dem colitogenen Gramm-positiven Bakterienstamm *Enterococcus faecalis* monoassoziiert, um so die Bakterien-Epithelzell-Interaktionen, sowie die vom Wirt abgeleiteten Mechanismen, die an der Regulierung der chronischen Darmentzündung im genetisch empfindlichen Wirt beteiligt sind, zu charakterisieren.

Zuerst wurde nachgewiesen, dass protektive Mechanismen der TGFβ/Smad Signaltransduktion in Darmepithelzellen von IL-10$^{-/-}$ Mäusen fehlen. Die Aktivierung der TGFβ/Smad-Signaltransduktion führte zum Abbau des Mustererkennungsrezeptors TLR2 in Darmepithelzellen und als Folge davon wurde die Expression von NF-κB-abhängigen pro-inflammatorischen Cytokinen im nativen Darmepithel der Wildtyp nicht aber der IL-10$^{-/-}$ Maus gehemmt.

Anschließend wurden Proteinexpressionprofile nativer Darmepithelzellen nach 2 und 14 Wochen Kolonisierung mit *E. faecalis* von Wildtyp und IL-10$^{-/-}$ Mäusen durchgeführt. Übereinstimmend mit dem Nachweis chronischer Entzündung im Darmgewebe und anhaltender TLR2-vermittelter NF-κB Aktivierung in Darmepithelzellen von IL-10$^{-/-}$Mäusen, zeigte die Proteomanalyse signifikante Unterschiede zwischen Wildtyp und IL-10$^{-/-}$ Mäusen. Zusätzlich zur Induktion von Endoplasmatischen Reticulum (ER) Stress wurden Proteine zur Regulation metabolischer, entgiftender und immunregulatorischer Prozesse identifiziert.

Galektin-3 und das Glukose-regulierte ER-Stress Protein 78 (grp-78) wurden molekular, durch die Verwendung spezifischer siRNA, auf ihre Funktion in Darmepithelzellen charakterisiert. Beide Proteine waren in IL-10$^{-/-}$ Mäusen nach 14 Wochen Bakterienkolonizierung im nativen Darmepithel reguliert.

Auf molekularer Ebene wurde ein neuer Mechanismus der Grp-78-abhängigen ER-Stress Regulierung durch IL-10 beschrieben. IL-10 hemmt die grp-78-Genexpression über die Blockierung des ATF6 Kerntransports und dessen Bindung an den grp-78 Promoter. Mittels spezifischer siRNA wurde nachgewiesen, dass IL-10 die TNF-induzierte Rekrutierung von grp-78 aus dem ER zum IκB Kinase Komplex (IKK) und die NF-κB Signaltransduktion in Darmepithelzelllinien hemmt. Der pharmakologische p38 MAPK Inhibitor verhindert die IL-10-

vermittelte Hemmung der ATF6 Bindung an den grp-78 Promoter. Übereinstimmend damit wurde phospho-p38 im nativen Darmepithel von Wildtyp, nicht aber von IL-$10^{-/-}$ Mäusen nachgewiesen. Darüber hinaus wurde mittels spezifischer siRNA-Inhibierung von Galektin-3 die Aktivierung der pro-apoptotischen Caspase-3 in Darmepithelzellen nachgewiesen. Die Aktivierung der Caspase-3 korrelierte mit der Reduktion von Galektin-3 im nativen Darmepithel von IL-$10^{-/-}$ Mäusen. Die Proteomanalyse zusammen mit der Charakterisierung molekularer Funktionen von grp-78 und Galektin-3 geben neue Hinweise darauf, dass ER-Stress, Energieerschöpfung und Störung von mitochondrialer Homöostase in nativen Darmepithelzellen an der Krankheitsentstehung der experimentellen Colitis in IL-$10^{-/-}$ Mäusen beteiligt sind.

Anschließend wurde die Proteomanalyse zur Untersuchung chronischer Entzündungsprozesse im Darm von Patienten mit Morbus Crohn (MC) und Colitis ulcerosa (CU) durchgeführt. In dieser Pilotstudie wurden native Darmepithelzellen aus entzündetem Darmgewebe von Patienten mit chronisch entzündlichen Darmerkrankungen (CED) isoliert. Dabei wurde das Protein Rho GDIα identifiziert, das in entzündetem Darmgewebe der MC und CU Patienten im Vergleich mit Kontrollproben von nicht-entzündeten Dickdarmkrebspatienten hochreguliert war. Zusätzlich wurde die Epithelzellproteomanalyse von entzündetem und nicht-entzündetem Schleimhautgewebe durchgeführt. Das Ziel dieser Studie war die Identifizierung von neuen Proteinen, die an der Pathogenese von chronisch entzündlichen Darmerkrankungen beteiligt sind.

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9 Supplements

9.1 List of Tables

9.2 List of Figures

Curriculum vitae

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Lebenslauf

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Berufliche Tätigkeiten

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Erklärung

Ich erkläre an Eides statt, dass ich die der Fakultät Wissenschaftzentrum Weihenstephan für Ernährung, Landnutzung und Umwelt der Technischen Universität München zur Promotionsprüfung vorgelegte Arbeit mit dem Titel:

Functional epithelial cell proteomics under conditions of chronic intestinal inflammation

im Fachgebiet für Experimentelle Ernährungsmedizin der Technischen Universität München unter der Anleitung und Betreuung durch Prof. Dr. Dirk Haller ohne sonstige Hilfe erstellt und bei der Abfassung nur die gemäß § 6 Abs. 5 angegebenen Hilfsmittel benutzt habe.
Ich habe die Dissertation in keinem anderen Prüfungsverfahren als Prüfungsleistung vorgelegt. Ich habe den angestrebten Doktorgrad noch nicht erworben und bin nicht in einem früheren Promotionsverfahren für den angestrebten Doktorgrad endgültig gescheitert.

Die Promotionsordnung der Technischen Universität München ist mir bekannt.

Anna Shkoda