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Molecular mechanisms for the inhibition of the nuclear factor κB signal transduction in intestinal epithelial cells under conditions of chronic inflammation

Pedro Antonio Ruiz-Castro

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I. Ruiz, PA, Kim, SC, Sartor, RB and Haller, D
"15-deoxy-delta12,14-prostaglandin J2-mediated ERK signaling inhibits gram-negative bacteria-induced RelA phosphorylation and interleukin-6 gene expression in intestinal epithelial cells through modulation of protein phosphatase 2A activity" **J. Biol. Chem.**, 2004, Aug 20; 279(34):36103-11

II. Ruiz, PA*, Shkoda, A*, Kim, SC, Sartor, RB and Haller, D
"IL-10 gene-deficient mice lack TGF-β/Smad signaling and fail to inhibit pro-inflammatory gene expression in intestinal epithelial cells after the colonization with colitogenic *Enterococcus faecalis*" **J. Immunol.**, 2005, 174: 2990-2999
*Authors contributed equally

III. Ruiz, PA, Hoffmann, M, Szcesny, S, Blaut, M and Haller, D
"Innate mechanisms for Bifidobacterium lactis to activate transient pro-inflammatory host responses in intestinal epithelial cells after the colonization of germfree rats" **Immunology**, 2005, 115, 441-450

IV. Ruiz, PA and Haller, D
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V. Shkoda, A*, Ruiz, PA*, Daniel, H, Kim, SC, Rogler, G, Sartor, RB and Haller, D
"Interleukin 10 blocked endoplasmic reticulum stress in intestinal epithelial cells: impact on chronic inflammation" **Gastroenterology**, 2007, 132(1): 190-207
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VI. Ruiz, PA, Braune, A, Hölzlwimmer, G, Quintanilla-Fend and Haller, D
"Quercetin inhibits TNF-induced NF-κB transcription factor recruitment to pro-inflammatory gene promoters in murine intestinal epithelial cells" Submitted to **J. Nutr.**

“What are the facts? Again and again and again—what are the facts? Shun wishful thinking, ignore divine revelation, forget what "the stars foretell," avoid opinion, care not what the neighbors think, never mind the unguessable "verdict of history"—what are the facts, and to how many decimal places? You pilot always into an unknown future; facts are your single clue. Get the facts!”

Robert A. Heinlein
(1907-1988)

TABLE OF CONTENTS

SUMMARY

Intestinal bowel disease (IBD) is an inflammatory disorder which results from an exacerbated mucosal immune response induced by the indigenous microbiota. The nuclear factor (NF)-κB is responsible for the expression of many genes involved in immunity and inflammation. In the present work, animal models as well as cell lines were used to characterize the molecular mechanisms for the downregulation of the NF-κB signalling pathway in intestinal epithelial cells (IECs), which play a crucial role in mucosal immune homeostasis. Non-pathogenic Gram-negative *Bacteroides vulgatus* was found to induce NF-κB activation in germ-free rats as well as in cultured IECs. Studies *in vitro* evidenced a mechanism by which the anti-inflammatory mediator 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) reversed Gram-negative bacteria-induced NF-κB phosphorylation through activation of protein phosphatase (PP)2A. Paradoxically, the probiotic bacterium *Bifidobacterium lactis* strain BB12 triggered a higher transient pro-inflammatory gene expression than *B. vulgatus* in the intestinal epithelium of germ-free rats. This response was concomitant with suppressive mechanisms, pointing to tolerance-inducing processes in the intestinal epithelium. Reconstitution of the IEC line Mode K with Toll-like receptor (TLR)4 and stable transfection of non-functional TLR2 helped us to identify the latter as the target for *B. lactis* BB12 activation of NF-κB. The same was found to be true for the colitogenic *Enterococcus faecalis*. Transforming growth factor (TGF)-β had the ability to abrogate *E. faecalis*-induced interleukin (IL)-6 expression by blocking NF-κB recruitment to its promoter as shown by chromatin immunoprecipitation (ChIP) analysis *in vitro*. Together with TGF-β, IL-10 plays a predominant role in the control of inflammation. Experiments with IL-10 receptor (IL10R)-reconstituted Mode K cells demonstrated the ability of IL-10 to inhibit tumor necrosis factor (TNF)-induced recruitment of activating transcription factor (ATF)-6 to the promoter of the glucose regulated protein (Grp)78, a crucial mediator of endoplasmatic reticulum (ER) stress response. An additional major focus in the present work was the characterization of the molecular anti-inflammatory effects of flavonoids. These plant-derived polyphenols presented a striking functional diversity in the inhibition of TNF-induced NF-κB signalling cascade. In addition, the flavonol quercetin inhibited pro-inflammatory gene expression in $TNF^{\Delta ARE}$ mice, an animal model for chronic ileitis, pointing to its possible therapeutic use in chronic inflammation.

PROLOGUE

There is a war going on. An unheeded war taking place on the surfaces of the intestinal mucosa from the very moment of our birth. Lined up on the one side we have the enteric bacteria, outnumbering by a factor of ten the cells of our whole body. They are faded on the other side by the mucosal immune system, always on the edge and in a constant pre-inflammatory status, ready to launch a devastating attack on the intruders. In the middle of this battlefield are the epithelial cells, sentinels taking active part in the decision of whether or not to wage war. Normally there are repressive mechanisms which curb the aggression, peacemakers abrogating the total war. This is fortunate, because not only is this a war we cannot hope to win, but also a war we do not want to win. However, some people either harbour bacteria which are too vicious, have an immune system which is too aggressive, or perhaps have peacemakers performing their duties insufficiently. This results in the break out of hostilities and the inevitable rise of war in periodic waves with dramatic consequences for the host.

Approximately 4 million people worldwide suffer from IBD, an intestinal inflammatory condition of unknown origin. Therapies which rely on anti-inflammatories and immunosuppressants are effective in inducing and maintaining remission, but side effects and lack of response in some patients limit their utility. The inflammatory response consists of the sequential release of mediators and the recruitment of circulating leukocytes, which become activated at the inflammatory site and in turn release more pro-inflammatory mediators. In most cases, the inflammatory response is resolved by the action of anti-inflammatory cytokines as well as the increased production of intracellular suppressive factors. Nevertheless, the persistent accumulation and activation of leukocytes are a hallmark of chronic inflammation, pointing to a dysfunction of negative regulatory mechanisms. An emerging line of research stresses the crucial contribution of IECs to intestinal homeostasis and inflammation due to their strategic location in the complex network of the mucosal immune system and their ability to respond to a wide range of stimuli. Rapid cellular response, which is critical for host defence, is coordinated by a regulatory network of signal transduction pathways leading to selective activation of transcription factors. These control the expression of a set of target genes, depending on the cell type and the nature of the stimuli. Molecular biology approaches leading to the understanding of cellular mechanisms involved in inflammation could potentially give rise to new targets for therapeutic intervention.

INTRODUCTION

1 Ecology of the gastrointestinal tract

The mucosal surfaces of the gastrointestinal (GI) tract are colonized by an extremely vast, complex and dynamic population of microorganisms. In fact, the number of microbes associated with the intestinal mucosa of an adult human is estimated to exceed the number of host cells by at least one order of magnitude: 10^{14} total bacteria compared to the 10^{13} total cells making up the human body (Savage 1977; Berg 1996).

The fetus develops in sterile conditions. At birth the exposed surfaces immediately get colonized by the aerobic and anaerobic bacteria present in the birth canal and the surrounding environment. Initially, bacteria with a high rate of multiplication dominate, but when space and nutrients become a limiting factor, more specialized bacteria colonize the intestinal mucosa and the complexity of the microbiota increases. The term “normal microbiota” (also known as “indigenous microbiota”, “normal microflora” or “indigenous microflora”) denotes the population of microorganisms that normally inhabit the GI tract, in opposition to the “transient microbiota” (Berg 1996). The indigenous bacteria are not randomly distributed, but are found at characteristic population levels in particular regions of the GI tract (Savage, Dubos et al. 1968) (Figure 1). The large intestine harbors the vast majority of our bacteria, comprising 400-500 species in the fully assembled microbial community with 30-40 species making up to 99% of the total population (Savage, Dubos et al. 1968; Moore and Holdeman 1974; Gordon, Hooper et al. 1997). Obligate anaerobes are 100- to 1000-fold more numerous than facultative anaerobes, which makes many species very difficult to culture and identify (Moore and Holdeman 1974).

1.1 Host-microbial interactions

The intestinal ecosystem is molded by interactions between its microbes, epithelium, mucosal immune system, microvasculature and enteric nervous system. Relationships between gut bacteria and their host can be sorted into symbiosis, commensalisms and pathogenicity. Symbiotic interactions are mutually benefitial for both partners, whereas pathogenic relationships benefic one partner at expenses of the other. Commensal relationships benefic one partner without necessarily being harmful to the other (Hooper, Midtvedt et al. 2002)

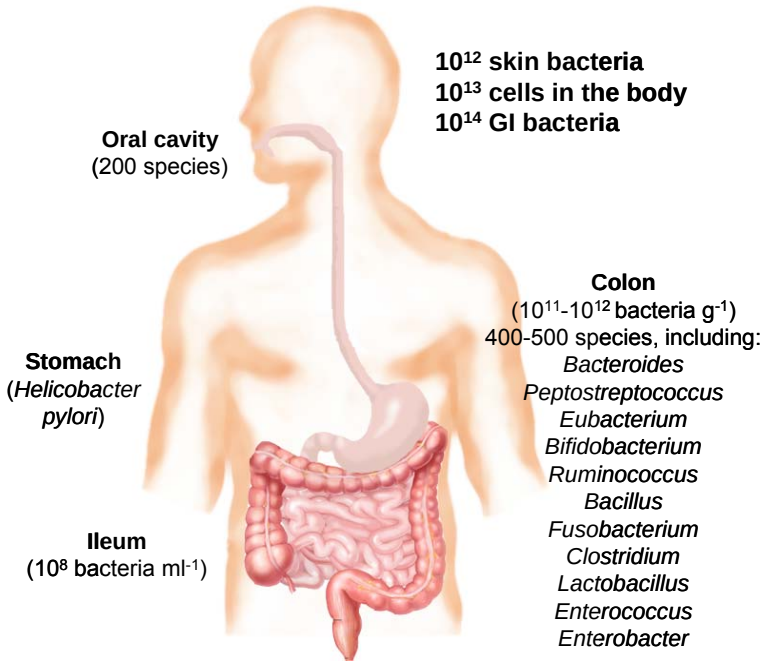

Figure 1: Distribution of the indigenous microbiota. Microbial densities in the proximal and middle small intestine are relatively low, but increase dramatically in the distal small intestine (approximately 108 bacteria ml-1 of luminal contents) and colon (1011-1012 bacteria g-1 intestinal contents) the last being the major reservoir of our bacteria (Savage 1977).

The true extent of biodiversity in the gut is difficult to define, not only because it is difficult to distinguish resident from transient species, but also because the major contributors to the microbiota have not yet been cultured ex vivo (Tannock, Crichton et al. 1987). The complexity of the normal microbiota makes it extremely difficult to determine the particular contribution of a species to a concrete biological process. Being able of controlling the population of bacteria in the gut became therefore a powerful tool to study individual contributions in the development of an organism.

The first report describing how animals could be reared under germ-free conditions appeared more than one century ago (Nuttal and Thierfelder 1895/1896). Over the years it became a scientific field of its own called Gnotobiology, from the Greek "γνῶσις" (gnosis), meaning knowledge, and "βιος" (bios), meaning life (Gustafsson 1948). Gnotobiology allow us to control the composition of the indigenous microbiota and functions, and can provide important information about the impact of a single bacterial species in the normal development and maintenance of the intestinal mucosa and its associated immune system.

Germ-free animals are delivered by cesarean section into sterile film isolators and maintained free of bacteria, viruses and other known life forms (MacDonald and Carter 1978). The introduction of microorganisms into germ-free animals is called association or colonization, implying that the microorganism will persist in the intestine without the need for periodic reintroduction. A gnotobiotic animal colonized with only one of the many spices of indigenous bacteria is also an artificial model. Nevertheless, comparisons between germ-free and conventional animals clearly show that the normal microbiota have a dramatic and profound impact on the morphological, biochemical, physiological and immunological development of the host. The most striking morphological singularity of germ-free rodents is a dramatic enlargement of the cecum, the segment of intestine that connects the distal small intestine with the proximal colon, which can be ten times larger than the conventional rodent cecum due to accumulation of undegraded mucus (Lindstedt, Lindstedt et al. 1965).

1.2 Assembling of the intestinal mucosa

Mammals have evolved together with their intestinal microbiota. This interaction results in a situation of mutualism or ‘microbial tolerance’ characterized by a homeostatic balance between the lack of responsiveness to the commensal gut microbiota and the necessary immune response to pathogens. Furthermore, in this permanent crosstalk established between the intestinal mucosa and the indigenous microbiota, the latter has a crucial role in the maturation of the gut mucosa and its humoral and cellular immune system.

The intestinal epithelium forms a tight barrier between the host and the luminal environment of the gut. In addition to its functions in digestion, nutrient transport, water and electrolyte exchange and hormone production, the intestinal epithelium must control the access of potential antigens and pathogens. Accessibility to the apical surface of the intestinal epithelium and rupture of the barrier are indeed the main properties that distinguish pathogenic from commensal microorganisms. The intestinal mucosa is constantly collecting bacteria and bacterial products. Intestinal epithelial cells (IECs) can process and present antigens to T cells present in the lamina propria, the meshwork of connective tissue underlying the gut epithelium. Dentritic cells (DCs), one type of professional antigens presenting cells (APCs), are also able to sample luminal antigen directly extending its dendrites through epithelial tight junctions without compromising the integrity of the epithelial barrier (Rescigno, Urbano et al. 2001). Nevertheless, the main

via of antigen sampling is a specialized type of cell of the mucosal epithelium called M cell. Instead of the characteristic brush border glycocalyx present in enterocytes, M cells have microfolds (hence “M cell”), which makes it easier for the luminal antigens to cross the epithelium and reach the APCs in the Peyer’s patches. Peyer’s patches are aggregations of B lymphoid follicles and interfollicular populations of T cells, and are primarily found in the distal ileum of the small intestine. Peyer’s patches constitute the inductive sites of the gut-associated lymphoid tissue (GALT) (Falk, Hooper et al. 1998) (Figure 2).

1.3 Gut-associated lymphoid tissue

The intestinal ecosystem has been shaped by the interaction between the microbiota, epithelium and GALT. Indeed, studies of germ-free and ex-germ-free mice have shown that the bacterial colonization during neonatal life affects the composition of the GALT. Bacterial-host interactions also maintain the physiologically normal state of inflammation or activation of the GALT throughout life (Cebra 1999).

The GALT is devoted to distinguish harmless antigens that are present in food or on commensal bacteria from pathogenic invasion of the mucosa. Antigens are collected in the inductive sites of the GALT where APCs initiate the maturation of T cells. T cells travel to the lymph nodes where they are differentiated into effector T cells and regulatory T cells. Effector T cells can be divided into T helper (Th)1 cells which produce interferon (IFN)γ, an important agent for cell-mediated immune responses and inflammation, and Th2 cells, which secrete interleukin (IL)-4, IL-5 and IL-13 and induce B-cell activation and differentiation. Th2 are also responsible for the B-cell differentiation into IgA-secreting plasma cells (van Ginkel, Wahl et al. 1999). On the other hand, regulatory T cells are devoted to the production of anti-inflammatory cytokines such as IL-10 and transforming growth factor (TGF)-β, the latter being central to two of the most distinctive functions of GALT: secretion of IgA and the generation of regulatory T cells. Just as important are macrophages, which remain in a quiescent state in the intestinal mucosa under normal circunstances and when activated, produce a wide array of pro-inflammatory cytokines, including tumor necrosis factor (TNF), IL-1, and IL-6 (Nagler-Anderson 2001).

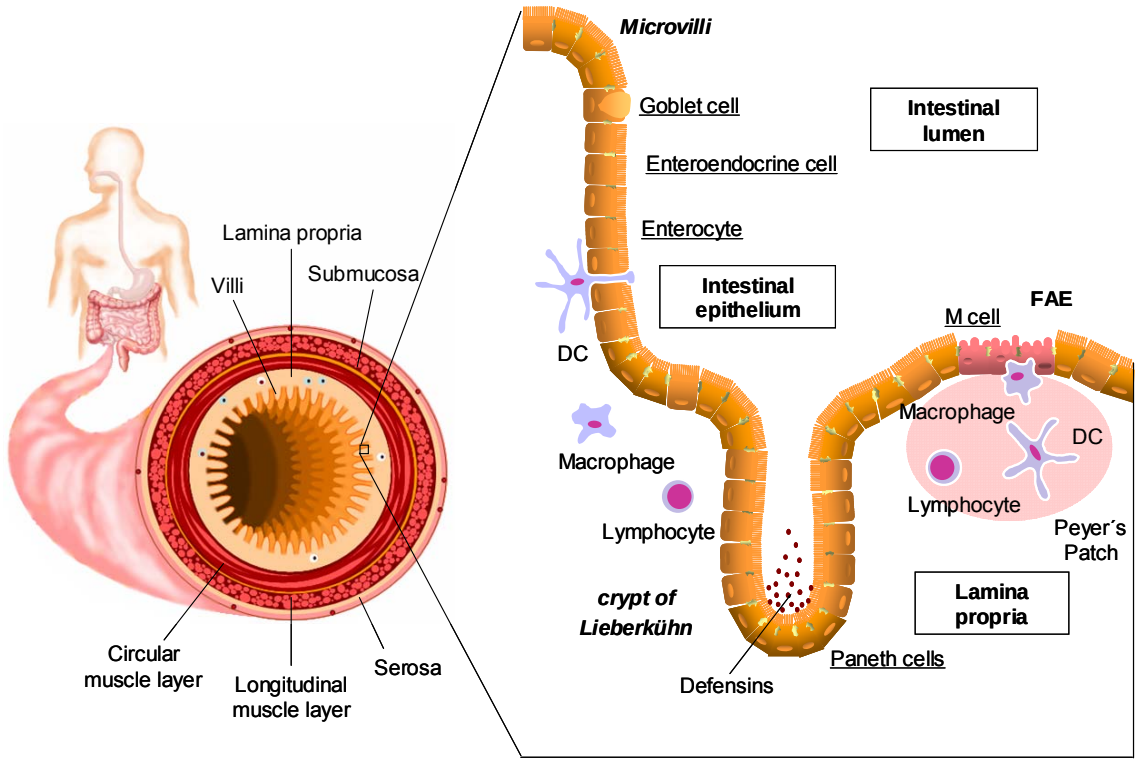

Figure 2: The intestinal mucosa. Proliferation of the epithelium occurs in the crypts of Lieberkühn. The pluripotent stem cells of the crypt differentiate during an upward migration into absorptive enterocytes (making up 80% of all epithelial cells), enteroendocrine cells and mucus producing goblet cells. Once the cells arrive at the top of the villus they are removed by apoptosis. Paneth cells differentiate during a downward migration to the base of the crypt and are responsible for the modulation of the microbiota through secretion of lysozyme and anti-microbial peptides (Ouellette and Selsted 1996). The intestinal mucosa collects luminal antigens through enterocytes, M cells and DC. M cells constitute the 10-20% of the specialized FAE and use transepithelial vesicular transport to carry microbes to the APCs present in the Peyer´s Patches, right under the FAE.
Abbreviations: FAE, follicle-associated epithelium

The activation of immune cells is also accompanied by the production of a large variety of nonspecific mediators of inflammation. These include many other cytokines, chemokines and growth factors as well as reactive oxygen metabolites such as nitric oxide (NO) and metabolites of arachidonic acid, including the prostaglandins (PGs) and thromboxanes (TXs) (Swidsinski, Ladhoff et al. 2002). Constitutive high-level production of arachidonic acid metabolites is unique to the intestinal mucosa and might represent one of the earliest developmental adaptations in settling the anti-inflammatory environment of the GALT. The spontaneous and constitutive production of the enzyme cyclooxygenase (COX)2 is also not dependent on the presence of the luminal microbiota or on inflammatory stimuli and leads to the production of PGE_2, which has immunomodulatory effects in the small intestinal lamina propria (Nagler-Anderson 2001).

2 Inflammatory bowel disease (IBD)

Human inflammatory bowel diseases (IBDs), Crohn's disease (CD) and ulcerative colitis (UC) are chronic inflammatory disorders of the GI tract of unknown etiology. IBD results from inappropriate and ongoing activation of the mucosal inmmune system to normal constituents of the mucosal microbiota in genetically predisposed individuals (Sartor 1997; Bouma and Strober 2003).

Ever since Samuel Wilks first described UC in 1875 and Crohn, Ginzburg and Oppenheimer described CD in 1932, enormous progress has been made in understanding the pathogenesis of these diseases. UC is a mucosal disease that always affects the rectum and extends proximately to involve a variable length of the colon, whereas CD can affect any part of the GI, preferentially ileum and colon. UC is associated with high proportion of leukocytes, macrophages and a different lymphocyte population from the normal gut. In contrast, CD also affects deeper layers of the mucosa as well as being a more lymphocyte dominated inflammation. Finally, through the study of patients and mouse models of chronic inflammation, it was discovered that UC is probably driven by the production of IL-13, whereas CD is characterized by the production of IL-12 and IFNγ (Podolsky 2002).

There is strong evidence that genetic factors as well as environmental factors such as diet and smoking habits play an important role in the pathogenesis of IBD. Susceptibility to the development of the disease is inherited (Bonen and Cho 2003), the genetic contribution to the development of disease being more important in CD that in UC. The most significant finding in this area was the identification of mutations in the gene that encodes the nucleotide-binding oligomerization domain (NOD)-2 protein in a subgroup of patients with CD. NOD-2 contains a caspase recruitment domain on one end and a leucine-rich domain on the other end, which is thought to be a binding region for microorganism products and where most of the mutations have been found (Beutler 2001). Nevertheless the driving force of the inflammation is the nonpathogenic commensal organisms resident on the mucosal surface, which provide the major stimulus for the induction of effector T-cells that cause the inflammation (Strober, Fuss et al. 2002), as evidenced in experimental models of mucosal inflammation (Blumberg, Saubermann et al. 1999)

2.1 Models of mucosal inflammation

An IBD animal model is characterized by chronic or relapsing inflammation of the GI tract with features resembling human IBD (Hoffmann, Pawlowski et al. 2002–03). Although studies in models of mucosal inflammation reflect wide variety of causes, mucosal inflammation is almost always mediated by one of two pathways, either an excessive Th1-cell response that is associated with increased secretion of IL-12, IFNγ and/or TNF, or an excessive Th2-cell response that is associated with increased secretion IL-5, but not IL-4 and/or IL-13 (Strober, Fuss et al. 2002). CD is in fact a Th1-mediated inflammation (Christ, Stevens et al. 1998; Liu, Geboes et al. 2000).

In murine models, the over-activity of Th1 cells may be caused by the simultaneous decrease in subgroups of suppressor T cells, variously designated Th3 or Tr1, which produce the down-regulatory cytokines IL-10 and TGF-β respectively (Toms and Powrie 2001). This imbalance between effective and suppressive T-cells leads to the classification of the models of mucosal inflammation into two broad categories: “type 1 models,” in which inflammation occurs as a result of excessive effector T-cell function and “type 2 models” wherein the effector T-cell response is normal, but there is a deficient regulatory T-cell function (Figure 3).

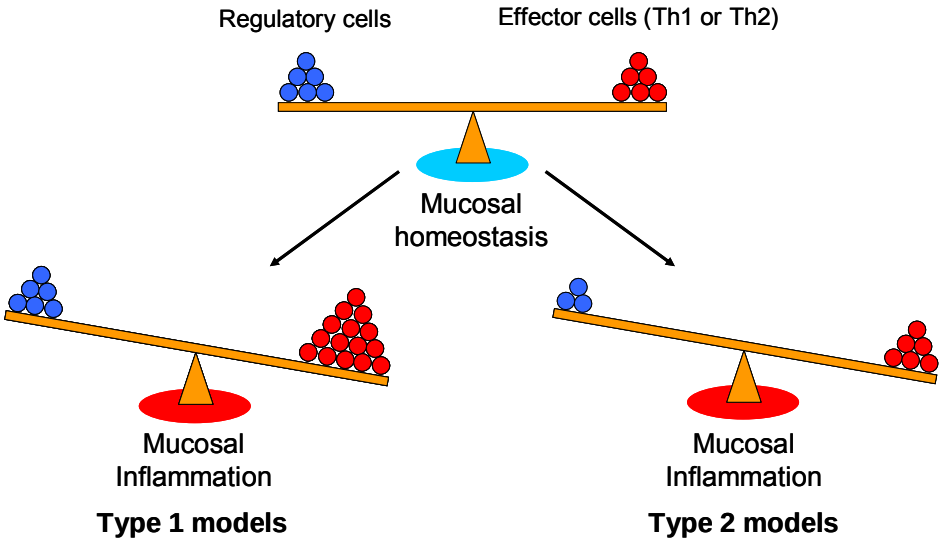

Figure 3: Effector T cells versus regulatory T cells. Mouse models of mucosal inflammation result from an imbalanced situation in which either regulatory T-cell function is impaired (type 1 models) or effector T-cell function is exacerbated (type 2 models).

The $TNF^{\Delta ARE}$ mouse is one of the best characterized type 1 models of colitis (Table 1). This model results from a targeted deletion of adenosine-uracil rich elements (AREs) of the TNF gene, which leads to dysregulation of the processing of TNF messenger ribonucleic acid (mRNA) and the overproduction of TNF protein. This situation gives rise to a phenotype very similar to that in CD (Kontoyiannis, Pasparakis et al. 1999). Type 2 models of colitis include colitis due to a deficiency or abnormalities of IL-10 signaling and defects in TGF-β function. The most important type 2 model is the IL-10 gene-deficient ($IL\text{-}10^{-/-}$) mice, in which the absence of IL-10, a potent downregulator of IL-12 and TNF, leads to a Th1-mediated inflammation (Blumberg, Saubermann et al. 1999).

Models of mucosal inflammation

Type 1 models	Type 2 models
$TNF^{\Delta ARE}$ mice	SCID-transfer colitis
TNBS colitis	IL-10 deficiency and IL-10 signaling defect colitis
?C_3H/HeJBir mice	?C_3H/HeJBir mice
Gi2α-deficient mice	IL-2-deficient mice
STAT4 Tg mice	TGF-β RII dominant-negative mice
N-cadherin dominant-negative mice	Tgε26 mice
IL-7 Tg mice	
DSS colitis	
Mice with NF-κB defects	

Table 1: Categories of mucosal models of inflammation. Models of mucosal inflammation can be divided into 2 groups: Type 1, which present a defect in the effector mechanism of the mucosal response and Type 2, presenting an inadequate regulatory T-cell response, but a normal mucosal response (Strober, Fuss et al. 2002). For a more comprehensive listing of models of mucosal inflammation see Table 2.
Abbreviations: SCID, severe combined immunodeficiency; STAT4, signal transduction and activators of transcription-4; TNBS, 2,4,6-trinitrobenzene sulfonic acid; DSS, dextran sulfate sodium.

Diverse models of colitis are also due to defects in epithelial cell barrier function. This includes inadequate secretion of immune mediators as in the case of $IL\text{-}10^{-/-}$ mice, as well as increased permeability of the intestinal epithelium. IECs are the first contacts for the commensal bacteria and restrict the passage of luminal antigens into the lamina propria and the APCs, which in turn stimulate lymphocytes (Blumberg, Saubermann et al. 1999). Regarding models that present breakdown of the epithelial barrier, one of the more relevant cases is the dominant-negative N-cadherin mouse, in which defective cell-cell adhesion leads to an excessive exposure of mucosal lymphoid elements to the intestinal microbiota (Hermiston and Gordon 1995). This is similar to what happens in the 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis, in which the rectal

administration of this chemical hapten in the presence of ethanol disrupts the epithelial barrier, resulting in an unbalanced immune response leading to chronic inflammation (Morris, Beck et al. 1989; Fuss, Marth et al. 1999). Another example of colitis related to barrier function is the multiple drug-resistant (mdr)1a-deficient mouse, in which colitis develops because of the deficiency of the mdr1a gene in epithelial cells. Mdr1a-deficient mice develop a severe, spontaneous Th1-mediated intestinal inflammation consisting of a dysregulated epithelial cell growth and T cell and B cell infiltration into the lamina propria of the large intestine which is similar to that found in CD (Panwala, Jones et al. 1998). Finally, oral administration of dextran sulfate sodium (DSS) also disrupts the epithelial cell barrier (Kitajima, Takuma et al. 1999), resulting in another important mouse model of intestinal inflammation. DSS-induced colitis is characterized by a Th1 response in the acute stages of DSS colitis and a mixed Th1/Th2 response in the later and chronic phases of the inflammation (Dieleman, Palmen et al. 1998). In both stages, large amounts of TNF and IL-6 are secreted, which are the main causes for the tissue damage in the disease (Strober, Fuss et al. 2002).

Although the experimental models of inflammation are quite different, none of them develop colitis under germ-free conditions (including IL-$10^{-/-}$ and IL-$2^{-/-}$ mice), sharing a remarkable dependence on the presence of normal non-pathogenic bacteria to develop inflammation (Song, Ito et al. 1999). For example, *Helicobacter hepaticus*, *Escherichia coli*, *Enterococcus faecalis* and *Bacteriodes vulgatus* have been found to be especially important in the induction of colitis in IL-$10^{-/-}$ mice (Sellon, Tonkonogy et al. 1998; Kim, Tonkonogy et al. 2003). Remarkably, probiotic species such as *Lactobacillus* prevent colitis in this model under pathogen-free conditions (Madsen, Doyle et al. 1999).

2.2 Probiotics and IBD

Besides the detrimental effects of GI microbiota, such as opportunistic infections or conversion of dietary non-carcinogens to carcinogens, the metabolic activity of normal microbiota can also be beneficial to the host. There is strong experimental evidence to support the idea that certain components of the gut microbiota are involved in protection of the host against infectious disease (Bengmark 1998; Dunne and Shanahan 2002; Ewaschuk and Dieleman 2006). However, little is known about the non-pathogenic bacteria-host interactions taking place in the GI tract.

Antigen-specific	Inducible	Genetic	Adoptive transfer	Spontaneous
	Chemical	*Transgenic*		
Peptidogylcan-polysaccharide[r]	Acetic acid[r,d]	Cytochrome c TCR tg[m]	CD4+CD45RB^{high}/SCID[m]	C3H/HeJBir[m]
Carageenan[m, gp, rb]	DNBS/ethanol[m, r]	HGF tg[m]	CD4+CD45RB^{high}/RAG-2 KO[m]	Cotton-top tamarin[p]
Complete Freund´s adjuvantt[r]	DSS[h, m, r]	HLA B27 X β_2-MG tg[r]	CD4+CD62L^{high}/SCID[m]	Grower/finisher pgs
Helicobacter hepaticus (rag-2 KO)[m]	Indomethacin[r]	HSV tyrosine kinase tg/gancyclovir[m]	CD4+CD45RB^{high}Yit/SCID[m]	HistiocyticUC[d]
Helicobacter hepaticus (p50$^{-/-}$ X p65$^{+/-}$)[m]	Lactulose[m]	HTF tg[m]	CD4+/SCID[m]	Samp1/Yit[m]
Hsp60[m]	Oxazolone[m]	IL-7 tg[m]	CD4+ con A blasts/SCID[m]	
Lymphogranuloma venereum proctitis[p]	Sulfhydryl blockers[r]	N-Cadherin dominant neg. tg[m]	CD4+ con A blasts/RAG-1 KO[m]	
Ovalbumin in OVA TCR tg mice[m]	TNBS/ethanol[r, m, rb]	STAT4 tg/TNP-KLH[m]	Bone marrow/DC3$_\varepsilon$Tg26[m]	
Ovalbumin/transfercolitis/OVA tg mice[m]		TGF-β receptor-II dominant	HSP60-CD8 clone/TCRβ-KO[m]	
TNBS/transfer/TCR KO[m]	*Immunological*		MuLV splenocytes/nude[m]	
	Cyclosporin A[m]	*Knock-Out*		
	Immune complex[rb, r]	A20 KO[m]		
		IκBα KO[m]		
	Physical	Gi-$_{2\alpha}$ KO[m]		
	Radiation (MHC II KO)[m]	Gpx 1 KO X Gpx 2 KO[m]		
		IL-2 receptor-β KO[m]		
		IL-2 receptor-α KO[m]		
		IL-2 KO X β_2-MG KO[m]		
		IL-2 KO[m]		
		IL-10 KO[m]		
		CRF2-4 KO[m]		
		Mdr1a KO[m]		
		MHC class-II KO[m]		
		Myloid STAT-3 KO[m]		
		TCR-α KO[m]		
		TCR-β KO[m]		
		TGF-β KO[m]		
		TNF$^{\Delta ARE}$[m]		
		WASP KO[m]		

Table 2: Classification of IBD animal models according to the Core Facility 'IBD animal models' of the German Competence Network IBD
Category and species are indicated (d=dog; gp=guinea pig; m=mouse; p=primate; pgs=piglets; r=rat; rb=rabbit). HGP=hepatocyte growth factor; HTF=human fucosyl transferase; mdr=multiple drug resistance gene; tg=transgenic.

Elli Metchnikoff, Pasteur´s collegue and father of innate immunity, was the first who at the beginning of the 20th century proposed manipulating the microbiota to benefit human health, giving a scientific basis to the idea that ingestion of fermented milk was related to health (Metchnikoff 1908). The word “probiotics”, literally “for life”, was first used by Lilley and Stillwell in 1965 to describe substances secreted by one microorganism which stimulate the growth of another (Lilly and Stillwell 1965). With the time the concept has evolved and the current definition given by the Food and Agriculture Organization (FAO) and the World Health Organization (WHO) is “probiotics are live microorganisms which when administered in adequate amounts confer a health benefit on the host” (FAO/WHO 2001; Sanders 2003). The most common microorganisms used as probiotics are from the *Lactobacillus* and *Bifidobacterium* genera, followed by other bacterial genera, including *Enterococcus*, *Streptococcus* and *Escherichia* (Silva, Barbosa et al. 2004; Bai and Ouyang 2006).

Four mechanisms have been attributed to probiotics to exert their beneficial effects: antagonism by the secretion of anti-microbial substances, which inhibit or kill the pathogen (Vandenbergh 1993; Lievin, Peiffer et al. 2000), inhibition of the production or action of bacterial toxin (Corthier, Dubos et al. 1985), competition with the pathogen for adhesion sites or nutritional resources (Bernet, Brassart et al. 1994) and immunomodulation of the host (Perdigon, Rachid et al. 1994; Neumann, Oliveira et al. 1998; Borruel, Carol et al. 2002). Regarding the last point, probiotics have been shown to increase resistance to infectious diseases in human and animal models by stimulating protective immune responses and mucosal barrier function, and modulating cytokine gene expression (Gill, Rutherfurd et al. 2001; Madsen, Cornish et al. 2001). Furthermore, probiotics have been probed to be useful in the treatment of chronic intestinal inflammation improving the condition of IBD patients significantly (Guandalini 2002; Reid, Jass et al. 2003; Sartor 2004; Bai and Ouyang 2006). Oral administration of bacterial species such as *lactobacillus* and *bifidobacterium* have been shown to downregulate the expression of the pro-inflammatory cytokines TNF, IL-6 and INFγ in inflamed mucosa of active IBD or experimental colitis (Dieleman, Goerres et al. 2003; Furrie, Macfarlane et al. 2005), as well as increase secretion of the anti-inflammatory cytokines IL-10 and IL-4 in human (Schultz, Linde et al. 2003).

2.3 Terapeutic role of flavonoids in IBD

The impact of environmental factors in IBD such as smoking and nutritional habits, is still difficult to define, but is clearly important as evidenced by the increasing occurrence of CD in recent decades, the changing incidences of IBD in migrant populations, as well as the lack of complete concordance among monozygotic twins in the development of the disease (Andus and Gross 2000; O'Sullivan and O'Morain 2006). Enteral nutrition continues to play an important role in the therapy of CD and has been the subject of recent studies that have focussed on the components of effective foods, and the mechanism by which they might act. Thus, nutrients such as butyrate, antioxidants and short chain fatty acids are able to ameliorate inflammation, the last being suggested to have an immunomodulatory effect in colonic inflammation (Belluzzi, Boschi et al. 2000; Bocker, Nebe et al. 2003; Kles and Chang 2006).

In the last years increasing interest has been driven to polyphenols, especially flavonoids, due to their antioxidant and free-radical scavenging properties *in vitro* (Rice-Evans, J. et al. 1996), as well as their ability to modulate important cellular signaling processes such as cellular growth, cellular differentiation and NF-κB activation (Yamamoto and Gaynor 2001). Flavonoids are produced as a result of the secondary metabolism of plants and are widely distributed in foods and beverages of plant origin (Figure 4). These polyphenols are frequently attached to sugars (glycosides) although they can also be found as aglycones (Ross and Kasum 2002). Flavonoids have been known as plant pigments for over a century, but Rusznyak and Szent-Gyorgyi were the first who discovered their biological effects in 1936 by using a mixture of the flavone hesperidin and eriodictiol glycoside for treating a patient with subcutaneous capillary bleedings (Rusznyak and Szent-Gyorgyi 1936). Besides their antioxidant abilities, flavonoids have been found to have various clinically relevant properties such as anti-thrombic, anti-ischemic, anti-tumor (Scalbert, Manach et al. 2005), anti-microbial, anti-ulcerogenic, anti-allergic (Ross and Kasum 2002) and anti-inflammatory effetcs (Rotelli, Guardia et al. 2003; Kim, Son et al. 2004). In adition, diverse flavonoids can interfere with various kinase signal transduction pathways regulating cell growth, cell cycle control and apoptosis (de Azevedo, Mueller-Dieckmann et al. 1996; Agullo, Gamet-Payrastre et al. 1997).

Both *in vivo* and *in vitro* studies suggest that the protective effects of flavonoids can be attributed to its antioxidant and free radical scavenging properties improving the colonic

oxidative stress characteristical of the inflammatory status. Thus several flavonoids including quercetin, apigenin and luteolin have been shown to inhibit secretion of inflammatory mediators such as nitrogen reactive species produced by inducible nitric oxide synthase (iNOS) as well as the expression of adhesion molecules (Choi, Choi et al. 2004) and pro-inflammatory cytokines such as TNF and IL-1β by inhibiting the NF-κB pathway (Tsai, Lin-Shiau et al. 1999; Comalada, Ballester et al. 2006). In addition, different flavonoids have been shown to exert intestinal anti-inflammatory effects in various experimental models of inflammation (Galvez, de la Cruz et al. 1994; Comalada, Ballester et al. 2006).

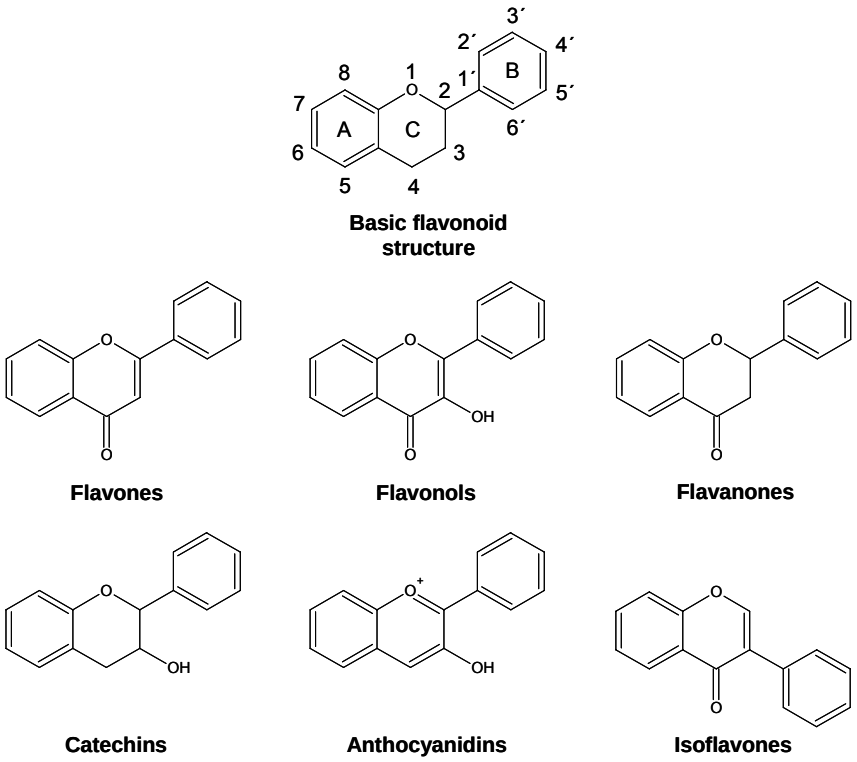

Figure 4: Basic structure and major categories of flavonoids. Flavonoids share a common structure of diphenylpropanes (C6-C3-C6), made up of two aromatic rings linked through three carbons. Variations in the heterocyclic C-ring give rise to six subclasses: flavonols, flavones, catechins, flavanones, anthocyanidins and isoflavonoids. To date, more than 5000 categories of flavonoids have been identified. The flavonol Quercetin is the most abundant flavonoid in foods. Also common are kaempferol, myricertin, and the flavones apigenin and luteolin.

3 IECs and the mucosal immune system

Enteric bacteria are crucial for the pathogenesis of IBD. However, no single species has been found to have a cause-and-effect relationship with UC or CD (Chandran, Satthaporn et al. 2003). Attention has now focused on understanding the interaction between the intestinal epithelium of the host and bacteria.

IECs form a single layer of cells that isolates the host from the luminal microbiota, being the first contacts for the commensal bacteria present in the gut. Besides their barrier function, IECs serve as sensors of the bacterial environment and can actively participate in the mucosal response to intestinal inflammation and infection having a clear role in host defense and gut immune homeostasis. Thus IECs are able to respond to a large repertoire of substances like cytokines, bacterial products and short chain fatty acids. In turn, IECs transmit signals to resident intraepithelial and lamina propria mononuclear cells that directly regulate mucosal inflammation and immune responses (Jobin and Sartor 2000; Haller, Russo et al. 2002). As shown by several animal models of experimental colitis, an exacerbate immune response to non-pathogenic commensal luminal bacteria can cause colitis in genetically predisposed individuals (Kagnoff 2005; Quigley 2005). The way IECs interact with the enteric comensal bacteria, a critical component in the initiation and regulation of chronic intestinal inflammation, may determine whether inflammation in the gut is induced or not (Haller 2006).

IECs stimulated with cytokines or bacterial products can secrete high levels of C-X-C chemokines, including growth-related oncogene (GRO)α, GROβ, GROγ, IL-8 and epithelial cell-derived neutrophil activating protein (ENA)-78 (Eckmann, Kagnoff et al. 1993; Ingalls, Heine et al. 1999). In addition IECs can express C-C chemokines such as macrophage-inflammatory protein (MIP)-1α, MIP-1β, regulated on activation, normal T cell expressed and secreted (RANTES) and monocyte chemoattractant protein (MCP)-1. IECs can also be induced to express pro-inflammatory cytokines such as TNF, IL-1α, IL-1β and granulocyte macrophage colony stimulating factor (GM-CSF) (Yang, Eckmann et al. 1997). IEC pro-inflammatory signals are both transient and fast upregulated. The high expression levels of chemoattract cytokines which are implicated in the recruitment of a variety of immune cells, suggests a participation of IECs in the initial acute inflammatory response whereas monocytes, macrophages and other immune cells have a more

important role in the maintenance of the inflammatory response (Kagnoff and Eckmann 1997).

Regarding proteins expressed in the cell membrane, IECs constitutively express or can be induced to express receptors involved in immune recognition such as major histocompatibility complex (MHC) class II molecules (Blumberg, Terhorst et al. 1991; Ruemmele, Gurbindo et al. 1998), as well as classical MHC class I molecules and non-classical MHC-related molecules (Panja, Blumberg et al. 1993). IECs are also able to present antigens to T cells *in vitro*, suggesting a participation of IECs in antigen specific mucosal immune responses. Additionally, IECs express receptors for signals coming from the mucosa such as IFNγ, IL-1, IL-12, IL-4, IL-7, IL-9, as well as TNF and TGF-β1 (Reinecker and Podolsky 1995). Besides pro-inflammatory cytokines, IECs can also produce physiologic mediators with an autocrine/paracrine function like PGE_2, $PGF_{2\alpha}$ and PG H synthase (PGHS)-2, whose expression is upregulated in the presence of enteroinvasive bacteria (Eberhart and Dubois 1995). The expression of iNOS-2 is also increased in conditions of intestinal inflammation and upon stimulation of IECs with cytokines (Ribbons, Zhang et al. 1995).

The type of immune response induced by luminal antigens depend on initial recognition by the innate immune system. IECs recognize pathogenic bacteria through TLRs and NOD, which play a central role in innate immune responses. The presence of TLRs in the cellular membrane of IECs is modulated by a variety of factors such as bacterial products, microbial invasion and cytokines. This fine regulation of TLRs in IECs might explain why pathogenic bacteria, but not commensal bacteria, trigger inflammatory responses in the intestine. In addition, it has been shown that the expression of some TLRs is remarkably increased in the intestinal epithelium of patients with IBD. This is consistent with the idea that IBD may result from an exaggerated inflammatory response to the normal microbiota (Cario, Gerken et al. 2004). Most of the immune genes that are upregulated in stimulated IECs are transcriptionally controlled, belonging to a class known as immediate-early genes for which induction occurs without new protein synthesis. One of the transcription factors that bind to the promoter/enhancer region of many immediate-early genes is NF-κB (Jobin and Sartor 2000).

4 NF-κB signaling

NF-κB was identified just two decades ago as a transcription factor that binds to an enhancer element in the κ light chain gene in mature B- and plasma cells (Sen and Baltimore 1986). Subsequent studies revealed that NF-κB is an inducible transcription factor ubiquitously expressed, which plays a central role in regulating the expression of many genes involved in immune, apoptotic and inflammatory processes (Baldwin 1996; Ghosh, May et al. 1998).

NF-κB is a dimeric transcription factor formed by the hetero or homodimerization of proteins of the Rel family of transcription factors, including p50 and p60 (Siebenlist, Franzoso et al. 1994). NF-κB is a pivotal regulator of the immune response modulating B lymphocyte survival, the differentiation of B lymphocytes into plasma cells and IL-2 production, which in turn enhances the proliferation and differentiation of T cells (Gerondakis, Grumont et al. 1998). Regarding regulation of apoptosis, NF-κB is a key mediator of anti-apoptotic genes such as the cellular inhibitor of apoptosis (IAP) c-IAP, c-IAP2 and IXAP, the TNF receptor (TNFR)-associated factors (TRAF)1 and TRAF2, the Bcl-2 homologue A1/Bfl-1, and IEX-IL (Wu, Ao et al. 1998; Barkett and Gilmore 1999). Activation of the NF-κB pathway plays a central role in inflammation and is involved in the pathogenesis of chronic inflammatory diseases such as asthma, arthritis and IBD (Schmid and Adler 2000). There is a large body of literature addressing the link between NF-κB activation and development of colitis in IBD patients and animal models of chronic inflammation, such as mice with TNBS-induced colitis (Neurath, Pettersson et al. 1996). Patients with active IBD present mucosal NF-κB activation, as well as an increased production of pro-inflammatory cytokines by both lymphocytes and macrophages (Podolsky 2002).

NF-κB is activated by a wide array of agents, such as pro-inflammatory cytokines, cyclic adenosine monophosphate (cAMP) and bacterial and viral components such as lipopolysaccharide (LPS) and double stranded ribonucleic acid (dsRNA) (Barnes and Karin 1997) (Table 3). As a result, activated NF-κB regulates the expression of more than 150 genes including many cytokines and chemokines such as IL-1β, IL-2, IL-6, IL-12, TNF, IFNγ and GM-CSF (Pahl 1999). Cytokines that are stimulated by NF-κB, such as IL-1β and TNF, can also directly activate the NF-κB pathway, establishing a positive autoregulatory loop that can amplify the inflammatory response increasing the duration

of chronic inflammation (Yamamoto and Gaynor 2001). NF-κB also regulates the expression of proteins involved in antigen presentation such as members of MHC proteins (Kagnoff and Eckmann 1997), as well as receptors required for neutrophil adhesion and transmigration such as intracellular adhesion molecule (ICAM)-1 (Chen, Hagler et al. 1995). In addition NF-κB stimulates the expression of enzymes whose products contribute to the pathogenesis of the inflammatory process, including iNOS, which generates NO and the inducible COX2, which produces prostanoids (Pahl 1999). Not all prostanoids produced are pro-inflammatory. During an inflammatory response, the profile of prostanoids changes remarkably and COX2 also directs the synthesis of anti-inflammatory cyclopentenone PGs (cyPGs) which are involved in the resolution phase of inflammation. Thus the cyPG 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15d-PGJ_2) has been shown to inhibit NF-κB regulated genes (Ricote, Li et al. 1998; Rossi, Kapahi et al. 2000). Since NF-κB regulates COX2 synthesis, the inhibition of NF-κB transactivation by cyPGs may be part of a negative feedback loop that contributes to resolution of inflammation.

Inducers of NF-κB

Cytokines and Growth Factors	**Bacteria and Bacterial Products**
IL-1β*	*Salmonella* and *Shigella**
IL-2*	Enteropathogenic *E. coli**
IL-17*	*Helicobacter pylori*
IL-18*	*Mycoplasma fermentans*
Lymphotoxin	*Listeria monocytogenes**
TNF*	Lactobacilli
Macrophage colony-stimulating factor	LPS*
Platelet-derived growth factor*	PG-PS*
	Toxic shock syndrome toxin 1*
T Cells Mitogens	
Antigen	**Viruses and Viral Products**
Anti-CD2	Adenovirus*
Anti-CD3	Epstein-Barr virus
Anti-CD28	Human immunodeficienty virus type 1
Calcium ionophores	Human T cell leukemia virus type 1
Lectins (PHA, ConA)	Hepatitis B virus B
	Herpes simplex virus type 1
Oxidative Stress	dsRNA
Hydrogen peroxide*	Latent membrane protein
Ozone	
Reactive oxygen intermediates*	

* Documented to stimulate IECs

Table 3: Inducers of NF-κB. NF-κB is activated by a wide variety of stimuli, including phorbol esters, IL-1β, TNF, LPS, dsRNA, cAMP, bacteria and viral transactivators (Jobin and Sartor 2000).
Abbreviations: PG-PS, peptidoglycan-polysaccharide; PHA, phytohemagglutinin; ConA, concavalin A

4.1 Rel/NF-κB and IκBα families

NF-κB is composed of homo- and heterodimers of subunits structurally related and evolutionary conserved referred to as the Rel family. To date, five members have been found in mammals: cRel, RelA, RelB, p50 and p52, the latter two synthesized as p105 and p100 precursors respectively (Ballard, Dixon et al. 1992; Baeuerle and Henkle 1994). While the generation of p52 from p100 is tightly regulated, p50 is produced by constitutively processing p105 (Karin and Ben-Neriah 2000)

Members of the NF-κB/Rel proteins are characterized by a highly conserved NH_2-terminal Rel homology domain (RHD) which contains a nuclear localization signal (NLS) and is responsible for the dimerization, sequence-specific deoxyribonucleic acid (DNA) binding and the association with inhibitory proteins known as IκBs (Ghosh, May et al. 1998) (Figure 5).

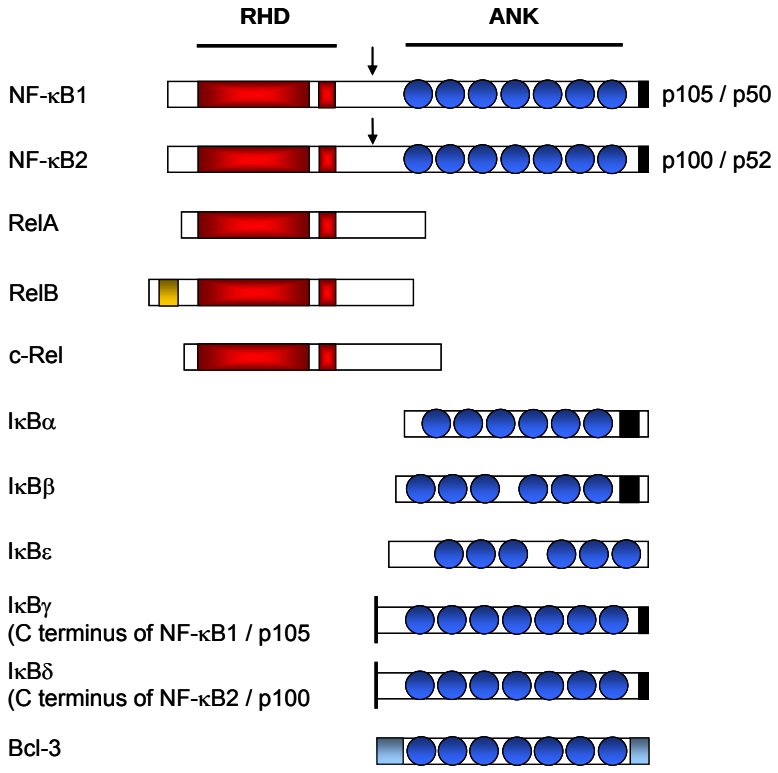

Figure 5: Members of the Rel/NF-κB and IκB families of proteins. All RelA proteins share a RHD domain in the NH2-terminal (red boxes). The arrows indicate the endoproteolytic cleavage sites of p105 and p100 which give rise to p50 and p52, respectively. IκBs are a family of related proteins that bear an NH_2-terminal regulatory domain followed by six or more ankyrin repeats (indicated by blue circles) and a PEST domain (black boxes) in their C-terminus, which function is enhancing protein degradation. Blue boxes on Bcl-3 indicate transactivation domains, and the yellow box on RelB indicate a leucine zipper domain (Ghosh, May et al. 1998; Caamaño and Hunter 2002).
Abbreviations: PEST, proline/glutamate/serine/threonine-rich

The balance between different combinations of homo- and heterodimers of Rel members is related with specific responses to different stimuli. Thus, although most NF-κB dimmers are activators of gene transcription, the p50/p50 and p52/p52 homodimers repress the transcription of their target genes (May and Ghosh 1998; Karin and Ben-Neriah 2000). In addition, NF-κB proteins are also expressed in a cell- and tissue-specific manner, providing an additional level of regulation. Whereas p50 and RelA are ubiquitously expressed, p52, RelB and cRel are exclusively present in lymphoid cells (Caamaño and Hunter 2002). RelA and p50 were the first NF-κB proteins identified and the most potent gene transactivator (Ballard, Dixon et al. 1992; Ruben, Narayanan et al. 1992), being also the most abundant activated NF-κB heterodimer found in IECs upon cytokine stimulation (Jobin, Haskill et al. 1997) (Figure 6).

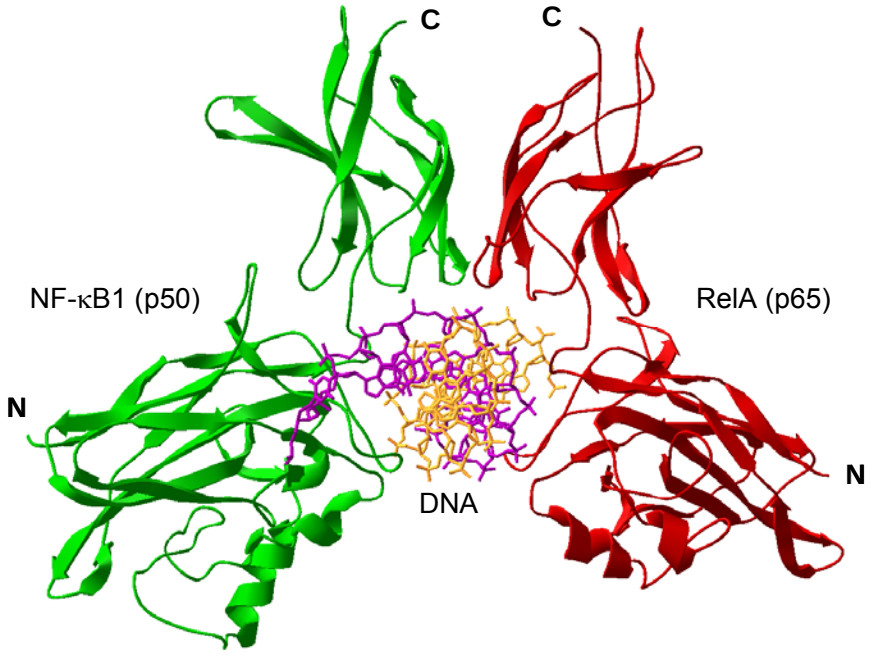

Figure 6: Crystal structure of the NF-κB heterodimer RelA/p50 bound to a DNA κB site. The RHD, which is located in the NH_2-terminal of all NF-κB/RelA proteins, is a highly conserved region which contains a NLS, a dimerization domain, a sequence-specific DNA binding domain and is also responsible for the association of NF-κB with IκBs. In mammals, only RelA, RelB and c-Rel bear a transactivation domain in the C-terminal, which consist of numerous serines, acidic and hydrophobic amino acids that are essential for transactivation activity. In contrast, p50 and p52 lack of transactivation domain and therefore cannot act as transcriptional activators by themselves (Baeuerle 1998; Chen, Huang et al. 1998).
Protein Data Bank Code: 1VKX. Figure generated with Swiss PDB Viewer 3.7.

In unstimulated cells, NF-κB dimmers are bound to the inhibitors IκB via non-covalent interactions and sequestered in an inactive form in the cytoplasm. IκB molecules are members of a multigene family containing seven known mammalian proteins including:

IκBα, IκBβ, IκBε, IκBγ, Bcl3, p100 and p105 (Figure 5). The IκB family is characterized by the presence of multiple copies of protein-protein interaction motifs called ankyrin repeats, which interact with NF-κB via the RHD masking its NLS (Figure 7). The IκB proteins bind with different affinities and specificities to NF-κB dimers, adding more complexity to the system. The better characterized and major NF-κB inhibitor is IκBα, which binds to the RelA subunit of NF-κB.

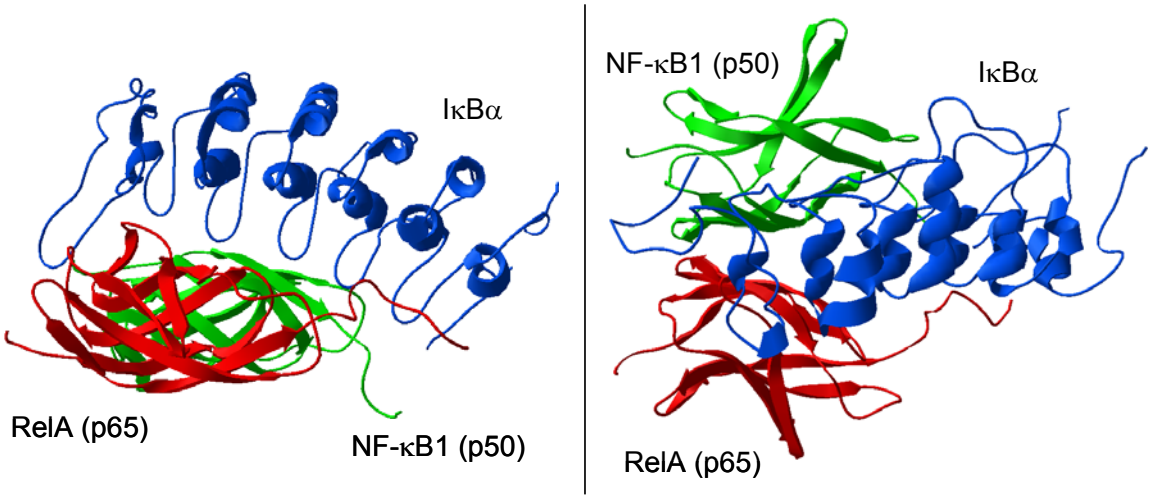

Figure 7: Crystal structure of the IκBα/NF-κB complex. IκBα binds to the RHD on the NH_2-terminal of Rel/NF-κB Proteins trough their ankyrin motifs (Huxford, Huang et al. 1998). In the Figure, only the NH_2-terminal of both RelA and p50 are shown.
Protein Data Bank Code: 1IKN. Figure generated with Swiss PDB Viewer 3.7.

Upon appropriate stimulation, IκBα is phosphorylated by IκB kinase (IKK) in the N-terminal domain at the conserved serine 32 and serine 36 (Brown, Gersberger et al. 1995). IκBα is then polyubiquinated on lysine 21 and lysine 22 by a ubiquitin ligase E3 complex (Verma, Stevenson et al. 1995) and rapidly degraded via nonlysosomal, adenosine triphosphate (ATP)-dependent 26S proteolytic complex composed of a 700-KDa proteasome (Palombella, Rando et al. 1994; Scherer, Brockman et al. 1995; Karin and Ben-Neriah 2000). This process is followed by NF-κB translocation into the nucleus where it binds to κB consensus sequences and induce gene expression of its target genes.

4.2 NF-κB signaling pathway

The regulatory step for IκBα degradation is the phosphorilation of its two N-terminal serines. The kinase responsible for IκB phosphorilation is IKK, a serine/threonine kinase that respond to several NF-κB activators like IL-1β and TNF and phosphorylate at serine

residues of the conserved motif DSGXXS, X being any amino acid (Hattori, Hatakeyama et al. 1999). The first identified and also most abundant IKK complex in most cells is made up of heterodimers of two catalytic subunits, IKKα and IKKβ (DiDonato, Hayakawa et al. 1997; Zandi, Chen et al. 1998) and the regulatory component IKKγ, also called NEMO (Rothwarf, Zandi et al. 1998; Yamaoka, Courtois et al. 1998). IKKα and IKKβ have very similar structures, sharing 52% sequence homology and both are exclusively phosphorylated at serines. Despite these similarities they exhibit differences in their function and regulation. Thus IKKβ and not IKKα seems to be responsible for IκBα phosphorylation and NF-κB activation after pro-inflammatory stimuli (Delhase, Hayakawa et al. 1999).

The binding of pro-inflammatory signals, mainly TNF, IL-1 or TLR ligands to their corresponding receptors, leads to the recruitment of adaptor proteins such as MyD88 and IL-1 receptor (IL-1R)-associated kinase (IRAK) in the case of IL-1R/TLR, and TNFR 1-associated death domain (TRADD) and receptor interacting protein (RIP)1 for TNFR. Subsequently, these receptor-associated proteins recruit TRAF6 or TRAF2 (Malinin, Boldin et al. 1997), both of which activate TGF-β-activated kinase (Tak)1. Activated Tak1 or other upstream signaling molecules such as NF-κB-inducing kinase (NIK) (Regnier, Song et al. 1997), mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) kinase (MEK) (Nakano, Shindo et al. 1998), protein kinase C (PKC) and protein kinase A (PKA) (Schouten, Vertegaal et al. 1997; Tando, Algul et al. 1999), may activate IKK complexes that in turn phosphorylate IκBs, leading to IκB proteasome-mediated degradation (Chen, Hagler et al. 1995). The degradation of IκBα exposes the NLS of NF-κB and allows it to translocate to the nucleus, where NF-κB binds to κB motifs in the promoter of its regulated genes and recruit histone acetyl transferase (HAT) coactivators. The consensus binding site of NF-κB is composed of the GGGRNNYYCC sequence, where R is purine, Y is pyrimidine and N is any bases (Miyamoto and Verma 1995). Acetylation of the core histones destabilizes chromatin folding facilitating the access of additional transcription factors to DNA and the ribonucleic acid (RNA) polymerase, which therefore begins the transcription of NF-κB regulated genes (Lee, Hayes et al. 1993) (Figure 8).

Another mechanism of NF-κB regulation is through proteolytic cleavage of the precursor proteins p105 and p100, which function as IκB-like molecules (Sun, Ganchi et al. 1994).

The processing of these precursor proteins results in the disruption of their IκB-like function and the generation of p50 and p52. Induction of p100 processing is mediated by a noncanonical NF-κB signaling pathway that relies on NIK (Xiao, Harhaj et al. 2001) as well as IKKα, but does not require IKKβ and IKKγ (Senftleben, Cao et al. 2001; Claudio, Brown et al. 2002), key components of the canonical NF-kB signaling pathway. The processing of p100 leads to the liberation of RelB, which in turn translocates to the nucleus where it exerts its gene regulation function (Solan, Miyoshi et al. 2002; Derudder, Dejardin et al. 2003).

One of the first genes induced by NF-κB is its repressor protein IκBα. IκBα contains an export sequence and mediates the exportation of NF-κB to the cytoplasm, establishing a regulatory loop for NF-κB activity (Brown, Park et al. 1993; Sun, Ganchi et al. 1993; Chiao, Miyamoto et al. 1994). Other factors that regulate the NF-κB pathway negatively include A20, a cytoplasmic zinc finger protein expressed in a wide range of cell types upon stimulation with cytokines such as TNF and IL-1β (Beyaert, Heyninck et al. 2000). A20 inhibits NF-κB and TLR signaling in cell lines and mice by inhibition of IKK activity as well as by deubiquitination of TRAF6 (Boone, Turer et al. 2004). In addition, the adaptor protein Toll-interacting protein (TOLLIP) has been shown to inhibit TLR4 and TLR2-mediated NF-κB activation in cultured IECs (Zhang and Ghosh 2002; Otte, Cario et al. 2004). Another important intrinsic negative regulator of NF-κB is IRAKM, a kinase induced upon TLR stimulation and able to inhibit TLR signalling (Kobayashi, Hernandez et al. 2002). Also the ubiquitination of the TLR signaling adaptor protein IRAK1 leads to the downregulation of TLR-mediated NF-κB activation in mice primary IECs (Lotz, Gutle et al. 2006). Peroxisome proliferator-activated receptor (PPAR)γ is a member of the steroid receptor superfamily with various cellular functions including differentiation, apoptosis, lipid metabolism and anti-inflammatory responses, and has been shown to interfere with the NF-κB pathway (Daynes and Jones 2002; Blanquart, Barbier et al. 2003). Furthermore, PPARγ has been also reported to mediate the nuclear export of NF-κB in cultured IECs (Kelly, Campbell et al. 2004).

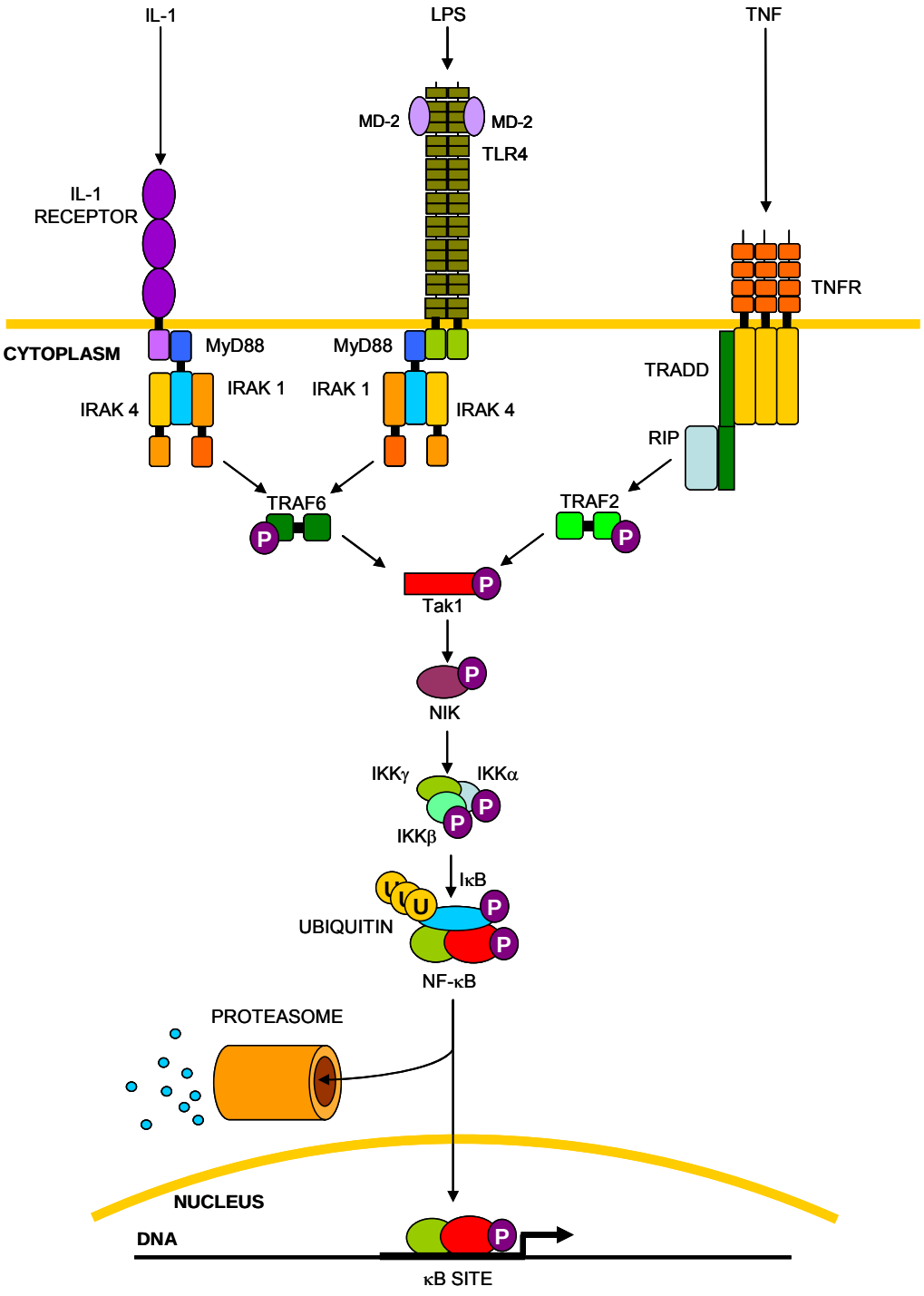

Figure 8: Simplified canonical signal transduction pathways of NF-κB activation. NF-κB is retained inactive in the cytoplasm by the inhibitor protein IκB. Appropriate stimuli elicit a cascade of transductional signals that converge on IKK. Activation of IKK leads to selective IκB phosphorylation, which is then ubiquitinated and targeted for degradation by the proteasome pathway. Free NF-κB translocates to the nucleus by virtue of its NLS and induces transcription of κB-dependent genes.

5 TLR-mediated NF-κB activation

The immune system is responsible for the recognition of any foreign substance in the organism. Adaptive immunity provides highly sophisticated mechanisms for recognition of antigens and is only present in vertebrates, appearing relatively late in evolutionary terms. In contrast, innate immunity is a non-specific ancient form of host defense present in almost all multicellular organisms, which not only constitute the first line of rapid host defense, but is also crucial for the development and function of adaptive immunity (Fearon and Locksley 1996; Iwasaki and Medzhitov 2004). Innate immunity is designed to recognize a few highly conserved structures present in many different microorganisms known as pathogen-associated molecular pattern (PAMPs), such as LPS, peptidoglycan-polysaccharide (PG-PS) or unmethylated CpG DNA of bacteria. Recognition of PAMPs is mediated by germline-encoded pattern recognition receptors (PRRs) including CD14, C-type lectins, complement receptors and TLR (Medzhitov and Janeway 1997).

TLRs are the most important group of PRRs and are characterized by their ability to recognize and discriminate between different classes of microbial components, triggering the activation of cellular immune responses (Anderson 2000). The first Toll receptor was originally identified in *Drosophila* as an essential receptor for the establishment of the dorso-ventral pattern in developing embryos (Hashimoto, Hudson et al. 1988). A year after the discovery of the role of the *Drosophila* Toll in the host defense against fungal infection, a mammalian homologue of the *Drosophila* Toll (now termed TLR4) was identified and shown to induce the expression of genes involved in inflammatory responses (Medzhitov, Preston-Hurlburt et al. 1997). Subsequentely, a family of proteins structurally related to Drosophila Toll was identified, consisting so far of 10 members in human (TLR1-TLR10). TLRs bear an extracellular amino-terminal leucine-rich repeat (LRR) domain, probably involved in ligand-binding and TLR dimerization and a carboxy-terminal cytoplasmatic domain which shows high similarity to that of the IL-1R family and is therefore called the Toll/IL-1R (TIR) domain (O'Neill and Greene 1998) (Figure 9).

Consistent with their role in pathogen recognition and host defense, mammalian TLRs are strategically expressed in cell types that are first to interact with microorganisms upon infection, like monocytes/macrophages, neutrophile, DCs, IECs and endothelial cells (Muzio, Polentarutti et al. 2000). TLR and NOD systems do not discriminate between structurally related PAMPs from commensal and pathogenic microorganisms.

Therefore, innate host responses towards enteric bacteria seem to be determined not only by the nature of bacteria such as cell wall composition and presence of pathogenic factors, but also by the cellular distribution and expression levels of PRR in the mucosal epithelium, and activity of host-derived regulators of PRR signalling (Mowat 2003; Haller and Jobin 2004; Otte, Cario et al. 2004).

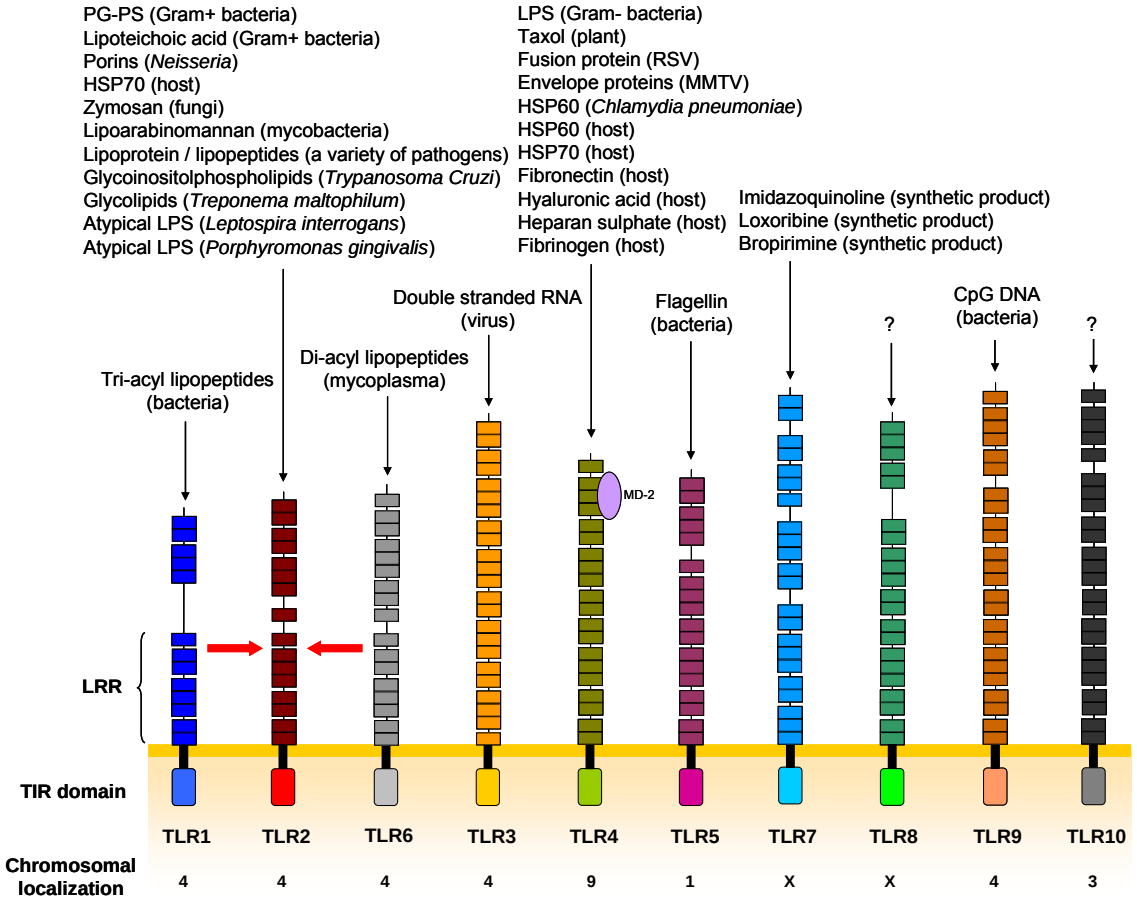

Figure 9: Human TLR protein family and their ligands. The TLR family is able to discriminate between specific patterns of microbial components. TLRs are characterized by an amino-terminal extracellular LRR domain involved in ligand binding and an intracellular TIR domain required for signal transduction. Red arrows indicate a possible dimerization between TLR1, TLR6 and TLR2.

Since comensal bacteria are able to induce colitis in a genetically predisposed host, an unbalanced activation of TLR-induced signalling pathways may turn a physiological response to enteric bacteria into a pathological situation such as chronic intestinal inflammation (Bell, Mullen et al. 2003). To date, the most extensively studied and best characterized TLRs are TLR4 and TLR2, which mediate cellular responses to cell wall components of Gram-negative and Gram-positive bacteria respectively.

5.1 TLR4

The most important PAMP recognized by TLR4 is LPS, a major cell wall component of Gram-negative bacteria. Two mouse strains, C3H/HeJ and C57BL10/ScCr, known for their hypo-responsiveness to LPS, have been found to have mutations in *Tlr4* gene (Poltorak, He et al. 1998; Qureshi, Lariviere et al. 1999). The C3H/HeJ mouse strain has a point mutation in the cytoplasmic region of the *Tlr4* gene leading to the replacement of a highly conserved proline with histidine at position 712. This mutation results in the generation of a dominant negative allele and in a lack of response to LPS. C57BL10/ScCr mice have a null mutation in the *Tlr4* gene. In addition, TLR4-deficient mice are hypo-responsive to LPS, confirming the crucial role of TLR4 as a receptor of LPS (Hoshino, Takeuchi et al. 1999).

Recognition of LPS requires other molecules in addition to TLR4. LPS binds to LPS-binding protein present in the serum and this LPS-LPS-binding protein complex is subsequently recognized by a glycosylphosphatidylinositol-linked protein known as CD14 (Fenton and Golenbock 1998). LPS stimulation leads to increased physical proximity between CD14 and TLR4, suggesting an interaction of CD14 and TLR4 in LPS signaling (Jiang, Akashi et al. 2000; da Silva Correia, Soldau et al. 2001). MD-2 was identified as a molecule that associates with the extracellular portion of TLR4 (Shimazu, Akashi et al. 1999). In MD-2-deficient cells, TLR4 remains in the Golgi apparatus instead of being on the cells surface, indicating that MD-2 is essential for the intracellular distribution of TLR4 (Nagai, Akashi et al. 2002).

TLR4 is also able to recognize other ligands (Figure 9). Among them, extracellular matrix components such as oligosaccharides of hyaluronic acid and heparan sulfate, which have been found to be important in activation and maturation of DCs via TLR4 (Termeer, Benedix et al. 2002). TLR4 also seems to be responsible for the inflammatory responses triggered by Hsps such as Hsp60 and Hsp70 (Gallucci and Matzinger 2001). Therefore, TLR4 is presumably involved in several aspects of the inflammatory response by recognizing endogenous ligands produced during inflammation.

5.2 TLR2

Analysis of TLR2-deficient mice showed that TLR2 is critical to the recognition of PG-PS and lipoproteins (Takeuchi, Hoshino et al. 1999). Additionally, TLR2 recognizes a wide variety of other microbial components such as lipoproteins from Gram-negative bacteria,

Mycoplasma and spirochetes (Aliprantis, Yang et al. 1999) or lipoarabinomannan from mycobacteria (Underhill, Ozinsky et al. 1999). Furthermore, TLR2 is able to recognize atypical types of LPS from *Leptospira interrogans* and *Porphyromonas gingivalis* in contrast to TLR4, which recognizes LPS from enterobacteria such as *E. coli* and *Salmonella spp.* (Hirschfeld, Weis et al. 2001; Werts, Tapping et al. 2001) (Figure 9). The two types of LPS differ structurally in the number of acyl chains in the lipid A component which presumably confers differential recognition (Netea, van Deuren et al. 2002).

TLR2 has also been shown to cooperate with other TLR family members, in particular TLR6 and TLR1 (Wyllie, Kiss-Toth et al. 2000). Macrophages from TLR6-deficient mice do not produce inflammatory cytokines in response to diacyl lipopeptides derived from mycoplasma, but show normal production of inflammatory cytokines in response to triacyl lipopeptides from Gram-negative bacteria (Takeuchi, Hoshino et al. 2000). In contrast, macrophages from TLR1-deficient mice show a normal response to diacyl lipopeptides, but an impaired response to triacyl lipopeptides (Takeuchi, Kaufmann et al. 2000). Therefore it seems like the functional association of TLR1 and TLR6 with TLR2 allows the cell to discriminate between subtle differences of diacyl and triacyl lipopeptides (Takeuchi, Kawai et al. 2001).

5.3 TLR-mediated signaling pathways

Upon activation with microbial products, TLR form homodimers leading to a conformational change in the cytoplasmic TIR domain and the recruitment of an adaptor protein known as MyD88 (O'Neill and Greene 1998). MyD88 has a TIR domain in its C-terminal portion which allows it to associate with TLR via homophilic interaction. The death domain in its N-terminal portion allows MyD88 to recruit IRAK to the receptor complex through interaction of the death domains of both molecules (O'Neill and Greene 1998). IRAK is then autophosphorylated and dissociated from the receptor complex, and recruits TRAF6 (Li, Strelow et al. 2002), which is in turn responsible for the activation of downstream kinases. Several of those kinases have been found to be involved in TLR/NF-κB signaling pathways including NIK and MEK kinase (MEKK)1 (Karin and Ben-Neriah 2000) (Figure 10).

MyD88 is crucial for the TLR-mediated cytokine production as shown by knockout mice after stimulation with TLR ligands (Adachi, Kawai et al. 1998; Alexopoulou, Holt et al. 2001; Hemmi, Kaisho et al. 2002; Li, Strelow et al. 2002; Suzuki, Suzuki et al. 2002). On

the other hand, recent accumulating evidence indicates that there is also a MyD88-independent pathway that is peculiar to the TLR3- and TLR4 signaling pathways (Akira, Takeda et al. 2001; Alexopoulou, Holt et al. 2001). In the MyD88-independent pathway, LPS stimulation leads to activation of the IFN regulatory factor (IRF)-3, which induces IFNβ. IFNβ in turn, activates signal transducer and activator of transcription (STAT)1, leading to the induction of several IFN-inducible genes (Doyle, Vaidya et al. 2002).

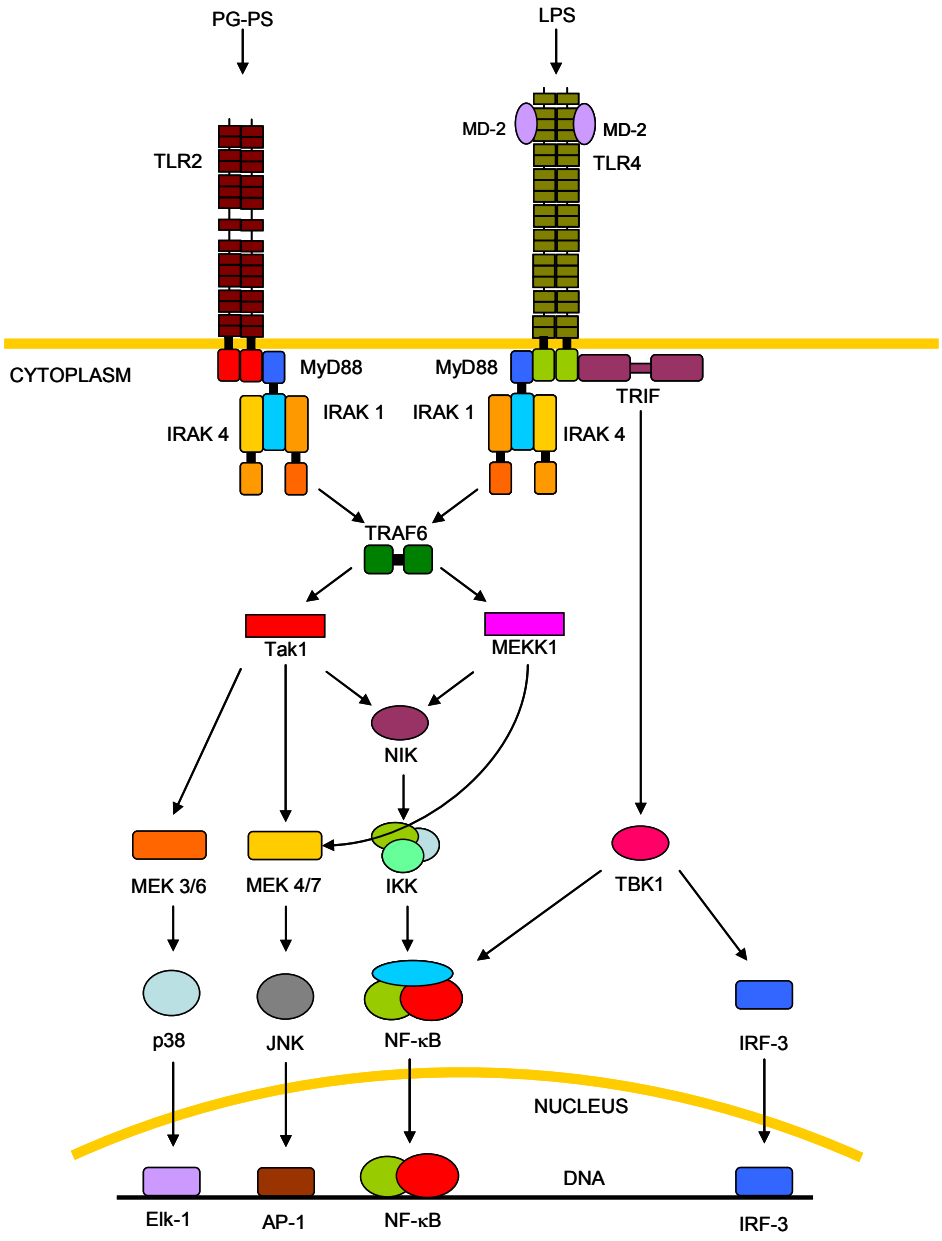

Figure 10: TLR signaling pathways. All TLRs utilize the adapter protein MyD88 leading to NF-κB and MAPKs activation in a TRAF6 dependent manner. In adition, TLR4 and TLR3 activate IRF-3 in a MyD88 independent manner using the TIR containing adaptor TRIF.
Abbreviations: TRIF, TIR domain containing adaptor inducing IFNβ; TBK, TRAF-associated NF-κB activator (TANK)-binding kinase

6 MAPKs

As pointed out in the previous chapters, cells recognize and respond to extracellular stimuli by engaging a complex network of interacting proteins and signaling pathways. One of these signalling cascades leads to the activation of several cytoplasmic protein kinases known as MAPKs. All eukaryotic cells have multiple MAPK pathways, which coordinately regulate a multitude of cellular processes including proliferation, differentiation, development and inflammation (Marshall 1995).

So far, five different groups of MAPKs have been characterized in mammals: ERKs 1 and 2 (ERK1/2), c-Jun amino-terminal kinase (JNK)1 and JNK2, the p38 isoforms α, β, γ and δ, ERKs 3 and 4, and ERK5 (Chen, Gibson et al. 2001; Kyriakis and Avruch 2001). To date, the best characterized groups of vertebrate MAPKs are the ERK1/2, JNKs, and p38 kinases. Although MAPKs can be activated by a wide variety of different stimuli, ERK1/2 are mainly activated by growth factors and phorbol esters, while JNK and p38 kinases are more responsive to stress stimuli, such as ionizing radiation and cytokines (Pearson, Robinson et al. 2001).

Several characteristics are sheared by all MAPK pathways. Each family of MAPKs is composed of a set of three evolutionarily conserved, sequentially acting kinases: a MAPK, a MAPK kinase (MAPKK), and a MAPKK kinase (MAPKKK). The MAPKKKs are serine/threonine kinases which activation leads to the phosphorylation and activation of MAPKKs which are members of the MEK family. MEKs in turn stimulate MAPK activity through dual phosphorylation on threonine and tyrosine residues. Once activated, MAPKs phosphorylate target substrates on serine or threonine residues, including phospholipases, cytoskeletal proteins and transcription factors (Figure 11).

In the last years, it has become clear that most of mammalian MAPK pathways, in conjunction with the NF-κB pathway, are central to stress and inflammatory responses rather than to mitogen responses. NIK, for instance, is an additional MAKKK that is a specific activator of the NF-κB pathway (Malinin, Boldin et al. 1997). ERK1/2 are expressed in all mammalian tissues and are activated by growth factors, serum, phorbol esters, ligands of the heterotrimeric G protein-coupled receptors and cytokines (Lewis, Shapiro et al. 1998). ERK1/2 signaling is a key regulator of cell proliferation and its activation leads to the phosphorylation of numerous substrates including nuclear

substrates such as steroid receptor coactivator (SRC)-1, Elk-1, c-Fos and STAT3. In mammalian cells, the p38 isoforms are strongly activated by environmental stresses including oxidative stress, UV irradiation and numerous mediators of inflammation, including cytokines such as IL-1 and TNF, chemokines and LPS. The MAPK p38 pathway has being shown to be crucial for normal immune and inflammatory responses and upon activation p38 phosphorylates several cellular targets, including cytosolic phospholipase A2 and transcription factors such as ATF-1 and -2, Elk-1 and NF-κB (Lee, Laydon et al. 1994; Roux and Blenis 2004). JNK1, JNK2, and JNK3 are ubiquitously expressed in mammalian cells and are strongly activated in response to cytokines, UV irradiation, DNA-damaging agents and growth factors. JNKs are know to phosphorylate several transcription factors such as c-Jun, ATF-2, heat shock factor (HSF)-1, and STAT3 (Weston and Davis 2002).

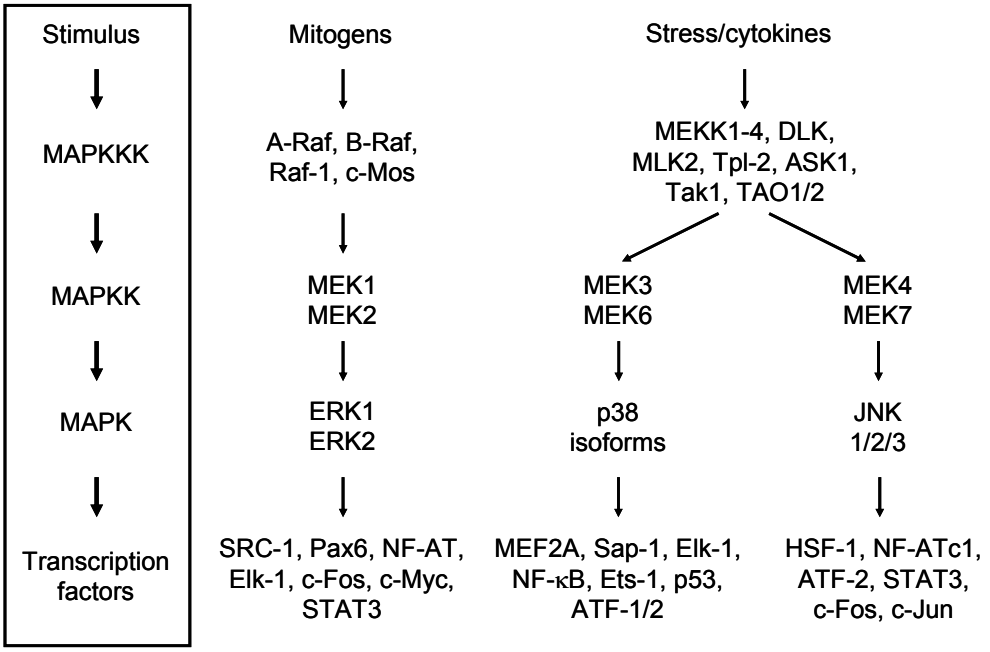

Figure 11: MAPK signalling pathways. Mitogens target cells surface receptors such as tyrosine kinases and G protein-coupled receptors, which transmit signals to the Raf/MEK/ERK cascade through the small GTP-binding protein. Likely, stress and pro-inflammatory signals activate a multitude of MAPKKK, for example, MEKKs 1 to 4 and Tak1, which in turn activate the MAPKK MEK3 and MEK6 leading to the activation of p38, or MEK4 and MEK7 which trigger JNK activation.
Abbreviations: Pax, paired homeobox; NF-AT, NF of activated T cells; DLK, dual leucine zipper bearing kinase; MLK, mixed lineage kinase; Tpl, tumor progression locus; ASK, apoptosis signal-regulating kinase; TAO, thousand and one amino acid protein kinase; Sap, SRF accessory protein; NF-ATc, NF-AT, cytoplasmic, calcineurin-dependent

7 Cytokine signaling and IBD

The inflammatory response consists of the sequential release of mediators such as pro-inflammatory cytokines, including IL-1, TNF, IFNγ, IL-12, IL-18 and GM-CSF, and the recruitment of circulating leukocytes, which become activated at the inflammatory site and in turn release further mediators. Activated cells require negative feedback mechanisms to terminate pro-inflammatory processes and in most cases the inflammatory response is resolved by the release of endogenous anti-inflammatory cytokines such as IL-4, IL-13, IL-10 and TGF-β, the latter being of high relevance in IBD. The persistent accumulation and activation of leukocytes are a hallmark of chronic inflammation, suggesting a dysfunction of these host-derived regulatory mechanisms in controlling the activation of the intestinal epithelium. Current clinical approaches to the treatment of inflammation focus mostly on the inhibition of pro-inflammatory mediator production and the suppression of the initiation of the inflammatory response.

The major producers of cytokines are macrophages, T-cells and mast cells, but other cells such as fibroblasts and epithelial cells are also capable of producing them. The secreted cytokines bind to their own receptors, activating signalling cascades which ultimately activate transcription factors such as Smads, STATs, IRFs and NF-κB (Hanada and Yoshimura 2002). Sequence-specific transcription factors recruit coactivators with intrinsic HAT activity, such as cAMP response element (CREB) binding protein (CBP)/p300. These coactivators destabilize chromatin by mechanisms including histone acetylation and interaction with others proteins allowing the binding of other cofactors and RNA polymerase II to the promoter of target genes (Goodman and Smolik 2000), thus the recruitment of cofactors modulate the transcriptional activity of transcription factors (Shikama, Chan et al. 2000).

7.1 TGF-β/Smad signaling

The TGF-β family, including TGF-β1, TGF-β2 and TGF-β3 in mammals, are a family of cytokines involved in many biologic processes including development, tissue repair and tumorigenesis on a wide range of cell types. Additionally the members of TGF-β factors have a unique and essential role in immune regulatory responses and inflammation, as shown in functional studies *in vitro* as well as *in vivo* experimental models of disease (Wahl 1992; Geiser, Letterio et al. 1993; Kulkarni, Huh et al. 1993). For instance, it has been shown that disruption of TGF-β signaling in the intestinal epithelium of mice

expressing a dominant-negative TGF-β type II receptor leads to colitis (Hahm, Im et al. 2001). In addition, TGF-β knockout mice develop massive inflammation under conventional conditions (Kulkarni, Ward et al. 1995; Hanada and Yoshimura 2002).

TGF-β signals by stimulating the formation of specific heteromeric complexes of type I and type II serine/threonine kinase receptors. This leads to the phosphorylation of the receptor-regulated Smad2 and Smad3 (Moustakas, Souchelnytskyi et al. 2001), which in turn associate with the common mediator Smad4. This complex translocates into the nucleus and binds to the promoter of TGF-β target genes (Figure 12).

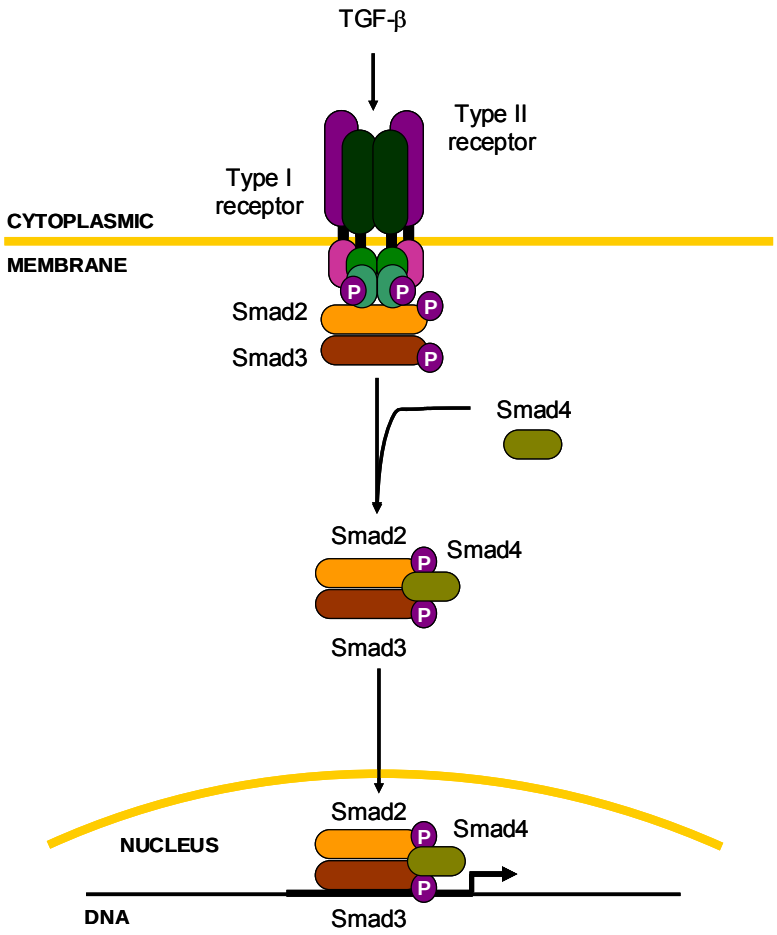

Figure 12: TGF-β/Smad signaling pathway. TGF-β induces the association of two type I and two type II receptors. Both receptor components have a serine/threonine protein kinase domain in the cytoplasmic region. The type II receptor phosphorylates the type I receptor, which in turn catalyses the phosphorylation in C-terminal serine residues of receptor-regulated Smad2 and Smad3 prior binding of the common mediator Smad4. The resulting Smad complex translocates into the nucleus together with DNA-binding cofactors and binds to the promoter of TGF-β target genes (Heldin, Miyazono et al. 1997).

The inhibitory Smad6 and Smad7 antagonizes TGF-β signaling by interfering with the binding of Smad2 and Smad3 with the activated receptor complex (Pulaski, Landstrom et al. 2001). Since expression of Smad7 is induced by TGF-β, it is apparently a negative-feedback regulator of the TGF-β/Smad pathway (Nakao, Afrakhte et al. 1997). The three principal MAPK pathways in mammalian cells, ERK, p38 and JNK have been shown to affect the TGF-β/Smad signalling pathway, by suppressive as well as inductive mechanisms. Conversely, TGF-β can stimulate JNK and p38 in various cell types (Massague and Chen 2000).

7.2 IL-10 signaling pathway

The anti-inflammatory cytokine IL-10 was first identified as an inhibitor of T cells, monocytes and macrophages activity. Although IL-10 is a multifunctional cytokine with diverse effects on a variety of cell types, such as cell growth and differenciation, the principal function of IL-10 is to restrict and ultimately terminate pro-inflammatory processes (Moore, de Waal Malefyt et al. 2001). As already indicated, IL-$10^{-/-}$ mice develop chronic intestinal inflammation, indicating the critical function of IL-10 as regulator of inflammation *in vivo* (Kuhn, Lohler et al. 1993). The functional IL-10 receptor (IL-10R) complexes are tetramers consisting of two IL-10R1 polypeptide chains and two IL-10R2 chains. Binding of IL-10 to the extracellular domain of IL-10R1 induces phosphorylation of the receptor-associated Janus kinase (JAK)1 and tyrosine kinase 2 (Tyk2), and activation of STAT3 (O'Farrell, Liu et al. 1998). STAT3 homodimerizes and translocates to the nucleus where it binds with high affinity to STAT-binding elements (SBE) in the promoters of IL-10-responsive genes (Donnelly, Dickensheets et al. 1999) (Figure 13). The JAK/STAT pathway is also crucial for the signal transduction of many other cytokines and growth factors, such as IL-6 (Hirano, Ishihara et al. 2000) and IFN (Nguyen et al., 1997; Stark et al., 1998) (Figure 13).

Although the molecular mechanisms of the inhibitory properties of IL-10 are still unclear, IL-10 has been shown to inhibit pro-inflammatory signaling cascades by inducing suppressors of cytokine signaling (SOCS)-mediated inhibitory mechanisms (Alexander and Hilton 2004). In addition, studies in mice have demonstrated that IL-10 induces the expression of heme oxygenase (HO)-1, a stress inducible protein with anti-inflammatory propperties (Shibahara 1988; Lee and Chau 2002). Interestingly, some studies have suggested interrelated roles for IL-10 and TGF-β in maintaining epithelial cell homeostasis. Thus, IL-10 protective mechanisms in TNBS-induced experimental colitis

have been shown to be mediated through its inductive effect on TGF-β secretion in lamina propria T cells (Fuss, Boirivant et al. 2002).

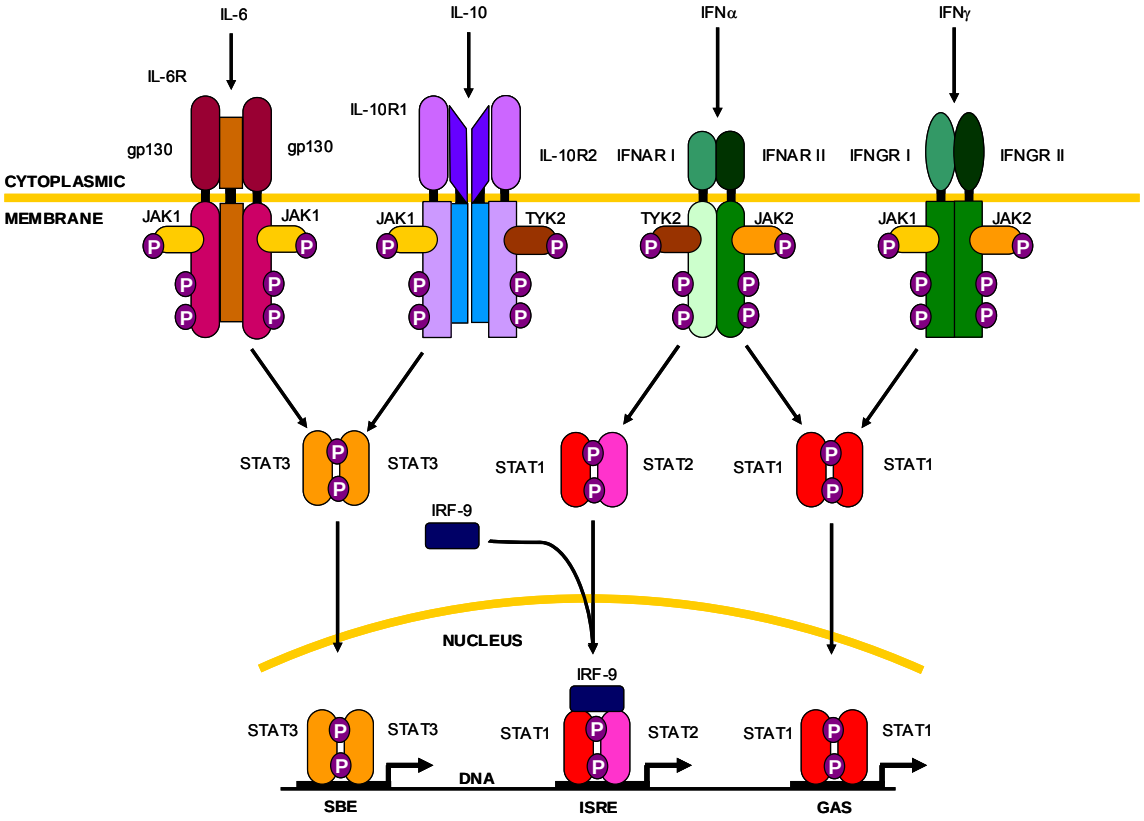

Figure 13: JAK/STAT signaling pathway. Binding of cytokines to their respective receptors, activate receptor-associated JAKs, which in turn phosphorylate STATs. Activated STATs form homodimers or heterodimers that translocate to the nucleus where bind to the promoter of cytokine-dependent genes. IL-10 triggers phosphorylation of JAK1 and Tyk2. These kinases phosphorylate specific tyrosine residues on the intracellular domain of the IL-10R1 chain comforming docking sites for the latent transcription factor STAT3, which in turn is tyrosine-phosphorylated by the receptor-associated JAKs. STAT3 homodimerizes and translocates the nucleus where it binds to SBE. Other cytokines, such as the pro-inflammatory cytokine IL-6 mainly target JAK1 and STAT3.
Abbreviations: IFNAR, IFNα receptor; IFNGR, IFNγ receptor; ISRE, IFN-stimulated response element; GAS, IFNγ-activated-sit.

AIMS OF THE WORK

The general objective of the present thesis was to gain insight in the molecular mechanisms involved in the inhibition of pro-inflammatory processes in IECs, with special emphasis in the downregulation of the NF-κB signaling pathway. For this purpose, germ-free animals as well as animal models of chronic intestinal inflammation such as $TNF^{\Delta ARE}$ mice where used to investigate the molecular events underlying activation of intestinal inflammation *in vivo*. Experiments *in vitro* were carried out in the mouse colon carcinoma epithelial cell line CMT-93 and the mouse small IEC line Mode K. The main strategies of this study can be summarized as follows: characterization of pro-inflammatory cellular mechanisms induced by enteric bacteria and pro-inflammatory cytokines in IECs, and their inhibition by anti-inflammatory prostanoids, suppressive cytokines and flavonoids (Figure 14). Our starting point was to study *Bacteroides vulgatus*-induced NF-κB activation in germ-free rats, with the subsequent analysis of the inhibitory abilities of the antidiabetic drug rosiglitazone and the prostanoid 15d-PGJ_2 in Gram-negative bacteria-induced inflammation in CMT-93 cells. We were also interested in the molecular mechanisms of the protective effects of probiotic bacteria. For this purpose we compared the pro-inflammatory response in the mucosal epithelium of germ-free rats after monoassociation with *B. vulgatus* and the probiotic *Bifidobacterium lactis* strain BB12. Furthermore, we sought to characterize *B. lactis* BB12-induction of NF-κB and MAPK signalling pathways in Mode K cells. We also explored *Enterococcus faecalis* colitogenic properties in IECs, with special interest in the TLR signalling pathways. Additionally, we analyzed the mechanisms for TGF-β-mediated inhibition of *E. faecalis*-induced pro-inflammatory gene expression in Mode K cells, with particular interest in recruitment of NF-κB to the promoter of pro-inflammatory cytokines. The signal transduction pathways induced with the anti-inflammatory cytokine IL-10 in Mode K cells were also object of this thesis, with special foccus on cellular processes related to the inflammatory condition, such as TNF-induced ER stress response and especially on the recruitment of transcription factors to the promoter of the ER stress marker glucose regulated protein (Grp)78. Finally, we also wanted to dissect the signal transduction events taking place after stimulation with flavonoids in TNF-activated Mode K cells. Since flavonoids are subjected to degradation by the intestinal microbiota, we explored the inhibitory activities of the flavonol quercetin and its bacterial metabolites in TNF-induced NF-κB pathway activation. In addition we used the animal model for chronic ileitis $TNF^{\Delta ARE}$ mice to study the potential therapeutic properties of quercetin *in vivo*.

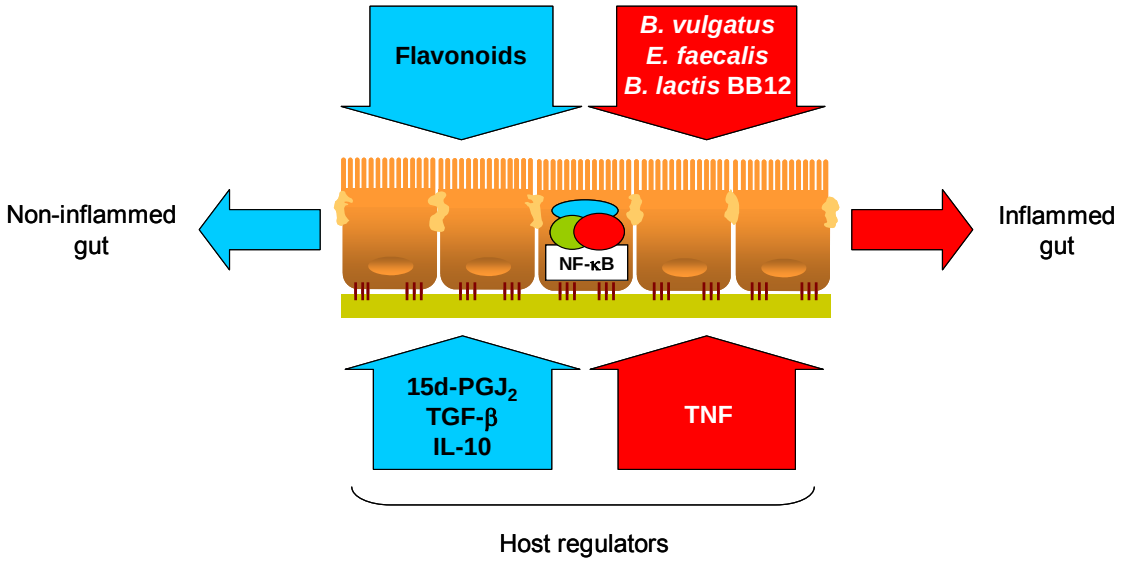

Figure 14: Aims of the work. The purposes of this work can be schematized as follows: Characterization of pro-inflammatory processes induced by pro-inflammatory cytokines and enteric bacteria, including colitogenic (*B. vulgatus*) and probiotic bacteria (*B. lactis* BB12) in IECs. The study of the molecular mechanisms implicated were conducted with special emphasis in regulation of NF-κB phosphorylation/activation as well as activation of other pro- and anti-inflammatory signaling cascades such as MAPK, recruitment of transcription factor to the promoter of diferent pro- and anti-inflammatory mediators, and gene expression. In addition we sought to investigate the suppressive effects of host-derived anti-inflammatory mediators, including prostanoids (15d-PGJ_2), suppressive cytokines, such as TGF-β and IL-10, and flavonoids in the context of chronic inflammation.

RESULTS AND DISCUSSION

1 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 inhibits Gram-negative bacteria-induced pro-inflammatory responses in IECs through modulation of protein phosphatase 2A activity

Non-pathogenic enteric bacteria are an important factor for the development of IBD, but studies in animal models of experimental colitis have shown that some resident enteric bacteria play a more important role than others in inducing chronic inflammation (Rath, Herfarth et al. 1996; Bhan, Mizoguchi et al. 1999). Identification of members of the indigenous microbiota that have a prominent participation in the induction of colitis is therefore of major importance in the treatment of IBD patients (Bhan, Mizoguchi et al. 1999). *Bacteroides vulgatus*, a Gram-negative enteric non-pathogenic bacterium, has been shown to be especially important in the development of IBD and immune responses. Thus, this commensal bacteria has been shown to be implicated in the induction of chronic inflammation in germ-free HLA-B27 transgenic rats (Rath, Wilson et al. 1999) and constituted the major stimulus in carrageenan-induced colitis in guinea pigs (Onderdonk, Franklin et al. 1981). Taken together, these results demonstrate a consistent role of *B. vulgatus* in the pathogenesis of chronic intestinal inflammation.

At the molecular level, it has been found that *B. vulgatus* also induces activation of NF-κB in native and IEC lines (Haller, Russo et al. 2002), as well as in the intestinal epithelium of monoassociated germ-free rats (Haller, Holt et al. 2003). Consistent with these results, our data showed that the monoassociation of germ-free Fischer F344 rats with *B. vulgatus* triggered transient phosphorylation of RelA at serine 536 at day 3 after colonization. Interestingly, the downregulation of NF-κB at day 7 coincided with the arise of stable expression of PPARγ, suggesting a regulatory role of this steroid receptor in NF-κB activation (*Appendix 1, Figure 1*).

PPARs are members of the nuclear hormone receptor superfamily. Upon stimulation, PPARs form a complex with the 9-*cis*-retinoic acid receptor (RXR) and translocate to the nucleus to bind to peroxisome proliferator response elements. So far, three PPARs isoforms have been described: PPARα, PPARβ/δ and PPARγ, the last being mainly expressed in adipose tissue and colonic epithelium (Fajas, Auboeuf et al. 1997). PPARγ was originally identified because of its critical participation in the expression of genes

involved in adipocyte differentiation, lipid storage and glucose homeostasis (IJpenberg, Jeannin et al. 1997; Barak, Nelson et al. 1999). Nevertheless, in the last years PPARγ has emerged to play an important role in inflammation. For instance, several studies have shown that activation of PPARγ can interfere *in vitro* with inflammatory pathways, such as NF-κB (Chung, Kang et al. 2000), as well as the activator protein (AP)-1 and the STAT1 signaling cascades (Delerive, De Bosscher et al. 1999; Li, Pascual et al. 2000). As a consequence, PPARγ activators such as fatty acids and arachidonic acid metabolites (Kliewer, Lenhard et al. 1995), have been found to modulate the production of inflammatory cytokines, chemokines and cell-adhesion molecules, therefore leading to the reduction of immune cell recruitment (Jiang, Ting et al. 1998; Auwerx 2002). Disruption of the PPARγ gene in mice ($PPAR^{-/-}$) results in embryonic lethality. Nonetheless, heterozygous $PPAR\gamma^{+/-}$ and $RXR\alpha^{+/-}$ mice have been shown to be more susceptible to TNBS-induced colon inflammation (Desreumaux, Dubuquoy et al. 2001). In addition, a remarkably reduced PPARγ expression has been reported in DSS-treated mice (Katayama, Wada et al. 2003) as well as in patients with UC, where members of the normal microbiota have been shown to counteract this effect enhancing PPARγ expression (Dubuquoy, Jansson et al. 2003). Further evidence for the negative regulation induced by commensal bacteria in the intestinal epithelium was the finding that the enteric bacterial species *Bacteroides thetaiotaomicron* induced PPARγ-mediated nuclear export of activated NF-κB in cultured IECs (Kelly, Campbell et al. 2004). Despite of the amount of data accumulated in the last decades, the function of PPARγ in the colonic epithelium is still unclear.

The antidiabetic agents termed thiazolidinediones (TZDs), such as ciglitazone, pioglitazone and rosiglitazone are highly specific PPARγ ligands, being also potent activators of this transcription factor (Lehmann, Moore et al. 1995). Treatment with these synthetic PPARγ agonists has been shown to attenuate colitis induced by oral administration of DSS (Tanaka, Kohno et al. 2001), as well as by intrarectal administration of TNBS in rodents (Desreumaux, Dubuquoy et al. 2001).

The PGD_2 metabolite 15d-PGJ_2 (Figure 15) is a potent and specifc low affinity PPARγ ligand (Khan 1995; Su, Wen et al. 1999). This cyclopentenone prostanoid has been shown to be produced *in vivo* during the resolution phase of acute inflammation (Gilroy, Colville-Nash et al. 1999) and it is also expressed in large quantities by macrophages *in vitro* (Shibata, Kondo et al. 2002). In addition it has also been found that

15d-PGJ_2 is able to inhibit iNOS expression and activity in murine macrophages (Saltiel 2001), as well as NO production in microglial cells (Kitamura, Kakimura et al. 1999).

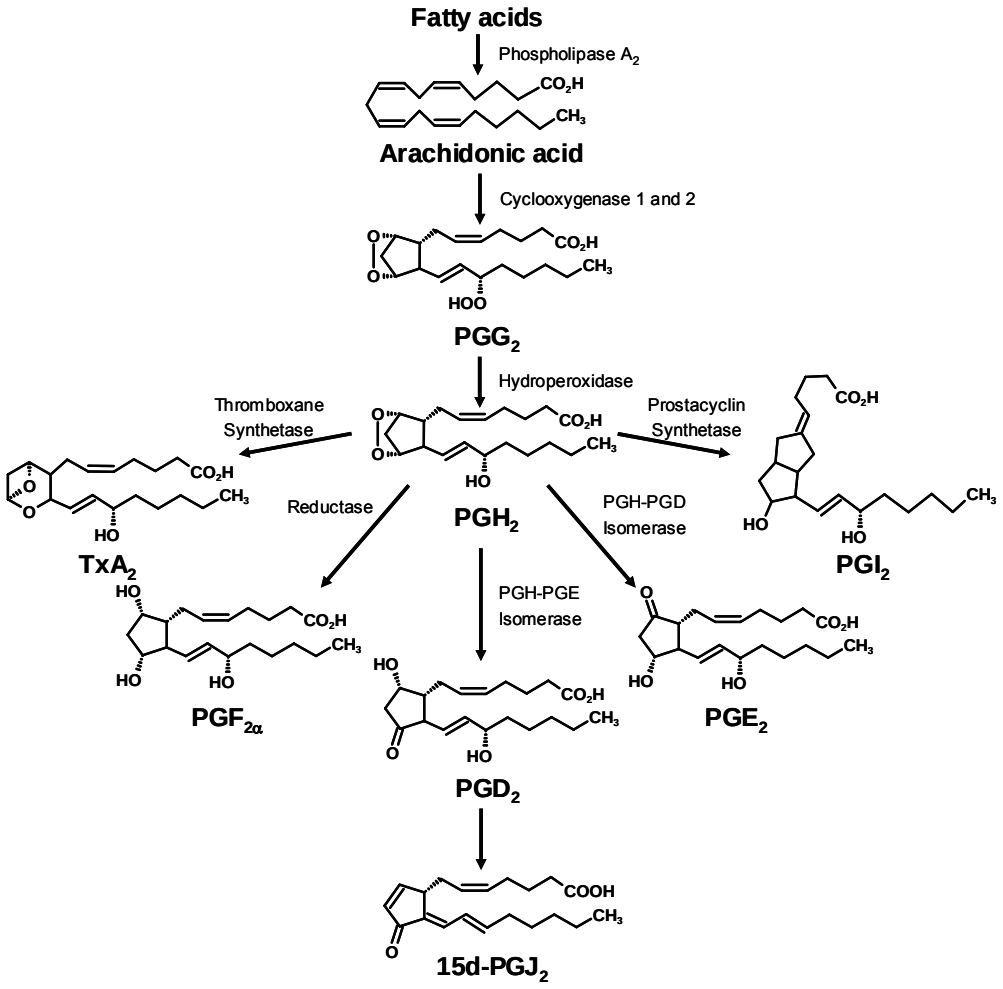

Figure 15: Arachidonic acid pathway. Upon stimulation, lipids are released from the plasma membrane by phospholipases and metabolized by COXs and other enzymes into prostanoids, including PGs and TXs. During an inflammatory response the production of prostanoids is much more increased prior leukocite recruitment and during tissue infiltration of immune cells. Also the profile of prostanoid production can change dramatically (Tilley, Coffman et al. 2001).

In order to gain further insight in the molecular mechanisms underlying the anti-inflammatory effects of PPARγ in IECs, we stimulated the mouse colonic epithelial cell line CMT-93 with the TZD rosiglitazone (also called BRL49653) and 15d-PGJ_2. As expected, the stimulation of CMT-93 cells with both *B. vulgatus* and *E. coli* LPS triggered NF-κB phosphorylation at serine 536 of RelA after 1 hour of stimulation. 15d-PGJ_2 was able to inhibit LPS-induced RelA phosphorylation after 1 and 2 hours of stimulation. Surprisingly, RelA phosphorylation was not inhibited in the presence of the high affinity

PPARγ ligand rosiglitazone upon stimulation with LPS (*Appendix 1, Figure 2*). 15d-PGJ_2 was also able to inhibit IκBα phosphorylation after 2 hours of LPS stimulation as well as IL-6 mRNA expression and protein secreted, but not IκBα degradation, RelA nuclear translocation or RelA binding activity in IECs. Rosiglitazone again showed no effect through any of these steps of NF-κB signaling (*Appendix 1, Figures 3-4*), suggesting a PPARγ-independent effect in the inhibition of NF-κB signaling pathway through 15d-PGJ_2.

Recent published work has suggested that PPARγ ligands may exert their anti-inflammatory effects independently of PPARγ. For instance, Hinz et al. showed inhibition of cytokine production in LPS-stimulated human blood monocytes through 15d-PGJ_2, but not TZDs, pointing again towards PPARγ-independent mechanisms of 15d-PGJ_2 (Hinz, Brune et al. 2003). Additionally, it has been reported that PPARγ is not required by 15d-PGJ_2 to inhibit the inflammatory response in LPS-stimulated wild-type and PPARγ-deficient macrophages (Chawla, Barak et al. 2001). Some PPARγ-independent mechanisms have been proposed for 15d-PGJ_2 to inhibit NF-κB activity, such as direct modification and inhibition of IKKβ activation (Rossi, Kapahi et al. 2000). In order to address this question, we knocked-down PPARγ by transfecting CMT-93 cells with short interfering RNA (siRNA). siRNA is a non-coding RNA 21-23 long base pairs, which after incorporation into a RNA-induced silencing complex (RISC), hybridize specifically with a mRNA target mediating its selective enzymatic degradation (Sontheimer 2005). Our results showed that 15d-PGJ_2 inhibited LPS-induced pro-inflammatory signaling trough PPARγ-independent mechanisms (Figure 16).

The inhibitory effects of 15d-PGJ_2 seem to be specific for this PG since PGE_2, a metabolite of PGH_2 (Figure 15) was unable to reproduce the suppressive effects of 15d-PGJ_2 on LPS-induced RelA phosphorylation and IL-6 mRNA expression (*Appendix 1, Figure 5*). These results are consistent with the finding that elevated expression levels of the pro-inflammatory PGE_2 was observed in a model for carrageenan-induced acute inflammation at early stages. In contrast, the resolution stage was associated with low expression levels of PGE_2 and high levels of anti-inflammatory PGs, such as PGD_2 and 15d-PGJ_2 (Gilroy, Colville-Nash et al. 1999).

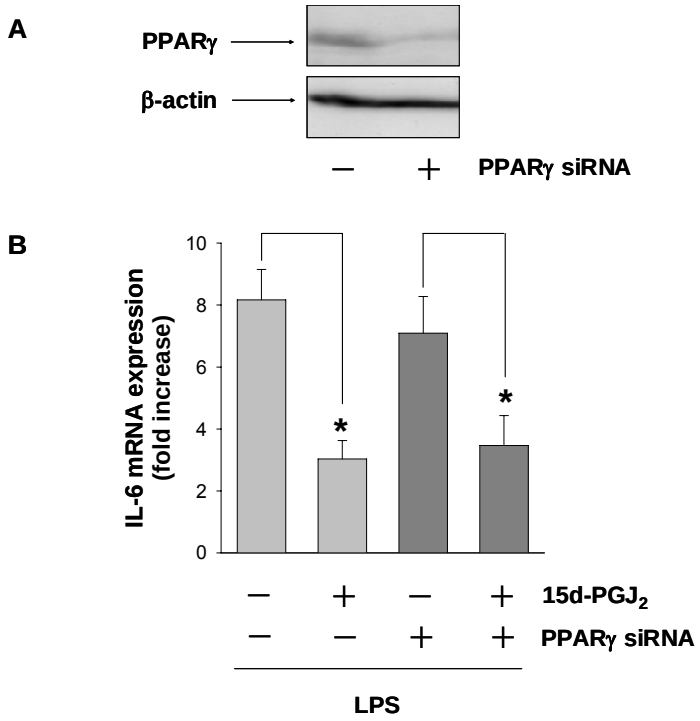

Figure 16: knock-down of PPARγ in CMT-93 cells. PPARγ was knocked-down (target sequence: AAAGACCCAGCTCTACAACAG) by transfecting cells with 16µl of TransMessenger Reagent (Quiagen) and 0.8 µg of the following siRNA sequences: sense r(AGACCCAGCUCUAC AACAG)d(TT), antisense r(CUGUUGUAGAGCUGGGUCU)d(TT) (Quiagen). 48 hours after siRNA transfection, PPARγ protein expression was significantly reduced. ß-actin shows equal sample loading (A). Real-time quantitative PCR analysis (Light cycler, Roche) using GAPDH as houskeeping gene for normalization, showed that 15d-PGJ_2 was able to inhibit LPS-induced IL-6 mRNA expression in the presence as well as in the absent of PPARγ (B) indicating PPARγ-independent mechanisms for 15d-PGJ_2-mediated inhibition of pro-inflammatory processes.
Abbreviations: PCR, polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

It has been reported that stimulation with 15d-PGJ_2 triggers phosphorylation of the MAPK ERK 1/2 in murine myoblasts (Huang, Chio et al. 2002), as well as in vascular smooth muscle cells (Takeda, Ichiki et al. 2001). Accordingly, our data showed phosphorylation of ERK 1/2 upon stimulation with LPS and 15d-PGJ_2. In the presence of the MEK1 pharmacological inhibitor PD98059, the 15d-PGJ_2-mediated inhibition of LPS-induced RelA phosphorylation was partially reversed and IL-6 mRNA expression was completely abolished, indicating a crucial role of the MEK/ERK cascade in 15d-PGJ_2-mediated inhibition (*Appendix 1, Figure 6*).

Serine/threonine phosphatases, including protein phosphatase (PP)1, PP2A, PP2B and PP2C, are involved in the regulation of various signal transduction in proceses including cell proliferation and metabolism, and also play a role in the regulation of the NF-κB

signaling pathway (Zolnierowicz 2000). The treatment of CMT-93 cells with Calyculin A, a serine/threonine phosphatase inhibitor, reversed 15d-PGJ_2-mediated inhibition of LPS-induced RelA phosphorilation and IL-6 mRNA expression (*Appendix 1, Figures 7 A-B*) suggesting that 15d-PGJ_2 inhibitory effects require the activity of one or more serine/threonine phosphatases. PP2A comprises a family of a non-calcium dependent serine/threonine phosphatases widely expressed in mammalian cells, which functions include cell cycle regulation, cell morphology and development, and regulation of specific signal transduction cascades. PP2A is composed of a 36-kDa catalytic subunit, a 65-kDa regulatory subunit A and several variable regulatory subunits that confer substrate specificity to the enzymatic activity (Mumby and Walter 1993; Janssens and Goris 2001). *In vivo*, PP2A has been shown to dephosphorylate preferently protein kinases and transcription factors, and is constitutively associated with RelA (Janssens and Goris 2001). In addition, the specific inhibition of PP1 and PP2A with okadaic acid induced the activation and translocation of NF-κB to the nucleus in Jurkat cells (Thevenin, Kim et al. 1990). Furthermore, Yang et. al presented the first evidence for a specific physical association between RelA and the regulatory subunit A of PP2A, which was involved in RelA dephosphorylation (Yang, Fan et al. 2001). Seeking for an inhibitory mechanism of 15d-PGJ_2 on the NF-κB signalling pathway, we addressed the question whether 15d-PGJ_2 could also activate PP2A in CMT-93 cells. As shown in *Figure 7C* (*Appendix 1),* the immunoprecipitation of P-RelA and PP2A from LPS- and 15d-PGJ_2-treated samples respectively and their posterior co-incubation, leaded to the dephosphorylation of RelA after 1 hour of co-incubation. This result represents a novel mechanism by which 15d-PGJ_2 regulates the activity of PP2A that in turn directly dephosphorylate LPS-induced phospho-RelA.

2 Mechanisms for *Bifidobacterium lactis* BB12-induced transient pro-inflammatory processes in the intestinal epithelium

Bacteria- and host-derived signals are integrated into the intestinal epithelium in order to maintain intestinal homeostasis. Besides the deleterious impact of the intestinal microbiota, many studies have shown that certain members of the normal microbiota exert protective effects on the host against the actions of pathogens (Shanahan 2000). The molecular mechanisms underlying the activation of the intestinal epithelium by probiotic bacteria at early stages of bacterial colonization may play an important role in the initiation and mainteinance of the complex homeostasis between non-pathogenic enteric bacteria and the host (Haller 2006).

There is an extensive amount of literature addressing the possible health benefits associated with the consumption of probiotic microorganisms. Lactic acid bacteria (Streptococcus, Lactobacillus, Lactococcus) and bifidobacteria are the most used as probiotic because of their known inmmunemodulatory effects (Silva, Barbosa et al. 2004). Bifidobacteria are especially attractive as potential probiotics as they constitute one of the predominant populations of the normal microbiota in humans and animals and are extremely well adapted to this ecosystem (Gomes and Malcata 1999).

Reported beneficial effects of Bifidobacteria include stabilizing the gut mucosal barrier, prevention and treatment of viral diarrhoea, modulation of intestinal microbiota, antibacterial activities, and alleviation of IBD (Kailasapathy and Rybka 1997; Ouwehand, Salminen et al. 2002; Reid, Jass et al. 2003; Saarela, Hallamaa et al. 2003). These anaerobic Gram-positive bacteria have been shown to enhance humoral immune responses (Lee, Ametani et al. 1993; Ko, Goh et al. 1999; Shu, Qu et al. 2001), as well as cellular immune responses *in vivo* (Ulisse, Gionchetti et al. 2001). In addition some bifidobacteria strains have been found to have the ability of inhibiting binding of enteropathogens onto human intestinal mucus and cultured IECs (Collado, Gueimonde et al. 2006; Trejo, Minnaard et al. 2006). The treatment of IL-$10^{-/-}$ mice with VSL no. 3, a probiotic product based on bifidobacteria, induces normalization of colonic physiological function and barrier integrity, as well as reduction in secretion of the pro-inflammatory cytokines TNF and IFN-c (Madsen, Cornish et al. 2001). Furthermore the oral

administration of VSL no. 3 induced remision in patients with UC (Bibiloni, Fedorak et al. 2005).

Species of bifidobacteria used for probiotic purposes include *B. breve*, *B. longum* and *B. animalis* (*lactis*) (Fasoli, Marzotto et al. 2003; Temmerman, Pot et al. 2003), the last two being related to an increased resistance to intestinal infection with Salmonella typhimurium in mice (Shu, Lin et al. 2000; Silva, Barbosa et al. 2004). In addition, *B. infantis* attenuates colitis in IL-$10^{-/-}$ mice (McCarthy, O´Mahony et al. 2003) and has been shown to prevent inflammation in DSS-induced rat colitis (Osman, Adawi et al. 2004). *B. lactis* has been shown to enhance immunity in animal models (Arunachalam, Gill et al. 2000) as well as in human (Gill, Rutherfurd et al. 2000). Furthermore, *B. lactis* strain BB12 enhance cellular immune responses, including phagocytosis, lymphocyte proliferation and cytokine production *in vivo* (Schiffrin, Brassart et al. 1997) and yogurt containing *B. lactis* BB12 had a suppressive effects *in vitro* on *Helicobacter pylori*, a bacterium related to severe gastritis (Wang, Li et al. 2004).

In order to understand host-bacteria interactions at IEC level and gain insight into the molecular mechanisms for the probiotic activity of *B. lactis* BB12 *in vivo*, germ-free Fisher rats were monoassociated with *B. lactis* BB12 and *B. vulgatus*, a Gram-negative bacterium especially important in the pathogenesis of chronic inflammation *in vitro* and *in vivo* as discussed in *Appendix 1*.

Both *B. vulgatus* and *B. lactis* BB12 induced RelA phosphorylation at serine 536 in isolated primary IECs after 5 days of bacterial monoassociation (*Appendix 3, Figure 1a*). Of note, *B. lactis* BB12, but not *B. vulgatus* induced phosphorylation of the MAPK p38, also after 5 days of colonization (*Appendix 3, Figure 1b*). Consistent with the activation of NF-κB, *B. vulgatus* and *B. lactis* BB12 induced IL-6 mRNA expression in IECs. Interestingly, the probiotic *B. lactis* BB12 induced a much higher expression of this pro-inflammatory cytokine than *B. vulgatus*, reaching the maximal expression on day 5 and decreasing to basal levels on day 7 of monoassociation (*Appendix 3, Figure 2a*). Although *B. lactis* BB12 induced pro-inflammatory signal transduction and gene expression in IECs, *B. lactis* BB12-monoassociated rats presented no histological signs of inflammation, suggesting that the host developed negative feed-back mechanisms to maintain the mucosal immune homeostasis. Indeed, *B. lactis* BB12 induced a much higher mRNA expression of the NF-κB inhibitory protein A20 than *B. vulgatus*, also after

5 days of bacterial monoassociation (*Appendix 3, Figure 2b*). This seems to be part of the mechanisms that regulate the NF-κB signal transduction pathway.

To further study the molecular mechanisms underlying *B. lactis* strain BB12-induced pro-inflammatory processes in IECs, Mode K cells were stimulated with this Gram-positive bacterium at multiplicity of infection (moi) 30. As shown in *Appendix 3, Figure 3*, *B. lactis* BB12 was also able to reproduce RelA phosphorylation in Mode K cells after 30 minutes of treatment as well as transient p38 phosphorylation. Moreover, *B. lactis* BB12 also induced IL-6 and A20 mRNA expression, with a maximal expression after 3 hours of stimulation (*Appendix 3, Figure 4*), showing the ability of this bacterium to reproduce the pro-inflammatory processes *in vitro*.

Previous studies have already demonstrated that bifidobacteria enhance production of pro-inflammatory cytokines by human and murine cell lines (Marin, Lee et al. 1997), althought different species of bifidobacteria have been found to vary greatly as to the induction of cytokine production (He, Morita et al. 2002). Although still unclear, the correlation between probiotic activities and early induced pro-inflammatory cytokine expression seems to be analogous to tolerance induced by repeatedly stimulation of bacterial products to human beings, animal models and cell lines (Mengozzi and Ghezzi 1993).

The impairment of NF-κB signalling through the adenoviral delivery of dominant negative IKKβ, an upstream kinase involved in RelA phosphorylation, leaded to a significant reduction of *B. lactis* BB12-induced IL-6 protein secretion, providing evidence of the crucial role of NF-κB pathway in *B. lactis* BB12 pro-inflammatory gene expression (*Appendix 3, Figure 5*). The concomitant induction of p38 phosphorylation and NF-κB activation leads to the idea that activation of this MAPK pathway may play a role in the induction of IL-6 expression. Indeed, *B. lactis* BB12-activated Mode K cells showed a significant reduction of IL-6 protein expression in the presence of the specific p38 pharmacological inhibitor SB203580, showing the major role of this MAPK pathway in the induction of pro-inflammatory gene expression in *B. lactis* BB12-stimulated Mode K cells (*Appendix 3, Figure 5*).

Mode K cells are a IEC line immortalized by simian virus (SV)40 large T gene transfer through a murine ecotropic virus (Vidal, Grosjean et al. 1993). Mode K cells were

established from the small intestine of C3H/HeJ mice, which carry a spontaneous point mutation in the cytoplasmic region of the TLR4 gene. This mutation results in the generation of a dominant negative allele and in a lack of response to LPS (Poltorak, He et al. 1998). The fact that Mode K cells are deficient in TLR4-mediated signaling makes these cells especially interesting for studies in which it is important to exclude the role of contaminating LPS in TLR-induced signal transduction.

PGs are polymers of sugars and aminoacids present in the cellular wall of eubacteria. Although also present in Gram-negative bacteria, PGs layer is substantially thicker in Gram-positive bacteria, constituting the major component of their cellular wall. PGs produced by bifidobacteria have been shown to stimulate innate immune responses in a wide variety of hosts (Sasaki, Samegai et al. 1996). In addition, the ability of bifidobacteria to induce cytokine expression varies greatly among the different species of bifidobacteria and also among strains of the same species because of the differences in the PGs (Zhang, Rimpilainen et al. 2001). TLR2 is the receptor responsible for recognition of PGs of Gram-positive bacteria prior induction of innate responses. As showed in *Appendix 3, Figure 7*, this is the case in *B. lactis* BB12-induced pro-inflammatory processes since this Gram-positive bacterium was unable to induce IL-6 protein secretion in TLRΔTIR stable transfected Mode K cells, carrying a non-functional TLR2.

In conclusion, *B. lactis* BB12 triggered pro-inflammatory processes through TLR2 in mode K cells. Bifidobacteria colonize the GI tract shortly after birth comprising the predominant intestinal microbiota in infants (Mitsuoka 1992). The early and transient activation of TLR-mediated pro-inflammatory host responses through probiotic bacteria may be part of the mechanisms for initiating mucosal homeostasis at early stages of bacterial colonization.

3 TGF-β1 inhibits TLR2-mediated *Enterococcus faecalis* induction of NF-κB signaling pathway in IECs

As pointed out in the introduction, diverse studies in models of experimental colitis have shown that an exacerbate immune response to enteric bacteria can cause colitis in a genetically susceptible host. *Enterococcus faecalis*, a common inhabitant of the intestinal tract, is one of the bacterial species reported to induce chronic intestinal inflammation. This conclusion is mainly supported by studies in which germ-free IL-$10^{-/-}$ mice developed colitis after monoassociation with *E. faecalis*, demonstrating the colitogenic potential of this Gram-positive bacteria in a genetically susceptible host (Balish and Warner 2002; Kim, Tonkonogy et al. 2003). Consistent with the ability of *E. faecalis* to induce pro-inflammatory processes in the intestinal mucosa, stimulation of CMT-93 cells with *E. faecalis* at moi 100 triggered RelA phosphorylation at serine 536 after 1 hour of incubation (*Appendix 2, Figure 3A*). In addition, *E. faecalis* induced RelA phosphorylation in the IEC line Mode K, as well as IκBα phosphorylation and subsequent IκBα degradation after 10 and 60 minutes respectively (*Appendix 2, Figure 3B*). Accordingly with the activation of the NF-κB signalling pathway, *E. faecalis* also triggered the expression of the NF-κB regulated pro-inflammatory cytokines IL-6 and INFγ-inducible protein (IP)-10 in Mode K cells with maximal protein secretion after 12 hours of stimulation (*Appendix 2, Figure 4*).

In accordance with the fact that Mode K cells are deficient in TLR4 signaling, LPS was not able to induce IL-6 or IP-10 protein expression in Mode K cells after 12 hours of stimulation, in contrast to *E. faecalis* (*Appendix 2, Figure 5A*). This result indicates that this Gram-negative bacterium does not require TLR4 to induce pro-inflammatory gene expression.

TLR2 is known for its capacity to recognize of lipoteichoic acid, which is present in the cell membrane of Gram-positive bacteria including *E. faecalis* (Takeda, Kaisho et al. 2003). Since Mode K cells have been reported to lack of TLR4, we generated a stable transfected cell line reconstituting Mode K cells with TLR4 and MD2, using a TLR4/MD2 expression vector system. As shown in *Figure 5B (Appendix 2)*, LPS was able to induce IL-6 and IP-10 protein expression in TLR4/MD2 reconstituted Mode K cells, in sharp contrast with non-transfected Mode K cells. This result clarifies that the lack of

responsiveness to LPS in Mode K cells is strictly due to lack of a functional TLR4 and not to an intrinsic defect in the downstream signaling cascade.

Stable transfected Mode K cells with a TLR2ΔTIR expression vector, which results in the expression of TLR2 without TIR domain, presented a significant reduction of RelA phosphorylation as well as IL-6 protein secretion after *E. faecalis* stimulation (*Appendix 2, Figure 7*). In addition, the use of wild-type and TLR2 gene-deficient ($TLR2^{-/-}$) myoembryogenic fibroblasts (MEFs) as a control helped us to further clarify whether *E. faecalis* specifically targets TLR2 to induce NF-κB activation. Thus, whereas *E. faecalis* triggered RelA phosphorylation as well as IL-6 expression in wild-type MEFs, $TLR2^{-/-}$ MEFs did not respond to the stimulation with *E. faecalis* (*Appendix 2, Figure 6*). All together these results showed that *E. faecalis* induces NF-κB activation specifically targeting TLR2.

The over-expression of the dominant negative form of IKKβ has been found to inhibit NF-κB activity, as well as expression of pro-inflammatory proteins in endotelial cells (Oitzinger, Hofer-Warbinek et al. 2001). To further study the implication of NF-κB in *E. faecalis* induced proinflamatory gene expression the NF-κB signaling cascade was impaired by adenoviral delivery of a dominant negative IKKβ protein, which was not functional due to the replacement of a lysine by an alanine at position 44. As a consequence, *E. faecalis*-induced IL-6 and IP-10 mRNA expression was significantly reduced (*Appendix 2, Figure 8*). To further study the implication of NF-κB in *E. faecalis*-induced proinflammatory gene expression, we also used adenoviral delivery of a mutant IκBα, which can not be phosphorylated or degradated. These results demonstrate the crucial role of the IκB/NF-κB system in *E. faecalis* induced pro-inflammatory signaling (*Appendix 2, Figure 8*).

The inhibition of the inflammatory response is a clinically relevant strategy in the treatment of IBD. Thus, the study of mechanisms involved in the resolution of inflammation, like the production of anti-inflammatory cytokines such as TGF-β may provide new targets in the treatment of chronic inflammation. Anna Shkoda´s analysis of *E. faecalis*-monoassociated $IL\text{-}10^{-/-}$ mice under germ-free conditions showed not only induction of pro-inflammatory gene expression in IECs (*Appendix 2, Figure 1*), but also absence of TGF-β/Smad signaling cascade suggesting a role of this pathway in the negative regulation of colitis in $IL\text{-}10^{-/-}$ mice *(Appendix 2, Figure 9)*. There is strong

evidence that the disruption of TGF-β signaling pathway in mice leads to inflammatory disease. For example, expression of a dominant negative TGF-β receptor type II causes T-cell hyperactivity and autoimmunity (Gorelik and Flavell 2000) and the deletion of the TGF-β1 gene itself leads to systemic inflammation and early death in mice (Shull, Ormsby et al. 1992). The administration of TGF-β can also abrogate chronic inflammation in TNBS-treated mice (Neurath, Fuss et al. 1996). Paradoxically, upregulation of TGF-β expression has been documented in IBD patients (Babyatsky, Rossiter et al. 1996) questioning the role of TGF-β in the control of chronic inflammation. The reason why this condition fails to control IBD remains unclear, but studies in IBD patients provided evidence that the blockade of TGF-β signaling by excessive Smad7 expression is the reason for low TGF-β activity in IBD patients (Monteleone, Kumberova et al. 2001), providing a good example of the complex and fine-tuned regulation of TGF-β signalling and its crucial role in gut homeostasis.

TGF-β-triggered signals are transduced by Smad proteins, a family of transcription factors with serine/threonine kinase activity in their cytoplasmic domain (Massague 2000). As expected, the stimulation of Mode K cells with TGF-β1 in the presence of *E. faecalis* triggered Smad2 phosphorylation at Serines 465 and 467, and of more relevance, inhibition of *E. faecalis*-induced RelA phosphorylation after 2 hours of stimulation with the bacteria (*Appendix 2, Figure 11A*). Several studies showed evidence of the relationship between TNF-β-induced Smad activation and the inhibition of inflammation. For example, mice with targeted disruption of Smad3 develop chronic infection and inflammation at the mucosal surfaces (Yang, Letterio et al. 1999). In addition, Smad2 activation has been shown to be necessary for the inhibition of pro-inflammatory gene expression in cell lines (Walia, Wang et al. 2003). Activation of the TGF-β pathway by *E. faecalis* has crucial consequences in *E. faecalis*-induced pro-inflammatory processes. Chromatin inmunoprecipitation (ChIP) analysis showed that *E. faecalis*-induced recruitment of phospho-RelA to the IL-6 promoter was inhibited in the presence of TGF-β (*Appendix 2, Figure 11B*). This may constitute part of the mechanisms underlying TGF-β suppressive effects on IL-6 and IP-10 mRNA expression in *E. faecalis*-activated Mode K cells (*Appendix 2, Figure11C*).

4 IL-10 abrogates ER stress response by blocking ATF-6 recruitment to the Grp78 promoter

The regulation of the suppressive cytokines IL-10 and TGF-β plays a crutial role in the treatment of chronic intestinal inflammation. In fact, their protective effects have been shown to be tightly interrelated (Fuss, Boirivant et al. 2002). According to our previous results, TGF-β abrogated the recruitment of activated NF-κB to the promoter of pro-inflammatory cytokines, therefore inhibiting gene expression in *E. faecalis*-activated Mode K cells (*Appendix 2*). The anti-inflammatory effects of IL-10 as well as its ability in inhibiting intestinal inflammation have been extensively demonstrated *in vivo* studies (Kuhn, Lohler et al. 1993). Asseman et al. have provided evidence of the pivotal role of IL-10 in the function of regulatory T-cells, which are crucial for the control of pro-inflammatory response in the intestinal mucosa (Asseman, Mauze et al. 1999). In addition, the administration of IL-10-secreting *Lactococcus lactis* reduced colitis in DSS-treated IL-$10^{-/-}$ mice (Steidler, Hans et al. 2000). As pointed out along this work IL-$10^{-/-}$ mice develop colitis with similarities to IBD (Balish and Warner 2002), stressing the major role of this suppressive cytokine in the maintenance of mucosal homeostasis.

IL-10 regulates inflammatory processes in the mucosal epithelium by blocking the production of pro-inflammatory cytokines (Denning, Campbell et al. 2000). Although IL-10 has been shown to control the activity of NF-κB in diverse cell types, results from different groups are controversial and so far, the molecular mechanism for IL-10-mediated NF-κB inhibition remains undetermined. Previous studies had clearly shown that *E. faecalis*-monoassociated IL-$10^{-/-}$ mice develop chronic intestinal inflammation (*Appendix 2*). As showed by Anna Shkoda using proteomic analysis, the expression of the glucose-regulated protein (Grp)78 is upregulated in the intestinal epithelium of IL-$10^{-/-}$ 129 SvEv mice monoassociated for 14 weeks with *E. faecalis* strain OG1RF (*Appendix 5, Figure 1*). Grp78, also referred to as the immunoglobulin heavy chain-binding protein (BiP), is the most abundant member of the ER heat shock protein (Hsp)70 family (Harding, Calfon et al. 2002; Zhao and Ackerman 2006) and the induction of Grp78 gene expression has been typically used as a marker for the ER stress response (Lee 2001). The upregulation of Grp78 in the intestinal epithelium of this model of colitis points to a possible role of the endoplasmic reticulum (ER) stress response in the control of inflammation.

In all eukaryotic cells, the ER is the site where the folding and assembly of proteins takes place. Various biochemical and physiologic stimuli, such as perturbation in calcium homeostasis or redox status, elevated protein synthesis and secretion, glucose deprivation, viral infection and altered glycosylation can induce stress to the ER and subsequently lead to accumulation of unfolded or misfolded proteins in the ER lumen. The ER has evolved an evolutionary conserved response called the unfolded protein response (UPR) to cope with the accumulation of unfolded or misfolded proteins (Zhang and Kaufman 2006).

In order to adapt to the changing environment, the UPR triggers adaptive mechanisms that involve the expression of genes that attenuate translation of mRNAs and enhance protein folding and secretion, as well as degradation of misfolded proteins. When adaptation fails, ER-dependent pathways activate NF-κB, which in turn induces the expression of host defense genes. Excessive and prolonged ER stress triggers apoptosis, which involves mitochondria-dependent or -independent mechanisms (Orrenius, Zhivotovsky et al. 2003; Xu, Bailly-Maitre et al. 2005).

The mammalian UPR pathway has three branches, with the critical transmembrane signaling proteins ATF-6, type-I ER transmembrane protein kinase (IRE)1 and RNA-dependent protein kinase (PKR)-like ER kinase (PERK), acting as stress sensors (Rao and Bredesen 2004; Shen, Zhang et al. 2004). Normally, the N-termini of these ER proteins are held by Grp78, preventing their aggregation. Upon ER stress, Grp78 is released into the lumen of the ER and become sequestrated by unfolded proteins inducing oligomerization and autophosphorylation of IRE1 and PERK, and relocation of the transcription factor ATF-6 to the Golgi compartment, where it undergoes protease-mediated cleavage. Active ATF6 translocates into the nucleus where it controls expression of UPR genes, included Grp78.

ER stress occurs under both physiological and pathological conditions. There is a large body of literature addressing the relationship between ER stress and the pathogenesis of different diseases such as cancer (Ma and Hendershot 2004), diabetes (Harding and Ron 2002), ischemia/reperfusion injury, or hearth disease (Xu, Bailly-Maitre et al. 2005). In addition, ER stress activation has also been associated with the initiation of the immune response (Brewer and Hendershot 2005) and inflammatory diseases *in vivo* (Turner, Sowders et al. 2005). For instance, mice deficient in IRE1β (IRE1$\beta^{-/-}$) in the

intestinal epithelium have been shown to express higher levels of Grp78 compared to normal mice and develop colitis earlier when orally administered with DSS (Bertolotti, Wang et al. 2001). However, little is known about the role of ER stress in IBD patients.

To further study wheter IL-10 targets IECs and its suppressive molecular mechanisms, Mode K cells, which lack IL-10R, were reconstituted with the receptor-signaling complex of IL-10. As showed in *Appendix 5, Figure 5*, IL-10 triggered phosphorylation of p38 and STAT3 after 3 hours of stimulation with IL-10, whereas non-reconstituted Mode K cells showed no effect. IL-10 signals through STAT and the MAPK p38. Thus, IL-10 has been found to target both STAT1 and STAT3 to exert its anti-proliferative properties in macrophages (O'Farrell, Liu et al. 1998) and to require STAT3 activation for the inhibition of pro-inflammatory gene expression in LPS-stimulated macrophages and neutrophils (Riley, Takeda et al. 1999; Takeda, Clausen et al. 1999). In contrast the effects of IL-10 on p38 are controversial, with studies reporting IL-10 suppressive effects on pro-inflammatory gene expression through p38-dependent and -independent mechanisms (Donnelly, Dickensheets et al. 1999; Denys, Udalova et al. 2002). Nonetheless, the cross-talk existing between the MAPK p38 and JAK/STAT signaling pathways has been clearly exposed, with the activation of p38 being shown to be required for STAT3 transcriptional activity in cultured fibroblasts (Turkson, Bowman et al. 1999).

As shown by Anna Shkoda *(Appendix 5, Figure 6)*, IL-10 inhibited Grp78 at the level of mRNA and protein expression in IL-10R-reconstituted Mode K cells, reproducing the results observed *in vivo*. This downregulation of Grp78 may form part of the anti-inflammatory mechanisms of IL-10 as already suggested by Shibahara et al. This group showed that IL-10 contribute to the resolution of inflammation and suppression of IBD through HO-1, a stress-responsive protein induced by stimulus such as LPS and oxidative stress, via activation of the p38 MAPK pathway (Shibahara 1988; Naito, Takagi et al. 2004).

TNF is a pro-inflammatory cytokine, produced mainly by activated macrophages and T lymphocytes that plays a central role in diverse immune and inflammatory processes (Rutgeerts, Van Assche et al. 2004). TNF targets a variety of cells including IECs, where it is able to induce NF-κB activation and pro-inflammatory gene expression. The amount of TNF secreted as well as the number of TNF-secreting cells is substantially increased in the bowell in patients of IBD (Reinecker, Steffen et al. 1993; Breese, Michie et al.

1994). Further evidence for the relevant role of TNF in IBD is the fact that $TNF^{\Delta ARE}$ mutant mice, which over-express TNF due to the production of a more stable TNF mRNA, develop CD (Kontoyiannis, Pasparakis et al. 1999). Antibodies to TNF have been shown to be an effective treatment for patients with CD (Rutgeerts, Van Assche et al. 2004). In addition it has been demonstrated that $IL\text{-}10^{-/-}$ x $TNF^{-/-}$ mice do not develop chronic inflammation (Kontoyiannis, Kotlyarov et al. 2001). Additionally Schottelius et al. showed that IL-10 inhibited TNF-induced pro-inflammatory gene expression in cultured monocytic and IECs (Schottelius, Mayo et al. 1999), although the molecular target for IL-10-induced inhibition of pro-inflammatory gene expression had not been characterized.

Considering our initial data *in vivo* and *in vitro*, IL-10 may exert its suppressive effects on TNF-induced pro-inflammatory processes in the intestinal epithelium by using the STAT3 and p38 pathways through mechanisms that involve the regulation of the ER stress response and in particular gene expression of the chaperone Grp78. Since the impact of diverse environmental factors, including bacterial infection has the potential to induce ER stress, the UPR has in turn been found to activate signal transduction pathways associated with innate immunity.

Several studies have suggested mechanisms relating TNF and UPR. Like many members of the TNFR family, IRE1 can bind the adapter protein TRAF2, which in turn may contribute to the activation of JNK, the upstream regulator of the c-Fos and c-Jun transcription factors, which together comprise the transcription complex AP-1 (Yoneda, Imaizumi et al. 2001; Pillai 2005). Furthermore, it has been demonstrated that TNF induces PERK-mediated eukaryotic translation initiation factor (eIF)2α phosphorylation, as well as ATF-6- and IRE1-mediated induction of X box protein (XBP)1 (Xue, Piao et al. 2005).

In mammalian cells, Grp promoters contain multiple units of ER stress response element (ERSE) motifs, which consist of a tripartite structure $CCAAT(N_9)CCACG$ (where N is any bases) (Yoshida, Haze et al. 1998; Lee 2001). The release of Grp78 in the lumen of the ER liberates the transcription factor ATF-6, which undergo ER-stress-induced cleavage in the Golgi apparatus by Site 1 and Site 2 proteases. The cytosolic activated form of ATF-6 translocates to the nucleus where it binds to ERSEs and activates the transcription of UPR target genes (Roy and Lee 1999; Shen, Chen et al. 2002), included Grp78 (Yoshida, Okada et al. 2001). ChIP analysis showed that IL-10 blocked TNF-

induced ATF-6 recruitment to the Grp78 promoter after 2 hours of stimulation, revealing a suppressive effect of IL-10 in the ER stress response (*Appendix 5, Figure 11B*). Interestingly, IL-10 showed no effect in TNF-induced c-Fos recruitment, which has also been reported to bind ERSEs and is implicated in Grp78 induction (He, McColl et al. 2000), indicating a selective effect for IL-10 targeting only ATF-6. IL-10 was also unable to inhibit acetylation/phosphorylation of histone 3 at the Grp78 promoter indicating recruitment of other transcription factors and cofactors with HAT activity and supporting the idea of a selective IL-10 mediated inhibition on ATF-6 promoter binding (*Appendix 5, Figure 11B*).

ATF-6 is a substrate of p38 MAPK and its activity can also be regulated by phosphorylation (Thuerauf, Arnold et al. 1998). Therefore it is likely that the inhibition of the p38 MAPK pathway through IL-10 may play an important role in the blockade of ATF-6 activity. The treatment of IL-10R reconstituted Mode K cells with the p38 specific inhibitor SB203580 prior to IL-10 and TNF stimulation showed a markedly reversion of IL-10-mediated inhibition of ATF-6 recruitment to the Grp78 promoter, providing evidence of the relevant role of this MAPK in IL-10-mediated inhibition of TNF-induced ATF-6 recruitment to Grp78 promoter (*Appendix 5, Figure 11D*).

5 Functional diversity of flavonoids and its bacterial metabolites in the inhibition of pro-inflammatory signaling pathways in IECs

As a key transcription factor for the pro-inflammatory response, NF-κB constitutes an important therapeutic target for drugs in the treatment of inflammatory disorders. Recently, a great number of plant-derived substances, such as polyphenols, have been evaluated as possible inhibitors of the NF-κB pathway (Nam 2006). Flavonoids exhibit a variety of biological activities, including cancer chemoprevention, protection from vascular disease and suppression of inflammation. In addition, flavonoids have been found to modulate different signaling pathways involved in crucial cellular proceses (Birt, Hendrich et al. 2001) such as cellular growth and differentiation, chromatin structure, glutathione biosynthesis and, as a consequence, regulate pro-inflammatory gene expression in a variety of cellular types (Tsai, Lin-Shiau et al. 1999). Studies *in vitro* and *in vivo* have provided evidence of the anti-inflammatory properties of flavonoids suggesting a therapeutic use of these plant-derived substances in IBD (Middleton, Shorthouse et al. 1993; Kandaswami and Middleton 1994). The widely probed antioxidant and free-radical properties of flavonoids have been proposed to counteract the oxidative stress characteristical of the mucosa of patients of IBD (Kwon, Murakami et al. 2005; Muia, Mazzon et al. 2005). In addition, some studies suggest that several of the biological activities of flavonoids may be mediated by the downregulation of the NF-κB pathway (Holmes-McNary and Baldwin 2000; Yamamoto and Gaynor 2001). Thus some flavonoids haven been found to block NF-κB activation by inhibiting IKK activity (Holmes-McNary and Baldwin 2000) and in the model of experimental colitis IL-$2^{-/-}$ mice (Varilek, Yang et al. 2001).

The pro-inflammatory cytokine TNF has a predominant role in the induction of chronic intestinal inflammation as evidenced by diverse animal models (Kollias, Douni et al. 1999). The stimulation of Mode K cells with TNF induces RelA phosphorylation as well as IκBα phosphorylation and posterior degradation in a time dependent manner (*Appendix 6, Figure 1*). In addition, the inhibition of TNF-induced IP-10 and MIP-2 protein expression by pyrrolidinedithio-carbamate ammonium (PDTC), a pharmacological inhibitor of the NF-κB pathway, demonstrates the crucial role of NF-κB in TNF-induced pro-inflammatory gene expression in IECs (*Appendix 6, Table 2*).

The screening of 10 flavonoids belonging to the subclases of flavones, flavonols, flavanones and isoflavones showed notable differences affecting pro-inflammatory gene expression. Some of the tested flavonoids significantly inhibited IP-10 and IL-6 protein expression in Mode K cells (Figure 17) consistent with the already shown ability of flavonoids to inhibit TNF-induced pro-inflammatory molecules in epithelial and endothelial cells (Gerritsen, Carley et al. 1995; Chen, Chow et al. 2004; Choi, Choi et al. 2004).

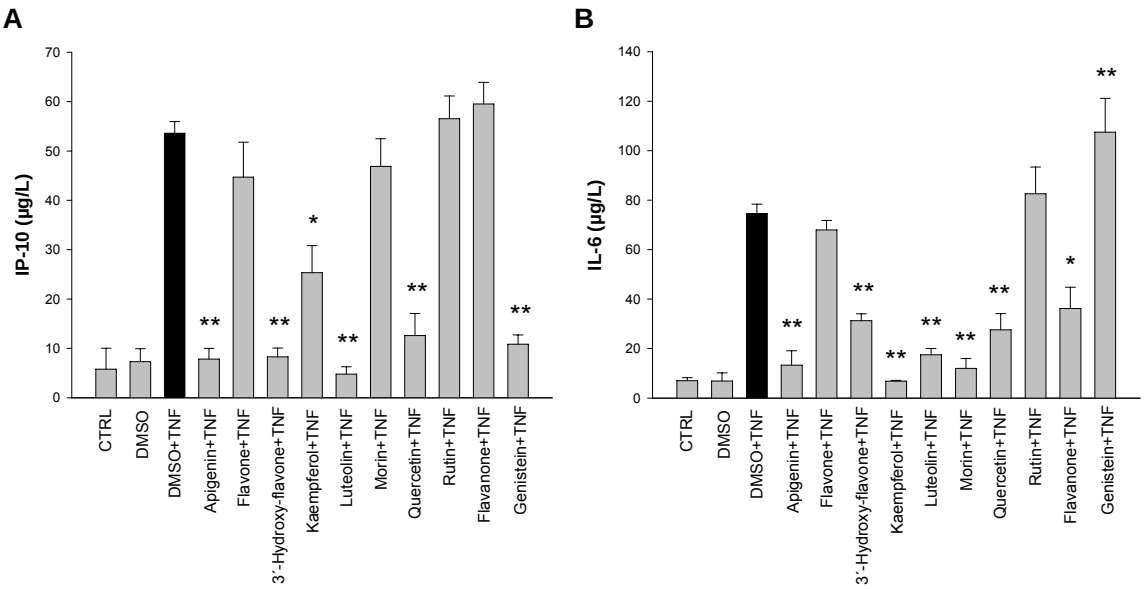

Figure 17: Screening of flavonoids. Mode K cells were pre-incubated with TNF (20ng/ml) for 1 hour prior stimulation with 10 different flavonoids (100µmol/L) dissolved in dimethyl sulfoxide (DMSO) for 24 hours. IP-10 protein (A) and IL-6 protein (B) were measured in the cell culture supernatant using ELISA technique. Bars represent the combined mean value ± standard deviation (SD) of triplicate stimulation from 3 independent experiments. Statitical analysis was performed using One Way ANOVA followed by Tukey's test. *, p value < 0.05; **, p value < 0.01

The flavones apigenin and luteolin, the flavonols 3´-hydroxy-flavon and quercetin, and the isoflavon genistein (Figure 18) showed the strongest inhibitory effects on TNF-induced IP-10 expression with effective concentrations for 50% of the inhibitory effect $(EC)_{50}$ of IP-10 of 23, 27, 20, 40 and 27 µmol/L respectively (*Appendix 4, Figure 1, and Appendix 6, Table 1 and Figure 2*). In addition, quercetin was also able to inhibit TNF-induced MIP-2 expression with EC_{50} of 44 µmol/L (*Appendix 6, Table 1 and Figure 2*).

Although all the selected flavonoids with exception of flavone share the ability of inhibiting pro-inflammatory gene expression, they targeted the NF-κB signaling pathway in different manners. Thus, whereas apigenin, luteolin and 3´-hydroxy-flavone

significantly inhibited NF-κB reporter activity, only apigenin and 3´-hydroxy-flavone inhibited NF-κB binding activity in TNF-stimulated Mode K cells (*Appendix 4, Table 1*). Surprisingly, only 3´-hydroxy-flavone was able to block phosphorylation of RelA as well as phosphorylation of IκBα and IκBα proteasomal degradation after 20 minutes of TNF stimulation (*Appendix 4, Figure 2*). Moreover, 3´-hydroxy-flavone was also able to inhibit TNF-induced IKK activity (*Appendix 4, Figure 3*), consistent with the reported suppressive effects of other flavonoids on IKK activity in IECs (Yang, Oz et al. 2001). These data demonstrate that 3´-hydroxy-flavone already exerts its inhibitory effects upstream on the pro-inflammatory NF-κB signalling cascade.

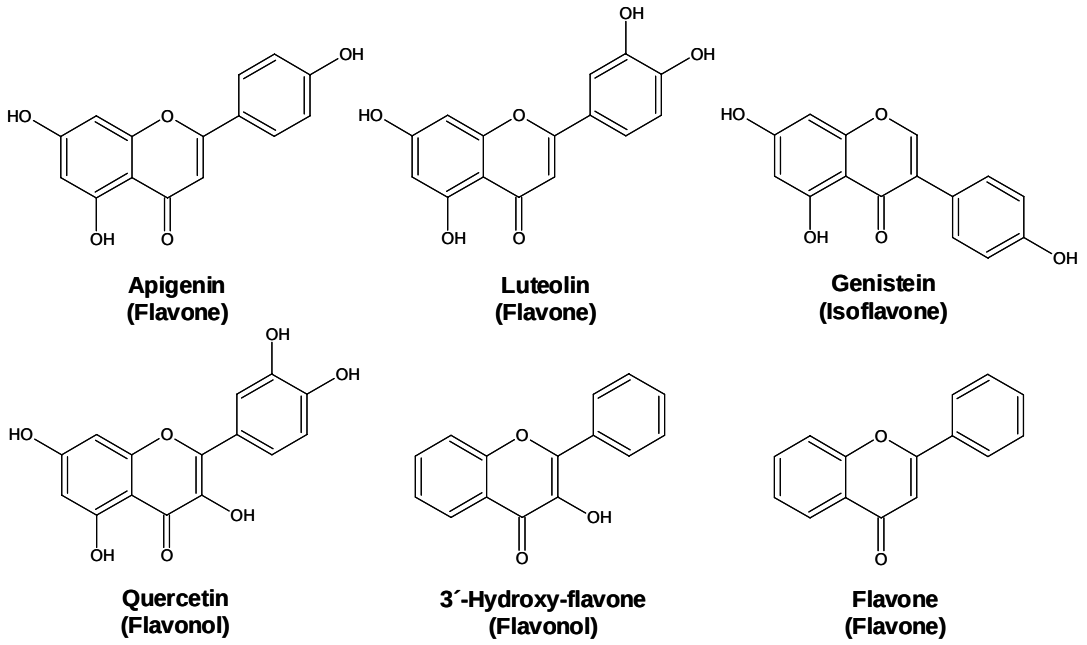

Figure 18: Chemical structures of the studied flavonoids. The flavones apigenin and luteolin, the flavonols quercetin and 3´-hydroxy-flavone, and the isoflavone genistein presented the strongest inhibitory effect on IP-10 and IL-6 expression in TNF-activated Mode K cells whereas flavone showed no effect.

Regarding the dietary therapeutic use of flavonoids, it muss be considered that a major part of ingested flavonoids are not absorbed, but to a great extend degraded by the normal microbiota. Several human studies have been conducted to addres the question of the absorption and bioavailability of flavonoids (Hollman, van Trijp et al. 1997). For instance, recent studies have shown that the absorption of orally administered quercetin in healthy individuals is approximately 24%, with the absortion of quercetin glycosides being 52% (Hollman, de Vries et al. 1995; Hollman, van Trijp et al. 1997), and absorption and bioavailability of flavonoids varying greatly depending of the dietary source (de Vries, Hollman et al. 2001). Since a considerable amount of the consumed flavonoids

are degraded in the gut, it is necessary to take into account the biological effects of their bacterial metabolites in order to gain insight in the potential beneficial health effects of flavonoids in the human intestinal tract. *Eubacterium ramulus* is a common member of the intestinal microbiota and a flavonoid-degrading anaerobe whose sole carbon and energy source is quercetin-3-glucoside (isoquercitrin) (Schneider, Simmering et al. 2000). The degradation of quercetin by this Gram-positive bacteria results in the metabolite intermediates taxifolin, alphitonin, 3,4-dihydroxy-phenylacetic acid and phloroglucinol (Braune, Gütschow et al. 2001) (*Appendix 6, Figure 1*). After the treatment of Mode K cells with quercetin and its metabolites taxifolin, alphitonin and 3,4-dihydroxy-phenylacetic acid, only quercetin was able to significantly inhibit TNF-induced IP-10 and MIP-2 protein expression (*Appendix 6, Table 1*), suggesting that none of quercetin bacterial products conserved its ability to inhibit pro-inflammatory signal transduction.

The serine/threonine protein kinase Akt, also called protein kinase B (PKB), is an important effector for phosphatidylinositol 3-kinase (PI3K) signaling and can be activated by growth factors and cytokines (Downward 1998). Besides playing a role in cell growth, gene expression and oncogenesis (Aoki, Batista et al. 1998), Akt has been shown to promote cell survival *in vivo* and *in vitro* by different mechanisms that block apoptosis, including activation of NF-κB and the MAPK p38 (Datta, Brunet et al. 1999; Madrid, Mayo et al. 2001). Furthermore, Akt has been found to enhance the degradation of the IκBs and to cooperate with other factors to induce NF-κB-mediated activation of NF-κB target genes (Kane, Shapiro et al. 1999). Moreover, Akt can associate with the IKK complex *in vivo* and has been shown to phosphorylate and activate IKKα (Ozes, Mayo et al. 1999). All these data implicate Akt as part of a survival signaling pathway to NF-κB. As shown in *Appendix 6, Table 2*, the presence of the synthetic inhibitor of the PI3K/Akt signalling pathway, LY294002, completely abrogated TNF-induced IP-10 and MIP-2 expression, showing the key role of this pathway in the expression of this pro-inflammatory chemokines. Correlating with the inhibition of NF-κB activity, luteolin and to a lower extend apigenin inhibited Akt phosphorylation at serine 473, in the Akt regulatory domain after 30 minutes and 1 hour, whereas 3´-hydroxy-flavone showed no effect (*Appendix 4, Figure 4*). The inmunoprecipitation of Akt and posterior kinase assay with the Akt substrate, glycogen synthase kinase (GSK), showed that inhibition of Akt phosphorylation through apigenin and luteolin leaded to a partial loss of Akt activity after 30 minutes in TNF-stimulated Mode K cells (*Appendix 4, Figure 5*). Similarly to LY294002, quercetin is a broad-spectrum protein kinase inhibitor which phisically

interacts with PI3K, leading to the inhibition of the Akt pathway (Walker, Pacold et al. 2000). As expected and consistent with the inhibitory activity this flanonol on Akt activity in primary human epithelial cells (O'Prey, Brown et al. 2003), quercetin was able to inhibit Akt phosphorylation in TNF-treated Mode K cells, whereas its bacterial metabolites showed no effect (*Appendix 6, Figure 3*).

IP-10 is a complex regulated gene, which promoter contains the binding sites of different transcription factors including NF-κB and IRF-1 (Ohmori and Hamilton 1993). IRF-1 plays an important role in the transcriptional control of growth regulatory and immune regulatory genes (Merika, Williams et al. 1998; Lin and Hiscott 1999), and its expression and activation has been found to be induced by TNF in immune cells and fibroblast (Ohmori, Schreiber et al. 1997; Gupta, Xia et al. 1998). Luteolin and 3´-hydroxy-flavone induced degradation of constitutive IRF-1 after 24 hours in TNF-stimulated Mode K cells, whereas apigenin did not affect IRF-1 expression levels. Interestingly, IRF-3, which also posses a potent transactivation domain and acts as a coactivator of NF-κB in the induction of expression of many chemokines (Falvo, Thanos et al. 1995; Mamane, Heylbroeck et al. 1999; Wietek, Miggin et al. 2003) was not degradated in Mode K cells supporting the notion of the selective inhibition of flavonoids.

3´-hydroxy-flavone induced transient phosphorylation of the MAPK p38 in TNF-stimulated Mode K at 30 minutes and 24 hours of TNF stimulation. Interestingly, this polyphenolic compound could not induce p38 phosphorylation in the absence of TNF after 24 hours (*Appendix 4, Figures 7 and 9*). The presence of the specific pharmacological inhibitor of p38, SB203580 was not able to inhibit TNF-induced IP-10 and MIP-2 expression (*Appendix 6, Table 2*), indicating that this pathway was not essential for TNF-induction of pro-inflammatory cytokines in Mode K cells. Flavonoids have been shown to have pro-apoptotic activity. Nevertheless, luteolin and genistein induced cleavage of caspase-3 only in the presence of TNF and after 24 hours of TNF stimulation. On the contrary, 3´-hydroxy-flavone induced caspase-3 cleavage, also after 24 hours of stimulation of Mode K cells (*Appendix 4, Figures 8-9*). Interestingly, apigenin triggered neither p38 phosphorylation nor caspase-3 cleavage, in the presence and in the absence of TNF (*Appendix 4, Figures 7- 9*). All these data provide evidence that cellular death was not the mechanism by which flavonoids inhibited pro-inflammatory signal transduction in TNF-stimulated IECs.

Genistein, a well known broad-spectrum tyrosine kinase inhibitor, has already been shown to inhibit cytokine-induced pro-inflammatory gene expression. Interestingly and despite its ability to inhibit IP-10 expression, genistein did not target the NF-κB signalling pathway at any level (*Appendix 4, Table 1 and Figure 2*), suggesting a different mechanisms for this isoflavone in the inhibition of pro-inflammatory gene expression. This result does not correlate with previous studies reporting an inhibitory effect mediated by this isoflavone on NF-κB activation in prostate and breast cancer cells (Li and Sarkar 2002; Gong, Li et al. 2003), suggesting a cell type-dependent effect. In contrast with the other studied flavonoids, genistein did not inhibit TNF- and IL-1β-induced IL-6 expression, but significantly increased it, indicating a selective effect for this flavonoid in the inhibition of TNF induced pro-inflammatory cytokine expression (*Appendix 4, Table 2*).

Despite of the ability of quercetin to inhibit TNF-induced IP-10 and MIP-2 protein expression and Akt phosphorylation, quercetin failed to inhibit RelA phosphorylation and IκBα proteasomal degradation as well as NF-κB reporter gene activity in TNF-activated Mode K cells (*Appendix 6, Figure 4B and Table 3*) indicating that the inhibition of Akt phosphorylation had no effect on IKK activity. On the other hand, quercetin showed a significant inhibition of HAT activity in Mode K cells after 20 minutes of TNF stimulation (*Appendix 6, Table 1*), suggesting that quercetin may exert its inhibitory effects downstream of the pro-inflammatory signaling cascade. Efficient gene transcription requires the binding of activated transcription factors to DNA promoter elements. Transcription factors recruit coactivators with HAT activity, such as CBP/p300 proteins, which in turn potentiate transcription by acetylation-dependent loosening of the chromatine structure (Chan and La Thangue 2001). ChIP analysis showed that quercetin inhibited the recruitment of TNF-induced phospho-RelA to IP-10 and MIP-2 promoters (*Appendix 6, Figure 5A-B*). Activated NF-κB has been shown to recruit CBP/p300 proteins to initiate gene transcription (Zhong, May et al. 2002). Consequentely, ChIP analysis showed that the recruitment of CBP/p300 proteins to IP-10 and MIP-2 promoters was also abrogated in the presence of quercetin, as well as subsequent acetylation and phosphorylation of histone 3 (*Appendix 6, Figure 5A-B*). These results provide a mechanism for quercetin inhibition of TNF infuced pro-inflamatory gene expression.

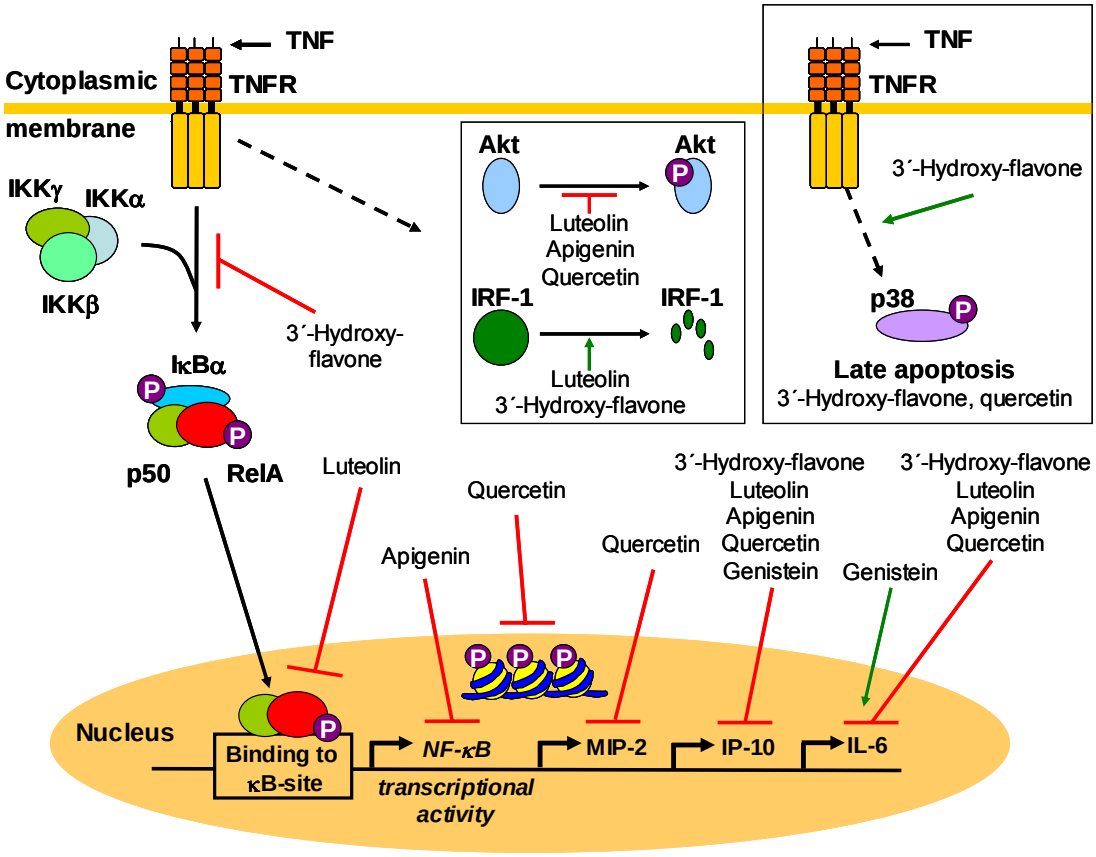

Figure 19: Primary inhibitory effects of flavonoids in IECs after stimulation with TNF. 3´-Hydroxy-flavone blocks pro-inflammatory signal transduction upstream of NF-κB signaling, whereas the rest of flavonoids seem to abrogate pro-inflamatory cytokine expression at nuclear level. Luteolin blocked NF-κB binding to κB consensus sequences, whereas apigenin inhibited NF-κB transcriptional activity. Quercetin inhibited HAT activity as well as recruitment of phospho-RelA to the promoter of the pro-inflammatory cytokines IP-10 and MIP-2. In addition, 3´-Hydroxy-flavone induced p38 phosphorylation as well as IRF-1 degradation. Luteolin triggered IRF-1 degradation and Akt phosphorylation, which was also inhibited by apigenin and quercetin.

In order to investigate quercetin suppressive effects on inflammatory processes in vivo, $TNF^{\Delta ARE}$ transgenic mice and wild-type mice were fed with quercetin for 10 weeks at 8 weeks of age (Figure 20). The deletion of the AREs present in the 3´untranslated region of the TNF gene confers stability to the transcripted mRNA, resulting in the enhanced expression of TNF in these mice. $TNF^{\Delta ARE}$ mice constitute a model of experimental ileitis with a phenotype resembling CD (Kontoyiannis, Pasparakis et al. 1999). The expression levels of IP-10 and MIP-2 mRNA in primary IECs isolated from the ileum of $TNF^{\Delta ARE}$ mice were significantly reduced in comparation to the IECs from wild-type mice (*Appendix 6, Figure 6*), supporting our previous results in Mode K cells and pointing to a posible terapeutic effect of quercetin in the treatment of colitis.

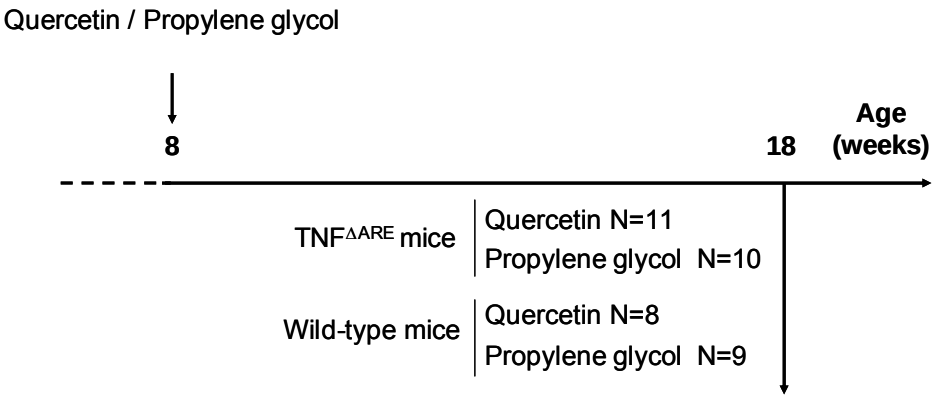

Figure 20: Experimental design of oral application of quercetin to TNF$^{\Delta ARE}$ and wild-type mice. TNF$^{\Delta ARE}$ mice and wild-type mice were fed with quercetin and the vehicle propylene glycol for 10 weeks. Mice were killed at 18 weeks of age and IECs from ileum was isolated. N indicates the number of mice in every group.

ZUSAMMENFASSUNG

Chronisch entzündliche Darmerkrankungen (CED) entstehen durch eine übersteigerte mukosale Immunreaktion, ausgelöst durch die intestinale Mikroflora. Eine wichtige Rolle bei diesen Entzündungsprozessen spielt der Transkriptionsfaktor NF-κB. In dieser Arbeit wurden molekulare Mechanismen zur Hemmung des NF-κB-Signalweges in intestinalen Epithelzellen (IECs) mittels Tiermodellen und Zelllinien aufgeklärt. Es konnte gezeigt werden, dass nicht-pathogene Bakterien der Gattung *Bacteroides vulgatus* den Transkriptionsfaktor NF-κB in keimfreien Ratten und in kultivierten IECs aktivieren. Über *in vitro* Experimente wurde ein Mechanismus identifiziert, welcher die Inhibierung der Bakterien-induzierten NF-κB-Phosphorylierung durch 15-desoxy-$\Delta^{12,14}$-Prostaglandin J_2 (15d-PGJ_2) erklärt. Dabei induziert 15d-PGJ_2 die Proteinphosphatase (PP)2A Aktivität, welche NF-κB deaktiviert. Paradoxerweise löste das probiotische Bakterium *Bifidobacterium lactis* Stamm BB12 eine höhere pro-inflammatorische Genexpression im Darmepithel keimfreier Ratten aus als *B. vulgatus.* Zusätzlich wurden durch *B. lactis* BB12 suppressive Mechanismen aktiviert, was auf Toleranz-induzierte Prozesse im Darmepithel hinweist. Die NF-κB-Aktivierung durch *B. lactis* BB12 erfolgte durch TLR2. Dies wurde gezeigt, durch Mode K IEC-Zellen, welche mit Toll-like-Rezeptor (TLR)4 oder mit nicht-funktionellem TLR2 transfiziert wurden. Auch bei *Enterococcus faecalis* erfolgte die NF-κB-Aktivierung über TLR2. Der transformierende Wachstumsfaktor (TGF)-β inhibierte die *E. faecalis*-induzierte Interleukin (IL)-6-Expression. Durch Chromatin-Immunopräzipitation (ChIP) wurde nachgewiesen, dass die Rekrutierung von NF-κB zum IL-6-Promotor blockiert wird. Zusammen mit TGF-β spielt IL-10 eine Schlüsselrolle bei der Entzündungskontrolle. IL-10 inhibierte die Tumornekrosefaktor (TNF)-induzierte Rekrutierung des aktivierenden Transkriptionsfaktors (ATF)-6 zum Promotor des Glukose-regulierten Proteins (Grp)78. Grp78 ist ein bedeutender Mediator der Stressreaktion des endoplasmatischen Retikulums (ER). Diese Zusammenhänge wurden bestätigt durch Experimente mit IL-10-Rezeptor (IL-10R) rekonstituierten Mode K-Zelle. Ein weiterer bedeutender Teil dieser Arbeit war die molekulare Charakterisierung der anti-inflammatorischen Effekte von Flavonoiden. Diese sekundären Pflanzenstoffe zeigten eine funktionelle Vielfalt bei der Hemmung des durch TNF-induzierten NF-κB-Signalweges. Es konnte nachgewiesen werden, dass das Flavonol Quercetin die pro-inflammatorische Genexpression in $TNF^{\Delta ARE}$-Mäusen, einem Tiermodell für chronische Ileitis, inhibiert. Dies deutet auf einen möglichen therapeutischen Nutzen von Quercetin bei chronischen Entzündungsprozessen hin.

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LIST OF ABBREVIATIONS

15d-PGJ_2	15-deoxy-$\Delta^{12,14}$-PGJ_2
AP	activator protein
APC	antigens presenting cell
ARE	adenosine-uracil rich element
ASK	apoptosis signal-regulating kinase
ATF	activating transcription factor
ATP	adenosine triphosphate
BiP	immunoglobulin heavy chain-binding protein
cAMP	cyclic adenosine monophosphate
CBP	CREB binding protein
CD	Crohn's disease
ChIP	chromatin inmmunoprecipitation
ConA	concavalin A
COX	cyclooxygenase
CREB	cAMP response element binding protein
cyPG	cyclopentenone PG
DC	dentritic cell
DLK	dual leucine zipper bearing kinase
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dsRNA	double-stranded RNA
DSS	dextran sulfate sodium
EC_{50}	effective inhibitory concentration
eIF	eukaryotic translation initiation factor
ENA	epithelial cell-derived neutrophil activating protein
ER	endoplasmic reticulum
ERSE	ER stress response element
ERK	extracellular signal-regulated kinase
FAE	follicle-associated epithelium
FAO	Food and Agriculture Organization
GALT	gut-associated lymphoid tissue
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GAS	IFNγ-activated-site

GI	gastrointestinal
GM-CSF	granulocyte-macrophage colony-stimulating factor
GRO	growth-related oncogene
Grp	glucose-regulated protein
GSK	glycogen synthase kinase
HAT	histone acetyl transferase
HO	heme oxygenase
HSF	heat shock factor
Hsp	heat shock protein
IAP	inhibitor of apoptosis
IBD	inflammatory bowel diseases
ICAM	intracellular adhesion molecule
IEC	intestinal epithelial cell
IFN	interferon
IFNAR	IFNα receptor
IFNGR	IFNγ receptor
IKK	IκB kinase
IL	interleukin
IL-1R	IL-1 receptor
IL-10R	IL-10 receptor
iNOS	inducible NO synthase
IP	INFγ-inducible protein
IRAK	IL-1R-associated kinase
IRE	type-I ER transmembrane protein kinase
IRF	IFN regulatory factor
ISRE	IFN-stimulated response element
JAK	Janus kinase
JNK	c-Jun amino-terminal kinase
LRR	leucine-rich repeat
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
MAPKK	MAPK kinase
MAPKKK	MAPKK kinase
MCP	monocyte chemoattractant protein
mdr	multiple drug-resistant

MEF	myoembryogenic fibroblast
MEK	MAPK/ERK kinase
MEKK	MEK kinase
MHC	major histocompatibility complex
MIP	macrophage-inflammatory protein
MLK	mixed lineage kinase
moi	multiplicity of infection
mRNA	messenger RNA
NF	nuclear factor
NF-AT	NF of activated T cells
NF-ATc	NF-AT, cytoplasmic, calcineurin-dependent
NIK	NF-κB-inducing kinase
NLS	nuclear localization signal
NO	nitric oxide
NOD	nucleotide-binding oligomerization domain
PAMP	pathogen-associated molecular pattern
Pax	paired homeobox
PCR	Polymerase chain reaction
PDTC	pyrrolidinedithio-carbamate ammonium
PERK	PKR-like ER kinase
PEST	proline/glutamate/serine/threonine-rich
PG	prostaglandin
PGHS	PG H synthase
PG-PS	peptidoglycan-polysaccharide
PHA	phytohemagglutinin
PI3K	phosphatidylinositol 3-kinase
PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C
PKR	RNA-dependent protein kinase
PP	protein phosphatase
PPAR	peroxisome proliferator-activated receptor
PRR	patter recognition receptor
RANTES	regulated on activation, normal T cell expressed and secreted
RHD	Rel homology domain

RIP	receptor interacting protein
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
RXR	retinoic acid receptor
Sap	SRF accessory protein
SBE	STAT-binding elements
SCID	severe combined immunodeficiency
SD	standard deviation
siRNA	short interfering RNA
SOCS	suppressor of cytokine signaling
SRC	steroid receptor coactivator
STAT	signal transducer and activator of transcription
SV	simian virus
Tak	TGF-β-activated kinase
TANK	TRAF-associated NF-κB activator
TAO	thousand and one amino acid protein kinase
TBK	TANK-binding kinase
TGF	transforming growth factor
Th	T helper
TIR	Toll/IL-1R
TNBS	2,4,6-trinitrobenzene sulfonic acid
TNF	tumor necrosis factor
TNFR	TNF receptor
TOLLIP	Toll-interacting protein
Tpl	tumor progression locus
TRADD	TNF receptor 1-associated death domain
TRAF	TNFR-associated factor
TRIF	TIR domain containing adaptor inducing IFNβ
TX	thromboxane
Tyk2	tyrosine kinase2
TZD	thiazolidinedione
UC	ulcerative colitis
UPR	unfolded protein response
WHO	World Health Organization
XBP	X box protein

LIST OF FIGURES

LIST OF TABLES

APPENDIX 1

Ruiz, PA, Kim, SC, Sartor, RB and Haller, D.

"15-deoxy-delta12,14-prostaglandin J2-mediated ERK signaling inhibits gram-negative bacteria-induced RelA phosphorylation and interleukin-6 gene expression in intestinal epithelial cells through modulation of protein phosphatase 2A activity"

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[*In vitro* experimental work by Ruiz, PA *(Figures 2-7)*; maintenance of the animal facility by Kim, SC and Sartor, RB; writing by Haller, D with editorial help of the co-authors]

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15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2-mediated ERK Signaling Inhibits Gram-negative Bacteria-induced RelA Phosphorylation and Interleukin-6 Gene Expression in Intestinal Epithelial Cells through Modulation of Protein Phosphatase 2A Activity*

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Pedro A. Ruiz‡, Sandra C. Kim§¶, R. Balfour Sartor¶‖, and Dirk Haller‡**

From the ‡Centre for Nutrition and Food Research, Immunobiology of Nutrition, Technical University of Munich, 85350 Freising-Weihenstephan, Germany and §Department of Pediatrics, ‖Departments of Medicine, Microbiology, and Immunology, and ¶the Center for Gastrointestinal Biology and Disease, University of North Carolina, Chapel Hill, North Carolina 27599

We have previously shown that non-pathogenic Gram-negative *Bacteroides vulgatus* induces transient RelA phosphorylation (Ser-536), NF-κB activity, and pro-inflammatory gene expression in native and intestinal epithelial cell (IEC) lines. We now demonstrate that 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) but not prostaglandin E_2 inhibits lipopolysaccharide (LPS) (*B. vulgatus*)/LPS (*Escherichia coli*)-induced RelA phosphorylation and interleukin-6 gene expression in the colonic epithelial cell line CMT-93. This inhibitory effect of 15d-PGJ_2 was mediated independently of LPS-induced IκBα phosphorylation/degradation and RelA nuclear translocation as well as RelA DNA binding activity. Interestingly, although *B. vulgatus* induced nuclear expression of peroxisome proliferator-activated receptor γ (PPARγ) in native epithelium of monoassociated Fisher rats, PPARγ-specific knock-down in CMT-93 cells using small interference RNA failed to reverse the inhibitory effects of PPARγ agonist 15d-PGJ_2, suggesting PPARγ-independent mechanisms. In addition, 15d-PGJ_2 but not the synthetic high affinity PPARγ ligand rosiglitazone triggered ERK1/2 phosphorylation in IEC, and most importantly, MEK1 inhibitor PD98059 reversed the inhibitory effect of 15dPGJ_2 on LPS-induced RelA phosphorylation and interleukin-6 gene expression. Calyculin A, a specific phosphoserine/phosphothreonine phosphatase inhibitor increased the basal phosphorylation of RelA and reversed the inhibitory effect of 15d-PGJ_2 on LPS-induced RelA phosphorylation. We further demonstrated in co-immunoprecipitation experiments that 15d-PGJ_2 triggered protein phosphatase 2A activity, which directly dephosphorylated RelA in LPS-stimulated CMT-93 cells. We concluded that 15d-PGJ_2 may help to control NF-κB signaling and normal intestinal homeostasis to the enteric microflora by modulating RelA phosphorylation in IEC through altered protein phosphatase 2A activity.

The mucosal surfaces and cavities of the gastrointestinal tract in humans and animals are populated by a complex mixture of non-pathogenic microorganisms of more than 400 species having spatial differences in population size and relative species predominance along the digestive tract (1–4). The host has evolved various homeostatic mechanisms to acquire tolerance (hyporesponsiveness) to resident enteric microorganisms, whereas protective cell-mediated and humoral immune responses to enteropathogens are maintained. This complex homeostasis toward the normal enteric microflora is broken under conditions of chronic intestinal inflammation including the chronically relapsing, immune-mediated idiopathic disorders ulcerative colitis and Crohn's disease (5, 6). The selective effect of microbial factors in initiating and perpetuating chronic intestinal inflammation is extensively supported in comparative studies using germ-free and gnotobiotic rodent models for experimental colitis (7). For example, reconstitution studies with various non-pathogenic bacteria implicate *Bacteroides vulgatus* as particularly important to the induction of experimental colitis in monoassociated HLA-B27 transgenic rats (8, 9).

Increased NF-κB activity has been well documented in intestinal epithelial cell (IEC)[1] and lamina propria cells of inflammatory bowel disease patients with active disease (10–12), and accordingly, pharmacological NF-κB blockade may become potentially important in the treatment of chronic intestinal inflammation (13, 14). Indeed, local administration of antisense RelA oligonucleotides abrogated clinical and histological signs of trinitrobenzene sulfonic acid-induced experimental colitis, suggesting a mechanistic role for sustained NF-κB activity in the pathogenesis of chronic mucosal inflammation (15). On the other hand, blocking NF-κB activity with pharmacological inhibitors during the resolution phase of carrageenan-induced acute inflammation is deleterious to the host (16), suggesting dual functions of activated NF-κB including protective and detrimental mechanisms during the course of inflammation.

The induction of the IκB/NF-κB system and NF-κB-dependent gene expression is a complex process that involves the participation of multiple adaptor proteins and kinases acting in a coordinate fashion to give specificity to the cell surface stimuli. We have previously shown that *B. vulgatus* and lipopoly-

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** To whom correspondence should be addressed. Tel.: 49-8161-71-2026; Fax: 49-8161-71-3999; E-mail: haller@wzw.tum.de.

[1] The abbreviations used are: IEC, intestinal epithelial cell(s); NF-κB, nuclear factor κB; LPS, lipopolysaccharide; ERK, extracellular signal-regulated kinase; 15d-PGJ_2, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2; PPARγ, peroxisome proliferator-activated receptor γ; PP2A, protein phosphatase 2A; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; IKK, IκB kinase complex; TZD, thiazolidinedione; IL-6, interleukin 6; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; siRNA, small interference RNA; ELISA, enzyme-linked immunosorbent assay.

saccharide (LPS) signal through the TLR4 cascade to trigger IRAK1 degradation and IκBα phosphorylation/degradation as well as NF-κB DNA binding activity and NF-κB transcriptional activity in native and IEC lines (17). In addition to the activation of the IκB/NF-κB system and nuclear translocation of transcriptionally active RelA, the modification of NF-κB transcriptional activity by phosphorylation of RelA at various serine residues (Ser-276, Ser-529, Ser-536) has been shown to be an important regulatory element of this signaling pathway (18–21). Potential kinases involved in signal-induced RelA phosphorylation are the casein kinase II, Akt, and IKK. We showed that IKKβ and the phosphatidylinositol 3-kinase/Akt pathway participate in *B. vulgatus*-induced phosphorylation of serine 529 and/or 536 of the RelA transactivating domain 1 (TAD1) in IEC (17). Most importantly for the physiological relevance, we showed that monoassociation of wild type rats with *B. vulgatus* triggered transient nuclear localization of phosphorylated (Ser-536) and transcriptionally active NF-κB subunit RelA (p65) in the intestinal epithelium (17, 22). The absence of colitis and pathological immune responses in *B. vulgatus*-monoassociated wild type rats confirmed the non-pathogenic nature of this obligate anaerobic Gram-negative bacterial strain and suggests that the normal host developed mechanisms to control NF-κB activity in IEC (23).

Protein serine/threonine phosphatases including PP1, PP2A, PP2B, and PP2C are involved in the regulation of signaling pathways with a variety of protein kinases (24, 25). PP2A has been shown to form a complex with calcium/calmodulin-dependent protein kinase IV (26), casein kinase (27), p21-activated kinase-1 and -3, and p70 S6 kinase (28) as well as certain G-protein-coupled receptors (29, 30). Recently, PP2A was shown to interact with and directly dephosphorylate RelA, suggesting PP2A as an important regulator of NF-κB signaling (31, 32). The predominant heterotrimeric form of PP2A consists of the 36-kDa catalytic subunit (PP2Ac) and a 65-kDa regulatory subunit (PP2Aa or PR65). In addition, several regulatory subunits are associated with the core enzyme, conferring substrate specificity to its dephosphorylating activity (24, 25).

Peroxisome proliferator-activated receptor γ (PPARγ) is a member of the steroid receptor superfamily with various cellular functions including differentiation, apoptosis, lipid metabolism, and anti-inflammatory responses (33–35). Although PPARγ is expressed in multiple tissues, the highest levels are found in adipose tissue and colonic epithelium (36). Ligand-specific activation of the PPARγ transcription factor has been shown to inhibit pro-inflammatory gene expression and experimental colitis for synthetic anti-diabetic thiazolidinediones (TZDs) including rosiglitazone and troglizitazone as well as the endogenous prostaglandin D$_2$ metabolite 15-deoxy-$\Delta^{12,14}$-prostaglandin J$_2$ (15d-PGJ$_2$) (37–42). It appears from several studies that TZD and 15d-PGJ$_2$, which is present *in vivo* during the resolution phase of acute inflammation (43), mediate their anti-inflammatory effects also through PPARγ-independent mechanisms affecting the NF-κB signaling pathway at the level of IKK activity and IκBα degradation as well as RelA DNA binding activity (37, 44, 45).

In this study we characterized the molecular mechanism for the inhibitory effect of 15d-PGJ$_2$ on RelA phosphorylation (Ser-536) and IL-6 gene expression in IEC. Consistent with the transient induction of phospho-RelA (Ser-536) in the intestinal epithelium of *B. vulgatus*-monoassociated Fisher rats, 15d-PGJ$_2$ but not the synthetic high affinity PPARγ ligand rosiglitazone inhibited LPS (*B. vulgatus*)/LPS (*Escherichia coli*)-induced RelA phosphorylation and IL-6 gene expression in CMT-93 cells. Although *B. vulgatus* triggered nuclear expression of PPARγ in native epithelium of monoassociated Fisher rats, PPARγ-specific knock-down in CMT-93 cells using small interference RNA failed to reverse the inhibitory effects of PPARγ agonist 15d-PGJ$_2$, suggesting PPARγ-independent mechanisms. Finally, we could demonstrate that 15d-PGJ$_2$ inhibits LPS-induced RelA phosphorylation and IL-6 gene expression in IEC through the induction of the ERK signaling cascade by modulating PP2A activity.

MATERIALS AND METHODS

Animals and Bacterial Monoassociation—Germ-free Fisher F344 rats were monoassociated at 10–12 weeks of age with *B. vulgatus* (a generous gift from Dr. A. B. Onderdonk, Harvard University, Cambridge, MA) and maintained in the Gnotobiotic Animal Core at the College of Veterinary Medicine, North Carolina State University (Raleigh, NC). Bacterial monoassociation and the absence of contamination by other bacterial species were confirmed by culturing samples from the large intestine at necropsy and culturing serial fecal samples. Animal use protocols were approved by the Institutional Animal Care and Use Committee (IACUC), North Carolina State University. Rats were killed 3, 7, 14, and 35 days after initial bacterial colonization. Germ-free mice were used as controls. Sections of the ileum, cecum, proximal, and distal colon were fixed in 10% neutral buffered formalin. The fixed tissue was embedded in paraffin. Histology scoring was analyzed by blindly assessing the degree of lamina propria mononuclear cell infiltration, crypt hyperplasia, goblet cell depletion, and architectural distortion.

Immunohistochemistry and Isolation of Primary Rat Intestinal Epithelial Cells—B. vulgatus-monoassociated and germ-free rats were euthanized, and the cecum as well as colon were removed and placed in Dulbecco's modified Eagle's medium (Invitrogen) containing 5% fetal calf serum. Cecum and colon were cut longitudinally, washed three times in calcium/magnesium-free Hanks' balanced salt solution (Invitrogen), cut into pieces 0.5 cm long, and incubated at 37 °C in 40 ml of Dulbecco's modified Eagle's medium containing 5% fetal calf serum and 1 mM dithiothreitol for 30 min in an orbital shaker. The supernatant was filtered and centrifuged for 5 min at 400 $\times$ *g*, and the cell pellet was resuspended in Dulbecco's modified Eagle's medium containing 5% fetal calf serum. The remaining tissue was incubated in 30 ml of phosphate-buffered saline (1$\times$) containing 1.5 mM EDTA for an additional 10 min. The supernatant was filtered and centrifuged for 5 min at 400 $\times$ *g*, and the cell pellet was resuspended in Dulbecco's modified Eagle's medium containing 5% fetal calf serum. Finally, primary IEC were collected by centrifugation through a 25/40% discontinuous Percoll gradient at 600 $\times$ *g* for 30 min. Cell viability and purity was assessed by trypan blue exclusion and fluorescence-activated cell sorter analysis using mouse anti-CD3 monoclonal antibody (BD Biosciences Pharmingen, clone G4.18). Cells were >80% viable and >90% pure. Primary rat IEC from cecum and colon were combined and collected in sample buffer for subsequent Western blot analysis. Immunohistochemistry on paraffin-embedded tissue sections was performed using anti-PPARγ Ab (Cell Signaling, Beverly, MA) according to the protocol of the manufacturer, and sections were counterstained with hematoxylin.

Cell Culture and Bacterial Infection—The IEC line CMT-93 (passage 10–30) (ATCC CRL 223, American Type Culture Collection) was grown in a humidified 5% CO_2 atmosphere at 37 °C to confluency in 6-well tissue culture plates (Cell Star, Greiner bio-one, Frickenhausen, Germany) as previously described (17). *B. vulgatus* was anaerobically grown at 37 °C in brain-heart infusion broth. Bacteria were harvested by centrifugation (3000 $\times$ *g*, 15 min) supplemented with cysteine (0.05%), hemin (5 mg/liter), and resazurin at stationary growth phase. For LPS purification, *B. vulgatus* was killed by the addition of 1% phenol (Fluka, Heidelberg) and washed thoroughly with twice-distilled water. Endotoxin was extracted and purified by the method of Westphal *et al.* (26). Purity was checked by SDS-PAGE (46). Confluent epithelial cell monolayers were stimulated with *B. vulgatus* LPS (10 μg/ml) and *E. coli* LPS (10 μg/ml; from *E. coli* serotype O111:B4, Sigma) in the presence or absence of 20 μM 15d-PGJ$_2$ (BioMol, Plymouth Meeting, PA) and 20 μM rosiglitazone (BioMol). Where indicated, cells were treated with 20 μM MEK1 inhibitor PD98059 (Calbiochem) or 1–5 nM calyculin A (Cell Signaling).

RNA Isolation and Real-time Reverse Transcription-PCR—RNA from IEC was extracted using Trizol reagent (Invitrogen) according to the manufacturer's instructions. Extracted RNA was dissolved in 20 μl of water containing 0.1% diethyl pyrocarbonate. For reverse transcription, 1 μg of total RNA was added to 30 μl of reaction buffer containing 8 μl of 5$\times$ first-strand buffer, 4 μl of dithiothreitol (100 mM), and 6 μl of deoxyribonucleoside triphosphate mixture (300 μM) (all reagents from Invitrogen) and incubated for 5 min at 65 °C. After adding 10 μl of a

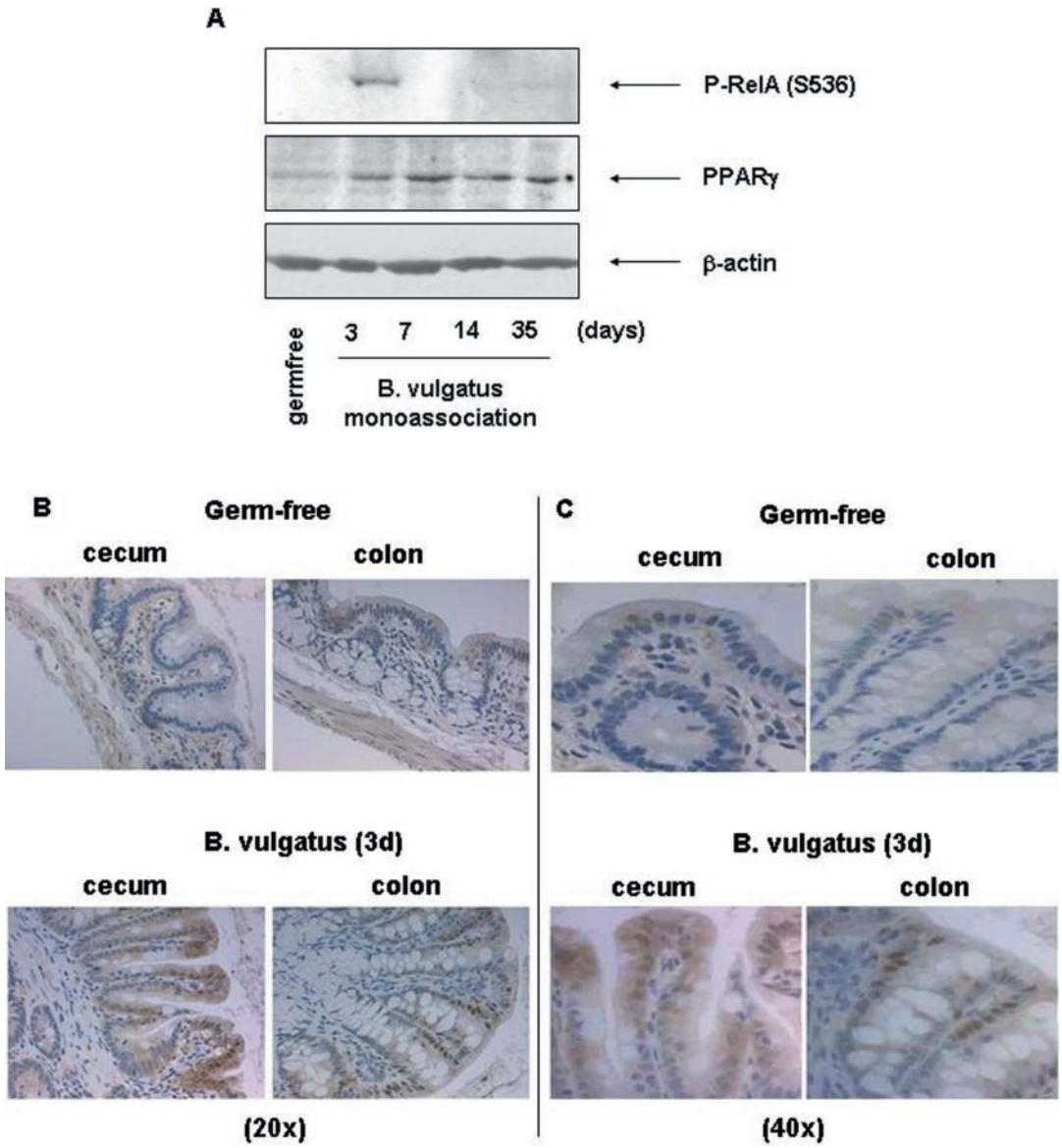

FIG. 1. **RelA phosphorylation and PPARγ expression in intestinal epithelium of *B. vulgatus*-monoassociated germ-free Fisher rats.** Germ-free Fisher rats (n = 2) were monoassociated with *B. vulgatus*. Rats were killed at days 3, 7, 14, and 35 after initial bacterial colonization. Native IEC were isolated from cecum and colon. Total protein was extracted, and 20 μg protein were subjected to SDS-PAGE followed by phospho-RelA, PPARγ, and β-actin immunoblotting using ECL technique (*A*). Sections of cecum and colon were fixed in 10% neutral-buffered formalin. Immunohistochemistry was performed on paraffin-embedded tissue sections using anti-PPARγ antibody and analyzed at 20× or 40× magnification (*B* and *C*). Sections were counterstained with hematoxylin.

solution containing 0.2 μg of random hexamers, 40 units of RNase Out, and 200 units of Moloney murine leukemia virus reverse transcriptase (all reagents from Invitrogen), the total mixture was incubated for an additional 60 min at 37 °C followed by a final 1-min heating step at 99 °C.

Real-time PCR was performed in glass capillaries using a Light Cycler™ system (Roche Diagnostics). Primer sequences and amplicon sizes are as follows: IL-6, 5′-acaacgatgatgcactt-3′ (forward) and 5′cttg-gtccttagccact-3′ (reverse) (334 bp); IκBα, 5′-gtgactttgggtgctg-3′ (forward) and 5′-gctgtatccgggtactt-3′ (reverse) (193 bp); PPARγ, 5′-tccgta-gaagccgtgc-3′ (forward) and 5′-ggatgtcctcgatggg-3′ (reverse) (364 bp); GAPDH, 5′-atcccagagctgaacg-3′ (forward) and 5′-gaagtcgcaggagaca-3′ (reverse) (198 bp). For real-time PCR, 1 μl of reverse-transcribed cDNA was added in a total volume of 10 μl of PCR reaction buffer containing 1× LC-FastStart DNA Master Mix (Roche Applied Science), $MgCl_2$ (4 μM), and forward and reverse primers (20 μM). The PCR program was one cycle of denaturation at 95 °C for 10 min followed by 50 cycles of 95 °C for 15 s, annealing at 60 °C for 10 s, and extension at 72 °C for 20 s. The amplified product was detected by the presence of a SYBR green fluorescent signal. Melting curve analysis and gel electrophoresis was used to document the amplicon specificity. 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 determined using the calculation $E^{\Delta Cp\ (\text{control samples} - \text{treated samples})}$ and normalized for the expression of GAPDH mRNA (47). Triplicate samples were measured in duplicate and blotted as -fold increase between treated and untreated control samples.

Western Blot Analysis—IEC were lysed in 1× Laemmli buffer, and 20–50 μg of protein was subjected to electrophoresis on 10% SDS-PAGE gels. Where indicated IEC cells were pretreated for 1 h with 20 μM proteasome inhibitor MG132 (BioMol). Anti-IκBα (C21, Santa Cruz Biotechnology), anti-NF-κB RelA (Santa Cruz), anti-phospho-IκBα (Ser-32), anti-phospho-RelA (Ser-536), anti-phospho-ERK1/2 (p44/p42) (Tyr-202/204), ERK1/2, anti-PPARγ (all antibodies from Cell Signaling, Beverly, MA), anti-PP2A (Abcam, Cambridge, UK), and anti-β-actin (ICN, Costa Mesa, CA) were used to detect immunoreactive phospho-IκBα, total IκBα, total RelA, phospho-RelA, phospho-ERK1/2, ERK1/2, PPARγ, PP2A, and β-actin, respectively, using an enhanced chemiluminescence light-detecting kit (Amersham Biosciences) as previously described (17).

Small Interference RNA and Transfection—Synthetic PPARγ-specific (accession number NM_011146) siRNA was designed and pur-

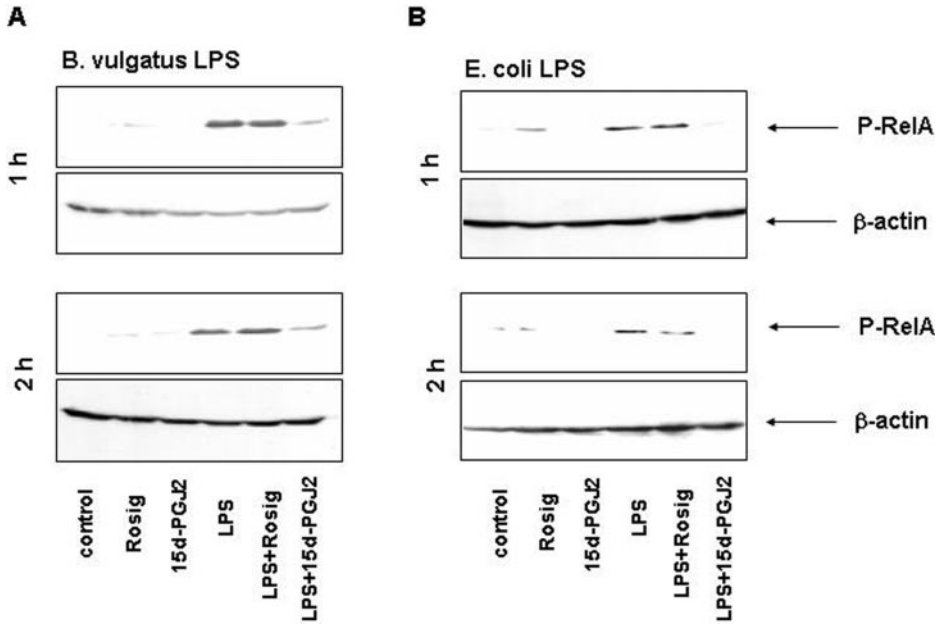

FIG. 2. **15d-PGJ$_2$ inhibits LPS-induced RelA phosphorylation in CMT-93 cells.** CMT-93 cells were stimulated with 10 μg/ml LPS from *B. vulgatus* (*A*) and *E. coli* (*B*) for 1 and 2 h in the presence of 20 μM 15d-PGJ$_2$ and 20 μM rosiglitazone (*Rosig*). Total protein was extracted, and 20 μg of protein were subjected to SDS-PAGE followed by phospho-RelA and β-actin immunoblotting using ECL technique. The results are representative of three independent experiments.

chased from Qiagen (Hilden, Germany) according to the protocol of the manufacturer. The sequence was as follows: sense, 5′-AGACCCAG-CUCUACAACAG(TT)-3′; reverse 5′-CUGUUGUAGAGCUGGGUCU-(TT)-3′. The annealed double stranded ribooligonucleotides were dissolved in RNase-free buffer and stored at −20 °C in a concentration of 20 μM. Before performing the experiments, the siRNA was heat-treated for 1 min at 90 °C and then incubated for an additional 60 min at 37 °C. CMT-93 cells, which were grown to 80–90% confluency, were transfected with single-stranded siRNA (0.4, 0.8, or 1.6 μg of total siRNA) according to the protocol of the manufacturer using TransMessanger reagent (Qiagen). Finally, the transfected CMT-93 cells were cultured for an additional 48 h and then stimulated with LPS (10 μg/ml) for 12 h in the presence or absence of 15d-PGJ$_2$. Rhodamine-stained siRNA was used to visualize the cellular distribution in transfected CMT-93 epithelial monolayers using fluorescence microscopy.

Nuclear Extracts and NF-κB p65 Protein/DNA Binding Activity—CMT-93 cells were stimulated for various times (0–4 h) with *E. coli* LPS (10 μg/ml), and nuclear extracts were prepared according to the manufacturer's instructions (Active Motif, Carlsbad, CA). Extracts (5 μg) were used to determine nuclear RelA binding activity to the κB-nucleotide consensus sequence 5′-GGGACTTTCC-3′ by using the TransAM ELISA-based NF-κB transcription factor assay (Active Motif). Protein/oligonucleotide binding activity was quantified by colorimetric analysis using a MultiScan spectrophotometer.

Co-immunoprecipitation and Phosphatase Activity Assay—CMT-93 cells were stimulated with either *E. coli* LPS (10 μg/ml) or 15d-PGJ$_2$ (20 μM). Cells were lysed in lysis buffer (50 mM Tris at pH 8.0, 2 mM EDTA, 0.1% Nonidet P-40, and 10% glycerol supplemented with protease inhibitors. Cell debris was removed by centrifugation at 12,000 × *g*, and the supernatant was precleared for 1 h with 20 μl of protein A/G-agarose (Santa, Cruz, Europe). Total protein concentration was normalized, and immunoprecipitation was carried out overnight at 4 °C using 5 μl of rabbit anti-phospho-RelA and goat anti-PP2A antibody (Abcam). Immune complexes were collected with 30 μl of protein A/G-agarose for 30 min, washed, and resuspended in reaction buffer (100 μl) containing 20 mM HEPES (pH 7.4), 10 mM $MgCl_2$, and 1 μM dithiothreitol. To assay the phosphatase activity, 30 μl of beads from LPS-stimulated cells were co-incubated with 50 μl of beads from 15d-PGJ$_2$-treated cells. Reactions were stopped after 1 and 2 h of incubation at 37 °C with the addition 2× Laemmli sample buffer. Western blot analysis for phospho-RelA and PP2A were carried out as described above.

ELISA Analysis—Protein concentrations were determined in spent culture supernatants of IEC cultures using an ELISA technique. IL-6 protein production was determined by mouse-specific ELISA assay kits according to the manufacturer's instructions (R&D Systems, Heidelberg, Germany).

Statistical Analysis—Data are expressed as the mean ± S.D. of triplicates. Statistical analysis was performed by the two-tailed Student's *t* test for paired data and considered significant if *p* values were <0.05 (*) or <0.01 (**).

RESULTS

B. vulgatus-monoassociated Fisher Rats Trigger Transient Phosphorylation of RelA and Persistent Expression of PPAR-γ in Native IEC—We investigated RelA phosphorylation and PPAR-γ expression in the intestinal epithelium of *B. vulgatus*-monoassociated and germ-free Fisher rats. The rats were killed after 3, 7, 14, and 35 days of initial bacterial colonization, and native IEC were isolated from cecal and colonic tissue. Phospho-RelA (Ser-536) and PPARγ protein expression were measured in isolated native IEC using Western blot analysis. As shown in Fig. 1, although *B. vulgatus* monoassociation of germ-free rats triggered transient phosphorylation of RelA at day 3 in native IEC, PPARγ protein expression was persistently induced 3–28 days after bacterial colonization. Nuclear localization of PPARγ in cecal and colonic epithelium was confirmed in intestinal tissue sections by performing immunohistochemical analysis (Fig. 1, *B* and *C*).

15d-PGJ$_2$ but Not Rosiglitazone Inhibits RelA Phosphorylation in CMT-93 Cells after Stimulation with B. vulgatus and E. coli LPS—We have previously shown that whole *B. vulgatus* cells and *E. coli*-derived LPS trigger RelA phosphorylation, NF-κB activity, and pro-inflammatory gene expression in IEC lines. Based on these results, we next asked the question whether PPARγ agonists inhibit RelA phosphorylation in CMT-93 cells. We used the endogenous prostaglandin D_2 metabolite 15d-PGJ$_2$ and the synthetic PPARγ ligand rosiglitazone to antagonize *B. vulgatus* LPS- and *E. coli* LPS-induced RelA phosphorylation in CMT-93. Fig. 2 shows that 15d-PGJ$_2$ inhibits RelA phosphorylation in CMT-93 cells after 1 and 2 h of stimulation with *B. vulgatus* LPS (Fig. 2*A*) as well as *E. coli* LPS (Fig. 2*B*). Of note, the synthetic high affinity PPARγ ligand rosiglitazone did not inhibit LPS-induced RelA phosphorylation. To further characterize the inhibitory mechanisms of 15d-PGJ$_2$ on LPS-induced NF-κB signaling, we used the commercially available *E. coli* LPS.

15d-PGJ$_2$ Failed to Inhibit LPS-induced IκBα Degradation, RelA Nuclear Translocation, and RelA DNA Binding Activity in IEC—We next investigated the effect of 15d-PGJ$_2$ and rosiglitazone on LPS-induced IκBα degradation, RelA nuclear translocation, and RelA DNA binding activity in CMT-93 cells. We stimulated the cells with LPS in the presence or absence of 15d-PGJ$_2$ and rosiglitazone for 1 and 2 h. Fig. 3*A* shows that

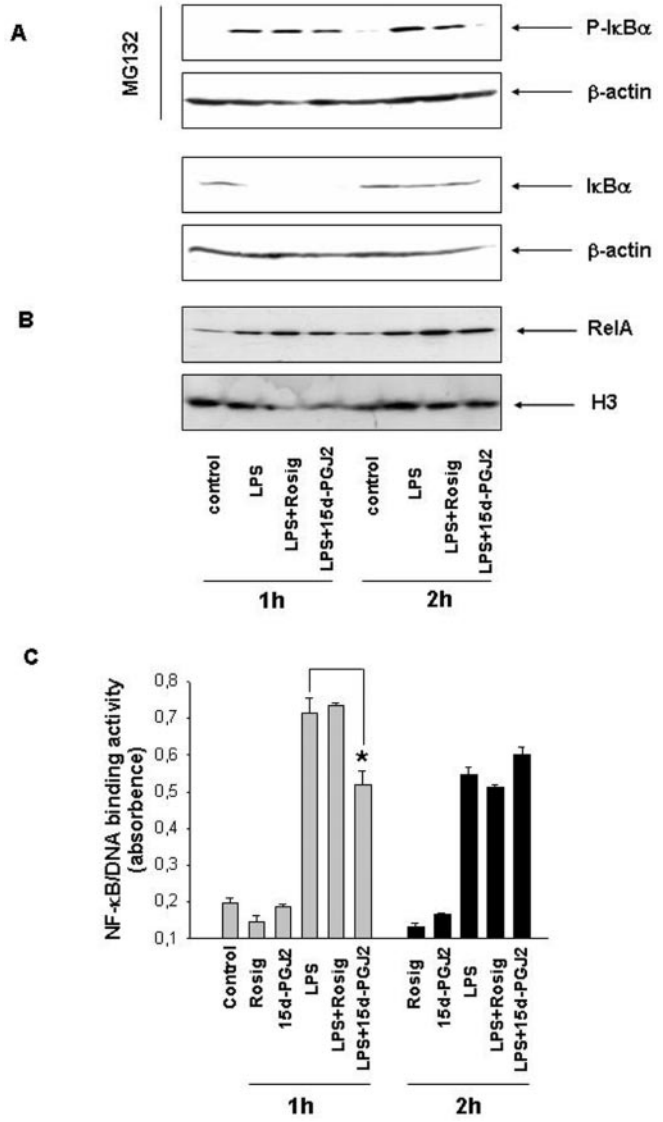

FIG. 3. **Effects of 15d-PGJ$_2$ on LPS-induced IκBα phosphorylation/degradation, RelA nuclear translocation, and RelA DNA binding activity.** CMT-93 cells were stimulated with 10 μg/ml LPS from *E. coli* for 1 and 2 h in the presence or absence of 20 μM 15d-PGJ$_2$ and 20 μM rosiglitazone (*Rosig*). Total protein was extracted, and 20 μg of protein were subjected to SDS-PAGE followed by phospho-IκBα, IκBα, and β-actin immunoblotting using an ECL technique (*A*). Nuclear extracts were isolated, and 20 μg of nuclear protein were subjected to SDS-PAGE followed by RelA and β-actin immunoblotting using ECL technique (*B*). Nuclear extracts were isolated, and RelA DNA binding activity was quantified using a TransAM ELISA-based NF-κB transcription factor assay. Protein/oligonucleotides binding activity was determined by colorimetric analysis using 5 μg of nuclear extracts (*C*). The results are representative three independent experiments.

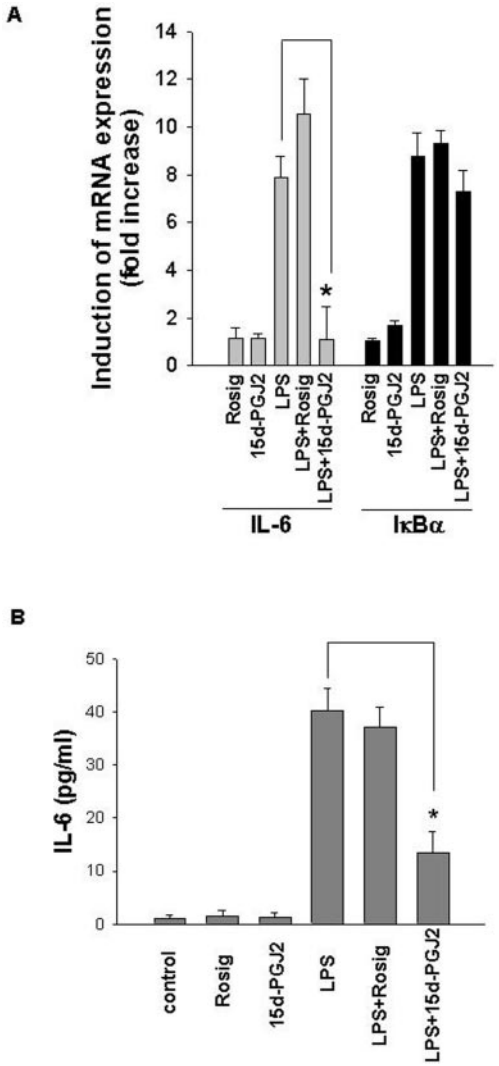

FIG. 4. **15d-PGJ$_2$ inhibits LPS-induced IL-6 mRNA but not IκBα gene expression.** CMT-93 cells were stimulated with 10 μg/ml LPS from *E. coli* for 12 h in the presence or absence of 20 μM 15d-PGJ$_2$ and 20 μM rosiglitazone (*Rosig*). Total RNA was extracted and reverse-transcribed, and real-time PCR was performed using the Light Cycler system with specific primers for murine IL-6, IκBα, and GAPDH. The induction of IL-6 and IκBα mRNA expression was calculated relative to untreated controls (-fold increase) using the crossing point of the log-linear portion of the amplification curve after normalization with GAPDH (*A*). CMT-93 cells were stimulated with 10 μg/ml LPS from *E. coli* for 36 h in the presence or absence of 20 μM 15d-PGJ$_2$ and rosiglitazone. IL-6 was measured in the spent culture supernatant using the ELISA method. The *bars* represent the combined mean values (±S.D.) of triplicate samples. *B*: *, *p* value <0.05.

LPS-induced IκBα phosphorylation as well as IκBα degradation in CMT-93 cells after 1 h of stimulation followed by complete IκBα resynthesis after 2 h of stimulation. Of note, 15d-PGJ$_2$ and rosiglitazone failed to inhibit LPS-induced IκBα phosphorylation/degradation after 1 h of stimulation. Interestingly and in contrast to rosiglitazone, 15d-PGJ$_2$ blocked IκBα phosphorylation after 2 h of stimulation. IκBα protein resynthesis was not affected in the presence of any of the two PPARγ agonists.

We next studied LPS-induced nuclear translocation and RelA DNA binding activity in the presence or absence of 15d-PGJ$_2$ and rosiglitazone in CMT-93 cells. As shown in Fig. 3*C*, whereas LPS-induced RelA DNA binding activity was slightly reduced after 1 h of stimulation, this moderate inhibitory effect was completely abolished after 2 h of stimulation. Rosiglitazone did not affect RelA nuclear translocation or RelA DNA binding activity (Fig. 3*B*).In summary, these results suggest that 15d-PGJ$_2$ did not affect LPS-induced IκBα degradation, IκBα resynthesis, or RelA nuclear translocation but slightly delayed RelA DNA binding activity in LPS-stimulated IEC.

15d-PGJ$_2$ Selectively Inhibited IL-6 but Not IκBα Gene Expression in LPS-stimulated IEC—We next sought to investigate the effect of 15d-PGJ$_2$ and rosiglitazone on LPS-induced IL-6 and IκBα gene expression in CMT-93 cells. Therefore, we stimulated the cells for 12 h with LPS in the presence or absence of 15d-PGJ$_2$ and rosiglitazone. Interestingly, reverse transcription and real-time PCR analysis revealed that 15d-PGJ$_2$ but not rosiglitazone inhibited IL-6 mRNA expression in LPS-stimulated CMT-93 cells (Fig. 4*A*). Accordingly, IL-6 protein production was significantly inhibited in the presence of

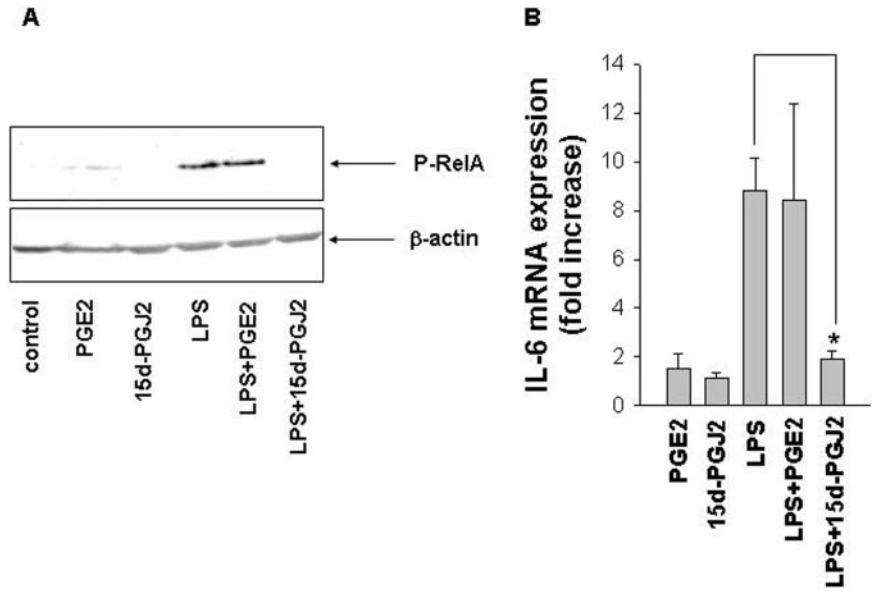

FIG. 5. **15d-PGJ$_2$ but not PGE$_2$ inhibit LPS-induced RelA phosphorylation and IL-6 gene expression.** CMT-93 cells were stimulated with 10 μg/ml LPS from *E. coli* in the presence or absence of 20 μM 15d-PGJ$_2$ and 20 μM PGE$_2$. *A*, total protein was extracted after 1 and 2 h of stimulation, and 20 μg of protein were subjected to SDS-PAGE followed by phospho-RelA and β-actin immunoblotting using ECL technique. *B*, total RNA was extracted after 12 h of stimulation, reverse-transcribed, and real-time PCR was performed using the Light Cycler system with specific primers for murine IL-6 and GAPDH. The induction of IL-6 mRNA expression was calculated relative to untreated controls (fold increase) using the crossing point of the log-linear portion of the amplification curve after normalization with GAPDH. The *bars* represent the combined mean values ($\pm$S.D.) of triplicate samples. *, p value <0.05.

15d-PGJ$_2$ but not rosiglitazone (Fig. 4*B*). Consistent with our previous results, IκBα mRNA expression was not affected in the presence of 15d-PGJ$_2$ or rosiglitazone.

Because 15d-PGJ$_2$ but not the synthetic high affinity PPARγ ligand rosiglitazone displayed inhibitory effects on IEC activation, we next transfected CMT-93 cells with PPARγ-specific siRNA oligonucleotides (0.8 μg). Western blot and real-time PCR analysis confirmed PPARγ-specific knock-down (>80%) in CMT-93 cells in the presence of siRNA oligonucleotides. Most importantly, functional analysis in the presence PPARγ-specific siRNA revealed no effect on 15d-PGJ$_2$-mediated inhibition of IL-6 gene expression, confirming PPARγ-independent mechanisms for the inhibitory effects 15d-PGJ$_2$ in LPS-activated IEC (data not shown).

To further elucidate the specificity of 15d-PGJ$_2$-mediated inhibition of RelA phosphorylation, we stimulated CMT-93 cells with LPS in the presence or absence of 15dPGJ$_2$ and PGE$_2$. As shown in Fig. 5, 15d-PGJ$_2$ but not PGE$_2$ blocked LPS-induced RelA phosphorylation (Fig. 5*A*) as well as IL-6 mRNA expression (Fig. 5*B*).

15d-PGJ$_2$-mediated Inhibition of LPS-induced RelA Phosphorylation and IL-6 Gene Expression Is Reversed in the Presence of MEK1 Inhibitor PD98059—The mechanisms of 15d-PGJ$_2$ to inhibit RelA phosphorylation and IL-6 gene expression were independent from the presence of PPARγ. It has been previously shown that 15d-PGJ$_2$ triggers ERK1/2 phosphorylation in murine myoblast cell line C2C12 (48). Based on these results, we next investigated the role of the MAP kinase pathway ERK1/2 to mediate the inhibitory effect of 15d-PGJ$_2$ on LPS-induced IEC activation. Interestingly, 15d-PGJ$_2$ and LPS but not rosiglitazone (data not shown) induced ERK1/2 phosphorylation in CMT-93 cells after 1 h of stimulation, whereas a weak expression of phospho-ERK1/2 remained after 2 h of stimulation in the presence of 15d-PGJ$_2$ but not LPS or LPS/15d-PGJ$_2$ (data not shown). We then used the MEK1 inhibitor PD98059 to inhibit ERK1/2 phosphorylation in CMT-93 cells after the stimulation with LPS for 1 h. Fig. 6*A* shows that the addition of PD98059 inhibited LPS-induced ERK phosphorylation in the presence (*lane 5*) and absence (*lane 7*) of 15d-PGJ$_2$. Interestingly, LPS stimulation in the presence of 15d-PGJ$_2$ further increased ERK1/2 phosphorylation in CMT-93 cells after 1 h of stimulation (*lane 6*). Consistent with our previous results (Figs. 2 and 5), 15d-PGJ$_2$ completely inhibited LPS-induced RelA phosphorylation (*lane 13*). Most importantly, although the presence of PD98059 revealed no effect on LPS-induced RelA phosphorylation (*lane 12*), the presence of the MEK1 inhibitor at least partially reversed 15d-PGJ$_2$-mediated inhibition of RelA phosphorylation (*lane 14*).

To further elucidate the role of the ERK signaling pathway on the inhibitory effects of 15d-PGJ$_2$, we measured LPS-induced IL-6 gene expression in the presence or absence of 15d-PGJ$_2$ after 12 h of stimulation. Real-time RT-PCR analysis revealed that the presence of PD98059 completely reversed 15d-PGJ2-mediated inhibition of LPS-induced IL-6 mRNA expression, whereas PD98059 alone did not affect LPS-induced IL-6 gene expression (Fig. 6*B*). In conclusion, these results suggest that 15d-PGJ$_2$ inhibits LPS-induced RelA phosphorylation as well as IL-6 gene expression in IEC through induction of the ERK-signaling cascade.

15d-PGJ$_2$ Triggers Protein Phosphatase 2A Activity, Which Directly Dephosphorylates RelA—Protein serine/threonine phosphatase activity plays an important role in the regulation of transcription factor activity (24, 25). To further elucidate the role of protein serine/threonine phosphatase activity in 15d-PGJ$_2$-mediated inhibition of RelA phosphorylation and IL-6 gene expression, we used the specific inhibitor calyculin A. Interestingly, the treatment of CMT-93 cells with calyculin A (5 nM) dramatically increased the basal level RelA phosphorylation after 1 h of stimulation (Fig. 7*A*, *lane 2*). The additional treatment of CMT-93 cells with LPS did not further increase RelA phosphorylation (Fig. 7*A*, *lane 5*). In addition and consistent with our previous results, LPS-induced RelA phosphorylation was inhibited in the presence of 15d-PGJ$_2$ (Fig. 7*A*, compare *lane 4* and *6*), and most importantly, calyculin A completely reversed this inhibitory effect of 15d-PGJ$_2$ (Fig. 7*A*, *lane 7*). Total PP2A was similar in all samples, suggesting that PP2A expression remained unchanged during the different treatments. As shown in Fig. 7*B*, LPS-induced IL-6 mRNA expression was significantly increased in the presence of calyculin A. Similar to our previous results, 15d-PGJ$_2$ inhibited LPS-induced IL-6 gene expression. This inhibitory effect was reversed in the presence of calyculin A, suggesting a role of protein phosphatases for the inhibitory effects of 15d-PGJ$_2$ on RelA phosphorylation as well as IL-6 gene expression in CMT-93 cells.

To further specify the role of 15d-PGJ$_2$ in triggering phosphatase activity, we co-immunoprecipitated endogenous phospho-RelA and PP2A after the treatment of CMT-93 cells with

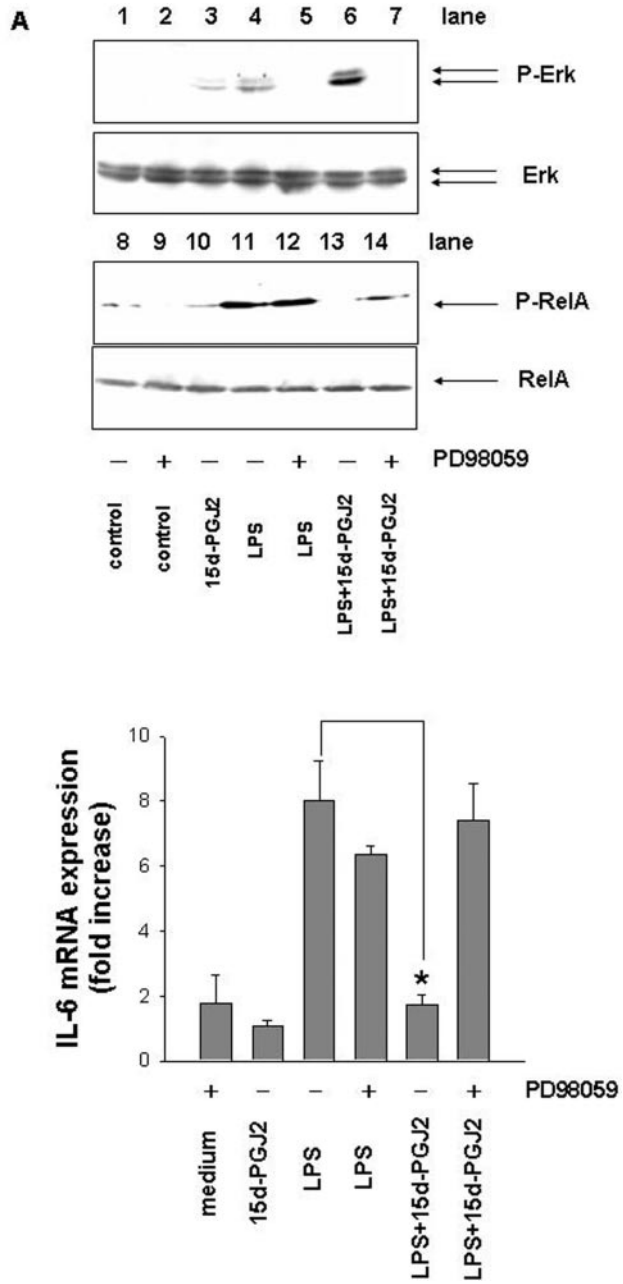

FIG. 6. **The inhibitory effect of 15d-PGJ2 on LPS-induced RelA phosphorylation and IL-6 gene expression was reversed in the presence of PD98059.** CMT-93 cells were stimulated with *E. coli* LPS (10 μg/ml) for 1 h in the presence or absence of 20 μM 15d-PGJ$_2$. Where indicated the cells were pretreated for 1 h with 50 μM PD98059. Total protein was extracted, and 20 μg of protein were subjected to SDS-PAGE followed by phospho-ERK1/2, ERK1/2, phospho-RelA, and RelA immunoblotting using an ECL technique (*A*). CMT-93 cells were stimulated with *E. coli* LPS (10 μg/ml) for 12 h in the presence or absence of 20 μM 15d-PGJ$_2$. Where indicated the cells were pretreated for 1 h with 50 μM PD98059. Total RNA was extracted after 12 h of stimulation and reverse-transcribed, and real-time PCR was performed using the Light Cycler system with specific primers for murine IL-6 and GAPDH. The induction of IL-6 mRNA expression was calculated relative to untreated controls (fold increase) using the crossing point of the log-linear portion of the amplification curve after normalization with GAPDH. The *bars* represent the combined mean values (±S.D.) of triplicate samples. *, p value <0.05.

LPS and 15d-PGJ$_2$, respectively. Immunoprecipitated phospho-RelA was then co-incubated with PP2A-loaded A/G-agarose beads for additional 1, 2, and 4 h. As shown in Fig. 7*C*, PP2A directly dephosphorylated RelA after 1, 2, and 4 h of co-incubation, suggesting that 15d-PGJ$_2$ triggers PP2A activity in CMT-93 cells. Of note, total RelA and PP2A confirmed equal loading of the samples. In conclusion, we demonstrated that 15d-PGJ$_2$ triggered PP2A activity, which directly dephosphorylated RelA in LPS-stimulated CMT-93 cells.

DISCUSSION

In this study, we demonstrate that 15d-PGJ$_2$ inhibits LPS (*B. vulgatus*)/LPS (*E. coli*)-induced RelA phosphorylation and IL-6 gene expression in the colonic epithelial cell line CMT-93 through induction of the MEK/ERK signaling cascade. Consistent with the transient expression of phospho-RelA in native IEC of *B. vulgatus*-monoassociated Fisher rats, 15d-PGJ$_2$ triggers PP2A activity, which directly dephosphorylates RelA in LPS-stimulated CMT-93 cells.

It appears from several studies that PPARγ agonists including synthetic TZD and 15d-PGJ$_2$ attenuate colonic inflammation in different models of experimental colitis (41, 42, 49–51) and antagonize NF-κB signaling through covalent modification and inhibition of IKKβ in PPARγ-independent mechanisms (37, 44, 45). Indeed, we show PPARγ-independent mechanisms for the inhibitory effect of 15d-PGJ$_2$ in CMT-93 cells using siRNA-mediated knock-down of PPARγ. In addition, several studies now point toward ligand-independent anti-inflammatory effects of PPARγ. For example, studies with mice heterozygous for a deficiency of PPARγ (PPAR$\gamma^{-/+}$) were significantly more susceptible to the development of experimental colitis when compared with wild type mice (42, 50, 51), and accordingly, PPARγ expression in colonic epithelium was substantially reduced in patients with ulcerative colitis (52) as well as dextran sodium sulfate-treated mice (53). Interestingly, *Bacteroides thetaiotaomicron* triggered PPARγ-mediated nuclear export of transcriptionally active RelA and directly abolished *Salmonella enteriditis*-induced inflammatory effects in IEC (54). These results provide compelling evidence that PPARγ plays an important role in the regulation of mucosal inflammation. Taken together, two mechanisms are apparent from these studies. First, PPARγ inhibits inflammatory processes in a ligand-independent manner, and second, PPARγ agonists inhibit NF-κB activity as well as experimental colitis through PPARγ-independent mechanisms. Consistent with previously published results (54), we demonstrate that *B. vulgatus* monoassociation of germ-free Fisher rats triggered persistent nuclear but not cytoplasmic expression of the transcription factor PPARγ in the middle/upper part of the crypts of IEC from large intestinal epithelium. In addition, we show that 15d-PGJ$_2$ but not synthetic TZD inhibits RelA phosphorylation and IL-6 gene expression, confirming previously published results that demonstrate different effects between 15d-PGJ$_2$ and synthetic TZD (38, 55, 56). It remains to be seen whether PPARγ expression is directly involved in the negative regulation of *B. vulgatus*-induced NF-κB signal transduction in the intestinal epithelium.

We also demonstrate that 15d-PGJ$_2$ but not the synthetic high affinity ligand rosiglitazone triggered ERK1/2 phosphorylation in CMT-93 cells and, most importantly, MEK1 inhibitor PD98059 reversed LPS-induced RelA phosphorylation and IL-6 gene expression in CMT-93 cells. Gilroy *et al.* (43) show in a model for carrageenan-induced acute inflammation that healing and survival in these mice during the resolution phase of inflammation were associated with increased levels of 15d-PGJ$_2$ and decreased levels of PGE$_2$ (43). Concordently, we show no inhibitory effects of PGE$_2$ on LPS-induced RelA phosphorylation and IL-6 gene expression in CMT-93 cells. Furthermore, previous studies demonstrated that 15d-PGJ$_2$, which has no known plasma membrane receptor, activates the MEK/ERK signaling cascade through different mechanisms in various cell types including superoxide anion-dependent induction of the Raf/MEK-signaling pathway in myoblasts (48) as well as induction of the phosphatidylinositol 3-kinase pathway in smooth

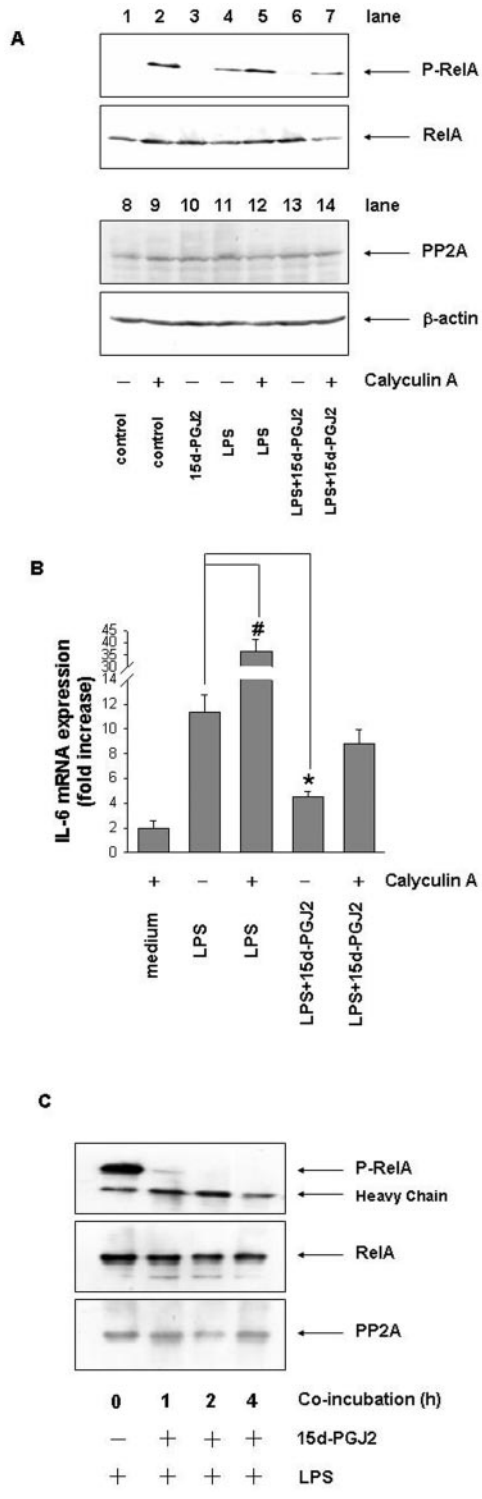

FIG. 7. **Calyculin A reversed the inhibitory effect of 15d-PGJ$_2$ on LPS-induced RelA phosphorylation and IL-6 gene expression in CMT-93 cells; 15d-PGJ$_2$ triggers PP2A activity and directly dephosphorylates RelA.** CMT-93 cells were stimulated with *E. coli* LPS (10 μg/ml) for 1 h in the presence or absence of 20 μM 15d-PGJ$_2$. Where indicated the cells were pretreated for 1 h with 5 nM calyculin A. Total protein was extracted, and 20 μg of protein were subjected to SDS-PAGE followed by phospho-RelA, RelA, PP2A, and β-actin immunoblotting using ECL technique (*A*). CMT-93 cells were stimulated with *E. coli* LPS (10 μg/ml) for 12 h in the presence or absence of 20 μM 15d-PGJ$_2$. Where indicated the cells were pretreated for 1 h with 1 nM calyculin A. Total RNA was extracted after 12 h of stimulation and reverse-transcribed, and real-time PCR was performed using the Light Cycler system with specific primers for murine IL-6 and GAPDH. The induction of IL-6 mRNA expression was calculated relative to untreated controls (-fold increase) using the crossing point of the log-linear portion of the amplification curve after normalization with GAPDH. The *bars* represent the combined mean values (±S.D.) of triplicate samples. *, reduction; #, increase; p value<0.05. *B*, CMT-93 cells were stimulated for 1 h with either *E. coli* LPS (10 μg/ml) or 20 μM 15d-PGJ$_2$. We then immunoprecipitated phospho-RelA and PP2A from lysed cells using A/G-agarose beads. To assay the phosphatase activity, 30 μl of beads from LPS-stimulated cells were co-incubated with 50 μl of beads from 15d-PGJ$_2$-treated cells. Reactions were stopped after 1, 2, and 4 h of incubation at 37 °C with the addition 2× Laemmli sample buffer. Total protein was extracted from beads and subjected to SDS-PAGE followed by phospho-RelA, RelA, and PP2A immunoblotting using ECL technique (*C*).

muscle cells (57). Inhibition of LPS-induced NF-κB activity in the presence of 15d-PGJ$_2$ was demonstrated in native and macrophage cell lines at the level of IKKβ activity, IκBα degradation, and NF-κB DNA binding activity as well as chemokine expression (55, 56, 58). Interestingly, we show that 15d-PGJ$_2$ does not affect LPS-induced IκBα phosphorylation/degradation, IκBα resynthesis, and RelA nuclear translocation in CMT-93 cells. It seems also unlikely that the slight delay in LPS-induced RelA DNA binding activity fully accounts for the complete inhibition of IL-6 gene expression in IEC but, rather, suggests that the 15d-PGJ$_2$-mediated inhibition of RelA phosphorylation directly affects IL-6 gene expression.

Phosphorylation and dephosphorylation of transcription factors regulates their DNA binding properties as well as their transactivating potential, and apparently, complexes containing both protein kinases and phosphoprotein phosphatases are important in maintaining the phosphorylation state of intracellular substrates. PP2A, which regulates a diverse set of cellular processes including signal transduction and transcription, constitutively associates with RelA in the cytoplasm (24, 25). Calyculin A, which is a serine/threonine phosphatase inhibitor, increased base levels of phospho-RelA in untreated CMT-93 cells, suggesting that the basal phosphatase activity has to be relatively active to maintain a low state of phosphorylated RelA in IEC. Upon stimulation of CMT-93 cells with LPS, the constitutive cycle of phosphorylation/dephosphorylation is disrupted presumably through induction of IKKβ, casein kinase, or phosphatidylinositol 3-kinase/Akt activity, resulting in RelA serine phosphorylation (Ser-536). Most importantly, calyculin A, which has higher inhibitory efficiency for PP2A than for PP1 or PP2B, reversed the inhibitory effects of 15d-PGJ$_2$ on LPS-induced RelA dephosphorylation and IL-6 gene expression in CMT-93 cells. Based on these results, we then show for the first time that immunoprecipitated PP2A from 15d-PGJ$_2$-treated CMT-93 cells directly dephosphorylates endogenous RelA (Ser-536) from LPS-treated cells, suggesting that 15d-PGJ$_2$ triggered PP2A activity in epithelial cells. It is not clear whether PP2A binds to the RelA serine phosphorylation sites, but studies on the interaction of PP2A with protein kinase Cζ, casein kinase-2α, and CXCR2 suggest that PP2A binds to other sites than the phosphorylation sites in these proteins (27–29).

Non-pathogenic enteric bacteria play an important role in initiating and perpetuating chronic intestinal inflammation in the susceptible host. It appears from our gnotobiotic studies in normal animals that enteric non-pathogenic bacteria including Gram-negative *B. vulgatus* (17, 22) and Gram-positive *Enterococcus faecalis* (59) trigger transient NF-κB signaling in the intestinal epithelium in normal rats and mice, respectively. In these studies the physiological importance for the induction of protective TGF-β/Smad signaling was clearly demonstrated in native IEC (59). Although TGF-β/Smad signaling blocked non-pathogenic bacteria-induced NF-κB transcriptional activity and IL-6 gene expression in IEC (22), the molecular mechanism for this inhibitory effect was identified at the level of altered

histone acetylation/phosphorylation rather than inhibition of RelA phosphorylation. Based on the results of this study, we may hypothesize that 15d-PGJ$_2$-mediated ERK1/2 signaling contributes to the inhibition of NF-κB-dependent gene expression in IEC through directly acting at the level of RelA phosphorylation by modulating PP2A activity. An attractive hypothesis is that under normal physiological conditions the coordinate induction of various protective mechanisms in IEC including TGF-β/Smad as well as 15d-PGJ$_2$/ERK signaling helps to control NF-κB activity after initial bacterial colonization to maintain normal epithelial cell homeostasis in the interplay with commensal bacteria.

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APPENDIX 2

Ruiz, PA*, Shkoda, A*, Kim, SC, Sartor, RB and Haller, D

"IL-10 gene-deficient mice lack TGF-β/Smad signaling and fail to inhibit pro-inflammatory gene expression in intestinal epithelial cells after the colonization with colitogenic *Enterococcus faecalis*"

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*Authors contributed equally

[Preparation and experimental work in TLR2ΔTIR and TLR4/MD2 stable transfected cell lines, ELISA analysis, adenoviral delivery into Mode K cells and ChIP analysis by Ruiz, PA *(Figures 3A CMT-93, 3B, 4B, 5-8 and 11)*; analysis of *in vivo* samples, studies in TLR2 expression and real time quantitative PCR by Shkoda, A; maintenance of the animal facility and bacterial monoassociation of IL-10 gene-deficient mice by Kim, SC and Sartor, RB; writing by Haller, D with editorial help of the co-authors]

IL-10 Gene-Deficient Mice Lack TGF-β/Smad Signaling and Fail to Inhibit Proinflammatory Gene Expression in Intestinal Epithelial Cells after the Colonization with Colitogenic *Enterococcus faecalis*[1]

Pedro A. Ruiz,[2]* Anna Shkoda,[2]* Sandra C. Kim,[†] R. Balfour Sartor,[‡] and Dirk Haller[3]*

Nonpathogenic enteric bacterial species initiate and perpetuate experimental colitis in IL-10 gene-deficient mice (IL-10$^{-/-}$). Bacteria-specific effects on the epithelium are difficult to dissect due to the complex nature of the gut microflora. We showed that IL-10$^{-/-}$ mice compared with wild-type mice fail to inhibit proinflammatory gene expression in native intestinal epithelial cells (IEC) after the colonization with colitogenic Gram-positive *Enterococcus faecalis*. Interestingly, proinflammatory gene expression was transient after 1 wk of *E. faecalis* monoassociation in IEC from wild-type mice, but persisted after 14 wk of bacterial colonization in IL-10$^{-/-}$ mice. Accordingly, wild-type IEC expressed phosphorylated NF-κB subunit RelA (p65) and phosphorylated Smad2 only at day 7 after bacterial colonization, whereas *E. faecalis*-monoassociated IL-10$^{-/-}$ mice triggered persistent RelA, but no Smad2 phosphorylation in IEC at days 3, 7, 14, and 28. Consistent with the induction of TLR2-mediated RelA phosphorylation and proinflammatory gene expression in *E. faecalis*-stimulated cell lines, TLR2 protein expression was absent after day 7 from *E. faecalis*-monoassociated wild-type mice, but persisted in IL-10$^{-/-}$ IEC. Of note, TGF-β1-activated Smad signaling was associated with the loss of TLR2 protein expression and the inhibition of NF-κB-dependent gene expression in IEC lines. In conclusion, *E. faecalis*-monoassociated IL-10$^{-/-}$, but not wild-type mice lack protective TGF-β/Smad signaling and fail to inhibit TLR2-mediated proinflammatory gene expression in the intestinal epithelium, suggesting a critical role for IL-10 and TGF-β in maintaining normal epithelial cell homeostasis in the interplay with commensal enteric bacteria. *The Journal of Immunology,* 2005, 174: 2990–2999.

The normal mucosal immune system acquires tolerance (hyporesponsiveness) to enteric indigenous flora, while protective cell-mediated and humoral immune responses to enteropathogens are maintained. This complex homeostasis presents an intriguing immunological paradox, and is broken under conditions of chronic intestinal inflammation, including ulcerative colitis and Crohn's disease (1, 2). Microbial agents of the normal enteric microflora are involved in each of the current etiologic theories of these chronically relapsing, immunologically mediated idiopathic disorders (3, 4). Duchmann et al. (5–7) reported a loss of immunologic tolerance in active inflammatory bowel diseases (IBD)[4] with mucosal T lymphocytes proliferating in response to commensal enteric bacteria. These findings are consistent with the clinical observation that inflammation in bypassed distal ileal or colonic segments of Crohn's disease patients is absent after proximal diversion of the fecal stream (8, 9), but immune responsiveness and inflammation are reactivated within 1 wk of perfusing ileostomy effluent into the bypassed ileum (10). In addition, the selective impact of certain nonpathogenic commensal luminal enteric bacteria on the development of chronic intestinal inflammation is elegantly illustrated in comparative studies using germfree and gnotobiotic rodent models for experimental colitis (11). For example, reconstitution studies with various nonpathogenic bacteria implicate *Enterococcus faecalis* as particularly important to the induction of colitis in selectively colonized gnotobiotic IL-10 gene-deficient (IL-10$^{-/-}$) mice. IL-10$^{-/-}$ 129 SvEv mice developed experimental colitis after 12–16 wk of *E. faecalis* monoassociation (12, 13). The absence of colitis and pathologic immune responses in *E. faecalis*-colonized wild-type (WT) mice demonstrated the nonpathogenic nature of this Gram-positive enteric bacterial species, and, most importantly, suggests that normal hosts develop immunosuppressive mechanisms that control mucosal immune responses to the constant challenge of commensal bacterial Ags.

Intestinal epithelial cells (IEC), which isolate the host from the gut luminal environment, constitutively express or can be induced to express TLR, costimulatory molecules, components of the human MHC, and a wide range of inflammatory and chemoattractant cytokines when activated by enteric pathogens or inflammatory products (14–17). Most of these molecules are in part transcriptionally regulated by the transcription factor NF-κB (18). We demonstrated that nonpathogenic Gram-negative *Bacteroides vulgatus* induce RelA (NF-κB p65 subunit) phosphorylation and NF-κB activation in vivo

*Centre for Nutrition and Food Research, Immunobiology of Nutrition, Technical University of Munich, Freising-Weihenstephan, Germany; and Departments of [†]Pediatrics and [‡]Medicine, Microbiology, and Immunology, and Center for Gastrointestinal Biology and Disease, University of North Carolina, Chapel Hill, NC 27599

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[2] P.A.R. and A.S. equally contributed to this manuscript.

[3] Address correspondence and reprint requests to Dr. Dirk Haller, Centre for Nutrition and Food Research, Immunobiology of Nutrition, Technical University of Munich, 85350 Freising-Weihenstephan, Germany. E-mail address: haller@wzw.tum.de

[4] Abbreviations used in this paper: IBD, inflammatory bowel disease; ChIP, chromatin immunoprecipitation; dn, dominant negative; HA, hemagglutinin; IEC, intestinal epithelial cell; IKK, I-κB kinase; IP-10, IFN-γ-inducible protein-10; MEF, myoembryogenic fibroblast; moi, multiplicity of infection; TIR, Toll/IL-1R; TNBS, trinitrobenzene sulfonic acid; WT, wild type.

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in the epithelium as well as in primary and IEC lines (19, 20). Monoassociation of germfree Fisher rats with *B. vulgatus* induced transient nuclear localization of phosphorylated RelA in the intestinal epithelium in a TLR4- and NF-κB-dependent manner, demonstrating the physiological relevance of the NF-κB signaling cascade in Gram-negative bacterial communication with IEC. In separate studies, Hornef et al. (21) showed that *Escherichia coli*-derived LPS was internalized by murine epithelial cells to stimulate the IκB/NF-κB system via intracellular TLR4.

Although bacteria trigger host responses by multiple mechanisms, the cornerstone of innate signaling is mediated by a set of well-conserved pattern recognition receptors, including TLR, of which >10 isoforms have been identified (22–24). Specific microbial ligands have been assigned to at least five different molecules. TLRs are transmembrane proteins characterized by an extracellular domain containing leucine-rich repeats and an intracellular domain homologous to the IL-1R, or Toll/IL-1R (TIR). Ligand-specific binding to TLR promotes interaction of the cytoplasmic TIR domain with adaptor proteins, including the MyD88, followed by the recruitment of multiple kinases and activation of downstream target effector systems, including the MAPK as well as the IκB/NF-κB transcriptional system (24, 25). Of note, under conditions of chronic intestinal inflammation, increased expression of TLRs was shown in lamina propria macrophages (26) and epithelial cells (27). TLR2, either alone or in conjunction with TLR1 or TLR6, recognizes pathogen-associated molecular patterns from Gram-positive bacteria, including lipoteichoic acid, peptidoglycans, neisserial porins, bacterial tripalmytoylated, and mycoplasmal diacetylated lipoproteins as well as yeast products (24).

In this study, we characterized the molecular mechanisms of Gram-positive *E. faecalis*-induced signal transduction in native IEC from gnotobiotic WT and IL-$10^{-/-}$ mice. Most importantly, and in contrast to wild-type animals, we show that *E. faecalis*-monoassociated IL-$10^{-/-}$ mice lack protective TGF-β/Smad2 signaling and exhibit persistent RelA phosphorylation through the TLR2 cascade at early stages of bacterial colonization (days 3, 7, 14, and 28), well before the onset of histologic colitis. Proinflammatory gene expression was transiently induced after 1 wk of *E. faecalis* monoassociation in WT mice, but persisted after 14 wk of bacterial colonization in IL-$10^{-/-}$ IEC, suggesting that initial bacteria-epithelial cell interactions may contribute to the induction of immunopathology at the late/chronic phase of intestinal inflammation in the genetically susceptible host. Interestingly, TLR2 protein expression was absent after 7 days in WT mice, but persisted in IL-$10^{-/-}$ IEC. Important for the mechanistic basis of altered TLR/NF-κB signaling in IEC from WT vs IL-$10^{-/-}$ mice, we could demonstrate that TGF-β1-activated Smad signaling was directly associated with the loss of TLR2 protein expression and the inhibited *E. faecalis*-induced NF-κB activity as well as proinflammatory gene expression in Mode-K cells.

Materials and Methods

Animals and bacterial monoassociation

Germfree 129 SvEv TAC mice and germfree IL-10 gene-deficient (−/−) 129 SvEv TAC mice (derived by E. Balish, University of Wisconsin, Madison, WI) were monoassociated at 12–16 wk of age with a human oral isolate of *E. faecalis* (a generous gift from M. Huycki, University of Oklahoma, Oklahoma City, OK) and maintained in the Gnotobiotic Animal Core at the College of Veterinary Medicine, North Carolina State University. Bacterial monoassociation and absence of contamination by other bacterial species were confirmed by culturing samples from the small and large intestine at necropsy and culturing serial fecal samples. Animal use protocols were approved by the Institutional Animal Care and Use Committee, North Carolina State University. Mice were killed 3, 7, 14, and 28 days and 14 wk after initial bacterial colonization. Germfree mice were used as controls. Sections of the ileum, cecum, proximal, and distal colon were fixed in 10% neutral buffered Formalin. The fixed tissue was embedded in paraffin. Histology scoring (0–4) was analyzed by blindly assessing the degree of lamina propria mononuclear cell infiltration, crypt hyperplasia, goblet cell depletion, and architectural distortion, as previously described (28).

Isolation of primary mouse IEC

E. faecalis-monoassociated and germfree mice were euthanized, and the cecum as well as colon were removed and placed in DMEM (Invitrogen Life Technologies) containing 5% FCS. Cecum and colon were cut longitudinally, washed three times in calcium/magnesium-free HBSS (Invitrogen Life Technologies), cut into pieces 0.5 cm long, and incubated at 37°C in 40 ml of DMEM containing 5% FCS and 1 mM DTT for 30 min in an orbital shaker. The supernatant was filtered and centrifuged for 5 min at 400 × *g*, and the cell pellet was resuspended in DMEM containing 5% FCS. The remaining tissue was incubated in 30 ml of PBS (1×) containing 1.5 mM EDTA for additional 10 min. The supernatant was filtered and centrifuged for 5 min at 400 × *g*, and the cell pellet was resuspended in DMEM containing 5% FCS. Finally, primary IEC were collected by centrifugation through a 25/40% discontinuous Percoll gradient at 600 × *g* for 30 min. Cell viability and purity were assessed by trypan blue exclusion and FACS analysis using mouse anti-CD3 mAb (BD Pharmingen; clone G4.18). Cells were >80% viable and >90% pure. Primary mouse IEC from cecum and colon were combined and collected in sample buffer for subsequent RNA isolation as well as Western blot analysis.

Cell culture and bacterial infection

The mouse IEC lines CMT-93 (passage 10–30) (ATCC CRL 223; American Type Culture Collection) and Mode-K (passage 10–30) (a generous gift from I. Autenrieth, University of Tübingen, Tübingen, Germany) were grown in a humidified 5% CO_2 atmosphere at 37°C to confluency in six-well tissue culture plates (Cell Star; Greiner Bioscience), as previously described (20). We also transfected Mode-K cells with the following plasmids, according to the manufacturer's instructions (InvivoGen): 1) pDUO vector (InvivoGen) for murine TLR4/MD-2 coexpressing TLR4 and the accessory molecule MD-2; 2) pZERO vector (InvivoGen) for murine TLR2ΔTIR lacking the cytoplasmic TIR domain. Stable transfected cells were then selected for their blasticidin (1 μg/ml) and puromycin (1 μg/ml) resistence, respectively. WT and TLR2$^{-/-}$ myoembryogenic fibroblast (MEF) were a generous gift from C. Kirschning (Technical University of Munich, Munich, Germany). *E. faecalis* was aerobically grown at 37°C in Luria broth containing tryptone (1%), yeast extract (0.5%), and NaCl (0.5%). Bacteria were harvested by centrifugation (3000 × *g*, 15 min) at stationary growth phase, washed in PBS (1× PBS, pH 7.4), and diluted in DMEM (Invitrogen Life Technologies). Confluent epithelial cell monolayers were infected with *E. faecalis* at a bacterium-to-epithelial cell ratio (multiplicity of infection (moi)) of 100 for various time points. Where indicated, Mode-K cells were treated with TGF-β1 (20 ng/ml; R&D Systems).

RNA isolation and real-time RT-PCR

RNA from isolated native IEC and IEC lines was extracted using TRIzol reagent (Invitrogen Life Technologies), according to the manufacturer's instructions. Extracted RNA was dissolved in 20 μl of water containing 0.1% diethyl-pyrocarbonate. For reverse transcription, 1 μg of total RNA was added to 30 μl of reaction buffer containing 8 μl of 5× first-strand buffer, 4 μl of DTT (100 mM), and 6 μl of desoxyribonucleoside triphosphate mixture (300 μM) (all reagents from Invitrogen Life Technologies), and incubated for 5 min at 65°C. After adding 10 μl of a solution containing 0.2 μg of random hexamers, 40 U of RNase Out, and 200 U of Moloney murine leukemia virus reverse transcriptase (all reagents from Invitrogen Life Technologies), the total mixture was incubated for an additional 60 min at 37°C, followed by a final 1-min heating step at 99°C.

Real-time PCR was performed in glass capillaries using a Light Cycler system (Roche Diagnostics). Primer sequences and amplicon sizes are provided in Table I. For real-time PCR, 1 μl of reverse-transcribed cDNA was added in a total volume of 10 μl of PCR buffer containing 1× LC-FastStart DNA Master Mix (Roche), $MgCl_2$ (4 μM), and forward and reverse primers (20 μM). The PCR program was one cycle of denaturation at 95°C for 10 min, followed by 50 cycles of 95°C for 15 s, annealing at 60°C for 10 s, and extension at 72°C for 20 s. The amplified product was detected by the presence of a SYBR green fluorescent signal. Melting curve analysis and gel electrophoresis were used to document the amplicon specificity. 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

Table I. *Primer sequences for real-time PCR*

	Forward Primer	Reverse Primer	Product Length (bp)
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-βR2	5′-catttggttccaaggtgc-3′	5′-tggtagtgttcagcgag-3′	282
GAPDH	5′-atcccagagctgaacg-3′	5′-gaagtcgcaggagaca-3′	198

equation: $E^{\Delta Cp\ (control\ samples\ -\ treated\ samples)}$ and normalized for the expression of GAPDH mRNA (29). Triplicate samples were measured in duplicates and blotted as fold increase between treated and untreated control samples.

Western blot analysis

Purified primary IEC or *E. faecalis*-stimulated Mode-K cells were lysed in 1× Laemmli buffer, and 20–50 μg of protein was subjected to electrophoresis on 10% SDS-PAGE gels. Where indicated, IEC cells were pretreated for 1 h with 20 μM proteasome inhibitor MG132 (BIOMOL). Anti-IκBα (C21; Santa Cruz Biotechnology); anti-phospho-IκBα (Ser^{32}), anti-phospho-RelA (Ser^{536}), RelA, and anti-phospho-Smad2 ($Ser^{465/467}$) (all Abs from Cell Signaling Technology); TLR2 (a generous gift from C. Kirschning); Smad1/2/3 (Santa Cruz Biotechnology); and anti-β-actin (Valeant Pharmaceuticals) were used to detect immunoreactive phospho-IκBα, total IκBα, phospho-RelA, RelA, phospho-Smad2, total Smad, TLR2, and β-actin, respectively, using an ECL light-detecting kit (Amersham), as previously described (20).

Chromatin immunoprecipitation (ChIP) analysis

Mode-K cells were stimulated with *E. faecalis* for 30 min in the presence or absence of TGF-β1 (20 ng/ml). Mode-K cells were fixed by adding formaldehyde to a final concentration of 1%, and nuclear extraction and ChIP were performed by using a kit from Active Motif. As previously described (19), the cells were lysed after formaldehyde fixation, and chromatin of isolated nuclei was sheared by sonication. Extracts were normalized according to their DNA concentration. Immunoprecipitations were conducted overnight at 4°C using 5 μl of anti-phospho NF-κB p65 Ab (Cell Signaling Technology). Immune complexes were collected with salmon sperm-saturated protein A/G agarose for 30 min and washed three times in high salt buffer, followed by three washes with no salt buffer. DNA cross-links of the immune complexes were reverted by heating. After proteinase K digestion, the DNA was extracted with phenol-chloroform and precipitated in ethanol. DNA isolated from an aliquot of the total nuclear extract was used as a loading control for the PCR (input control). PCR was performed with total DNA (1 μl, input control) and immunoprecipitated DNA (1 μl) using the following IL-6 promoter-specific primers: IL-6A (5′) 5-GACATGCTCAAGTGCTGAGTCAC-3; IL-6B (3′) 5-AGATTGCACAATGTGACGTCG-3. The length of the amplified product was 125 bp. The PCR products (10 μl) were subjected to electrophoresis on 2% agarose gels.

Adenoviral infection

IEC lines were infected overnight with adenoviral dominant-negative (dn) I-κB kinase (IKK)β (Ad5dnIKKβ) and Ad5IκBαAA vectors (a generous gift from C. Jobin, University of North Carolina, Chapel Hill, NC) in serum-reduced (2%) cell culture medium in the absence of antibiotics at an moi of 50, as previously described (20). Ad5Smad7 (a generous gift from D. Brenner, Columbia University, New York, NY) was used to infect Mode-K cells for 24 h. Expression of hemagglutinin (HA)- and FLAG-tagged mutant molecules was controlled by Western blot analysis using mouse anti-HA (Roche Molecular Biochemicals) and mouse anti-FLAG2 (Sigma-Aldrich) mAbs. Ad5GFP was used as a viral negative control. The adenovirus was removed by washing, and fresh cell culture medium was added. Cells were stimulated with *E. faecalis* (100 moi) for various times.

ELISA analysis

Protein concentrations were determined in spent culture supernatants of IEC cultures using an ELISA technique. IL-6 and IFN-γ-inducible protein-10 (IP-10) production were determined by mouse-specific ELISA kits, according to the manufacturer's instructions (R&D Systems).

Statistical analysis

Data are expressed as the mean ± SD of triplicates. Statistical analysis was performed by the two-tailed Student's *t* test for paired data and considered significant if *p* values were <0.05 (*) or <0.01 (**).

Results

Differential NF-κB RelA phosphorylation and IP-10 gene expression in native IEC from E. faecalis*-monoassociated WT vs IL-10$^{-/-}$ mice*

We investigated *E. faecalis*-induced proinflammatory gene expression and signal transduction in the intestinal epithelium of WT and IL-10$^{-/-}$ mice sequentially after bacterial colonization. First, *E. faecalis*-monoassociated WT and IL-10$^{-/-}$ mice (n = 3–5) were killed after 1 and 14 wk of initial bacterial colonization, and IP-10 mRNA expression was measured using Light Cycler real-time PCR. Luminal contents were plated, confirming equal colonization with *E. faecalis* between WT and IL-10$^{-/-}$ mice. Histological analysis was performed on paraffin-embedded tissue sections from the cecum and distal colon and RNA was isolated from native IEC of WT and IL-10$^{-/-}$ mice (cecum + colon). As shown in Fig. 1*A*, real-time PCR analysis showed that *E. faecalis* monoassociation

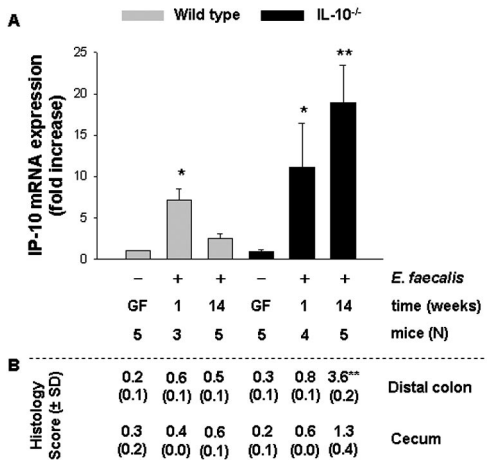

FIGURE 1. *A* and *B*, Differential IP-10 gene expression in native IEC from wild-type vs IL-10$^{-/-}$ mice. Germfree WT and IL-10$^{-/-}$ mice were colonized with *E. faecalis* at 12–16 wk of age. Mice (n = 3–5) were killed at days 3, 7, 14, and 28 as well as 14 wk after initial bacterial colonization, and native IEC from large intestine (cecum + colon) were isolated, as described in *Materials and Methods*. IEC from germfree mice were used as controls. *A*, Total RNA was extracted and reverse transcribed, and real-time PCR was performed using the Light Cycler system with specific primers for murine IP-10 and GAPDH. The induction of IP-10 mRNA was calculated relative to germfree WT controls (mean fold increase ± SD) using the crossing point of the log-linear portion of the amplification curve after normalization with GAPDH. *B*, Blinded histological analysis (score 0–4) was performed in paraffin-embedded tissue sections of the cecum and distal colon, as described in *Materials and Methods*.

triggered IP-10 gene expression in both WT (7.3-fold increase) and IL-10$^{-/-}$ IEC (11.1-fold) after the first week of bacterial colonization, well before the onset of histologic colitis. Interestingly and consistent with the lack of histopathology (Fig. 1*B*), IP-10 gene expression returned to low levels (2.5-fold) after 14 wk of *E. faecalis* monoassociation in WT mice. In contrast to WT mice, the initial induction of IP-10 gene expression in IL-10$^{-/-}$ IEC after 1 wk of bacterial colonization (11.1-fold) further increased after 14 wk of *E. faecalis* monoassociation (18.9-fold). *E. faecalis*-monoassociated IL-10$^{-/-}$ mice developed severe colitis after 14 wk of bacterial colonization (histological score of 3.8). IL-6 gene expression was not induced in IEC from WT nor IL-10$^{-/-}$ mice. Bacterial colonization of the cecum and colon was similar in WT and IL-10$^{-/-}$ mice ($4-6 \times 10^9$ CFU/g luminal content).

It is well established in various cell systems that Gram-positive bacteria trigger NF-κB activation and proinflammatory gene expression through the TLR2 signaling cascade (24). We next measured phospho-RelA, RelA, TLR2, and β-actin in isolated native IEC from WT and IL-10$^{-/-}$ mice after 3, 7, 14, and 28 days of monoassociation using Western blot analysis. As shown in Fig. 2 (*upper blot*), *E. faecalis*-monoassociated WT mice displayed transient RelA (p65) serine phosphorylation in native large IEC at days 3 (weak expression) and 7, but not at days 3, 14, and 28, or in germfree controls. Conversely, persistent RelA phosphorylation at days 3, 7, 14, and 28 was found in IEC of *E. faecalis*-monoassociated IL-10$^{-/-}$ mice. Interestingly, phospho-RelA levels steadily increased in IEC of *E. faecalis*-monoassociated IL-10$^{-/-}$ mice with maximal induction by 14 days after initial bacterial colonization. In contrast to WT mice, increased levels of phospho-RelA were detectable in IEC of germfree IL-10$^{-/-}$ mice. Of note, total RelA was equally expressed in IEC from WT and IL-10$^{-/-}$ mice. As shown in Fig. 2 (*lower blot*), TLR2 protein was present in IEC from germfree WT and IL-10$^{-/-}$ mice. Interestingly, while TLR2 protein expression was completely diminished in IEC from WT mice at days 14 and 28 of *E. faecalis* monoassociation, TLR2 expression persisted in IL-10$^{-/-}$ IEC. β-actin protein bands confirmed equal loading of samples (50 μg of protein).

E. faecalis *signals through the TLR2 cascade to induce RelA phosphorylation and proinflammatory gene expression in IEC*

We next asked the question as to whether *E. faecalis* can directly induce RelA phosphorylation and NF-κB signal transduction in IEC lines. We first stimulated Mode-K and CMT-93 mouse epithelial cell lines with *E. faecalis* (moi of 100) for 0–4 h. Interestingly, *E. faecalis* transiently induced phospho-RelA in both cell lines (Fig. 3*A*). To further dissect the signal transduction pathway of Gram-positive *E. faecalis* in IEC, we measured phospho-RelA, phospho-IκBα, total IκBα, and β-actin in Mode-K cells. As shown in Fig. 3*B*, *E. faecalis* (moi of 100) induced phosphorylation of the cytoplasmic NF-κB inhibitor IκBα after 10–20 min of stimulation, followed by the induction of phospho-RelA after 30 min and complete IκBα degradation after 1 h of stimulation. This clearly demonstrates the capability of *E. faecalis* to activate the IκB/NF-κB complex in Mode-K cells.

We next sought to examine *E. faecalis*-induced expression of IL-6 and IP-10 genes in Mode-K cells after the stimulation with *E. faecalis* (moi of 100) for 0–48 h. We measured IL-6 and IP-10 mRNA expression using real-time PCR and ELISA techniques. Fig. 4*A* shows that *E. faecalis* transiently induced IL-6 and IP-10 mRNA expression with maximal induction (20- to 40-fold increase) after 12 h of bacterial stimulation (100 moi). Accordingly, *E. faecalis*-induced IL-6 and IP-10 protein secretion was maximal at 12 h of bacterial stimulation (Fig. 4*B*).

Mode-K cells were established from C3H/He (H-2^K) mice (30). Later reports from Denning et al. (31) further specified the LPS-unresponsive mouse strain C3H/HeJ as the origin of Mode-K cells. To validate the characteristics of Mode-K cells with respect to their LPS responsiveness, we used normal Mode-K and TLR4/MD-2 stable transfected Mode-K cells. We stimulated the cells with *E. faecalis* (100 moi), LPS (10 μg/ml), and IL-1β (10 ng/ml) for 12 h and measured IL-6 and IP-10 protein secretion in the cell culture supernatants. Fig. 5*A* shows that Mode-K cells were hyporesponsive to the stimulation with LPS, whereas *E. faecalis* and IL-1β significantly triggered IL-6 and IP-10 protein secretion. Interestingly, TLR4/MD-2-reconstituted Mode-K cells fully regained LPS responsiveness (Fig. 5*B*), suggesting that Mode-K cells are indeed deficient in their TLR4 signaling cascade. These results validated Mode-K cells as a useful tool to characterize Gram-positive *E. faecalis*-induced signal transduction.

We showed that the pattern recognition receptor TLR2 was differentially expressed in native IEC from *E. faecalis*-monoassociated WT and IL-10$^{-/-}$ mice (Fig. 2). To dissect the role of TLR2 in *E. faecalis*-induced signaling in IEC, we first stimulated WT and TLR2$^{-/-}$ MEF for 0–2 h with *E. faecalis* (moi of 100). As shown in Fig. 6*A*, *E. faecalis* triggered RelA phosphorylation in WT, but not TLR2$^{-/-}$ MEF. Of note, IL-1β induced RelA phosphorylation

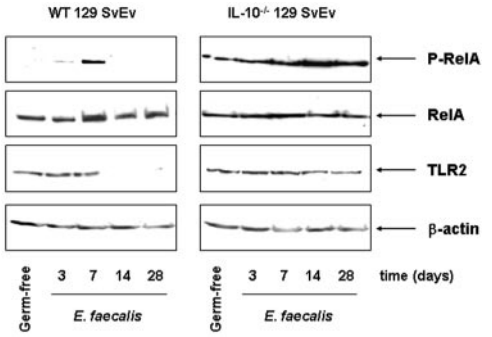

FIGURE 2. Differential RelA phosphorylation and TLR2 protein expression in native IEC from wild-type vs IL-10$^{-/-}$ mice. Germfree WT and IL-10$^{-/-}$ mice were colonized with *E. faecalis* at 12–16 wk of age. Mice (n = 3–5) were killed at days 3, 7, 14, and 28 as well as 14 wk after initial bacterial colonization, and native IEC from large intestine (cecum + colon) were isolated, as described in *Materials and Methods*. IEC from germfree mice were used as controls. Total protein was extracted, and 50 μg of protein was subjected to SDS-PAGE, followed by phospho-RelA, RelA, TLR2, and β-actin immunoblotting using the ECL technique. Gels represent the combined protein samples from each group.

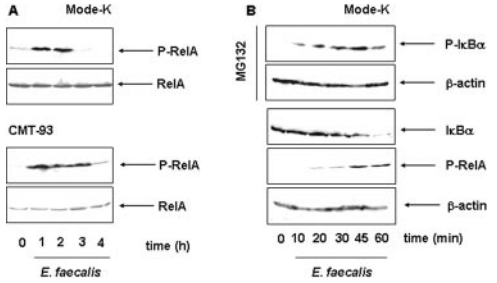

FIGURE 3. *A* and *B*, *E. faecalis* triggers RelA phosphorylation and IκB/NF-κB activation in CMT-93 and Mode-K mouse epithelial cell lines. Mode-K or CMT-93 cell lines were stimulated with *E. faecalis* for various times at an moi of 100. Total protein was extracted, and 20 μg of protein was subjected to SDS-PAGE, followed by immunoblotting using the ECL technique with Abs for phospho-RelA, RelA, phospho-IκBα, IκBα, and β-actin. Where indicated, the cells were pretreated for 1 h with the proteasome inhibitor MG132 (20 μM). Representative gels from at least three different experiments are shown.

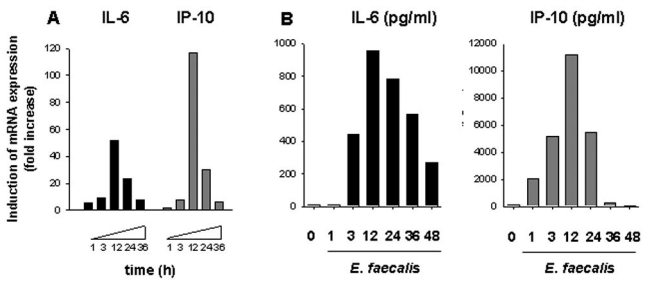

FIGURE 4. *A* and *B*, *E. faecalis* triggers IL-6 and IP-10 gene expression in Mode-K mouse epithelial cells. Mode-K or CMT-93 cell lines were stimulated with *E. faecalis* for various times at an moi of 100. *A*, Total RNA from Mode-K cells was extracted and reverse transcribed, and real-time PCR was performed using the Light Cycler system with specific primers for murine IL-6, IP-10, and GAPDH. The induction of IL-6 and IP-10 mRNA in Mode-K cells was calculated relative to germfree controls (fold increase) using the crossing point of the log-linear portion of the amplification curve after normalization with GAPDH. *B*, IL-6 and IP-10 protein were measured in the spent culture supernatant from stimulated Mode-K cells using ELISA methods. The bars represent the combined mean value of duplicate samples measured in duplicates.

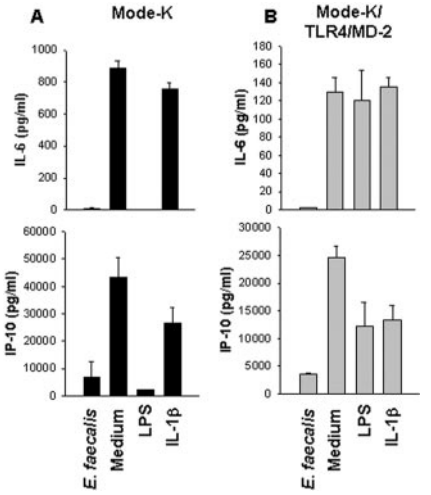

FIGURE 5. *A* and *B*, Reconstitution of the TLR4/MD-2 complex reverted LPS hyporesponsiveness in Mode-K cells. Mode-K cells were transfected with a TLR4/MD-2 expression vector system, as described in *Materials and Methods*. Wild-type Mode-K cells (*A*) and stable transfected Mode-K TLR4/MD2 cells (*B*) were then stimulated with *E. faecalis* (moi 100), LPS (10 μg/ml), and IL-1β (20 ng/ml) for 12 h. IL-6 and IP-10 protein were measured in the cell culture supernatant using ELISA technique. The bars represent the combined mean value ($\pm$SD) of three experiments.

in TLR2$^{-/-}$ MEF, confirming the specificity for the inhibition of *E. faecalis*-induced NF-κB signaling in TLR2$^{-/-}$ cells. In addition, *E. faecalis*-induced IL-6 secretion was completely abolished in TLR2$^{-/-}$ MEF compared with WT MEF (Fig. 6*B*), demonstrating that nonpathogenic *E. faecalis* signals through the TL2R cascade to induce RelA phosphorylation and to activate NF-κB-dependent proinflammatory gene expression. Interestingly, IL-1β-induced IL-6 protein secretion was significantly increased in TLR2$^{-/-}$ MEF compared with WT cells (Fig. 6*B*).

To further address the question as to whether *E. faecalis* also signals through the TLR2 cascade in IEC, we established stable transfected Mode-K cells expressing mutant TLR2. We used a TLR2ΔTIR expression vector system and selected the transfected cells by adding nontoxic concentrations of puromycin to the medium. We then stimulated normal Mode-K and Mode-K TLR2ΔTIR cells with *E. faecalis* and IL-1β and measured phospho-RelA in total cell extracts as well as IL-6 protein in the cell culture supernatants. As shown in Fig. 7*A*, *E. faecalis*-induced RelA phosphorylation was significantly reduced in Mode-K TLR2ΔTIR (*lanes 5* and *6*) compared with wild-type Mode-K cells (*lanes 2* and *3*). In contrast to TLR2$^{-/-}$ MEF (Fig. 6*A*, *lane 4*), phospho-RelA background levels were increased in untreated TLR2ΔTIR Mode-K cells (*lane 4*). In addition and consistent with TLR2$^{-/-}$ MEF cell results, *E. faecalis*-induced IL-6 protein secretion was significantly reduced in Mode-K TLR2ΔTIR cells compared with wild-type Mode-K cells (Fig. 7*B*). Of note, IL-1β stimulation revealed comparable responsiveness of Mode-K TLR2ΔTIR and normal Mode-K cells. The residual IL-6 response in *E. faecalis*-stimulated Mode-K TLR2ΔTIR cells may reflect the low transfection efficiency in IEC lines as well as an incomplete selection of TLR2ΔTIR-transfected cells. Nevertheless, these results clearly demonstrate that *E. faecalis* targets the TLR2 signal transduction pathway to induce proinflammatory gene expression in IEC. Similar results were obtained at the mRNA level for both MEF and Mode-K cells (data not shown).

We next used adenoviral delivery of mutant IκB (Ad5IκBαAA), which cannot be phosphorylated or degraded, and dn IKKβ (Ad5dnIKKβ) to investigate the role of the IκB/NF-κB system to mediate bacteria-induced proinflammatory gene expression in IEC. We infected Mode-K cells with Ad5IκBαAA, Ad5dnIKKβ, and Ad5GFP as a viral control at an moi of 50 overnight and washed to remove the adenoviruses, and then stimulated Mode-K cells with *E. faecalis* (100 moi) for 12 h. Protein expression of HA- and FLAG-tagged mutant molecules was confirmed by Western blot analysis of cell lysates (Fig. 8*A*). Interestingly, *E. faecalis*-induced IL-6 and IP-10 gene expression was significantly inhibited by the presence of either Ad5IκBαAA or Ad5dnIKKβ (Fig. 8*B*), suggesting that *E. faecalis* triggers IκB/NF-κB activation to induce proinflammatory gene expression in IEC.

Differential TGF-β/Smad signaling in IEC from E. faecalis*-monoassociated WT vs IL-10$^{-/-}$ mice*

It is well established that the immunosuppressive cytokine TGF-β activates the Smad pathway in numerous cell types (32, 33). Interestingly, phospho-Smad2 was induced in *E. faecalis*-monoassociated WT (at day 7), but not IL-10$^{-/-}$ mice (Fig. 8*A*, *upper blot*). It appeared from these results that the absence of phospho-Smad2 expression was associated with persistent TLR2 expression and RelA phosphorylation in native IEC from IL-10$^{-/-}$ mice (Fig. 2), suggesting a potential role for the TGF-β/Smad signaling cascade in inhibiting TLR2-mediated NF-κB activity in native IEC. Of note, total Smad2/3 was equally expressed in IEC from WT and IL-10$^{-/-}$ mice. In addition, while the expression of the endogenous inhibitor of the TGF-β/Smad signaling cascade Smad 7 was induced in WT IEC at days 14 and 28 of *E. faecalis* monoassociation, IL-10$^{-/-}$ mice completely lack Smad7 expression in IEC (Fig. 9*A*, *lower blot*).

To further elucidate the mechanisms for the lack of Smad2 phosphorylation, we measured TGF-β1 and TGF-βR2 mRNA expression in native IEC and total mucosal tissue from WT and IL-10$^{-/-}$ mice. As shown in Fig. 9*B*, TGF-β1 and TGF-βR2 mRNA expression were similar in native IEC as well as mucosal tissue from both WT and IL-10$^{-/-}$ mice. Of note, TGF-β1 and TGF-βR2 levels were constitutively expressed in colonic tissue from germfree and *E. faecalis*-monoassociated mice. Interestingly, *E. faecalis* monoassociation of WT and IL-10$^{-/-}$ mice induced TGF-β1 and to a lesser extent TGF-βR2 mRNA expression in colonic IEC after bacterial colonization.

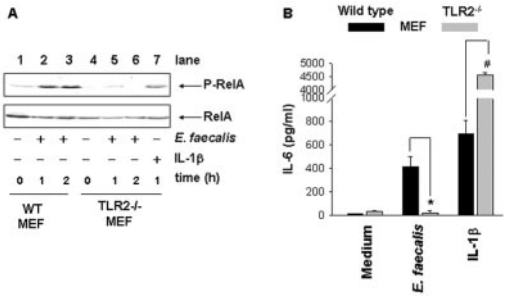

FIGURE 6. *A* and *B*, *E. faecalis* signals through the TLR2 cascade to induce RelA phosphorylation and proinflammatory gene expression: proof of principle in wild-type and TLR2$^{-/-}$ MEF. WT and TLR2$^{-/-}$ MEF were stimulated with IL-1β (20 ng/ml) and *E. faecalis* at an moi of 100 for various times. *A*, Total protein was extracted, and 20 μg of protein was subjected to SDS-PAGE, followed by phospho-RelA and RelA immunoblotting using the ECL technique. *B*, IL-6 protein was measured in the cell culture supernatant after 12 h of stimulation using ELISA technique. The bars represent the combined mean value ($\pm$SD) of three experiments. The bars represent the combined mean value ($\pm$SD) of three experiments. $*$, p value <0.05; #, p value <0.05.

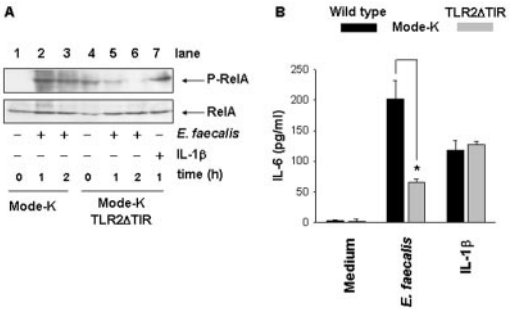

FIGURE 7. *A* and *B*, *E. faecalis* signals through the TLR2 cascade to induce RelA phosphorylation and proinflammatory gene expression in IEC. Mode-K cells were transfected with a TLR2ΔTIR expression vector system, as described in *Materials and Methods*. Wild-type Mode-K cells and stable transfected Mode-K TLR2ΔTIR cells were then stimulated with *E. faecalis* (moi 100) and IL-1β (20 ng/ml). *A*, Total protein was extracted, and 20 μg of protein was subjected to SDS-PAGE, followed by phospho-RelA and RelA immunoblotting using the ECL technique. *B*, IL-6 protein was measured in the cell culture supernatant after 12 h of stimulation using ELISA technique. The bars represent the combined mean value ($\pm$SD) of three experiments. The bars represent the combined mean value ($\pm$SD) of three experiments. $*$, p value <0.05.

To further verify whether the selective lack of Smad2 phosphorylation in IEC from IL-10$^{-/-}$ is attributed to intrinsic defects of TGF-β/Smad signaling in IEC, we stimulated primary IEC from germfree WT and IL-10$^{-/-}$ mice with TGF-β1 ex vivo. As shown in Fig. 10*A*, TGF-β1 triggered transient Smad2 phosphorylation in both WT and IL-10$^{-/-}$ IEC. These results confirm the principal capability of TGF-β1 to trigger Smad signaling in primary IEC from IL-10$^{-/-}$ mice. Consistent with the transient Smad2 phosphorylation (day 7) and expression of the endogenous inhibitor of the TGF-β/Smad signaling cascade Smad7 (days 14 and 28) in native wild-type IEC (Fig. 9), we now demonstrate in Mode-K cells that adenoviral delivery of Smad7 blocked TGF-β1-induced Smad2 phosphorylation (Fig. 10*B*).

TGF-β1-activated Smad signaling induces the loss of TLR2 protein expression and the inhibition of E. faecalis*-induced NF-κB-dependent gene expression in IEC*

We showed that IL-10$^{-/-}$ mice compared with WT mice lack TGF-β/Smad signaling and fail to inhibit TLR2-mediated NF-κB activity in native IEC after initial colonization with nonpathogenic Gram-positive *E. faecalis*, well before the onset of histologic colitis. We next sought to investigate the effect of TGF-β1 on *E. faecalis*-induced RelA phosphorylation in Mode-K cells. Fig. 11*A* shows that TGF-β1 triggers transient Smad2 phosphorylation after 1 h of stimulation of Mode-K (*lower blot*, *lane 5*), followed by the down-regulation of *E. faecalis*-induced phospho-RelA after 2 h of stimulation (*upper blot*, *lane 6*). These results were consistent with the findings in vivo, demonstrating that TGF-β1 inhibited *E. faecalis*-induced RelA phosphorylation. To provide direct evidence that TGF-β1 inhibits NF-κB-dependent IL-6 and IP-10 gene expression, we measured phospho-RelA DNA-binding activity on native IL-6 promoter sites using ChIP analysis. As shown in Fig. 11*B*, TGF-β1 inhibited *E. faecalis*-induced phospho-RelA binding to IL-6 promoter binding sites, and accordingly, TGF-β1 inhibited *E. faecalis*-induced IL-6 and IP-10 gene expression in Mode-K cells (Fig. 11*C*).

We next sought to investigate the role of TGF-β1 on TLR2 protein expression in Mode-K cells. We first stimulated Mode-K cells with TGF-β1 alone as well as in combination with *E. faecalis* and measured TLR2 protein expression after 1 and 2 h of stimulation. Interestingly, while the treatment of Mode-K cells with *E. faecalis* (Fig. 12*A*, *lanes 4* and *5*) and TGF-β1 (Fig. 12*A*, *lanes 2* and *3*) alone did not affect TLR2 protein expression in Mode-K cells (Fig. 12*A*, *lane 1*), the combined stimulation with *E. faecalis* and TGF-β1 completely abolished TLR2 protein expression (Fig. 12*A*, *lanes 6* and *7*). To further characterize the role of the TGF-β/Smad signaling pathway in the regulation of TLR2 expression in *E. faecalis*-stimulated Mode-K cells, we used the adenoviral vector Ad5Smad7 to block the TGF-β/Smad pathway (see Fig. 10*B*) and MG132 to inhibit proteasome activity (see Fig. 3*B*) in Mode-K cells. As shown in Fig. 12*B*, the presence of Ad5Smad7 (*lanes 3* and *4*), but not MG132 (*lanes 5* and *6*), inhibited TGF-β-induced TLR2 expression in *E. faecalis*-stimulated Mode-K cells.

In conclusion, we showed that TGF-β/Smad signaling was associated with the loss of TLR2 protein expression and the inhibition of bacteria-induced RelA phosphorylation and NF-κB-dependent gene expression in native and IEC lines.

Discussion

In this study, we demonstrate that *E. faecalis*-monoassociated WT and IL-10$^{-/-}$ mice differentially induce NF-κB and TGF-β/Smad signaling in the intestinal epithelium at early stages of bacterial colonization. Consistent with the finding that *E. faecalis* triggers RelA phosphorylation and NF-κB-dependent IL-6 and IP-10 gene expression through the TLR2 cascade, TLR2 protein expression was absent after day 7 from *E. faecalis*-monoassociated WT mice, but persisted in IEC from IL-10$^{-/-}$ mice. Most relevant for the mechanistic basis of altered NF-κB/TLR signaling in IEC from WT and IL-10$^{-/-}$ mice is the finding that TGF-β1 triggers TLR2 protein degradation and *E. faecalis*-induced NF-κB-dependent gene expression in epithelial cell lines. It was previously shown that *E. faecalis*-monoassociated IL-10$^{-/-}$, but not WT mice develop experimental colitis after 12–16 wk after initial bacterial colonization (12, 13), demonstrating the ability of this nonpathogenic Gram-positive bacterial strain to induce immune-mediated colitis in a susceptible host. Despite the findings that *E. faecalis* monoassociation progressively induced enteric bacterial Ag-specific CD4$^+$ T cell activation and IFN-γ production in IL-10$^{-/-}$ mice, the underlying mechanisms responsible for initiating intestinal inflammation are essentially unknown (13).

E. faecalis induces persistent RelA phosphorylation in native IEC from monoassociated IL-$10^{-/-}$ mice at days 3, 7, 14, and 28 after bacterial colonization, while colonized WT mice transiently trigger phospho-RelA at day 7. Consistent with the development of histopathology, IP-10 gene expression was transient after 1 wk of *E. faecalis* monoassociation in IEC from WT, but persisted after 14 wk of bacterial colonization in IL-$10^{-/-}$ mice. This may suggest an intrinsic defect of IL-$10^{-/-}$ mice to control transient induction of NF-κB activity in the intestinal epithelium of WT mice following bacterial colonization, supporting the possibility that bacteria-epithelial cell interactions contribute to the induction of immunopathology, leading to chronic intestinal inflammation in the genetically susceptible host colonized with enteric bacteria.

Increased NF-κB activation has been well documented in IEC and lamina propria cells of IBD patients with active disease (34–36), and accordingly, pharmacological NF-κB blockade may become potentially important in the treatment of chronic intestinal inflammation (37, 38). In addition, NF-κB RelA was strongly activated in the intestinal mucosa of IL-$10^{-/-}$ and trinitrobenzene sulfonic acid (TNBS)-treated mice (39). Most importantly, local administration of antisense RelA oligonucleotides abrogated clinical and histological signs of TNBS-induced experimental colitis, suggesting a mechanistic role for sustained NF-κB activity in the pathogenesis of chronic mucosal inflammation (39). In contrast, the selective inhibition of NF-κB activity in enterocytes of IKKβ gene-deficient mice sensitized these animals to ischemia-reperfusion-induced apoptosis in IEC, which was associated with a loss of mucosal integrity (40). In addition, blocking NF-κB activity with pharmacological inhibitors during the resolution phase of carrageenan-induced acute inflammation is deleterious to the host (41), suggesting dual functions of activated NF-κB, including protective and detrimental mechanisms during the course of inflammation.

Although many pathways are involved in the regulation of

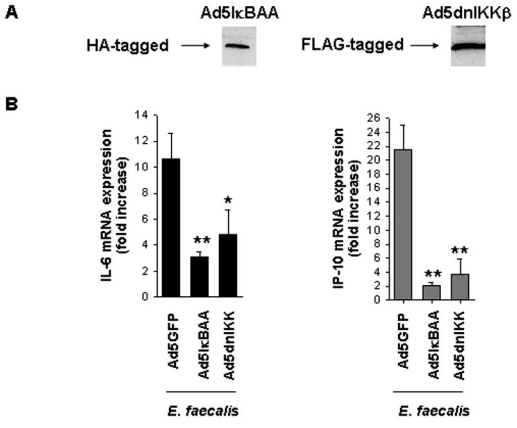

FIGURE 8. *A* and *B*, *E. faecalis* triggers IL-6 and IP-10 gene expression in Mode-K cells through the IκB/NF-κB system. Mode-K cells were infected overnight with adenoviral Ad5IκBαAA and dn IKKβ (Ad5dnIKKβ) in serum-reduced cell culture medium. Expression of HA- and FLAG-tagged mutant molecules was controlled by Western blot analysis using mouse anti-HA and mouse anti-FLAG2 mAbs. Ad5GFP was used as viral negative control. Mode-K were then stimulated with *E. faecalis* (moi of 100). Total RNA was extracted after 12 h of stimulation and reverse transcribed, and real-time PCR was performed using the Light Cycler system with specific primers for murine IL-6, IP-10, and GAPDH. The induction of IL-6 and IP-10 was calculated relative to untreated controls (fold increase) using the crossing point of the log-linear portion of the amplification curve after normalization with GAPDH. The bars represent the combined mean value (±SD) of three experiments. *, p value <0.05; **, p value <0.01.

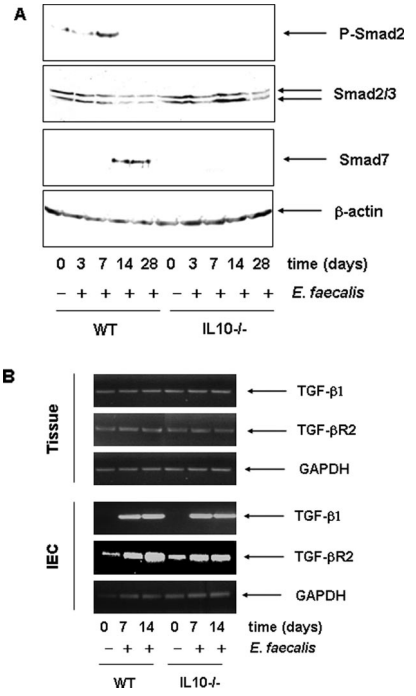

FIGURE 9. *A* and *B*, Differential TGF-β/Smad signaling in native IEC from *E. faecalis*-monoassociated wild-type vs IL-$10^{-/-}$ mice. Germfree WT and IL-$10^{-/-}$ mice were colonized with *E. faecalis* at 12–16 wk of age. Mice were killed at days 3, 7, 14, and 28 after initial bacterial colonization, and native IEC from large intestine (cecum + colon) were isolated, as described in *Materials and Methods*. IEC from germfree mice were used as controls. *A*, Total pooled protein was extracted, and 50 μg of protein was subjected to SDS-PAGE, followed by phospho-Smad2, Smad2/3, Smad7, and β-actin immunoblotting using the ECL technique. Gels represent the combined protein samples from each group. *B*, TGF-β1, TGF-βR2 mRNA expression was measured in native IEC and total mucosal tissue (cecum + colon) from WT and IL-$10^{-/-}$ mice using RT-PCR. Total RNA was extracted and reverse transcribed, and real-time PCR was performed using the Light Cycler system with specific primers for murine TGF-β1, TGF-βR2, and GAPDH. PCR products were run on 2% agarose gels.

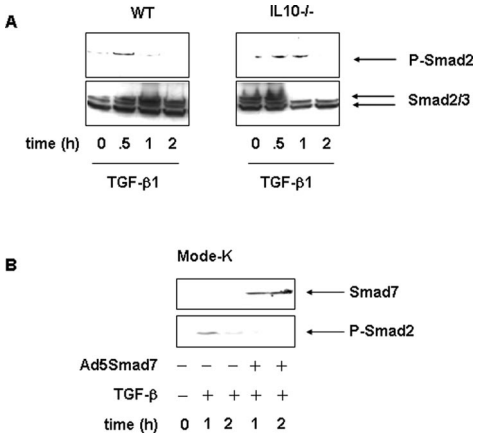

FIGURE 10. *A* and *B*, Ad5Smad7 inhibits TGF-β-mediated Smad2 phosphorylation in IEC. Primary IEC from the large intestine (cecum + colon) of germfree 129 SvEv WT and IL-$10^{-/-}$ mice were isolated and stimulated ex vivo with TGF-β1 (20 ng/ml) for 0–2 h (*A*). Mode-K cells were infected for 24 h with adenoviral Ad5Smad7 in serum-reduced cell culture medium. Mode-K cells were then stimulated with TGF-β1 (20 ng/ml) for 0–2 h in the presence and absence of Ad5Smad7 (*B*). Total protein was extracted, and 20 μg of protein was subjected to SDS-PAGE, followed by phospho-Smad2, Smad2/3, Smad7, and β-actin immunoblotting using the ECL technique.

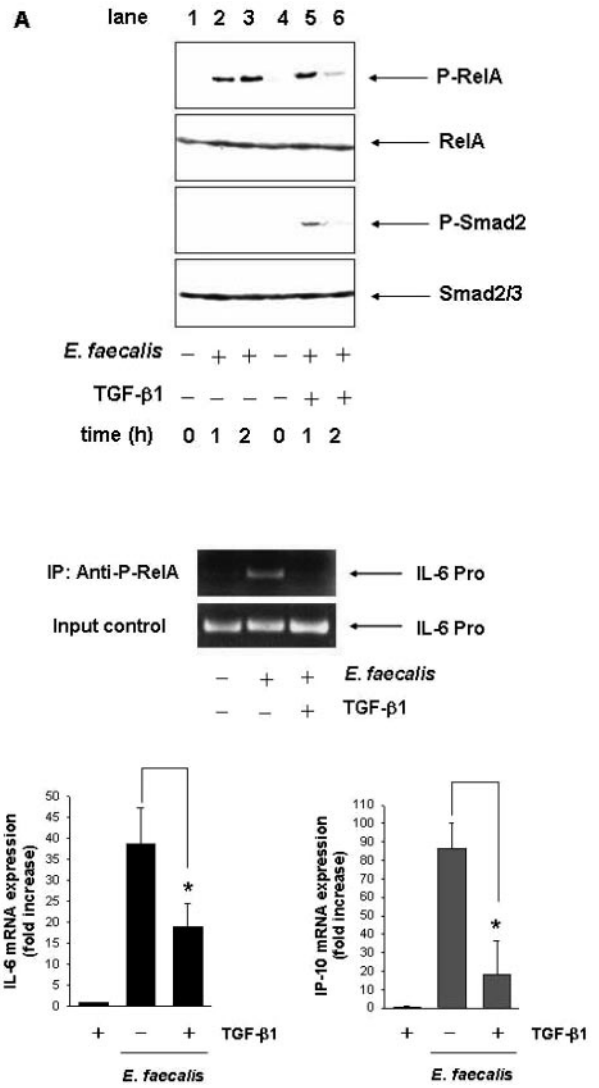

FIGURE 11. *A–C*, TGF-β inhibits RelA phosphorylation and NF-κB-dependent proinflammatory gene expression in *E. faecalis*-stimulated Mode-K cells. Mode-K cells were stimulated with *E. faecalis* (moi of 100) in the absence or presence of TGF-β1 (20 ng/ml). *A*, Total protein was extracted, and 20 μg of protein was subjected to SDS-PAGE, followed by phospho-RelA, RelA, phospho-Smad2, and Smad2/3 immunoblotting using the ECL technique. Representative results from two to three independent experiments are shown. *B*, Phospho-RelA DNA binding to IL-6 gene promoters was measured using ChIP analysis, as described in *Materials and Methods*. Input control shows equal immunoprecipitation using anti-phospho-RelA Ab. *C*, Mode-K cells were stimulated for 12 h. Total RNA was extracted and reverse transcribed, and real-time PCR was performed using the Light Cycler system with specific primers for murine IL-6, IP-10, and GAPDH. The induction of IL-6 and IP-10 mRNA was calculated relative to untreated controls (fold increase) using the crossing point of the log-linear portion of the amplification curve after normalization with GAPDH. The bars represent the combined mean value ($\pm$SD) of three experiments. $*$, p value <0.05.

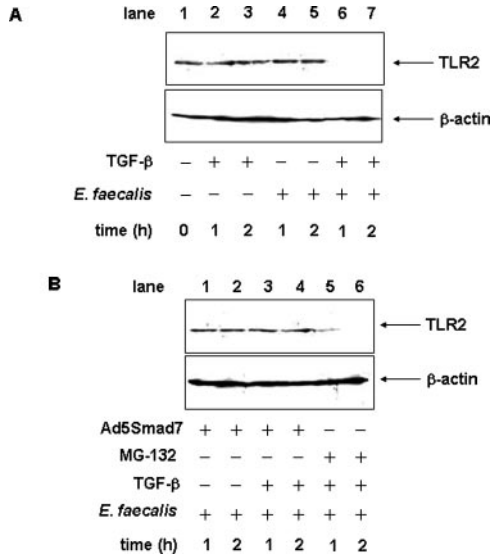

FIGURE 12. *A* and *B*, TGF-β/Smad signaling induces TLR2 protein degradation in *E. faecalis*-stimulated Mode-K cells. Mode-K cells were stimulated with *E. faecalis* (moi of 100) for 0–2 h in the absence or presence of TGF-β1 (20 ng/ml) (*A*). Mode-K cells were infected for 24 h with Ad5Smad7 in serum-reduced cell culture medium. Where indicated, Mode-K cells were pretreated with the proteasome inhibitor MG132 (20 μM). Ad5Smad7-infected Mode-K and MG132-pretreated Mode-K cells were stimulated with *E. faecalis* (moi 100) for 0–2 h (*B*). Total protein was extracted, and 20 μg of protein was subjected to SDS-PAGE, followed by TLR2 and β-actin by immunoblotting using the ECL technique. Representative Western blots from two different experiments are shown.

innate and/or adaptive immunity in the intestine, the immunoregulatory cytokines TGF-β and IL-10 are of high relevance to IBD, as elegantly demonstrated in TGF-β1- and IL-10-deficient mice that both spontaneously develop colitis (42). Accordingly, TGF-β1 overexpression in lamina propria immune cells inhibited Th1-mediated experimental TNBS-induced colitis (6, 43), and mucosal delivery of IL-10-secreting lactococci abrogated experimental colitis in IL-$10^{-/-}$ mice (44). The importance of TGF-β signaling in maintaining epithelial cell homeostasis was demonstrated in tissue-specific transgenic mice expressing a dn TGF-β receptor in IEC (TGF-R2). Molecular blockade of TGF-β1 signaling triggered colitis in TGF-R2 transgenic mice under conventional conditions and increased the susceptibility of these mice to dextran-sodium-sulfate-induced colitis (45).

Key features of our present study are the observations that the lack of Smad2 phosphorylation in IEC from *E. faecalis*-monoassociated IL-$10^{-/-}$ mice is associated with persistent TLR2 and IP-10 expression. Although the endogenous inhibitor of the TGF-β signaling cascade Smad7 is implicated in the development of chronic intestinal inflammation (46, 47) and blocked TGF-β-induced Smad2 phosphorylation in Mode-K (Ad5Smad7 infected) and other epithelial cell lines (48), Smad7 was not expressed in IL-$10^{-/-}$ IEC. In addition, the expression of TGF-β1 and the TGF-β-binding receptor TGF-βR2 mRNA was similar in WT and IL-$10^{-/-}$ IEC and, most importantly, TGF-β1 triggered Smad2 phosphorylation in ex vivo cultures from WT as well as IL-$10^{-/-}$ IEC. It appears from these findings that the differential Smad2 phosphorylation in IEC from *E. faecalis*-monoassociated WT vs IL-$10^{-/-}$ mice is not due to an intrinsic defect of responsiveness of the TGF-β/Smad cascade in IEC. Functional secretion of biologically active TGF-β requires posttranscriptional modifications and may be altered in IL-$10^{-/-}$ mice. In addition to the enormously difficult experimental conditions to measure secreted bioactive TGF-β from intestinal tissue and/or cells, functional TGF-β is also cell membrane bound, suggesting that the physiological inhibitory effects of TGF-β could also be locally mediated on the basis of cell-to-cell contact (49). Interestingly, Strober and colleagues (50, 51) demonstrated that the protective mechanisms of IL-10 in TNBS-induced experimental colitis are indirectly mediated through its inductive effect on TGF-β secretion in lamina propria T cells. Most importantly, CD45RB^{low} T cells from IL-$10^{-/-}$ mice fail to confer

protection in an adoptive transfer model in which CD45RB^{high} T cells trigger Th1-mediated murine experimental colitis in SCID mice (52, 53), supporting the possibility that IL-10 could be important in triggering TGF-β-producing T cells in the normal intestinal mucosa. An attractive hypothesis is that transient induction of NF-κB activity in epithelial cells triggers biologically active IL-10-mediated TGF-β responses in the lamina propria or the epithelium.

The intestinal epithelium must adapt to a constantly changing luminal environment by processing different biological information through multiple signaling cascades that target a defined set of genes, to provide an adequate effector response. We showed that Gram-positive *E. faecalis* can directly induce RelA phosphorylation in IEC lines and native IEC from WT mice, demonstrating the physiological relevance of *E. faecalis* in targeting the NF-κB cascade. Under normal conditions, *E. faecalis*-induced RelA phosphorylation and proinflammatory gene expression were transient, suggesting the presence of intrinsic mechanisms that antagonize *E. faecalis*-induced NF-κB activity in IEC. These regulatory mechanisms may be an intrinsic part of the NF-κB pathway such as Toll-interacting protein or IL-1R-associated kinase-M operating in a negative feedback loop fashion and/or alternatively be induced by independent signaling cascades that cross talk with NF-κB cascade, including TGF-β/Smad and 15-deoxy-$\Delta^{12,14}$ PGJ_2-ERK signaling (19, 54–56).

We performed a series of experiments to demonstrate that *E. faecalis* signals through TLR2 to trigger NF-κB activation, and, most importantly, to characterize the mechanistic basis for the inhibitory effects of TGF-β1. First, we show that *E. faecalis* induced IκBα and RelA phosphorylation, followed by complete IκBα degradation in Mode-K cells. Second and most relevant to elucidate the mechanisms of bacterial signaling in IEC, adenoviral delivery of IκBα superrepressor and dn IKK-β inhibited IL-6, IP-10, and TLR2 gene expression in Mode-K cells. This clearly demonstrates the pivotal role of the IκB/NF-κB system in *E. faecalis*-induced signal transduction in IEC. Third, functional evidence for the importance of TLR2 in *E. faecalis* signaling was achieved by using TLR2$^{-/-}$ MEF and TLR2 mutant Mode-K cells. We could demonstrate that *E. faecalis*-induced RelA phosphorylation as well as proinflammatory gene expression were abrogated in TLR2$^{-/-}$ MEF as well as Mode-K TLR2ΔTIR. It has been recently reported that the extracellular domain of TLR2 contains multiple binding domains for different Gram-positive bacteria-derived ligands (57), which may explain the differential ability of complex Gram-positive bacteria to induce TLR2 signaling and NF-κB activation in IEC (58). Finally and most relevant for a possible explanation of altered bacterial-induced TLR/NF-κB signaling in IL-10$^{-/-}$ IEC, we showed that TGF-β1 down-regulates TLR2 protein expression in IEC, followed by the inhibition of *E. faecalis*-induced RelA phosphorylation and proinflammatory gene expression. Interestingly, TGF-β-mediated degradation of TLR2 was induced in the presence, but not in the absence of *E. faecalis* stimulation. Similarly, Matsumura et al. (59) demonstrated that TGF-β up-regulated TLR2 expression in hepatocytes, but inhibited TLR2 expression induced by IL-1β. Together with our observation that the presence of Ad5Smad7, but not the pharmacological proteasome inhibitor MG132, blocked TGF-β-mediated TLR2 degradation, we may conclude from our findings that the TGF-β/Smad signaling cascade induces TLR2 degradation in *E. faecalis*-stimulated IEC most likely through a proteasome-independent pathway. Although TLR expression has been shown to be regulated through distinct E3 ubiquitin-protein ligases that selectively direct the proteasomal pathway to TLRs for proteolytic degradation (60), the recruitment of activated TLR1, 2, and 4 to endosomal/lysosomal degradation upon ligand-driven TLR activation may be an interesting new target of regulation (61, 62)

In conclusion, we demonstrate that Gram-positive nonpathogenic *E. faecalis* transiently induced TLR2-mediated RelA phosphorylation and NF-κB-dependent gene expression in IEC from WT mice, but persistent activation of the TLR/NF-κB pathway in IL-10-deficient mice at early stages of bacterial colonization preceding any histological evidence of colitis. Our results suggest the possibility that *E. faecalis* triggers persistently active TLR/NF-κB signaling in epithelial cells of IL-10$^{-/-}$ mice in the absence of the protective TGF-β/Smad cascade, which may lead indeed to the development of clinical and histological signs of intestinal inflammation. These results support the concept for an interrelated role of IL-10 and TGF-β1 in maintaining epithelial cell homeostasis to commensal enteric bacteria.

Disclosures

The authors have no financial conflict of interest.

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APPENDIX 3

Ruiz, PA, Hoffmann, M, Szcesny, S, Blaut, M and Haller, D.

"Innate mechanisms for Bifidobacterium lactis to activate transient pro-inflammatory host responses in intestinal epithelial cells after the colonization of germfree rats"

Immunology, 2005, 115, 441-450

[Isolation of intestinal epithelial cells from rat, analysis of *in vivo* samples and *in vitro* experimental work in Mode K cells by Ruiz, PA *(Figures 1-3, 4b, 5 and 7)*; stablishment of conditions for bacterial stimulation and *in vitro* experimental work in MEF cells by Hoffmann, M; maintenance of the animal facility and bacterial monoassociation of germ-free rats by Szcesny, S and Blaut, M; writing by Haller, D with editorial help of the co-authors]

Innate mechanisms for *Bifidobacterium lactis* to activate transient pro-inflammatory host responses in intestinal epithelial cells after the colonization of germ-free rats

Pedro A. Ruiz,[1] Micha Hoffmann,[1] Silke Szcesny,[2] Michael Blaut[2] and Dirk Haller[1]

[1]*Centre for Nutrition and Food Research, Immunobiology of Nutrition, Technical University of Munich, Freising-Weihenstephan, Germany and* [2]*Department of Gastrointestinal Microbiology, German Institute of Human Nutrition, Potsdam-Rehbrücke, Germany*

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Correspondence: Dr D. Haller, Centre for Nutrition and Food Research, Immunobiology of Nutrition, Technical University of Munich, 85350 Freising-Weihenstephan, Germany.
Email: haller@wzw.tum.de
Senior author: Dr D. Haller

Summary

Bifidobacteria comprise a dominant microbial population group in the human intestinal tract with purported beneficial health effects on the host. In this study, we characterized the molecular mechanisms for the initial interaction of probiotic *Bifidobacterium lactis* strain BB12 with native and intestinal epithelial cell (IEC) lines. We showed that *B. lactis*-monoassociated Fisher F344 rats transiently induce phosphorylation/activation of the NF-κB transcriptionally active subunit RelA and the mitogen-activated protein kinase (MAPK) p38 in native IEC at day 5 after initial bacterial colonization. In addition, Interleukin 6 (IL-6) gene expression was significantly increased at day 5, demonstrating the physiological relevance of transient transcription factor activation in IEC. In contrast, *Bacteroides vulgatus*-monoassociated Fisher rats revealed RelA but not p38 MAPK phosphorylation and failed to trigger significant IL-6 gene expression in native IEC. Moreover, we demonstrated that *B. lactis* triggers NF-κB RelA and p38 MAPK phosphorylation in IEC lines. Adenoviral delivery of mutant IKK-β (Ad5dnIKKβ) and inhibition of the p38 MAPK pathway through the pharmacological inhibitor SB203580 significantly blocked *B. lactis*-induced IL-6 gene expression in IEC, suggesting that *B. lactis* triggers NF-κB and MAPK signaling to induce gene expression in the intestinal epithelium. Regarding the mechanisms of bacteria epithelial cell cross-talk, *B. lactis*-induced IL-6 gene expression was completely inhibited in TLR2 deficient mouse embryogenic fibroblasts (MEF TLR2$^{-/-}$) as well as TLR2ΔTIR transfected Mode-K cells. In conclusion, we demonstrated that probiotic bacteria transiently trigger innate signal transduction and pro-inflammatory gene expression in the intestinal epithelium at early stages of bacterial colonization.

Keywords: intestinal epithelial cells; Toll-like receptor 2; probiotic bacteria; nuclear factor (NF)-κB; gnotobiology

Introduction

The mucosal surfaces and cavities of the gastrointestinal (GI) tract in humans and animals are populated by a complex mixture of more than 400 microbial species with spatial differences in population size and relative species predominance.[1–4] Studies in gnotobiotic animals have shown that association of germ-free rodents with single bacterial species has a profound impact on the anatomical, physiological, and immunological development of the host, including effects on epithelial cell functions and the gut-associated lymphoid tissue.[5–7] The complex homeostasis between non-pathogenic intestinal micro-organisms and the host is an intriguing immunological paradox as the normal mucosal immune system acquires tolerance (hyporesponsiveness) to the enteric microbiota, while protective cell-mediated and humoral immune responses to enteropathogens are maintained. Although the pattern of bacterial colonization in the premature neonatal or infant gut is different

Abbreviations: IEC, intestinal epithelial cells; NF-κB, nuclear factor κB; MAPK, mitogen-activated protein kinase; IL-6, interleukin-6; TLR2, Toll-like receptor 2; MEF, mouse embryogenic fibroblasts.

from the adult intestine, bifidobacteria are a dominant microbial population group in the human intestine.[8–10] Clinical and animal studies provide evidence that certain strains of *Bifidobacterium animalis* (*lactis*), *B. longum*, *B. infantis and B. breve* may be effective in the prevention and/or treatment of gastroenteritis, necrotizing enterocolitis and chronic intestinal inflammation.[11,12] The molecular mechanisms underlying these protective effects of probiotic bifidobacteria in the gut are completely unknown.

Intestinal epithelial cells (IEC), which make up the actual barrier that separates the host from the gut luminal environment, constitutively express or can be induced to express Toll-like receptors (TLR), costimulatory molecules, components of the human major histocompatibility complex (MHC) and a wide range of inflammatory and chemoattractant cytokines when activated by enteric pathogens or inflammatory products.[13–16] Most of these molecules are in part transcriptionally regulated by the transcription factor nuclear factor (NF)-κB.[17] Although bacteria trigger host responses by multiple mechanisms, the cornerstone of innate signalling is mediated by TLRs, a set of 10 well conserved pattern recognition receptors (PRR).[18–20] TLRs are transmembrane proteins characterized by an extracellular domain containing leucine-rich repeats and an intracellular domain homologous to the interleukin (IL)-1R, or Toll/IL-1R (TIR). Ligand-specific binding to TLR promotes interaction of the cytoplasmic TIR domain with adaptor proteins followed by the recruitment of multiple kinases and activation of downstream target effector systems, including the mitogen-activated kinases (MAPK) as well as the IκB/NF-κB transcriptional system.[20,21] We previously demonstrated that non-pathogenic Gram negative *Bacteroides vulgatus* induce RelA (NF-κB p65 subunit) phosphorylation and NF-κB activation in IEC through a TLR4-dependent manner.[22,23] Monoassociation of germ-free Fisher rats with *Bacteroides vulgatus* induced transient nuclear localization of phosphorylated-RelA in the intestinal epithelium, demonstrating the physiological relevance for bacteria–epithelial cell interaction at the mucosal surface of the gut.

In this study, we characterized the molecular mechanisms for the cellular interaction of the probiotic bacterial strain *B. lactis* BB12 with the intestinal epithelium in native epithelium and cell lines. We showed that *B. lactis* BB12 transiently triggered NF-κB RelA and p38 MAPK phosphorylation as well as IL-6 gene expression in native epithelium after the initial monoassociation of Fisher F344 rats. Similarly, *B. lactis* BB12 induced RelA and p38 phosphorylation in the IEC line Mode-K. In contrast, *B. vulgatus*-monoassociated Fisher rats revealed RelA but not p38 MAPK phosphorylation and failed to trigger significant IL-6 gene expression in IEC. The inhibition of the NF-κB and p38 MAPK pathways using the adenoviral vector Ad5dnIKKβ and the pharmacological inhibitor SB203580 significantly inhibited IL-6 protein secretion in *B. lactis* BB12-stimulated Mode-K cells. Interestingly, the mRNA expression level of A20 which is a negative regulator of the TLR-mediated NF-κB signal transduction cascade was significantly up-regulated in primary and IEC lines.[24–26] *In vitro*, the induction of IL-6 gene expression was completely blocked in TLR2 gene deficient mouse embryogenic fibroblast cells ($TLR2^{-/-}$ MEF) and in TLR2ΔTIR transfected Mode-K cells, suggesting an important role of this pattern recognition receptor to mediate the initial interaction of *B. lactis* BB12 with the intestinal epithelium.

Materials and methods

Animals and bacterial monoassociation

Germ-free Fisher F344 rats in the Gnotobiotic Animal Facility at the German Institute of Human Nutrition (DIFE Potsdam-Rehbrücke, Germany). They were obtained from the germ-free breeding colony of the department. Twice a month the germ-free status of the animals was monitored. The animals were maintained in positive-pressure isolators (Metall & Plastik, Radolfzell, Germany) and housed in polycarbonate cages on irradiated wood chips at 22 ± 2°, 55 ± 5% relative humidity on a 12 hr light–dark cycle. They had free access to irradiated diet (Altromin fortified type 1314; Altromin, Lage, Germany) and autoclaved distilled water. Rats were monoassociated at 12–16 weeks of age with *B. lactis* BB12 as well as *B. vulgatus* (a generous gift from Dr R. Balfour Sartor, University of North Carolina at Chapel Hill, NC) and maintained under gnotobiotic conditions. This *B. vulgatus* strain was used to induce chronic intestinal inflammation in the HLA-B27 transgenic rat model of experimental colitis.[27] Bacterial monoassociation and absence of contamination by other bacterial species were confirmed by culturing samples from the small and large intestine at necropsy and culturing serial faecal samples. The protocol for the animal experiment was approved by the Ministry of Nutrition, Agriculture and Forestry, Brandenburg, Germany. Rats were killed 3, 5, and 7 days after initial bacterial colonization. Germ-free rats were used as controls. Histological analysis of paraffin embedded colonic tissue sections assessed the degree of lamina propria mononuclear cell infiltration, crypt hyperplasia, goblet cell depletion and architectural distortion[27] and revealed no signs of inflammation in *B. lactis* BB12 monoassociated rats.

Isolation of primary rat intestinal epithelial cells

Gnotobiotic and germ-free rats were killed, and the cecum as well the as colon were removed and placed in

Dulbecco's modified Eagle's minimal essential medium (DMEM; Invitrogen Life Technologies, Karslruhe, Germany) containing 5% fetal calf serum (FCS). Caecum and colon were cut longitudinally, washed three times in calcium/magnesium-free Hank's balanced salt solution (Gibco BRL, Invitrogen, Karlsruhe, Germany), cut into pieces 0·5 cm long and incubated at 37° in 40 ml DMEM containing 5% FCS and 1 mM dithiothreitol (DTT) for 30 min in an orbital shaker. The supernatant was filtered, centrifuged for 5 min at 400 ***g*** and the cell pellet was resuspended in DMEM containing 5% FCS. The remaining tissue was incubated in 30 ml phosphate-buffered saline (PBS) (1×) containing 1·5 mM ethylenediaminetetra-acetic acid for an additional 10 min. The supernatant was filtered, centrifuged for 5 min at 400 ***g*** and the cell pellet was resuspended in DMEM containing 5% FCS. Finally, primary IEC were collected by centrifugation through a 25/40% discontinuous Percoll gradient at 600 ***g*** for 30 min. Primary rat IEC from caecum and colon were combined and collected in sample buffer for subsequent RNA isolation and Western blot analysis.

Cell culture and bacterial stimulation

The mouse IEC line Mode-K (passage 10–30; a generous gift from Dr Ingo B. Autenrieth, University of Tübingen, Germany) was grown in a humidified 5% CO_2 atmosphere at 37° to confluency in six-well tissue culture plates (Cell Star, Greiner bio-one, Frickenhausen, Germany) as previously described.[23] Mode-K cells that were generated from C3H/He mice are lipopolysaccharide unresponsive.[28] Reconstitution of Mode-K cells with the TLR4/MD2 complex conferred LPS responsiveness (data not shown). We used Mode-K cells to selectively characterize Gram-positive bacteria-induced activation of epithelial cells. In addition, Mode-K cells were transfected with pZERO vector (InvivoGen, San Diego, CA) for murine TLR2ΔTIR lacking the cytoplasmic TIR domain. Stable transfected TLR2ΔTIR Mode-K cells were established through serial plating in Puromycin-containing medium. Wild type (WT) and $TLR2^{-/-}$ MEF cells were a generous gift from Dr Carsten J. Kirschning (Technical University of Munich, Germany).

B. lactis BB12 was anaerobically grown at 37° in MRS broth (Sigma Aldrich, Taufkirchen, Germany) containing 1% Tween-80. Bacteria were harvested by centrifugation (3000 ***g***, 15 min) at stationary growth phase, washed in phosphate-buffered saline (1× PBS pH 7·4) and diluted in DMEM (Invitrogen). Confluent epithelial cell monolayers were infected with *B. lactis* BB12 at a bacterium-to-epithelial cell ratio (multiplicity of infection (m.o.i.)) of 3–30 at various time points. Based on the average number of Mode-K cells per six-well ($1{\cdot}5 \times 10^6$), we used $4{\cdot}5 \times 10^6$ (m.o.i. 3) or $4{\cdot}5\ 10^7$ (m.o.i. 30) total bacteria per 2 ml of medium. Where indicated, Mode-K cells were pretreated with the p38 MAPK inhibitor SB203580 (Merck Bioscience, Schwalboch, Germany) and the adenoviral vector Ad5dnIKKβ (a generous gift from Dr Christian Jobin, University of North Carolina at Chapel Hill, NC) as previously described.

RNA isolation and real-time reverse transcription–polymerase chain reaction (PCR)

RNA from IEC was extracted using Trizol Reagent (Invitrogen Life Technologies) according to the manufacturer's instructions. Extracted RNA was dissolved in 20 µl water containing 0·1% diethyl-pyrocarbonate. For reverse transcription, 1 µg total RNA was added to 30 µl of reaction buffer containing 8 µl 5× first-strand buffer, 4 µl DTT (100 mM), 6 µl desoxyribonucleoside triphosphate mixture (300 µM) (all reagents from Invitrogen Life Technologies) and incubated for 5 min at 65°. After adding 10 µl of a solution containing 0·2 µg random hexamers, 40 U RNase Out and 200 U murine Moloney leukaemia virus reverse transcriptase (all reagents from Invitrogen Life Technologies), the total mixture was incubated for an additional 60 min at 37° followed by a final 1 min heating step at 99°.

Real time PCR was performed in glass capillaries using a Light Cycler™ system (Roche Diagnostics, Mannheim, Germany). Primer sequences and amplicon sizes were as follows: rat IL-6-F 5′-ccactgccttccctac-3′, rat IL-6-R 5′-gtgcatcatcgctgtt-3′ (amplicon size 183 bp), rat A20-F 5′-catctgcccttcctgg-3′, rat A20-R 5′-tcttcttggttcttaggct-3′ (amplicon size 274 bp), rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-F 5′-ccaaggagtaagaaaccc-3′; rat GAPDH-R 5′-ggtgcagcgaactttatt-3′ (amplicon size 209 bp), mouse A20-F 5′-gaacaatgtcccgtgc-3′, mouse A20-R-5′-acctactcgttggctt-3′ (amplicon size 277 bp). For real time PCR, 1 µl reverse transcribed cDNA was added to a total volume of 10 µl PCR reaction buffer containing 1× LC-FastStart DNA Master Mix (Roche), $MgCl_2$ (4 µM), forward and reverse primers (20 µM). The PCR programme was one cycle of denaturation at 95° for 10 min followed by 50 cycles of 95° for 15 s, annealing at 60° for 10 s and extension at 72° for 20 s. The amplified product was detected by the fluorescent dye SYBR green. Melting curve analysis and gel electrophoresis were used to document the amplicon specificity. 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 EΔCp (control samples – treated samples) and normalized for the expression of GAPDH mRNA.[29] Triplicate samples were measured in duplicates and blotted as fold increase between treated and untreated control samples.

Western blot analysis

Purified primary IEC or Mode-K cells were lysed in 1× Laemmli buffer, and 20–50 μg of protein was subjected to electrophoresis on 10% sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis (PAGE) gels. Where indicated IEC cells were pretreated for 1 hr with 20 μM of the proteasome inhibitor MG132 (BioMol, Plymouth Meeting, PA). Anti-phospho-RelA (Ser536), RelA, anti-phospho-p38 (Thr180/Tyr182), p38, acetylated-phosphorylated-histone 3 (Ac-P-H3), histone 3 (all antibodies from Cell Signaling (Beverly, MA)), CD45 (Santa Cruz Biotechnology, Santa Cruz, CA) and β-actin (ICN, Costa Mesa, CA) were used to detect immunoreactive phospho-RelA, RelA, phospho-p38, p38, phospho-acetyl-histone 3, histone 3, CD45 and β-actin, respectively, using an enhanced chemiluminescence light-detecting kit (Amersham, Arlington Heights, IL) as previously described[23].

Enzyme-linked immunosorbent assay (ELISA) analysis

Protein concentrations were determined in spent culture supernatants of IEC cultures using an ELISA technique. IL-6 protein production was determined by mouse specific ELISA assay kits according to the manufacturer's instructions (R & D Systems, Heidelberg, Germany).

Statistical analysis

Data are expressed as the means ± S.D. of triplicates. Statistical analysis was performed by the two-tailed Student's *t*-test for paired data and considered significant if *P*-values were <0·05 (*) or <0·01 (**).

Results

NF-κB RelA and p38 phosphorylation as well as IL-6 gene expression in native IEC after bacterial colonization of germ-free rats with *B. lactis* BB12 and *B. vulgatus*

To investigate molecular mechanisms for the interaction of the Gram-positive probiotic bacterial strain *B. lactis* BB12 with the host after the initial colonization of the gut, we first associated germ-free Fisher F344 rats with *B. lactis* BB12 for 1 week. In parallel, we monoassociated germ-free rats with our reference strain *B. vulgatus*.[23] Monoassociated rats were killed after 3, 5 and 7 days after bacterial colonization. Primary IEC from the large intestine (caecum + colon) were isolated and pooled samples from two independent experiments were subjected to Western blot analysis. As shown in Fig. 1 *B. lactis* BB12 induced RelA (Fig. 1a) and p38 (Fig. 1b) phosphorylation in native IEC at day 5 but not at day 3 and day 7 after the association of the rats. In contrast, *B. vulgatus* triggered RelA but not p38 phosphorylation after 5 days of bacterial colonization. Total RelA and p38 protein expression was confirmed in IEC for germ-free control and monoassociated rats. Of note, germ-free control rats revealed no phospho-RelA and phospho-p38 expression in native IEC, suggesting that *B. lactis* BB12 transiently triggered activation of the NF-κB and p38 MAPK transcription factor systems in native epithelium. Histone acetylation and phosphorylation are post-transcriptional mechanisms leading to chromatin unfolding in order to provide access of transcrition factors to gene promoter binding sites and to achieve coordinate gene expression. As shown in Fig. 1(c) *B. lactis* BB12 induced histone 3 acetylation/phosphorylation in IEC on days 3, 5 and 7 after the initial bacterial colonization. The slightly elevated level of P-Histone 3 in germ-free IEC may reflect basic activation of gene expression. In addition, the absence of lymphocyte contaminations in the purified

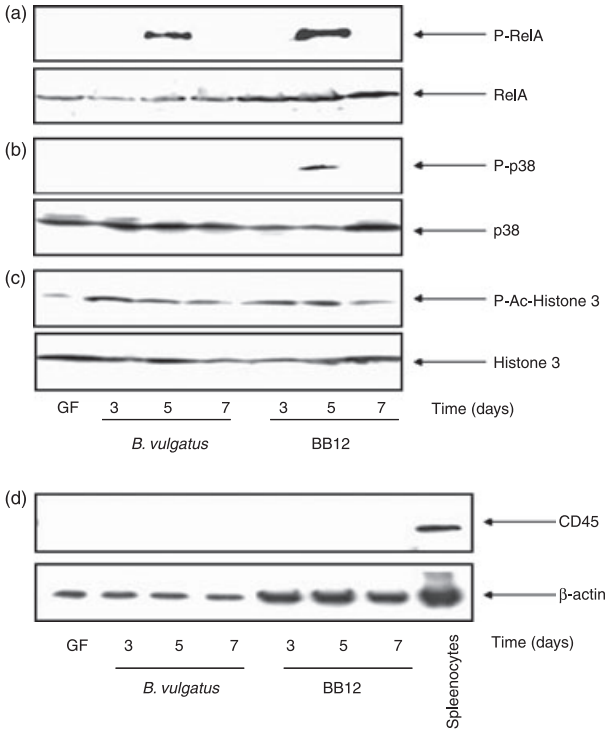

Figure 1. (a–c) NF-κB RelA and p38 MAPK phosphorylation in native IEC from *B. vulgatus* and *B. lactis* BB12-monoassociated Fisher rats. Germ-free Fisher F344 rats were monoassociated with *B. vulgatus* and *B. lactis* BB12 at 12–16 weeks of age. Rats ($n = 2$) were killed at day 3, 5, and 7 after initial bacterial colonization and native IEC from large intestine (caecum + colon) were isolated as described in Materials and Methods. IEC from germ-free rats were used as controls. Total protein was extracted and 50 μg of protein was subjected to SDS–PAGE followed by (a) phospho-RelA and RelA (b) phospho-p38 and p38 (c) actetylated-phosphorylated H3 and total H3 as well as (d) CD45 and β-actin immunoblotting using the ECL technique. Gels represent the combined protein samples from each group.

epithelial cell preparations was confirmed by determining the lymphocyte marker CD45 using Western blot analysis. As shown in Fig. 1(d), CD45 protein expression was absent in isolated epithelial cells but was clearly detectable in control samples from spleenocytes.

We next sought to investigate IL-6 gene expression in native IEC from gnotobiotic Fisher rats compared to germ-free controls using real-time quantitative Light Cycler reverse transcription–PCR. As shown in Fig. 2(a), the rats monoassociated with *B. lactis* triggered a significantly higher IL-6 mRNA expression on day 5 (20–23-fold increase) than on days 3 (three- to fivefold increase) and 7 (four- to fivefold increase) in both gnotobiotic experiments, confirming the transient activation of the NF-κB and MAPK signalling cascades in IEC. Interestingly, IL-6 gene expression in IEC was significantly lower in *B. vulgatus*-monoassociated rats.

A20 is an inducible molecule implicated in the negative regulation of NF-κB-dependent gene expression.[24] Parallel to the induction of RelA phosphorylation and IL-6 gene expression at day 5 after bacterial colonization, A20 mRNA expression was significantly higher in *B. lactis*-monoassociated rats (10- and eightfold increase) compared with *B. vulgatus*-colonized rats (two- and twofold increase) (Fig. 2b).

B. lactis BB12 triggers NF-κB RelA and p38 phosphorylation as well as IL-6 and A20 expression in Mode-K cells

Because *B. vulgatus* triggers RelA phosphorylation and IL-6 gene expression in IEC through the TLR4 signalling cascade, we next asked the question whether *B. lactis* BB12 can directly induce RelA and p38 phosphorylation in IEC. We first stimulated Mode-K cells with *B. lactis* BB12 at m.o.i. 30 for 0–4 hr. Interestingly, *B. lactis* BB12 transiently induced phospho-RelA (Fig. 3a) as well as phospho-p38 (Fig. 3b), suggesting that *B. lactis* directly triggers these signalling pathways in native epithelium (Fig. 1). We next measured IL-6 protein secretion in the culture supernatant of stimulated Mode-K cells using ELISA technique. As shown in Fig. 4(a). *B. lactis* BB12 induced IL-6 protein secretion in Mode-K cells in a dose- and time-dependent manner reaching maximal stimulation at m.o.i. 30 after 6 hr of stimulation.

At the level of mRNA expression, IL-6 was strongly induced after 3 hr of bacterial stimulation of Mode-K cells followed by a complete down-regulation after 6 hr to 24 hr of bacterial stimulation (Fig. 4b). Interestingly and in accordance with our *in vivo* data, the induction of A20 mRNA expression in Mode-K cells was associated with the induction of IL-6 gene expression. These data may suggest that A20 gene expression could be involved in the negative regulation of NF-κB activity and NF-κB-dependent gene expression in IEC.

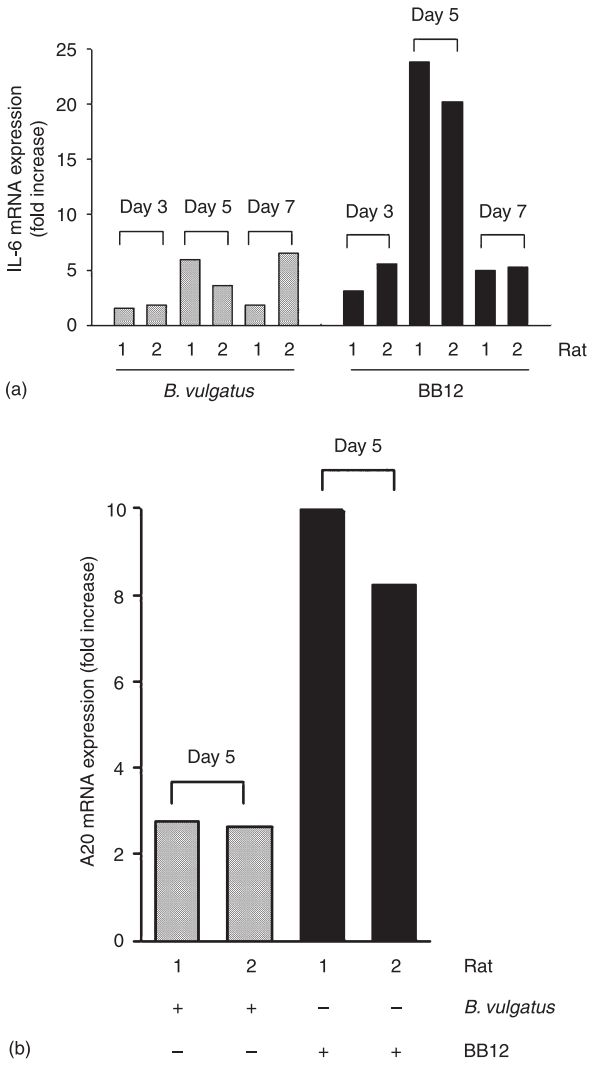

Figure 2. IL-6 gene and A20 gene expression in native IEC from *B. vulgatus* and *B. lactis* BB12-monoassociated Fisher rats. Germ-free Fisher F344 rats were monoassociated with *B. lactis* BB12 at 12–16 weeks of age. Rats ($n = 2$) were killed at day 3, 5, and 7 after initial bacterial colonization and native IEC from large intestine (caecum + colon) were isolated as described in Materials and methods. IEC from germ-free rats were used as controls. Total RNA was extracted, reverse transcribed and real-time PCR was performed using the Light Cycler system with specific primers for IL-6, A20 and GAPDH. The induction of IL-6 (a) and A20 (b) mRNA was calculated relative to germ-free controls (mean fold increase ± SD) using the crossing point of the log-linear portion of the amplification curve after normalization with GAPDH.

B. lactis BB12 induce TLR2 signalling to trigger IL-6 gene expression in IEC through the NF-κB and p38 MAPK pathways

To further dissect the role of NF-κB and p38 MAPK signalling in *B. lactis*-induced IEC activation, we measured IL-6 secretion in Mode-K cells in the absence or presence

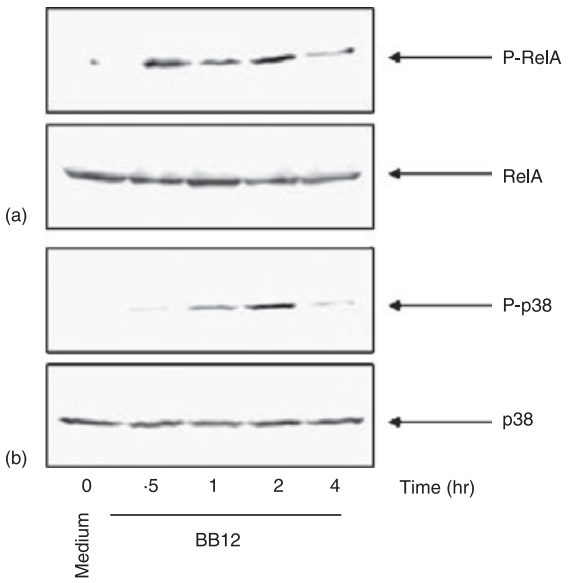

Figure 3. *B. lactis* BB12 triggers transient RelA and p38 phosphorylation in Mode-K epithelial cells. Mode-K cells were stimulated with *B. lactis* BB12 for various times at m.o.i. 30. Total protein was extracted and 20 μg of protein was subjected to SDS-PAGE followed by immunoblotting using the ECL technique with antibodies for (a) phospho-RelA and RelA as well as (b) phospho-p38 and p38phospho-RelA. Representative gels from at least three different experiments are shown.

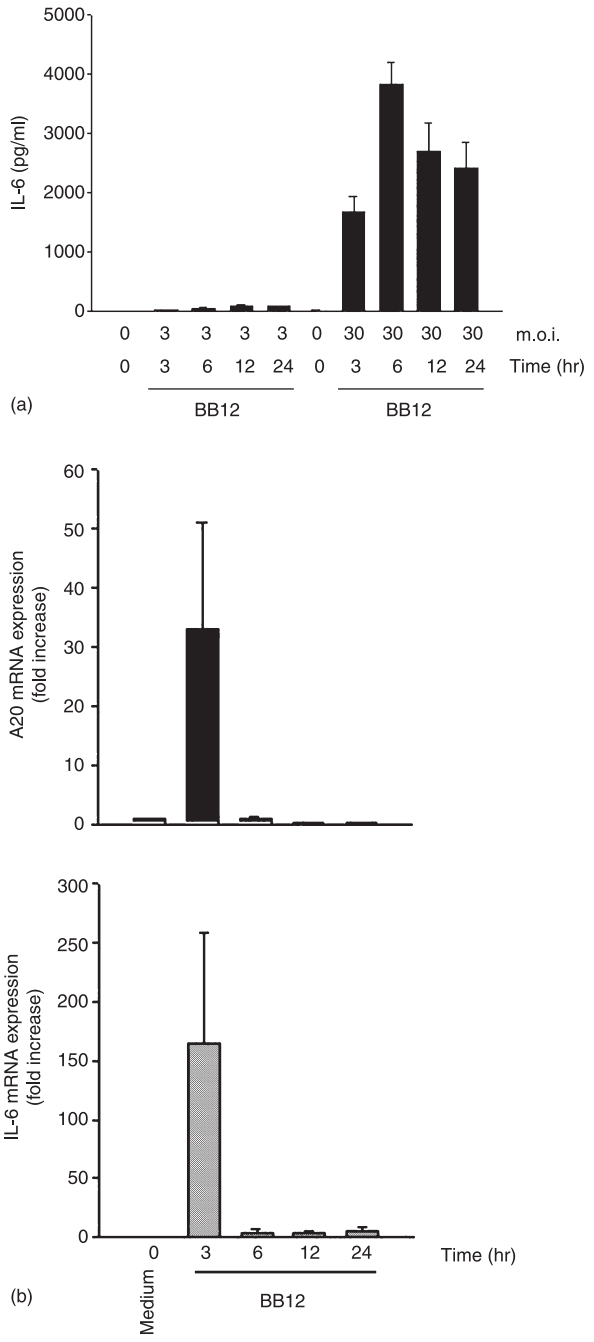

Figure 4. *B. lactis* BB12 triggers IL-6 and A20 gene expression in Mode-K epithelial cells. Mode-K cells were stimulated with *B. lactis* BB12 for various times at m.o.i. 3 and 30. (a) IL-6 protein was measured in the spent culture supernatant from stimulated Mode-K cells using ELISA. (b) IL-6 and A20 mRNA expression was measured in *B. lactis* BB12 stimulation (m.o.i. 30). Total RNA was extracted, reverse transcribed and real-time PCR was performed using the Light Cycler system with specific primers for IL-6, A20 and GAPDH. The bars represent the combined mean value (± SD) of three experiments.

of Ad5dnIKKβ as well as the pharmacological p38 inhibitor SB203580. Figure 5 shows that *B. lactis*-induced IL-6 production was significantly inhibited in the presence of dominant negative IKKβ as well as SB203580, suggesting an important role for both signalling pathways in mediating *B. lactis*-induced IL-6 gene expression.

Gram-positive bacterial products have been shown to trigger cell activation through the pattern recognition receptor TLR2. We first used wild type and TLR2$^{-/-}$ MEF cells to evaluate the role of TLR2 in triggering IL-6 production. As shown in Fig. 6, *B. lactis* BB12 induced significant IL-6 expression in wild type cells but completey failed to trigger IL-6 secretion in TLR2$^{-/-}$ MEF. In contrast, IL-1β-induced IL-6 secretion was intact in both cell types, demonstrating the specificity for the inhibition of *B. lactis* BB12-mediated IL-6 production in TLR2$^{-/-}$ MEF. To further confirm the capability of *B. lactis* to signal through the pattern recognition receptor TLR2 in IEC, we established stable transfected TLR2ΔTIR Mode-K cells as described in Materials and methods. As shown in Fig. 7, *B. lactis* BB12-induced IL-6 protein secretion in Mode-K cells was completely blocked in TLR2ΔTIR stable transfected cells. In contrast, IL-1β-induced IL-6 production was intact confirming the responsiveness of these cells.

In conclusion, these results demonstrate that the Gram-positive probiotic strain *B. lactis* BB12 signals through the TLR2 cascade to induce IL-6 gene expression. Of note, *B. lactis* BB12-induced IL-6 gene expression in IEC was mediated through the NF-κB and p38 MAPK pathways. In addition, we demonstrated that *B. lactis* BB12

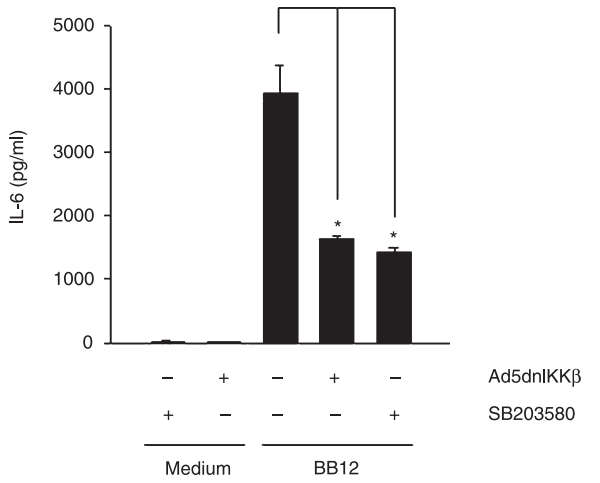

Figure 5. *B. lactis* BB12 triggers IL-6 secretion in Mode-K epithelial cells through the induction of NF-κB and p38 MAPK signalling. Mode-K cells were stimulated with *B. lactis* BB12 for 24 hr m.o.i. 30. Where indicated Mode-K cells were infected with adenoviral dominant negative (dn) IKKβ (Ad5dnIKKβ) and the pharmacological p38 MAPK inhibitor SB203580 (20 μM). IL-6 protein was measured in the spent culture supernatant from stimulated Mode-K cells using ELISA methods. The bars represent the combined mean value (± SD) of three experiments. $^{*}P < 0{\cdot}05$.

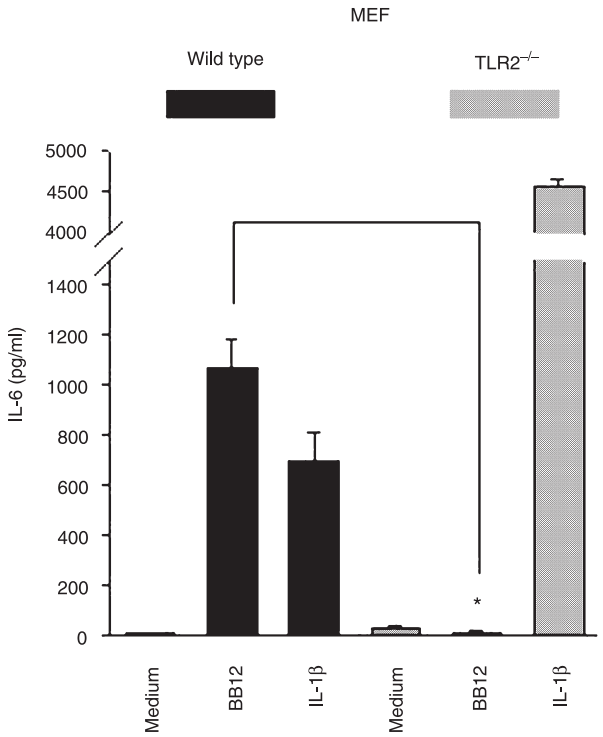

Figure 6. *B. lactis* BB12 signals through the pattern recognition receptor TLR2 cascade to induce IL-6 gene expression in MEF cells. Wild type and TLR2$^{-/-}$ MEF cells were stimulated with IL-1β (20 ng/ml) and *B. lactis* BB12 at m.o.i. 30 for 12 hr. IL-6 protein was measured in the spent culture supernatant from stimulated Mode-K cells using ELISA methods. The bars represent the combined mean value (± SD) of three experiments. $^{*}P < 0{\cdot}05$.

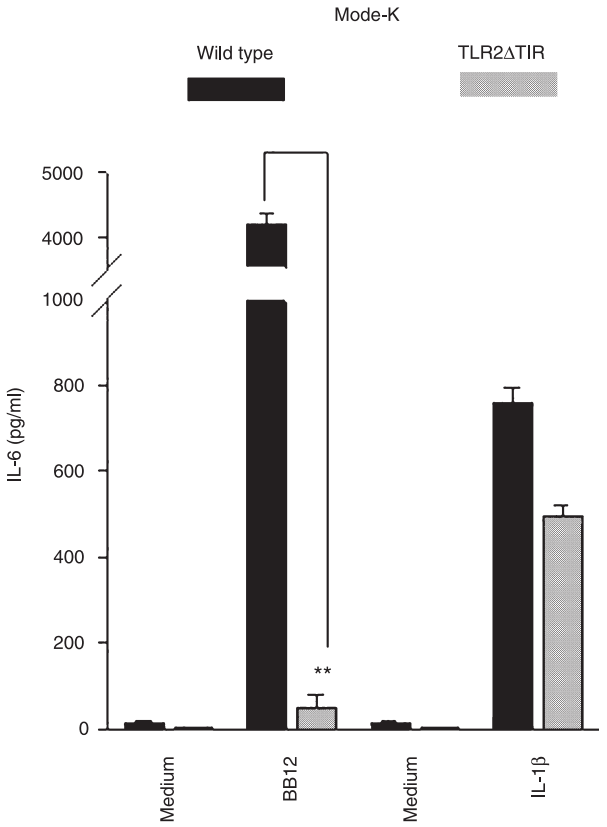

Figure 7. *B. lactis* BB12 signals through the pattern recognition receptor TLR2 cascade to induce IL-6 gene expression in IEC. Mode-K and stable transfected TLR2ΔTIR Mode-K cells were stimulated with IL-1β (20 ng/ml) and *B. lactis* BB12 at m.o.i. 30 for 6 hr. IL-6 protein was measured in the spent culture supernatant from stimulated Mode-K cells using ELISA methods. The bars represent the combined mean value (± SD) of three experiments. $^{**}P < 0{\cdot}01$.

transiently triggered NF-κB and p38 MAPK signalling as well as IL-6 gene expression in native IEC from monoassociated rats, demonstrating the physiological relevance of these mechanisms for bacteria–epithelial cell interaction *in vivo*.

Discussion

The molecular mechanisms of activating epithelial cell signal transduction in intestinal epithelium cells by probiotic bacteria may be relevant for initiating and maintaining intestinal homeostasis. We showed that the probiotic bacterial strain *B. lactis* BB12 signals through the NF-κB and p38 MAPK pathways to trigger IL-6 gene expression in both native and IEC lines. Most importantly, the monoassociation of germ-free Fisher F344 rats with *B. lactis* BB12 induced NF-κB RelA and p38 MAPK phosphorylation as well as IL-6 gene expression in native IEC. In contrast, *B. vulgatus*-monoassociated Fisher rats revealed RelA but not p38 MAPK phosphorylation and failed to trigger significant IL-6 gene expression in IEC. Of note,

B. vulgatus triggers chronic intestinal inflammation in gnotobiotic HLA-B27 transgenic but not normal rats.[27] Although *B. lactis* triggered pro-inflammatory signal transduction and gene expression in IEC, histological signs of inflammation were absent in *B. lactis*-monoassociated rats, suggesting that the normal host developed feedback mechanisms to control the mucosal immune responses to the constant challenge by commensal bacteria. We have previously shown that Gram-positive colitogenic *Enterococcus faecalis* initially triggered transient NF-κB signal transduction and pro-inflammatory gene expression in IEC from bacteria-monoassociated wild type and IL-$10^{-/-}$ mice. Interestingly and parallel to the development of chronic intestinal inflammation, NF-κB activity and pro-inflammatory gene expression was sustained in IEC IL-$10^{-/-}$ mice.[30] Considering these findings it seems important to evaluate and compare the physiological consequences of *B. lactis* and *B. vulgatus* signalling to the epithelium on the development of chronic intestinal inflammation in animal models for experimental colitis including HLA-B27 transgenic rats and IL-$10^{-/-}$ mice.

Miettinen *et al.* showed that the probiotic Gram-positive strain *Lactobacillus rhamnosus* GG induces NF-κB activation and IL-6 gene expression in freshly isolated human leucocyte cultures.[31] Here, we provide evidence that the Gram-positive *B. lactis* strain BB12 triggers IL-6 gene expression through TLR2-mediated activation of the NF-κB and p38 MAPK signalling cascades. Adenoviral delivery of dominant negative IKK-β and the addition of p38 MAPK inhibitor SB203580 significantly inhibited *B. lactis*-induced IL-6 gene expression in IEC, suggesting that both NF-κB and p38 signalling pathways contribute to the TLR2-meditaed activation of gene expression. Although *B. vulgatus* triggers TLR4-mediated RelA phosphorylation and IL-6 gene expression in epithelial cell lines[23] but failed to trigger p38 phosphorylation in native epithelium, the physiological consequences of p38 MAPK signalling in the epithelium for bacteria-mediated host responses remains to be elucidated. We previously characterized molecular mechanisms for the inhibition of *B. vulgatus* and *E. faecalis*-mediated NF-κB activity and IL-6 gene expression in IEC, including effects that involve pattern recognition receptor stability, chromatin remodelling and modulation of phosphatase activity.[32] It appears from several studies that transforming growth factor-β (TGF-β) and 15-deoxy-Δ12,14-prostaglandin J2 are important mediators in the negative regulation of acute and chronic inflammatory in the gut. Indeed, we showed that TGF-β-activated Smad signalling induced TLR2 degradation[30] and inhibited C basic protein/p300-mediated histone phosphorylation in IEC.[22] In addition, 15-deoxy-Δ12,14-prostaglandin J2-induced ERK signalling triggered phosphoprotein phosphatase (PP2A) activity, which directly dephosphorylated RelA and inhibited IL-6 gene expression in IEC.[33] In addition, we currently characterize the protective role of IL-10-induced p38 signalling in the intestinal epithelium (Ruiz and Haller, unpublished observation). Induction of the zinc finger protein A20 is also involved in the negative regulation of TLR-mediated activation of the NF-κB signal transduction cascade likely through the inhibition of IKK activity by binding to the TRAF/RIP proteins.[25,26,34,35] We showed that the induction of IL-6 mRNA expression by *B. lactis* was associated with the up-regulation of A20 mRNA expression in native and IEC lines suggesting a possible mechanism for the termination of NF-κB signalling and IL-6 gene expression.

Clinical and animal studies with probiotic micro-organisms provide some evidence that certain bifidobacterial strains are effective in the modulation of mucosal immune responses including the prevention and/or treatment of acute and chronic inflammation. For example, *B. lactis* BB12 antagonized *Helicobacter pylori* growth and/or metabolism *in vitro* as well as *in vivo*.[36] In addition, *B. lactis* BB12 was effective at least partially to maintain oral tolerance to β-lactoglobulin in mice.[37] Moreover, *B. lactis* HN019 and *B. longum* BB46[38] were shown to increase resistence to experimental salmonellosis in mice. *B. lactis* HN019 also revealed immune stimulatory functions in elderly people including effects on natural killer cell and granulocyte activation.[39] In addition, *B. infantis* strain 35624 attenuated experimental chronic colitis in IL-$10^{-/-}$ mice[40] and dextran sodium sulphate-induced acute colitis in Sprague–Dawley rats.[41] Furthermore, the probiotic mixture VSL#3 with eight different lactic acid bacterial species including *B. longum*, *B. infantis* and *B. breve* were effective in the treatment and/or treatment of experimental colitis in IL-$10^{-/-}$ mice[42] as well as inflammatory bowel disease patients with pouchitis.[43,44] The underlying cellular and molecular mechanisms for these beneficial health effects of bifidobacteria including their capability to activate and/or inhibit immune functions in the gut remain unclear.

Several studies now point to the fact that certain protective effects of probiotic bacteria may be mediated through the improvement of the gut epithelial cell barrier function. Indeed, ligand-specific activation of the TLR2 system greatly enhance transepithelial resistance in IEC lines associated with apical tightening and sealing of tight junction-associated ZO-1.[45] In addition, Yang and Polk suggested that *L. rhamnosus* GG inhibits tumour necrosis factor (TNF)-mediated pro-apoptotic mechanisms in IEC YAMC cultures through the blockade of TNF-induced p38 MAPK activation[46]. Interestingly and consistent with our data, one mechanism to trigger antiapoptotic signalling pathways includes the induction of the NF-κB signalling cascade.[47] Accordingly, the selective inhibition of NF-κB activity in enterocytes of IKKβ gene deficient mice sensitized these animals to ischaemia–reperfusion-induced apoptosis in IEC, which was associated with a loss of

mucosal integrity.[48] It appears from these results that although the pro-inflammatory NF-κB transcription factor system plays an important role in perpetuating the late/chronic state of certain inflammatory processes, the maintenance of normal mucosal homeostasis and epithelial cell integrity may require a fine-tuned balance between anti- and proapoptotic signals mediated at least in part by the presence of different bacterial species in the luminal gut microbiota.

In conclusion, we identified innate mechanisms for the probiotic *B. lactis* strain BB12 to activate transient pro-inflammatory host responses in the intestinal epithelium. We have shown that *B. lactis* BB12 triggers transient IL-6 gene expression in native epithelium and in cell lines. The initial and transient induction of TLR-mediated activation of pro-inflammatory transcription factor systems may play an important role in initiating epithelial cell homeostasis at early stages of bacterial colonization, while the persistent induction of NF-κB signalling associated with the lack of control mechanisms may lead to intestinal pathology including the development of chronic intestinal inflammation in the genetically susceptible host.

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APPENDIX 4

Ruiz, PA and Haller, D

"Functional Diversity of Flavonoids in the Inhibition of the Pro-inflammatory NF-κB, IRF, and Akt Signaling Pathways in Murine Intestinal Epithelial Cells"

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[Experimental work by Ruiz, PA *(Tables 1-2 and Figures 1-10)*; writing by Haller, D with editorial help of Ruiz, PA]

Functional Diversity of Flavonoids in the Inhibition of the Proinflammatory NF-κB, IRF, and Akt Signaling Pathways in Murine Intestinal Epithelial Cells

Pedro A. Ruiz and Dirk Haller[2]

Else Kroener-Fresenius-Centre for Experimental Nutritional Medicine, Technical University of Munich, Am Forum 5, 85350 Freising-Weihenstephan, Germany

ABSTRACT The molecular understanding of nutritional factors in the process of host factor–mediated activation of the intestinal epithelium may play an important role in the assessment of adjunct nutritional therapy for chronic intestinal inflammation. We characterized the molecular mechanisms of flavonoids including apigenin, luteolin, genistein, 3′-hydroxy-flavone, and flavone in inhibiting tumor necrosis factor-α (TNF)-induced interferon-induced protein (IP)-10 gene expression in the murine intestinal epithelial cell (IEC) line Mode-K. We demonstrated that 3′-hydroxy-flavone but not the chemical core structure flavone blocked TNF-α–induced nuclear factor (NF)-κB transcriptional activity and IP-10 expression at the level of NF-κB/IκBα phosphorylation/degradation by inhibiting IκB kinase activity. Although 3′-hydroxy-flavone effectively triggered p38 mitogen-activated protein kinase signaling and late caspase-3 cleavage, the induction of apoptotic cell death in TNF-activated IEC was not the primary mechanism inhibiting NF-κB transcriptional activity and IP-10 expression. In addition to the compound-specific inhibition of TNF-induced NF-κB DNA binding and NF-κB transcriptional activity, apigenin and luteolin selectively blocked Akt phosphorylation/activity. The ability of these polyphenolic compounds to target various signal transduction pathways was further supported by the observation that luteolin and 3′-hydroxy-flavone selectively induced interferon regulatory factor (IRF)-1 degradation. Finally, we showed that genistein blocked IP-10 but not IL-6 expression through NF-κB, IRF, and Akt independent mechanisms, demonstrating the functional diversity of flavonoids in inhibiting proinflammatory processes in IEC. In conclusion, we provide molecular evidence for the presence of characteristic inhibition patterns of these polyphenolic compounds to inhibit proinflammatory gene expression in IEC through the specific modulation of the NF-κB, IRF and Akt signaling pathways. J. Nutr. 136: 664–671, 2006.

KEY WORDS: • *intestinal epithelial cell pathology* • *inflammation* • *NF-κB signaling* • *flavonoids*

Gastrointestinal infections, the genetic predisposition to dysregulated mucosal immune responses, and the concurrent prevalence of certain environmental triggers in developed countries (e.g., nutritional habits) are strong etiologic factors for the development of chronic intestinal inflammation including ulcerative colitis and Crohn's disease, the 2 distinct pathologies of inflammatory bowel disease (IBD)[3] (1–3). Advancing knowledge about the molecular mechanisms of chronic intestinal inflammation has led to the development of specific biologic therapies (4); however, little is known about the anti-inflammatory effects of dietary components on disease pathology. Increased activity of the nuclear factor (NF)-κB transcription factor system was documented recently in the intestinal epithelium of animal models for experimental colitis (5) and IBD patients (6–8); accordingly, pharmacologic blockade may become particularly important in the treatment of chronic intestinal inflammation (9). Intestinal epithelial cells (IEC) must adapt to a constantly changing environment by processing the combined biological information of luminal enteric bacteria/nutritional factors (10) as well as host-derived immune signals (11–13) to maintain gut homeostasis; thus, IEC become an excellent target cell type with which to assess the anti-inflammatory effects of dietary components on the host (14).

Tumor necrosis factor-α (TNF) plays an important role in initiating and perpetuating NF-κB signaling and chronic intestinal inflammation (15,16). Indeed, the treatment of a subset of IBD patients with monoclonal antibodies to TNF (infliximab) induced clinical remission of the inflammatory disease status (17). Additional experimental evidence for the importance of TNF signaling in triggering intestinal inflammation was demonstrated in $TNF^{\Delta ARE}$ mice (18,19). These mice lack the translational repression of TNF due to the absence of TNF adenosine-uracil–rich elements in the 3′-untranslated region of the TNF mRNA transcripts; as a consequence, the mice develop experimental ileitis. At the cellular level, TNF activates

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[2] To whom correspondence should be addressed. E-mail: haller@wzw.tum.de.

[3] Abbreviations used: DMSO, dimethyl sulfoxide; EC_{50}, 50 % effective inhibitory concentration; GSK, glycogen synthase kinase; IBD, inflammatory bowel diseases; IEC, intestinal epithelial cells; IKK, IκB kinase; IP-10, interferon-γ-inducible protein 10; IRF, interferon regulatory factor; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor κB; SEAP, secreted alkaline phosphatase; TNF, tumor necrosis factor-α.

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the IκB/NF-κB transcription factor system through a TNF receptor 1 (TNFR1)-mediated cascade. Although controversial, the signal may converge on the NF-κB–inducing kinase, which then activates the IκB kinase (IKK) complex. The activation of IKK triggers NF-κB RelA (Ser536) and IκBα (Ser32/34) phosphorylation as well as IκBα ubiquitination and proteasomal degradation. The activation of the IκB/NF-κB complex is then followed by the nuclear translocation of transcriptionally active NF-κB subunits such as RelA and the induction of κB-dependent gene expression (20,21). Interestingly, the full activation of TNF-induced NF-κB activity and proinflammatory gene expression requires additional mechanisms including Akt serine-threonine kinase activation (22). Although TNF usually targets the cellular survival pathways in IEC through the activation of NF-κB and Akt signal transduction, TNF-mediated inflammatory signals can also be diverted to induce proapoptotic mechanisms through the induction of the p38 MAPK pathway (23–25).

Dietary flavonoids, which comprise the most common group of plant polyphenols particularly abundant in fruits and vegetables, were shown to mediate anti-inflammatory, antioxidative, antiproliferative, and proapoptotic effects in various cell types (26). Although certain flavonoids affect stress/cytokine-induced NF-κB signal transduction (27–29) and at least to some extent inhibit experimental colitis (30–32), the molecular mechanisms and the target specificity of these polyphenolic compounds in inhibiting epithelial cell activation is not well defined. We showed previously that under conditions of chronic experimental colitis, persistently activated NF-κB signal transduction and gene expression of the T cell CXC chemoattractant IFN-γ-inducible protein 10 (IP-10) in primary IEC was associated with the development of histopathologic changes in the colonic mucosa (5). In this study, we used preselected flavonoids that were shown to be highly efficient in inhibiting TNF-induced IP-10 expression in the murine IEC line Mode-K IEC. The aim of this study was to further characterize the molecular mechanisms of apigenin, luteolin, genistein, and 3′-hydroxy-flavone in inhibiting host factor-mediated activation of the intestinal epithelium.

MATERIALS AND METHODS

Cell culture and treatments. The mouse IEC line Mode-K (passage 10–30) (a generous gift from Dr. Ingo B. Autenrieth, University of Tübingen, Germany) was grown in a humidified 5% CO_2 atmosphere at 37°C to confluence in 6-well tissue culture plates (Cell Star, Greiner bio-one) as previously described (10). Mode-K cells were stimulated with TNF (5 μg/L; R&D Systems), IL-1β (5 μg/L; R&D Systems) in the absence or presence of apigenin, luteolin, 3′-hydroxy-flavone, flavone (all from Sigma-Aldrich), and genistein (Roth). Dose-response experiments were performed with the flavonoids in a concentration range of 1–200 μmol/L. The 50% effective inhibitory concentrations (EC_{50}) of these compounds were determined by calculating the inflection point of the inhibition curve. TNF-induced IP-10 protein concentrations were blotted against the flavonoid concentration. Where indicated, we used pharmacologic inhibitors including the p38 MAPK inhibitor SB203580 (20 μmol/L; Calbiochem, Merck Biosciences) and the proteasome inhibitor MG132 (20 μmol/L; BioMol). Cell viability was measured using MTT (thiazolyl blue tetrazolium bromide) (Sigma-Aldrich) and trypan blue exclusion.

Western blot analysis. Mode K cells were pretreated with flavonoids (100 μmol/L) for 1 h followed by stimulation with IL-1β and TNF. Cells were lysed in 1X Laemmli buffer and 20–50 μg of protein was subjected to electrophoresis on 10% SDS-PAGE gels. Anti-phospho-RelA (Ser536), anti-phospho-p38 (Thr180/Tyr182), anti-p38, anti-histone 3, anti-phospho-IκBα (Ser32), anti-phospho-Akt (Ser473), Akt, anti-phospho-glycogen synthase kinase (GSK)3α/β (Ser3/9), anti-cleaved caspase-3 (Asp175), anti-histone 3 (all from Cell Signaling), anti-interferon regulatory factor (IRF)-1, anti-IRF-3, anti-RelA, and anti-IκBα (all from Santa Cruz) were used to detect immunoreactive phospho-RelA, phospho-p38, p38, histone 3, phospho-IκBα, phospho-Akt, Akt, GSK-3α/β, IRF-1, IRF-3, RelA, and IκBα, respectively, using an enhanced chemiluminescence light-detecting kit (Amersham) as previously described (10).

IKKβ and Akt kinase assay. IKKβ and Akt kinase assays were performed according to the protocol from Cell Signaling. Mode-K cells were lysed in cell lysis buffer (Cell Signaling) after treatment with TNF in the absence and presence of 100 μmol/L flavonoids for 30 min. Total cellular protein (200 μg) was incubated overnight with 0.01 μL/L anti-IKKα (Cell Signaling) or anti-Akt (1G1, Cell Signaling) followed by incubation with 20 μL of A/G agarose beads (Santa Cruz) for an additional 2 h. The kinase reaction was performed in 40 μL of kinase buffer (Cell Signaling) supplemented with 200 μmol/L ATP and incubated in the presence of substrate including IκBα (1-317; Santa Cruz) or GSK-3 fusion proteins (Cell Signaling). Substrate protein was resolved by gel electrophoresis followed by the detection of phospho-IκBα and phospho-GSK-3α/β using immunoreactive antibodies.

ELISA analysis. Mode K cells were pretreated with flavonoids (100 μmol/L) for 1 h followed by the stimulation with TNF and IL-1β for an additional 24 h. Protein concentrations were determined in spent culture supernatants of IEC cultures. IL-6 and IP-10 production was determined by mouse-specific DuaSet Development ELISA assay kits, according to the manufacturer's instructions (R&D Systems).

Nuclear extracts and NF-κB RelA protein/DNA binding activity. Mode K cells were pretreated with flavonoids (100 μmol/L) for 1 h followed by the stimulation with TNF for an additional 30 min. Nuclear extracts were prepared according to the manufacturer's instructions (Active Motif). Extracts (5 μg) were used to determine nuclear RelA binding activity to the κB-nucleotide consensus sequence 5′-GGGACTTTCC-3′ using the TransAM ELISA-based NF-κB transcription factor assay (Active Motif). Protein/oligonucleotide binding activity was quantified by colorimetric analysis using a MultiScan spectrophotometer.

Cell transfection and selection. Mode K cells were grown to 80% confluence and then transfected with 2 μg of the NF-κB–inducible reporter plasmid pNiFty-secreted alkaline phosphatase (SEAP) (InvivoGen) in the presence of 6μL FuGENE 6 Transfection Reagent (Roche Diagnostics). The pNiFty-SEAP reporter construct contains an engineered ELAM promoter with 5 NF-κB binding sites (GGGGACTTTCC) and the SEAP as a reporter gene. Stable transfected cells were selected after the initial transfection (48 h) in the presence of the antibiotic zeocin (InvivoGen).

Reporter (SEAP) gene assay for NF-κB transcriptional activity. pNiFty-SEAP transfected Mode-K cells were pretreated with flavonoids (100 μmol/L) for 1 h followed by the stimulation with TNF for an additional 24 h. The spent culture supernatants (10 μL) were heated at 65°C for 5 min to eliminate the endogenous alkaline phosphatase activity. The SEAP secreted was measured according to the manufacturer's instructions (InvivoGen) at 405 nm in a MultiScan spectrophotometer.

Statistical analysis. Data are expressed as means ± SD of triplicate stimulations from 3 independent experiments. Dose-dependent analysis for the calculation of EC_{50} values was performed in duplicate stimulations. Statistical analysis was performed using 1-way ANOVA followed by Tukey's test. Differences were considered significant at $P < 0.05$.

RESULTS

Inhibition of IEC activation by flavonoids and their bacterial metabolites. To investigate anti-inflammatory mechanisms of flavonoids on IEC activation, we stimulated the murine noncarcinoma epithelial cell line Mode-K with the proinflammatory cytokine TNF for 24 h in the absence and presence of apigenin, luteolin, genistein, 3′-hydroxy-flavone, and flavone. Apigenin, luteolin, genistein, and 3′-hydroxy-flavone at a final concentration of 100 μmol/L completely blocked TNF-induced IP-10 protein secretion in Mode-K cells (**Table 1**).

TABLE 1

Differential effects of flavonoids on TNF-induced IP-10 expression, NF-κB DNA binding activity, and NF-κB reporter gene activity in Mode K cells[1]

	IP-10	NF-κB DNA binding	NF-κB reporter activity
	μg/L	*absorbance*	*fold of control*
CTRL	4.2 ± 0.4		
DMSO	5.0 ± 0.4	0.88 ± 0.01	1.5 ± 0.2
TNF + DMSO	53.6 ± 0.9[a]	2.79 ± 0.07[a]	20.0 ± 0.3[a]
TNF + apigenin	7.8 ± 1.2[b]	2.76 ± 0.11[a]	13.0 ± 0.6[b]
TNF + luteolin	4.8 ± 1.5[b]	2.05 ± 0.11[b]	1.8 ± 0.5[c]
TNF + genistein	11.2 ± 0.7[b]	2.74 ± 0.15[a]	20.5 ± 0.3[a]
TNF+3′-OH-flavone	9.4 ± 2.0[b]	1.30 ± 0.03[c]	2.5 ± 0.4[c]
TNF + flavone	53.4 ± 6.1[a]	3.24 ± 0.16[a]	19.9 ± 0.4[a]

[1] Values are means ± SD, $n = 9$. Means in a column without a common letter differ, $P < 0.05$.

Dose-response analysis revealed EC_{50} values for apigenin, luteolin, genistein and 3′-hydroxy-flavone in a concentration range of 20–27 μmol/L (**Fig. 1**). Of note, the maximal inhibition of TNF-induced IP-10 secretion was achieved for apigenin, luteolin, genistein, and 3′-hydroxy-flavone at a final concentration of 100 μmol/L without induction of significant cytotoxicity (<15%). Interestingly, nontoxic concentrations (1–200 μmol/L) of flavone also did not inhibit TNF-induced IP-10 secretion, suggesting that the inability of flavone to inhibit TNF-induced proinflammatory gene expression in IEC is not a concentration-dependent phenomenon.

3′-Hydroxy-flavone inhibits IKK activity. We next sought to evaluate the molecular mechanisms of flavonoid-mediated inhibition of TNF-induced proinflammatory gene expression in IEC. Therefore, we investigated the effects of apigenin, luteolin, genistein, 3′-hydroxy-flavone, and flavone on TNF-induced NF-κB/IκB activation in Mode-K cells. Time-response analysis revealed transient TNF-induced RelA phosphorylation after 20 min of stimulation followed by almost complete degradation of IκBα protein after 60 min (data not shown). The accumulation of phospho-IκBα was measured in the presence of the proteasome inhibitor MG-132 (1 μmol/L), reaching maximal IκBα phosphorylation levels after 20 min of TNF stimulation (data not shown). Based on the kinetic analysis of signal-specific protein phosphorylation/degradation, we measured TNF-induced RelA phosphorylation (after 20 min), IκBα phosphorylation (after 20 min), and IκBα degradation (after 60 min) in the presence of 100 μmol/L of apigenin, luteolin, genistein, 3′-hydroxy-flavone, and flavone. Interestingly, 3′-hydroxy-flavone but not apigenin, luteolin, genistein, or flavone inhibited RelA (**Fig. 2**A, lane 7) and IκBα phosphorylation (Fig. 2*B*, lane 7) after the stimulation of Mode-K cells with TNF. Most importantly, total RelA protein was present in 3′-hydroxy-flavone–treated Mode-K cells, suggesting compound-specific effects on RelA phosphorylation and not RelA degradation. Equal loading was confirmed using H3 immunoblotting. In addition, TNF-induced IκBα degradation was blocked in the presence of 3′-hydroxy-flavone but not by any of the other flavonoids (Fig. 2*C*, lane 7). Flavonoids alone did not change the expression pattern of IκBα in the absence of TNF (data not shown).

To further elucidate the inhibition of RelA and IκBα phosphorylation, we next measured TNF-induced IKKβ activity in the presence of 3′-hydroxy-flavone and flavone by determining phospho-IκBα in a kinase assay protocol. TNF-induced IκBα phosphorylation was inhibited in the presence of 3′-hydroxy-flavone but not the control compound flavone (**Fig. 3**).

Luteolin inhibits NF-κB RelA transcriptional activity. We next sought to investigate the inhibitory function of apigenin, genistein, luteolin, and flavone at the level of TNF-induced NF-κB DNA binding activity as well as NF-κB transcriptional activity. We measured NF-κB DNA binding activity to the

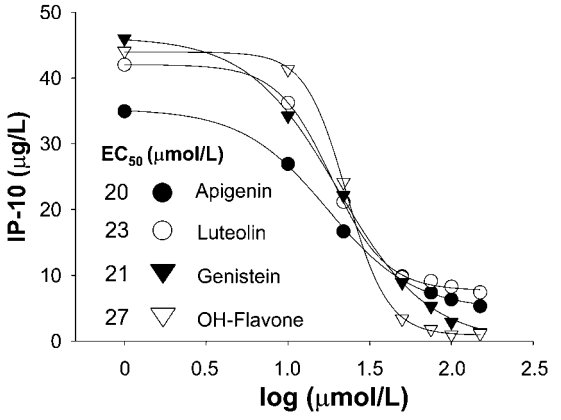

FIGURE 1 Effective flavonoid concentrations in the inhibition of TNF-induced IP-10 expression in IEC. Mode-K cells were stimulated with TNF (5 μg/L) in the presence of apigenin, luteolin, genistein, and 3′-hydroxy-flavone in a concentration range of 1–200 μmol/L. TNF-induced IP-10 protein concentrations were blotted against the flavonoid concentration and the EC_{50} were determined. Medium alone and medium with vehicle dimethyl sulfoxide (DMSO) were used as controls. The results represent the mean of 2 independent experiments performed in duplicate 6-well cultures. IP-10 protein concentrations (μg/L) were measured in the spent culture supernatant after 24 h of stimulation using ELISA analysis.

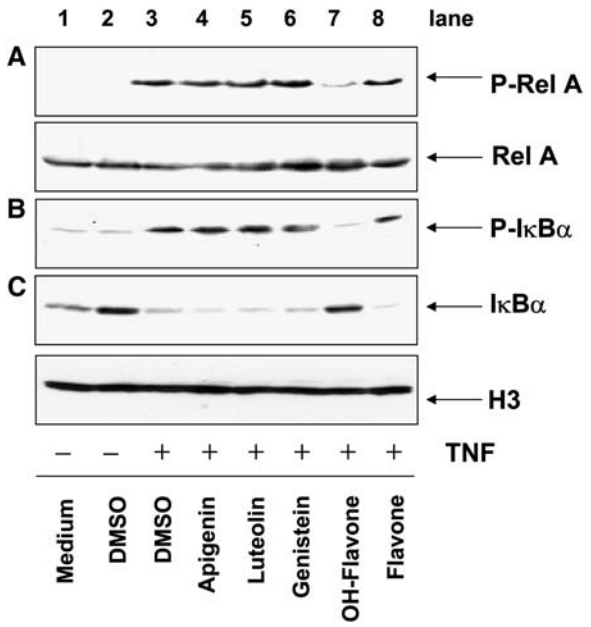

FIGURE 2 3′-Hydroxy-flavone inhibits TNF-induced NF-κB RelA and IκBα phosphorylation. Mode-K cells were stimulated with TNF (5 μg/L) in the presence of 100 μmol/L apigenin, luteolin, genistein, 3′-hydroxy-flavone, and flavone. Medium alone and medium with vehicle (DMSO) were use as controls; 20 μg of total protein was subjected to SDS-PAGE followed by (*A*) phospho-RelA, RelA, (*B*) phospho-IκBα, (*C*) IκBα and H3 immunoblotting. These results are representative of 3 independent experiments.

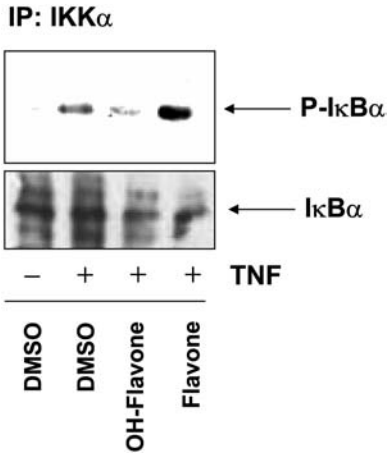

FIGURE 3 3′-Hydroxy-flavone inhibits IKK-activity. Mode-K cells were stimulated with TNF (5 μg/L) in the presence of 100 μmol/L 3′-hydroxy-flavone and flavone. The IκB kinase reaction was performed using IκBα (1-317) fusion protein. Substrate protein was resolved by SDS-PAGE followed by the detection of phospho-IκBα and total IκBα using immunoreactive antibodies. These results are representative of 2 independent experiments.

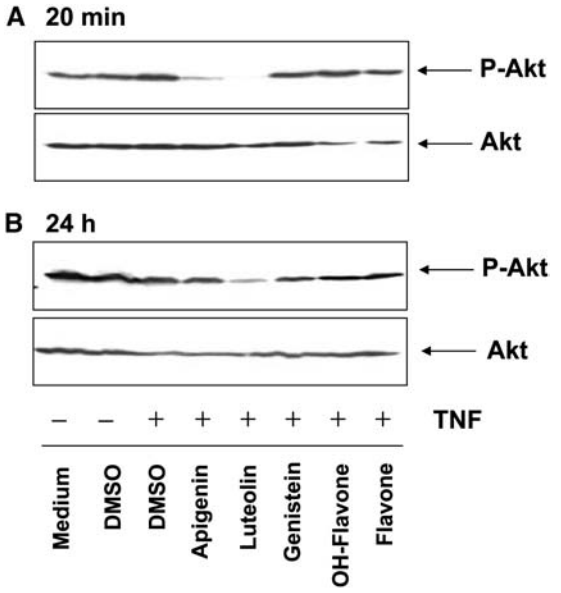

FIGURE 4 Luteolin and apigenin inhibit Akt phosphorylation/activity in TNF-stimulated IEC. Mode-K cells were stimulated with TNF (5 μg/L) in the presence of 100 μmol/L apigenin, luteolin, genistein, 3′-hydroxy-flavone, and flavone. Medium alone and medium with vehicle (DMSO) were used as controls; 20 μg of total protein was subjected to SDS-PAGE followed by phospho-Akt and total Akt immunoblotting for 20 min (*A*) or 24 h (*B*) These results are representative of 2 independent experiments.

κB-consensus DNA sequence by the colorimetric ELISA-based TransAM assay after 1 h of TNF stimulation. In addition, we used pNiFty-SEAP stable transfected cells to measure NF-κB–dependent reporter gene activity after 24 h of TNF stimulation. Interestingly, luteolin partially inhibited TNF-induced NF-κB DNA binding activity (Table 1) followed by the complete blockade of NF-κB transcriptional activity (Table 1). Consistent with the inhibition of the NF-κB/IκB complex, 3′-hydroxy-flavone blocked NF-κB DNA binding and NF-κB transcriptional activity. The inhibitory effect of apigenin on TNF-induced NF-κB transcriptional activity was significant but incomplete with no effects on NF-κB DNA binding activity. Of note, TNF-induced NF-κB DNA binding and NF-κB transcriptional activity were not affected in the presence of 100 μmol/L genistein and flavone.

Luteolin, apigenin, and hydroxy-flavone inhibit Akt phosphorylation and induce IRF transcription factor degradation. We next measured the level of Akt phosphorylation/activity and IRF-1/3 transcription factor stability in TNF-stimulated Mode-K cells in the presence and absence of flavonoids. Although phospho-Akt was already present in unstimulated control cells, the presence of luteolin and apigenin dramatically diminished Akt phosphorylation in IEC after stimulation with TNF for 20 min (**Fig. 4***A*). Interestingly, the level of phospho-Akt was restored to almost control levels in the presence of apigenin but not luteolin after 24 h of incubation (Fig. 4*B*), suggesting that luteolin had the most profound inhibitory effect on Akt phosphorylation. Consistent with the effects on phospho-Akt, apigenin and most particularly luteolin reduced Akt kinase activity in TNF-stimulated cells after 30 min of incubation (**Fig. 5**). The fact that 3-hydroxy-flavone did not affect the level of Akt kinase activity may further support the hypothesis for a characteristic inhibition pattern of flavonoids in TNF-activated IEC. In addition to the effects of these polyphenolic compounds on the survival pathway Akt, luteolin, and hydroxy-flavone selectively triggered IRF-1 but not IRF-3 transcription factor degradation in Mode-K cells after 24 h of stimulation (**Fig. 6**). Luteolin and hydroxy-flavone did not affect IRF-1 protein stability at the early stages (30 min) of signal transduction.

Inhibitory function of flavonoids are independent from p38 MAPK signaling and caspase-3 cleavage. We next measured phospho-p38 and cleaved caspase-3 in TNF-stimulated Mode-K cells in the presence and absence of 100 μmol/L apigenin, genistein, luteolin, 3′-hydroxy-flavone, and flavone. Although p38 phosphorylation was already induced in TNF-stimulated Mode-K cells after 30 min of co-incubation with 3′-hydroxy-flavone (**Fig. 7***A* and *B*), cleaved caspase-3 was present only at the late stimulation time point after 24 h (**Fig. 8***A* and *B*). These results strongly support the observation that the survival pathways NF-κB and Akt may represent the default signaling cascades in Mode-K cells after TNF stimulation. Although luteolin and genistein triggered caspase-3 cleavage after 24 h of TNF stimulation at least to some extent, these 2 polyphenolic

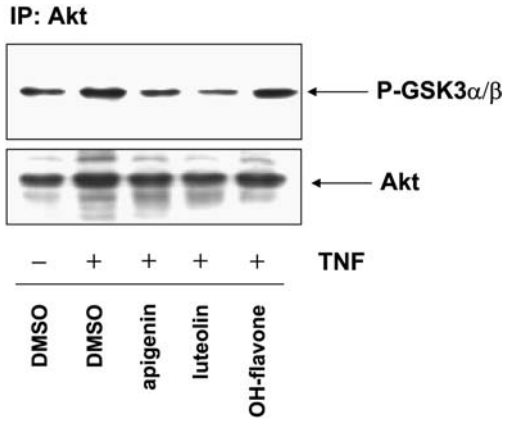

FIGURE 5 Luteolin and apigenin inhibit Akt activity. The Akt kinase reaction was performed in the presence of GSK3α/β fusion protein. Substrate protein was resolved by SDS-PAGE followed by the detection of phospho- GSK3α/β and total Akt using immunoreactive antibodies. These results are representative of 2 independent experiments.

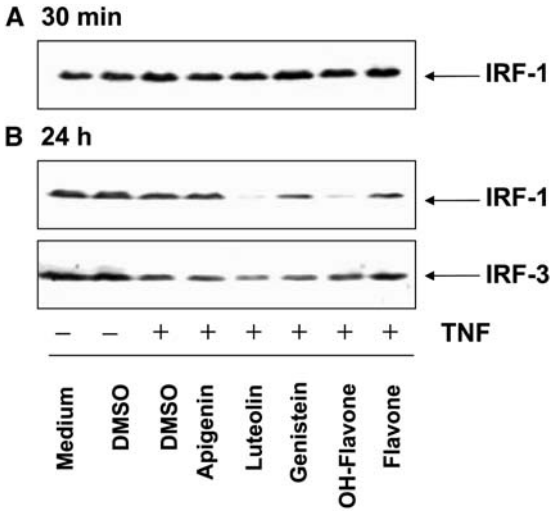

FIGURE 6 Luteolin and 3′-hydroxy-flavone trigger IRF-1 protein degradation. Mode-K cells were stimulated with TNF (5 μg/L) in the presence of 100 μmol/L apigenin, luteolin, genistein, 3′-hydroxy-flavone, and flavone. Medium alone and medium with vehicle (DMSO) were used as controls; 20 μg of total protein was subjected to SDS-PAGE followed by IRF-1 and IRF-3 immunoblotting. These results are representative of 2 independent experiments.

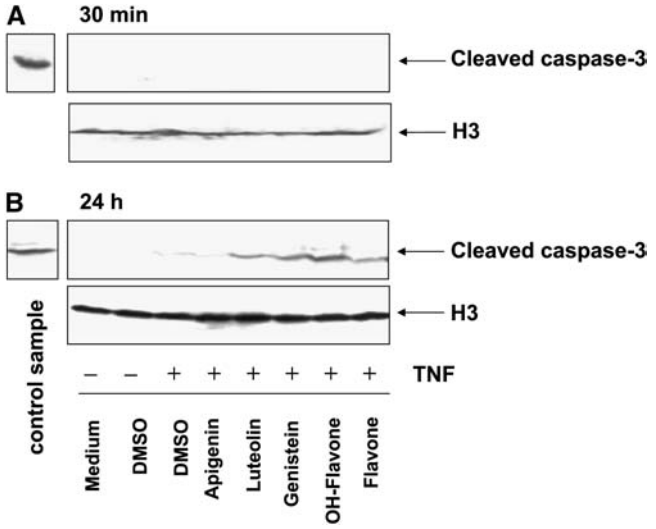

FIGURE 8 Caspase-3 cleavage in TNF-stimulated IEC. Mode-K cells were stimulated with TNF (5 μg/L) in the presence of 100 μmol/L apigenin, luteolin, genistein, 3′-hydroxy-flavone, and flavone. Medium alone and medium with vehicle (DMSO) were used as controls; 20 μg of total protein was subjected to SDS-PAGE followed by cleaved caspase-3 and H3 immunoblotting for 30 min (*A*) or 24 h (*B*). Positive controls for caspase-3 cleavage were generated from camptothecin-treated cells. These results are representative of 2 independent experiments.

compounds completely failed to trigger late proapoptotic activity in the absence of TNF (**Fig. 9**). In contrast, 3′-hydroxy-flavone triggered caspase-3 cleavage even in the absence of TNF (Fig. 9). Interestingly, apigenin completely lacked the capability to trigger p38 phosphorylation and caspase-3 cleavage in the presence and absence of the proinflammatory mediator TNF.

Interestingly, the pharmacologic p38 MAPK inhibitor SB203580 did not inhibit TNF-induced NF-κB transcriptional activity and, of note, SB203580 also did not reverse the inhibitory effect of 3′-hydroxy-flavone, luteolin, and apigenin (data not shown). These results did not support a significant role for the p38 MAPK signaling cascade in mediating TNF-induced IEC activation as well as in the inhibitory functions of flavonoids. Together with the fact that the cells were 85–90% viable after 24 h of stimulation, these results clearly suggest that the induction of apoptotic cell death in TNF-activated IEC was not the primary mechanism of flavonoids in inhibiting NF-κB transcriptional activity and IP-10 expression.

Differential effects of genistein in inhibiting IL-6 expression. To further evaluate whether the inhibitory effect of genistein is specific for TNF-induced IP-10 expression, we next measured the IL-1β– and TNF-induced IL-6 expression in Mode-K cells after 24 h of stimulation in the presence and absence of 100 μmol/L genistein. Genistein did not inhibit TNF-induced IL-6 expression and, consistent with previously

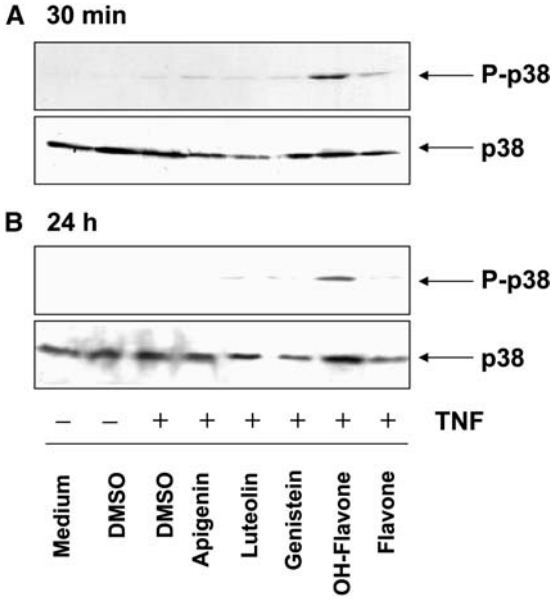

FIGURE 7 Induction of p38 phosphorylation in TNF-stimulated IEC. Mode-K cells were treated with 100 μmol/L apigenin, luteolin, genistein, 3′-hydroxy-flavone, and flavone in the presence of TNF (5 μg/L). Medium alone and medium with vehicle (DMSO) were used as controls; 20 μg of total protein was subjected to SDS-PAGE followed by phospho-p38 and p38 immunoblotting for 30 min (*A*) or 24 h (*B*). These results are representative of 2 independent experiments.

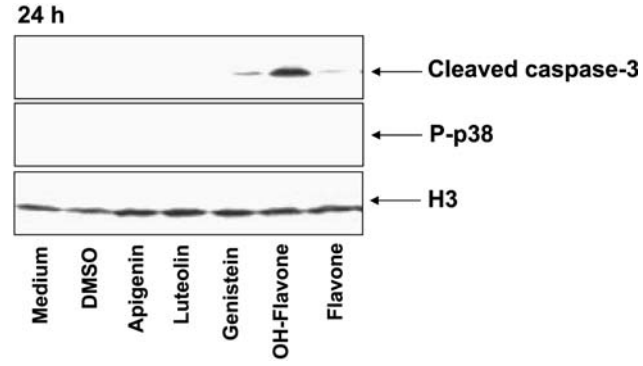

FIGURE 9 Induction of caspase-3 cleavage and p38 phosphorylation by flavonoids. Mode-K cells were stimulated with TNF (5 μg/L) in the presence of 100 μmol/L apigenin, luteolin, genistein, 3′-hydroxy-flavone, and flavone. Medium alone and medium with vehicle (DMSO) were used as controls; 20 μg of total protein was subjected to SDS-PAGE followed by cleaved phospho-p38, p38, caspase-3, and H3 immunoblotting. These results are representative of 2 independent experiments.

published results, genistein even increased IL-1β–induced IL-6 expression (**Table 2**).

DISCUSSION

In this study, we showed that polyphenolic plant-derived flavonoids display characteristic inhibitory patterns toward the NF-κB, IRF, and Akt signal transduction pathways using the murine noncarcinoma IEC line Mode-K. Apigenin, luteolin, and 3′-hydroxy-flavone specifically inhibited TNF-induced IP-10 expression by mechanisms that affect IKKβ and Akt activity, NF-κB DNA binding, NF-κB transcriptional activity, and IRF transcription factor stability. Although some flavonoids effectively triggered p38 MAPK signaling and late caspase-3 cleavage, the induction of apoptotic cell death in TNF-activated IEC was not the primary mechanism inhibiting NF-κB transcriptional activity and IP-10 expression. Interestingly, but in contrast to the human colon carcinoma cell line HT-29 (33), flavone did not induce proapoptotic mechanisms and completely failed to modulate TNF-induced NF-κB activation in Mode-K cells. Finally, the protein tyrosine kinase inhibitor genistein selectively blocked cytokine-induced IP-10 but not IL-6 expression, independently of the NF-κB, IRF, and Akt signaling pathways. These results clearly demonstrate the functional diversity of these polyphenolic compounds in inhibiting proinflammatory processes in IEC.

The 2 faces of TNF signaling and NF-κB activation in the epithelium were shown in intestinal ischemia-reperfusion studies of IKKβ−/− mice (34). The ablation of IKKβ activity in enterocytes resulted in severe apoptotic damage to the reperfused intestinal mucosa but was protective in the development of systemic inflammation, demonstrating the dual function of NF-κB inhibitors under conditions of acute inflammation. TNF mediates proinflammatory signals through the default survival pathways of the NF-κB and Akt cascades (20,22), but at the same time, the signal can be diverted to induce proapoptotic mechanisms in IEC through induction of the p38 MAPK pathway (35). In Mode-K cells, TNF did not induce p38 phosphorylation and caspase-3 cleavage, suggesting that TNF signal transduction per se was biased in these cells toward the interrelated survival pathways NF-κB and Akt. Although the inhibition of TNF-induced RelA and IκBα phosphorylation by 3′-hydroxy-flavone induced p38 phosphorylation, the inhibitory effect of 3′-hydroxy-flavone on NF-κB transcriptional activity was not reversed in the presence of the pharmacologic p38 inhibitor SB203580. Consistent with the observation that SB203580 had only moderate effects on TNF-induced NF-κB transcriptional activity and IP-10 expression, we concluded from these results that the inhibition of IKKβ activity by 3′-hydroxy-flavone was independent of the p38 MAPK signaling pathway. In addition, the lack of significant nuclear fragmentation and cell death suggests that proapoptotic effects were not the primary mechanisms for the inhibition of NF-κB transcriptional activity and IP-10 expression after 24 h of TNF stimulation. Nevertheless, the induction of late caspase-3 cleavage in the presence of 3′-hydroxy-flavone strongly indicates a late onset of proapoptotic effects in these cells. The pathological consequences of 3-hydroxy-flavone signaling to the epithelium under conditions of chronic inflammation remains to be determined.

TABLE 2

Genistein increased TNF- and IL-1β-induced IL-6 expression in Mode K cells[1]

	IL-6
	ng/L
CTRL	7.0 ± 1.2
DMSO	6.9 ± 3.3
TNF + DMSO	74.6 ± 3.8[c]
TNF + Genistein	107.5 ± 13.7[b]
IL-1β + DMSO	46.0 ± 11.6[c]
IL-1β + Genistein	130.0 ± 5.6[a]

[1] Values are means ± SD, $n = 9$. Means in a column without a common letter differ, $P < 0.05$.

Luteolin and apigenin inhibited TNF-induced IP-10 expression at the level of RelA nuclear translocation, NF-κB DNA binding, and NF-κB transcriptional activity. Although luteolin and apigenin differed in their ability to inhibit NF-κB signaling at the various control points of this cascade, Akt was completely dephosphorylated by all 3 polyphenolic compounds, with luteolin having the most profound and long-lasting effects. The serine/threonine kinase Akt was shown to stimulate the NF-κB signaling pathway at various levels including IKKβ activity, NF-κB DNA binding activity, and NF-κB transcriptional activity (10,36–38). Because luteolin and apigenin did not block TNF-induced RelA and IκBα phosphorylation, the inhibition of the PI3-kinase/Akt pathway may not specifically interfere with the induction of IKK activity and the subsequent phosphorylation/degradation of the IκB/NF-κB complex. Mayo et al. (39) showed in prostate cells that the reintroduction of PTEN, which is a lipid phosphatase responsible for the deactivation of PI3K/Akt signaling, resulted in the inhibition of TNF-induced NF-κB transcriptional activity by blocking the transactivation domain of the RelA/p65 subunit. Interestingly, the authors also showed that PTEN did not inhibit TNF-induced IKK activity, IκBα degradation, and NF-κB RelA nuclear translocation. Although the molecular mechanisms for the inhibition of TNF-induced IP-10 expression through the Akt cascade remain controversial, the inhibition of Akt activity by flavonoids may interfere with the full recruitment of transcriptionally active NF-κB RelA to the IP-10 promoter by partially inhibiting RelA nuclear translocation and NF-κB transcriptional activity.

The IP-10 promoter is regulated in a complex way with interrelated roles of the transcription factor binding sites for NF-κB and the family of IRF proteins. Promoter studies clearly indicated that the 2 NF-κB and additional IRF binding sites interrelate to fully activate IP-10 gene expression (40,41). In addition, IRF-3 is acting as coactivator for IP-10 transactivation by recruiting IRF-3 protein to κB-promoter sites where it can bind directly to NF-κB RelA (42,43). Interestingly, we showed that luteolin and 3′-hydroxy-flavone but not apigenin selectively triggered IRF-1 protein degradation at late stages of TNF-induced IEC activation. Considering that the IRF inhibitor ribavarin completely blocked TNF-induced IP-10 expression, we may speculate about a specific role of IRF-1 in mediating TNF-induced IP-10 expression. It is interesting that 3′-hydroxy-flavone, apigenin, and luteolin differentially affect the IKK, IRF, and Akt pathways, but at the same time all of the compounds inhibited IP-10 expression at the various levels of the TNF signaling cascade.

Two previous studies showed that luteolin also blocked lipopolysaccharide (LPS)-induced NF-κB signal transduction and proinflammatory gene expression at the level of IKKβ activity and IκBα phosphorylation in nontransformed IEC (28) and macrophages (44). Interestingly, Kim and Jobin (28) showed in IEC that the blockade of LPS-induced IKKβ activity by luteolin

resulted in the inhibition of IκBα but not RelA phosphorylation. In addition and consistent with findings that LPS-induced IKK activity was linked to the activation of the PI3-kinase/Akt pathway (10), Xagorari et al. (44) showed in LPS-stimulated macrophages that luteolin indeed inhibited IκBα and Akt phosphorylation. In contrast, we showed that the inhibition of TNF-induced IKKβ activity by 3′-hydroxyflavone resulted in the blockade of both RelA (Ser536) and IκBα phosphorylation. In addition, luteolin completely failed to block TNF-induced activation of the NF-κB cascade at the level of IKK and Akt activity. Signal-specific mechanisms may partially explain the discrepancy concerning the flavonoid-mediated inhibition of the NF-κB signal transduction pathway.

In addition, genistein inhibited the NF-κB signaling cascade through Akt-dependent mechanisms in PC3 prostate (45) and MDA-MB-231 breast (46) cancer cells. Interestingly, we showed that genistein completely failed to affect the NF-κB and Akt signaling pathways but effectively inhibited TNF-induced IP-10 expression. These results may support the hypothesis that the inhibitory mechanisms of flavonoids are not only signal specific but also cell type dependent. Indeed, Gustin et al. (47) provided some molecular insights for cell type–dependent differences in the cross-talk between the NF-κB and Akt signaling cascades. The authors showed that cells with a high proportion of IKKα relative to IKKβ were more sensitive to the inhibition of the PI3 kinase/Akt pathway; consequently, the transient overexpression of IKKβ diminished the capacity of the PI3 kinase/Akt inhibitors to block NF-κB DNA binding. The fact that genistein blocked cytokine-induced IP-10 expression but completely failed to inhibit IL-1β/TNF-induced IL-6 expression further supports the hypothesis that the specific capability of flavonoids to interfere with distinct signaling pathways characteristically modulates the inhibition profile of target genes depending on their requirements for transcription and co-activation factors.

In conclusion, these results provide molecular evidence for characteristic inhibition patterns of flavonoids in the regulation of cytokine-induced NF-κB/Akt signaling and IP-10 expression in noncarcinoma epithelial cells from the small intestine. Most interestingly, these polyphenolic compounds all exhibited inhibitory effects on proinflammatory cytokine expression but appeared to achieve their inhibitory effects by targeting distinct signaling pathways. The specific effects of the flavonoids on NF-κB, IRF, and Akt signal transduction are summarized schematically in **Figure 10**. It seems important to fully understand the functional diversity of these polyphenolic compounds in targeting epithelial cell–specific signal transduction pathways and gene expression profiles to select effective anti-inflammatory compounds for adjunct nutritional therapy of chronic intestinal inflammation with little or no side effects for the epithelium. It is therefore important to evaluate the physiologic consequences of anti-inflammatory but potentially proapoptotic flavonoids for the development of chronic intestinal inflammation with respect to the various disease pathologies in human IBD. Future experiments in animal models of experimental colitis (e.g., IL-10$^{-/-}$ mice) and ileitis (e.g., TNF$^{\Delta ARE}$) will add useful information about the therapeutic role of flavonoids in human IBD.

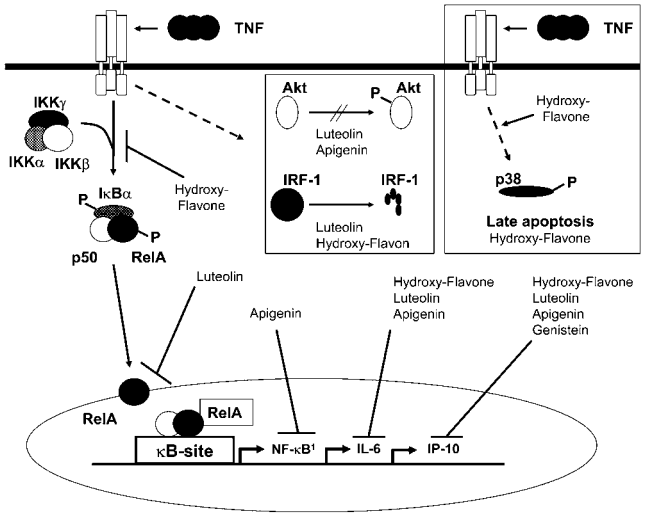

FIGURE 10 Schematic illustration for the inhibitory effects of flavonoids in IEC after stimulation with TNF. NF-κB^1 indicates NF-κB transcriptional activity; black stop lines indicate the primary mechanism of inhibition.

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APPENDIX 5

Shkoda, A*, Ruiz, PA*, Daniel, H, Kim, SC, Rogler, G, Sartor, RB and Haller, D

"Interleukin 10 blocked endoplasmic reticulum stress in intestinal epithelial cells: impact on chronic inflammation"

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*Authors contributed equally

[Reconstitution and characterization of Mode K cells with the IL-10 receptor-signaling complex and ChIP analysis by Ruiz, PA *(Figures 5, 11B and 11D)*; proteomic analysis, analysis of *in vivo* samples and *in vitro* experimental work by Shkoda, A; proteomic facility by Daniel, H; maintenance of the animal facility and bacterial monoassociation of IL-10 gene-deficient mice by Kim, SC and Sartor, RB; facilitation of samples from IBD patients by Rogler, G; immunohistochemistry by the Forschungszentrum für Umwelt und Gesundheit (GSF); writing by Haller, D with editorial help of the co-authors]

Interleukin-10 Blocked Endoplasmic Reticulum Stress in Intestinal Epithelial Cells: Impact on Chronic Inflammation

ANNA SHKODA,* PEDRO A. RUIZ,* HANNELORE DANIEL,‡ SANDRA C. KIM,§ GERHARD ROGLER,‖ R. BALFOUR SARTOR,§ and DIRK HALLER*

*Else-Kroener-Fresenius Center for Experimental Nutritional Medicine, ‡Molecular Nutrition, Department of Food and Nutrition, Technical University of Munich, Freising-Weihenstephan, Germany; §Department of Pediatrics and Medicine, Center for Gastrointestinal Biology and Disease, University of North Carolina, Chapel Hill, North Carolina; and ‖Department of Internal Medicine, University of Regensburg, Regensburg, Germany

Background & Aims: **The initiation of endoplasmic reticulum (ER)-mediated stress responses in intestinal epithelial cells (IEC) may contribute to the pathogenesis of chronic intestinal inflammation. The aim of the study was to use functional epithelial cell proteomics to characterize anti-inflammatory mechanisms of interleukin 10 (IL-10).** ***Methods:*** **Primary IEC were isolated from *Enterococcus faecalis*-monoassociated IL-10-deficient (IL-10−/−) and wild-type mice to perform 2D-gel sodium-dodecyl-sulfate polyacrylamide gel electrophoresis and matrix-assisted laser desorption/ionization-time of flight mass spectrometry. In addition, IEC from 6 patients with active Crohn's disease, ulcerative colitis, and sigmoid diverticulitis as well as noninflamed controls were purified. Molecular protective mechanisms of IL-10 were characterized in tumor necrosis factor (TNF)-stimulated IL-10 receptor (IL-10R) reconstituted epithelial cells.** ***Results:*** **Primary IEC from IL-10−/− mice as well as inflammatory bowel disease patients revealed increased expression levels of the glucose-regulated ER stress protein (grp)-78 under conditions of chronic inflammation. Consistent with the observation that TNF induced ER stress responses through grp-78 redistribution from the ER lumen to the cytoplasmic IκB kinase complex, grp-78 knockdown completely abolished TNF-induced nuclear factor-κB RelA phosphorylation in epithelial cell cultures. Interestingly, IL-10 inhibited grp-78 protein and messenger RNA expression in IL-10R reconstituted IEC. Chromatin immunoprecipitation analysis and immunofluorescence microscopy revealed that IL-10-mediated p38 signaling inhibited TNF-induced recruitment of the ER-derived activating transcription factor (ATF)-6 to the grp-78 promoter likely through the blockade of ATF-6 nuclear translocation.** ***Conclusions:*** **Primary IEC from inflamed IL-10−/− mice and inflammatory bowel disease patients revealed activated ER stress responses in the intestinal epithelium. IL-10 inhibits inflammation-induced ER stress response mechanisms by modulating ATF-6 nuclear recruitment to the grp-78 gene promoter.**

Inflammatory bowel diseases (IBD) including ulcerative colitis (UC) and Crohn's disease (CD) are spontaneously relapsing, immunologically mediated disorders of the distal gastrointestinal tract. Previous studies showed increased association of luminal enteric bacteria with the intestinal epithelium[1] and a loss of immunologic tolerance in patients with active IBD.[2,3] The normal enteric microbiota is involved in each of the current etiologic theories of this idiopathic disorder,[4] suggesting that the interaction of nonpathogenic enteric bacterial species with the epithelium may contribute to the initiation and/or perpetuation of chronic intestinal inflammation in the genetically susceptible host.

Selective colonization of germ-free rodent models for experimental colitis with defined commensal bacterial species confirmed these clinical observations regarding specific colitogenic mechanisms of certain bacteria at the epithelial cell level.[5,6] Indeed, the presence of colitogenic *Enterococcus faecalis* transiently induced toll-like receptor (TLR) 2-mediated RelA phosphorylation and NF-κB-dependent gene expression in native intestinal epithelial cells (IEC) from wild-type (WT) mice but persistent activation of the TLR/nuclear factor (NF)-κB pathway in interleukin (IL)-10-deficient (IL-10−/−) mice, suggesting a pathologic role for bacteria-epithelial cell signaling under chronic inflammation.[7] Of note, the colitogenic mechanism of the TLR/MyD88-dependent pathway in IL-10−/− mice was elegantly confirmed by Rakoff-Nahoum et al in IL-10 × MyD88−/− mice.[8] It seems important to understand that the absence of protective transforming growth factor (TGF)-β-activated Smad signaling at early stages of bacterial colonization (1 week) was associated with the development of chronic intestinal inflammation in IL-10−/− mice (14 weeks),[7] suggest-

Abbreviations used in this paper: ATF, activating transcription factor; ChIP, chromatin immunoprecipitation; ER, endoplasmic reticulum; grp-78, glucose-regulated protein 78; IEC, intestinal epithelial cells; IL-10−/−, interleukin-10 gene deficient; MALDI-TOF-MS, matrix-assisted laser desorption/ionization-time of flight mass spectrometry; NF-κB, nuclear factor κB; TNF, tumor necrosis factor α.

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ing the presence of additional molecular mechanisms that control the persistent challenge of bacteria- and host-derived signals at the epithelial cell level. Strober et al suggested an interrelated role for the protective mechanisms of TGF-β and IL-10.[9,10] Interestingly, the adoptive transfer of protective CD45RB^{low} T cells from IL-10−/− failed to inhibit the colitogenic effects of CD45RB^{high} T cells to trigger T-helper (Th)1-mediated experimental colitis in severe combined immunodeficiency disease (SCID) mice,[11,12] suggesting the possibility that IL-10 may confer protective mechanisms to the intestinal epithelium at the acute inflammatory stage of the disease process. The physiologic relevance and molecular mechanisms of IL-10 to inhibit proinflammatory processes in the epithelium are still unclear.

To define better the epithelial cell responses under the pathologic conditions of chronic intestinal inflammation in the absence of IL-10, we characterized the protein expression profile (proteome) in primary IEC from *E faecalis*-monoassociated WT and IL-10−/− mice at late stages of bacterial colonization. First, we performed 2D-gel electrophoreses (2D sodium-dodecyl-sulfate polyacrylamide gel electrophoresis [SDS-PAGE]) and peptide mass fingerprinting via matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry (MS) to identify 14 target proteins with significantly altered steady state expression levels in primary IEC from IL-10−/− mice. Although endoplasmic reticulum (ER) stress responses contribute to the initiation and regulation of various cellular stress mechanisms including the induction of inflammatory processes and apoptosis, the contribution of ER stress with respect to the glucose-regulated protein 78 (grp-78) is completely unknown under the pathologic conditions of experimental colitis and human IBD. Thus, we validated the increased grp-78 expression levels in IEC from IL-10−/− mice and IBD patients under conditions of chronic inflammation. In addition, we performed experiments in IL-10 receptor (IL-10R) reconstituted epithelial cells to identify the grp-78 as a potential target for IL-10. Finally and mechanistically of most importance, we characterized a novel anti-inflammatory mechanism for IL-10 by demonstrating that IL-10-mediated p38 signaling inhibited the recruitment of the ER transmembrane protein activating transcription factor (ATF)-6 to the grp-78 promoter using chromatin immunoprecipitation (ChIP) analysis. Interestingly, fluorescence microscopy revealed that IL-10 blocks nuclear translocation of ATF-6 upon tumor necrosis factor (TNF)-induced ER stress, suggesting that IL-10 inhibits ATF-6 recruitment to the grp-78 promoter through mechanisms that involve nuclear shuttling of this ER transcription factor. In conclusion, these results demonstrate for the first time that IL-10 modulates ER stress responses, suggesting that, in the absence of adequate control mechanisms, ER stress may contribute to the pathogenesis of IBD.

Materials and Methods

Animals and Bacterial Monoassociation

Germ-free 129 SvEv TAC mice and germ-free IL-10 gene deficient (−/−) 129 SvEv TAC mice (originally derived by Dr Edward Balish, University of Wisconsin, Madison, WI) were monoassociated at 12–16 weeks of age with the colitogenic *E faecalis* strain OG1RF (a generous gift from M. Huycki, University of Oklahoma, Oklahoma City, OK). The mice were maintained in the National Gnotobiotic Rodent Resource Center at the University of North Carolina at Chapel Hill. Bacterial monoassociation and absence of contamination by other bacterial species were confirmed by culturing samples from the small and large intestine at necropsy and culturing serial fecal samples. Animal use protocols were approved by the Institutional Animal Care and Use Committee (IACUC), University of North Carolina. Mice were killed 14 weeks after initial bacterial colonization. Germ-free mice were used as controls. Sections of the ileum, cecum, and proximal and distal colon were fixed in 10% neutral-buffered formalin. The fixed tissue was embedded in paraffin. Histology scoring was analyzed by blindly assessing the degree of lamina propria mononuclear cell infiltration, crypt hyperplasia, goblet cell depletion, and architectural distortion as previously described.[13]

Patients

Ileal and/or colonic tissue was obtained from 12 patients with active CD (n = 6) and active UC (n = 6) undergoing surgical resections. In addition, noninflamed and inflamed tissue regions of the same surgical specimens were included for further analysis. The severity of inflammation was histologically graded in the resected tissue specimens (Institute of Pathology, University of Regensburg). Normal epithelial cells were isolated from colonic samples from patients with colorectal carcinoma (control patients) used as noninflammatory controls (n = 6). The control tissue was taken at least 5-cm distance from the tumor. The used mucosal areas from the control patients were macroscopically and histologically normal. The study was approved by the Ethics Committee of the University of Regensburg and performed in accordance with the declaration of Helsinki.

Isolation of Primary Mouse and Human IEC

Primary IEC from the cecal and colonic epithelium of germ-free and *E faecalis*-monoassociated WT as well as IL-10−/− mice were purified as previously described.[7] Briefly, the cecal and colonic tissues were cut into pieces and incubated at 37°C in Dulbecco's modified Eagle medium (DMEM) containing 5% fetal calf serum (FCS) and 1 mmol/L dithiothreitol (DTT) for 30 minutes. The remaining tissue was incubated in 30 mL phosphate-buffered saline (PBS) (1X) containing 1.5 mmol/L EDTA for an additional 10 minutes. The supernatants were filtered, centrifuged for 5 minutes at 400*g*,

and the cell pellet was resuspended in DMEM containing 5% FCS. Finally, the primary IEC suspension was purified by centrifugation through a 25%/40% discontinuous Percoll gradient at 600*g* for 30 minutes.

The isolation of primary human IEC from the resected ileal and colonic tissue sections was performed as previously described.[14] Briefly, the mucosa was stripped from the submucosa within 30 minutes of intestinal resection, and the mucus was removed by treatment for 15 minutes with 1 mmol/L DTT. The mucosa was then incubated with 1.5 mmol/L EDTA in Hanks' balanced salt solution without calcium and magnesium and tumbled for 10 minutes at 37°C. The supernatant containing debris and mainly villus cells was discarded. The mucosa was incubated again with EDTA for 10 minutes at 37°C. The supernatant of this isolation step was collected into a 15-mL tube. Next, the remaining mucosa was vortexed, and the supernatant was again collected containing complete crypts, some single cells, and a small amount of debris. To separate IEC from contaminating nonepithelial cells, the suspension was allowed to sediment for 15 minutes. The sediment containing mainly complete crypts was collected and washed twice with PBS. Primary IEC from the resected intestinal surgical specimens were combined and collected in sample buffer.

Cell purity was assessed by determining the absence of $CD3^+$ T-cell contamination. Trypan blue exclusion confirmed the presence of at least 80% viable cells after the 2-hour isolation procedure. Primary mouse IEC from cecum and colon were combined and collected in sample buffer for subsequent RNA isolation, Western blot, and proteome analysis.

Sample Preparation for 2D-PAGE and Gel Analysis

Purified primary IEC from *E faecalis*-monoassociated WT and IL-10−/− mice were lysed in 200 μL buffer containing 7 mol/L urea, 2 mol/L thiourea, 2% CHAPS, 1% DTT (all from Roth, Karlsruhe, Germany), protease inhibitor (Roche Diagnostics, Mannheim, Germany), and 2% Pharmalyte (Amersham Biosciences, Freiburg, Germany). Homogenization of the cell extracts was achieved by ultrasonication (amplitude 35, cycle 0.5) using 10 impulses on ice. The lysed cells were then centrifuged for 30 minutes at 10,000*g* at 4°C. The total protein concentrations of the solubilized proteins in the supernatants were determined using the Bio-Rad protein assay (Munich, Germany) and used for further analysis or stored at −80°C.

Immobilized pH-gradient strips (IPG; pH 3–10, 18 cm, Amersham Biosciences) were rehydrated overnight in 350 μL buffer (8 mol/L urea, 0.5% CHAPS, 15 mmol/L DTT, 0.5% IPG buffer), and 500 μg of total protein was cup loaded onto the strip. Isoelectric focusing (IEF) in the first dimension and SDS-PAGE in the second dimension were performed. IEF was run by an Amersham IPGphor unit under the following conditions: 500 V (1 minute, gradient), 4000 V (1.5 hour, gradient), 8000 V (28,000 Vh, Step-n-hold). Subsequent to the first dimension and before loading onto SDS-PAGE gels, strips were incubated for 15 minutes in equilibration-buffer (1.5 mol/L Tris-HCL, pH 8.8, 6 mol/L urea, 26% glycerol, 2% SDS, 1% DTT), followed by additional equilibration in buffer for another 15 minutes (1.5 mol/L Tris-HCL, pH 8.8, 6 mol/L urea, 26% glycerol, 2% SDS, 4% iodoacetamide).

SDS-PAGE gel electrophoresis was performed by an Amersham Biosciences Ettan-Dalt II System with 4 mA per gel for 1 hour, followed by 12 mA per gel using 12.5% SDS-polyacrylamide gels (1 mm thick). For protein staining, gels were fixed in 40% ethanol and 10% acetic acid for 8 hours, followed by an overnight exposure to a Coomassie solution containing 10% $(NH_4)2SO_4$, 2% phosphoric acid, 25% methanol, and 0.625% Coomassie Brillant blue G-250. Destaining of the gels was performed in aqua bidest until the background was completely clear. All 12 gels from germ-free (pooled control) and *E faecalis*-monoassociated WT (n = 5) and IL-10−\− (n = 5) mice were simultaneously subjected to all steps of 2D-gel electrophoresis including IEF, SDS-PAGE, Coomassie Brillant blue staining, and quantitative analysis to minimize variability between samples.

Coomassie-stained gels were scanned (ImageScanner, Amersham Biosciences) and analyzed by ProteomWeaversoftware (Definiens, Munich, Germany) including background subtraction and volume normalization. Reference gels from the pooled samples of all 5 different germ-free WT and IL-10−/− mice were generated and compared with the IEC samples gels from *E faecalis*-monoassociated WT and IL-10−\− mice, respectively. Spots with at least 2-fold differences in protein intensity present in at least 3 out of 5 gels were picked for further MALDI-TOF-MS analysis.

Trypsin Digestion of Protein Spots and MALDI-TOF MS

Coomassie-stained spots were picked, washed alternately in acetonitrile and 50 mmol/L NH_4HCO_3, dried, and then digested using 0.1 μg sequencing grade modified trypsin in 50 mmol/L NH_4HCO_3 (Promega, Mannheim, Germany). The dried spots were rehydrated for 1 hour at 4°C by adding 5 μL 0.02 μg/μL trypsin and incubated for another 20 hours at 37°C. Finally, 5 μL 1% trifluouroacetic acid (TFA, Sigma, Germany) were added, and the tryptic peptide fragments were extracted using ultrasonication for 15 minutes at room temperature. Supernatants were stored at −80°C or directly used for MALDI-TOF-MS analysis.

Mass spectrometric analysis was performed according to the method of Bruker Daltonics (Leipzig, Germany) using the Autoflex Control software and the mass spectrometer from Bruker Daltonics. Briefly, 2–3 μL of the extracted protein sample together with 2 μL of 0.1% TFA

was spotted onto the target using the thin-layer affinity HCCA AnchorChipTM preparation by Bruker Daltonics. Proteins were identified by using the Mascot Server 1.9 (Bruker Daltonics) based on mass searches within murine sequences only. The search parameters allowed the carboxyamidomethylation of cystein and 1 missing cleavage. The minimum score of 61 and a mass accuracy of ± 250 ppm were selected as criteria for positive identification of proteins.

Generation of IL-10R Reconstituted Mode-K Cells

The mouse IEC line Mode-K (passage 10–30) (a generous gift from Dr Ingo B. Autenrieth, University of Tübingen, Germany) was grown in tissue culture plates as previously described.[7] Mode-K cells were transfected with the expression vector for murine IL-10R (a generous gift from Dr Heinz Baumann, Rosewell Park Cancer Institute, Buffalo, NY) at 50%–80% confluency using 2 μg of the 6505-base pair (bp) plasmid (MSCVneoEB) and 6 μL of FuGENE tranfection reagent (Roche Diagnostics). Stable transfected Mode-K cells were selected for their neomycin (1 μg/mL) resistance. Where indicated, the confluent epithelial cell monolayers were treated with TNF (R&D Systems, Heidelberg, Germany) and IL-10 (R&D Systems).

Cellular Fractionation

Qproteome mitochondria isolation kit (Qiagen, Hilden, Germany) was used for the separation of cytoplasmic (CP), ER, and mitochondrial (MC) protein fractions as described by the manufacturer. Briefly, IL-10R reconstituted Mode-K cells were cultured in 75-cm² flasks. The cells were scraped, washed with 0.9% sodium chloride, and centrifuged at 500*g* for 10 minutes at 4°C. The cell pellet was resuspended in 1 mL of ice-cold lysis buffer containing protease inhibitors and incubated on ice for 10 minutes. The cell lysate was then centrifuged at 1000*g* for 10 minutes at 4°C, and supernatant containing the CP fraction was collected. The cell pellet was then resuspended in 1.5 mL of ice-cold disruption buffer and centrifuged at 1000*g* for 10 minutes at 4°C. The supernatant was transferred in a new tube and centrifuged at 6000*g* for 10 minutes at 4°C. The ER protein fraction was collected in a separate tube. The pellet containing the MC fraction was resuspended in 100 μL mitochondria storage buffer. Proteins from the CP and ER fractions were concentrated by acetone precipitation. Therefore, 4 volumes of ice-cold acetone was added to each fraction and incubated for 15 minutes on ice. The tubes were then centrifuged at 12,000*g* for 10 minutes, and supernatants were discarded. Protein pellets were air-dried and then dissolved in 100 μL lysis buffer. Protein concentration was determined by Bradford assay. The protein extracts were mixed with 3X Laemmli buffer, and 50 μg of total protein was separated by SDS-PAGE and subjected to immunoblotting with specific antibodies.

Fluorescence Microscopy

IL-10R reconstituted Mode-K cells were grown on sterile glass cover slips in 6-well culture plates to 80% confluence. The cells were then treated with IL-10 (50 ng/mL) for 24 hours, followed by stimulation with TNF (10 ng/mL). After incubation, the cells were washed with 2 mL PBS and fixed for 5–10 minutes by adding 500 μL ice-cold methanol, followed by incubating with 250 μL blocking reagent (10% goat serum in PBS) for 30 minutes at room temperature. Next, 400 μL primary grp-78 antibody (Sigma Aldrich, Munich, Germany, dilution 1:600) or ATF-6α antibody (Santa Cruz, Heidelberg, Germany, dilution 1:250) was added, and the coverslips were incubated for 30 minutes. After washing with 2 mL PBS, the coverslips were incubated with 400 μL secondary antibody (anti-rabbit TRITC-conjugated IgG; Dianova, Hamburg, Germany) (dilution 1:100) for 30 minutes protected from the direct light. The coverslips were washed 3 times with 2 mL PBS and stained with 200 μL DAPI (Roche Diagnostics, Mannheim, Germany) (1 μg/mL) for 5 minutes. The glass coverslips were finally washed twice with PBS and placed upside down on a microscope slide with a drop of 50% glycerol in PBS. The slides were subjected to inverted microscopy. The images were analyzed by Leica software version 2.5.

Small Interference RNA and Cell Transfection

Synthetic grp-78 (NM_022310) specific siRNA was purchased from Qiagen. The target sequence for grp-78 was 5′-CCGATAATCAGCCAACTGTAA-3′. The sequences of grp-78-specific double-stranded ribo-oligonucleotides were as follows: sense r(AGACCCAGCUCUACAACAG)d(TT), reverse r(CUGUUGUAGAGCUGGGUCU)d(TT). The annealed double-stranded ribo-oligonucleotides including negative control siRNA (Qiagen) were heat treated for 1 minute at 90°C and then incubated for an additional 60 minutes at 37°C. Mode-K cells were grown in 24-well culture plates to 50%–80% confluency, followed by the transfection with 1.5 μg single-stranded grp-78-specific siRNA in 13.5 μL RNAi transfection reagent according to the protocol of the manufacturer (Qiagen).

RNA Isolation and Real-Time Reverse-Transcription Polymerase Chain Reaction

RNA from purified native IEC was extracted using Trizol Reagent (Invitrogen Life Technologies, Karlsruhe, Germany) according to the manufacturer's instructions. Extracted RNA was dissolved in 20 μL water containing 0.1% diethyl-pyrocarbonate. Reverse transcription was performed from 1 μg total RNA. Real-time polymerase chain reaction (PCR) was performed from 1 μL reverse

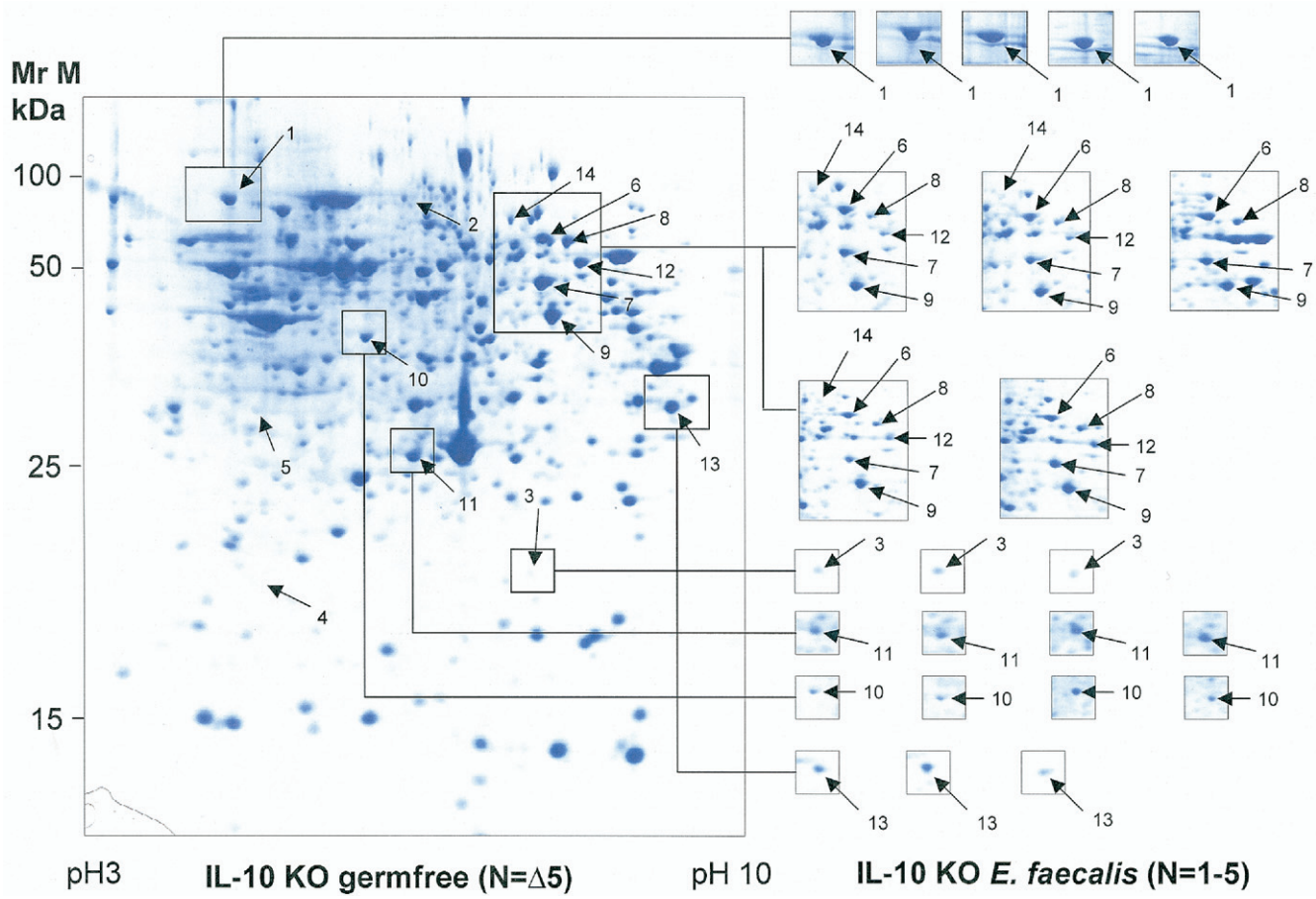


Figure 1. Proteome analysis in IEC from *E faecalis*-monoassociated IL-10−/− mice. Germ-free IL-10−/− mice were monoassociated with the colitogenic *E faecalis* for 14 weeks. Primary IEC were purified from the large intestine (cecum + colon), and 2D SDS-PAGE was performed. The reference gel (big gel) was generated from pooled IEC samples of germ-free mice and then separately compared with each of the 5 IEC samples from *E faecalis*-monoassociated IL-10−/− mice (small gels). Identified protein spots with at least 2-fold changes in steady state expression levels confirmed in at least 3 out of the 5 mice were considered as significant. Protein spots 1–5 were up-regulated, and protein spots 6–14 were down-regulated.

transcribed complementary DNA (cDNA) in glass capillaries using a Light Cycler system (Roche Diagnostics) as previously described.[7] Primer sequences were as follows: grp-78; sense 5′-TGACCAAAACCGCCTG-3′ and reverse 5′-CAATGTCCGCATCCTG-3′; GAPDH; sense 5′-ATCCCAGAGCTGAACG-3′ and reverse 5′-AAGT-CGCAGGAGACA-3′. The amplified product was detected by the presence of a SYBR green fluorescent signal. Melting curve analysis and gel electrophoresis were used to document the amplicon specificity. 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 equation $E^{\Delta Cp\ (\text{control samples} - \text{treated samples})}$ and normalized for the expression of GAPDH. All 5 samples from *E faecalis*-monoassociated mice were measured and blotted as fold increase over the 5 samples from germ-free control mice.

Western Blot Analysis

Purified primary IEC or Mode-K cells were lysed in 1X Laemmli buffer, and 20–50 μg protein was subjected to electrophoresis on 10% SDS-PAGE gels. Anti-phospho-RelA (Ser536), anti-RelA, anti-phospho-p38 (Thr180/Tyr182), anti-p38, phospho-STAT3 (Tyr705), STAT-3, anticleaved caspase-3, anti-CD3, anticreatine kinase, anti-IκB kinase (IKK)α (all antibodies from Cell Signaling, Beverly, MA), anti-E-cadherin (all Santa Cruz, Europe), anti-grp-78 (Calbiochem, Merk Biosciences, Schwalbach, Germany and Sigma Aldrich, Munich, Germany), and anti-β-actin (ICN, Costa Mesa, CA) were used to detect immunoreactive phospho-RelA, RelA, phospho-p38, p38, cleaved caspase-3, E-cadherin, grp-78, CD3, creatine kinase, IKKα, and β-actin, respectively, using an enhanced chemiluminescence light-detecting kit (Amersham, Arlington Heights, IL).

Coimmunoprecipitation

Mode-K cells (75-cm^2 flasks) were stimulated with TNF (10 ng/mL) for various time points. Cells were lysed in buffer containing protease inhibitors (Cell Signaling). Cell debris was removed by centrifugation at 12,000*g*, and supernatants were precleared for 3 hours with 100 μL

Table 1. Differentially Regulated Proteins in IEC From *E faecalis*-Monoassociated IL-10−/− Mice Compared With Germ-Free Control Mice

	Accession number	Name of protein	MrM (daltons)	pI	SCov %	Mean (fold changes)	SD	Frequency (mice)
1	A37048	DnaK type molecular chaperone grp78	72,491	5.12	27	2.42	0.5	5/5
2	Q922N3	Propionyl-CoA-carboxylase, α chain	80,517	7.0	15	2.73	0.28	3/5
3	Q6ZQH1	MKIAA0186	19,922	8.65	41	2.74	0.41	3/5
4	S31975	14-3-3 protein epsilon	29,326	4.63	24	2.85	0.76	3/5
5	BAB27292	AK010960 NID Tubulin β 5	50,064	4.78	28	3.43	0.50	3/5
6	CAA65761	Pyruvate kinase M2	58,448	7.18	38	0.34	0.08	5/5
7	B55729	Hydroxymethylglutaryl-CoA-synthase, mitochondrial	53,115	7.46	14	0.28	0.08	5/5
8	AAH06749	UDP-glucose dehydrogenase	55,482	7.49	37	0.35	0.06	5/5
9	S24612	Creatine kinase, mitochondrial	47,373	8.39	43	0.4	0.06	5/5
10	Q9D154	Serin protease inhibitor, clade B	42,719	5.85	16	0.37	0.08	4/5
11	Q99J99	3-mercaptopyruvate sulfotransferase	33,231	6.11	21	0.4	0.08	4/5
12	JQ1004	Aldehyde dehydrogenase NAD	55,131	7.89	41	0.3	0.07	4/5
13	A45983	Lactose-binding lectin Mac-2, galectin-3	27,455	8.57	20	0.39	0.02	3/5
14	Q9QYS0	Sulfurylase/APS kinase isoform SK2	70,991	7.31	13	0.39	0.01	3/5

MrM, molecular mass; pI, isoelectric point; SCov, sequence coverage for the peptide fingerprints.

protein A/G agarose (Santa Cruz). Total protein concentration was normalized, and immunoprecipitation was carried out overnight at 4°C using 25 μL rabbit anti-IKKα (Santa Cruz). Immune complexes were collected with 200 μL protein A/G agarose for 30 minutes, washed with lysis buffer, and resuspended in 3X Laemmli buffer. Western blot analysis for phospho-RelA, grp-78, and IKKα were carried out as described above.

ChIP Analysis

After the treatment of Mode-K cells with IL-10 and TNF, the cells were fixed by adding formaldehyde to a final concentration of 1%, and nuclear extraction and chromatin immunoprecipitation were performed by using the ChIP-IT Enzymatic kit from Active Motif (Carlsbad, CA) as previously described by the manufacturer. Extracts were normalized according to their DNA concentration, and immunoprecipitations were carried out overnight at 4°C using 5 μL anti-phospho NF-κB p65, anti-c-fos, antiacetylated-phosphorylated H3 antibodies (Cell Signaling), and anti-ATF-6α (Santa Cruz). Immune complexes were collected with salmon-sperm saturated protein A/G agarose for 30 minutes and washed 3 times in high salt buffer, followed by 3 washes with no salt buffer. DNA cross-links of the immune complexes were reverted by heating, followed by proteinase K digestion. The DNA was extracted with phenol-chloroform and precipitated in ethanol. DNA isolated from an aliquot of the total nuclear extract was used as a loading control for the PCR (input control). PCR was performed with total DNA (1 μL, input control) and immunoprecipitated DNA (1 μL) using the following grp-78 promoter-specific primers (5′) 5-AGAGCGCATTGACACCAGGAATGAA-3, (3′) 5-CCTCCACTTCCATAGAGTTTGCTGATA-3 as previously described.[15] The length of the amplified product was 187 bp. The PCR products (10 μL) were subjected to electrophoresis on 2% agarose gels.

Statistical Analysis

Data are expressed as the mean ± SD of triplicates (3 independent samples equally treated). Statistical analysis was performed by the 2-tailed Student *t* test for paired data and considered significant if *P* values were $< .05$.

Results

Protein Expression Profiling in the Intestinal Epithelium of WT and IL-10−/− Mice After the Colonization With E faecalis

Germ-free WT and IL-10−/− mice were monoassociated for 14 weeks with the *E faecalis* OG1RF strain shown to induce colitis. Histopathologic analysis of paraffin-embedded tissue sections confirmed the presence of severe inflammation in the distal colon of IL-10−/− but not of WT mice after 14 weeks of bacterial colonization, as previously shown.[7] The blinded colonic disease activity scores (0–4) were 0.5 ± 0.1 and 3.6 ± 0.2 for *E faecalis*-monoassociated WT and IL-10−/− mice, respectively. To identify novel protein targets in the epithelium that are differentially regulated under normal and inflamed conditions, we performed 2D-gel electrophoresis and MALDI-TOF-MS analysis in primary IEC from germ-free and *E faecalis*-monoassociated WT as well as IL-10−/− mice. We used pooled IEC samples from 5 different germ-free WT and IL-10−/− mice to generate reference 2D-gels. These reference 2D-gels (Figure 1, big gel) were then separately compared with each of

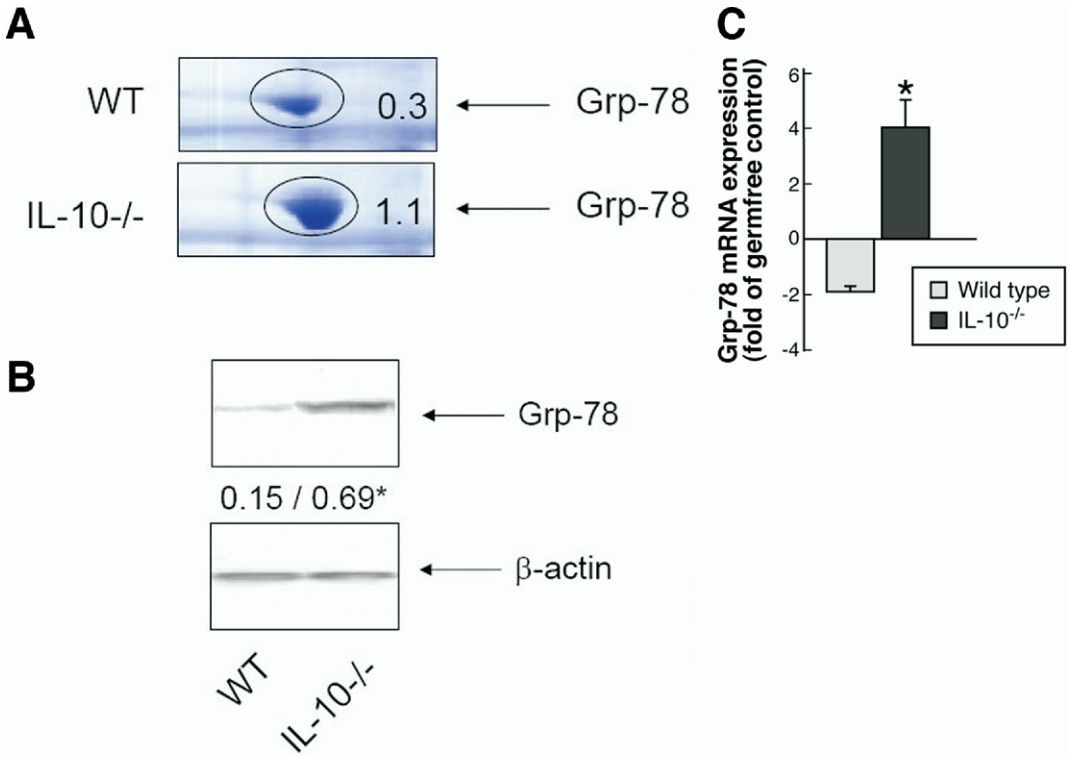


Figure 2. (*A–C*) Validation of grp-78 expression changes in primary IEC from *E faecalis*-monoassociated WT vs IL-10−/− mice. Proteome, Western blot, and RT-PCR analyses were performed from pooled IEC samples of the 5 *E faecalis*-monoassociated WT and IL-10−/− mice. (*A*) 2D SDS-PAGE was performed with 500 μg of total protein, and grp-78 protein spots were identified by MALDI-TOF MS. Expression intensities were given by ProteomeWeaver software. (*B*) Western blot analysis was performed with 50 μg of total protein using specific antibodies for grp-78 and β-actin. The expression intensities were calculated relative to β-actin using densitometric analysis. (*C*) Total RNA was extracted from primary IEC and reverse transcribed. Real-time PCR was performed using the Light Cycler system with specific primers for grp-78 and GAPDH. Expression changes (fold changes) of the specific mRNA were calculated relative to germ-free WT and IL-10−/− mice, using the crossing point of the log-linear portion of the amplification curve after normalization with GAPDH. The data represent mean fold changes ± SD from 5 different IEC samples.

the 5 IEC sample gels from *E faecalis*-monoassociated WT and IL-10−/− mice, respectively. The average number of Coomassie-stained protein spots that could be resolved on the 2D SDS-PAGE gels ranged between 400 and 600 spots. Regulation of protein spots with 2-fold changes in the steady state expression level confirmed in at least 3 out of the 5 different mice were considered to represent significant changes. We identified 14 proteins in IL-10−/− IEC compiled in Table 1 including the protein identification and accession number, the molecular mass (Mr M), their isoelectric point (pI), the sequence coverage for the peptide fingerprints (% SCov), the mean fold changes (± SD), and the frequency of changes in IL-10−/− mice. Representative gels from germ-free (reference gel) and *E faecalis*-monoassociated IL-10−/− mice (n = 3–5) are shown in Figure 1.

Differential Expression of the ER Stress Response Marker grp-78 Under Conditions of Chronic Intestinal Inflammation

We chose to investigate further the DnaK type molecular chaperone, grp-78 because this protein was consistently up-regulated in experimental colitis of IL-10−/− mice (Table 1) because grp-78-mediated ER stress responses contribute to the initiation and regulation of various cellular stress mechanisms including the induction of inflammatory processes and apoptosis.[16,17] Moreover, the role of grp-78 expression in the development of chronic intestinal inflammation is completely unknown. Because grp-78-specific antibodies were available for murine as well as human samples, we next studied grp-78 expression levels in primary IEC from noninflamed vs inflamed intestinal tissues using the murine model of experimental colitis (WT vs IL-10−/− mice) as well as IBD patients.

As shown in Figure 2, the significant induction of grp-78 protein and mRNA expression in IEC from inflamed (IL-10−/−) vs noninflamed (WT) colonic tissue was confirmed using proteome, Western blot, and RT-PCR analysis. First, we performed 2D-PAGE and MALDI-TOF MS analysis using the pooled IEC samples from all 5 germ-free and *E faecalis*-monoassociated WT as well as IL-10−/− mice, respectively. Consistent with the previous proteome analysis in single samples, *E faecalis* monoassociation of germ-free mice increased grp-78 protein expression in IL-10−/− IEC (2.2-fold change; blots not shown). The direct comparison between *E faecalis*-monoassociated WT and *E faecalis*-monoassociated IL-10−/−

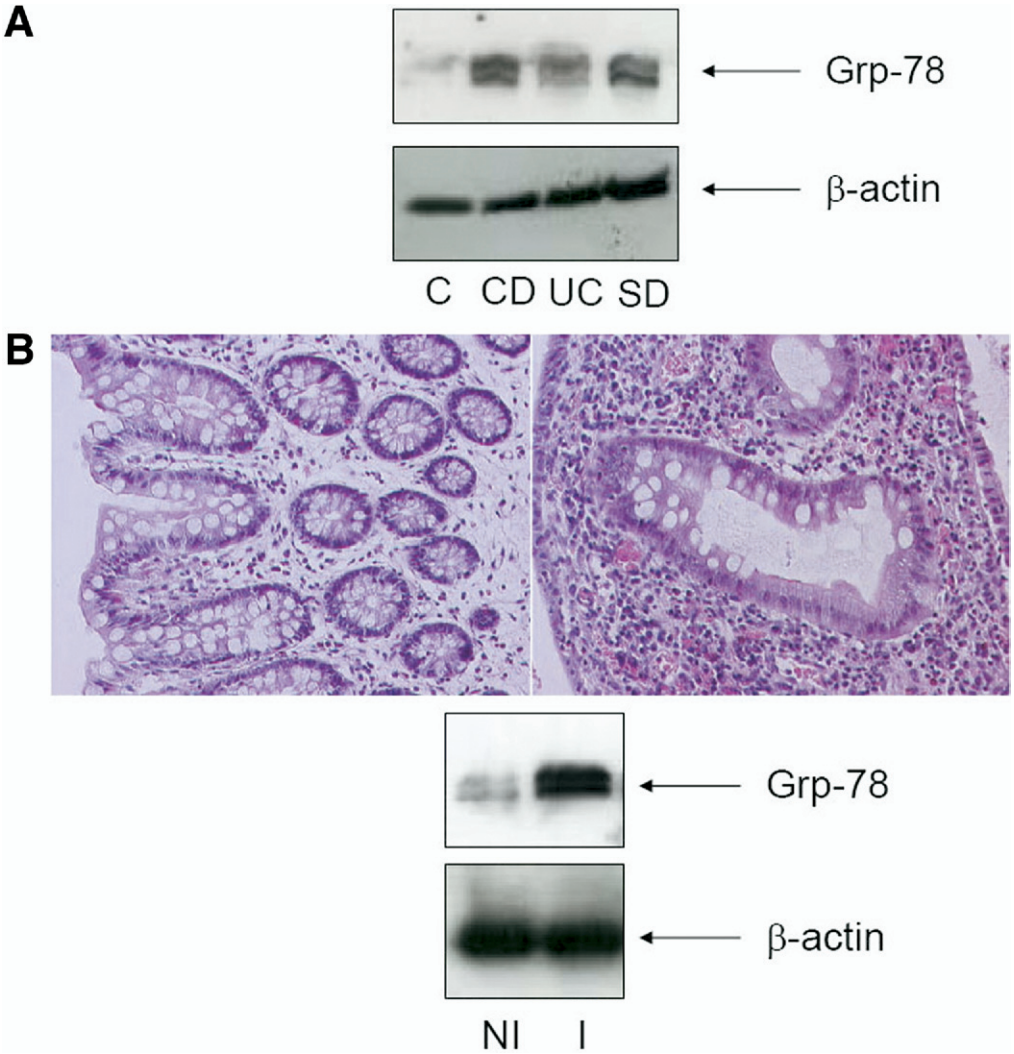


Figure 3. (*A* and *B*) Differential grp-78 protein expression in human IEC from noninflamed vs inflamed tissue sections under conditions of chronic intestinal inflammation. (*A*) Primary IEC were isolated from surgical specimens of 6 patients with active Crohn's disease, ulcerative colitis, and sigmoid diverticulitis. Tissue specimens from 6 colon cancer patients were used as noninflamed controls. Western blot analysis was performed with 50 μg of total pooled protein using specific antibodies for grp-78 and β-actin. (*B*) H&E staining of paraffin-embedded tissue sections including noninflamed (*left picture*) vs inflamed regions (*right picture*) from 1 single patient with ulcerative colitis. Western blot analysis from IEC of noninflamed vs inflamed tissue regions was performed with 50 μg of total pooled protein using specific antibodies for grp-78 and β-actin.

mice after 14 weeks of colonization clearly confirmed the significant increase in grp-78 protein expression (3.6-fold changes) between IEC from the noninflamed and inflamed colonic tissue (Figure 2*A*). These results support the fact that grp-78 is strongly up-regulated under the pathologic conditions of chronic inflammation. In accordance with the previous proteome analysis, Western blot analysis confirmed an increased steady state expression level of grp-78 (4.6-fold changes) in IEC from *E faecalis*-monoassociated IL-10−/− mice when compared with *E faecalis*-monoassociated WT mice after 14 weeks of colonization (Figure 2*B*). The relative expression intensities of grp-78 immunoblots were normalized to β-actin using densitometric analysis. Finally, we performed Light Cycler RT-PCR analysis in separate IEC samples from all mice to determine changes in grp-78 mRNA expression levels. The relative induction ratio was determined between *E faecalis*-monoassociated and germ-free controls. As shown in Figure 2*C*, grp-78 mRNA expression was significantly up-regulated (5.9-fold change) in IEC from IL-10−/− mice compared with WT control mice.

To validate and refine further these findings, we used purified IEC from patients with active CD, UC, and sigmoid diverticulitis. Noninflamed control cells were isolated from surgical specimens of colon cancer patients. The tissue was taken at 5-cm distance from the tumor. As shown in Figure 3*A*, the pooled IEC samples from 6 different patients clearly revealed increased expression levels of grp-78 comparing noninflamed and inflamed tissues. Interestingly, the sigmoid diverticulitis patients revealed similar expression levels of the grp-78 protein compared with IBD patients, suggesting that ER

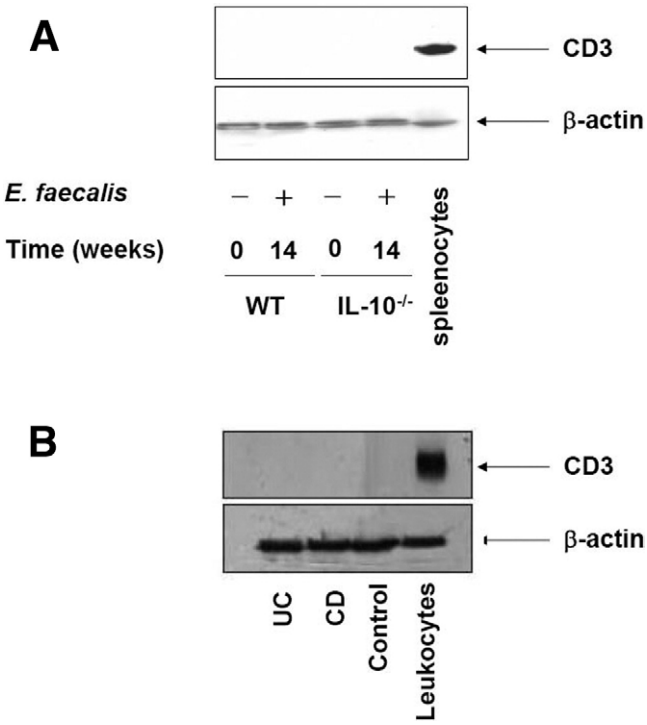


Figure 4. (*A* and *B*) Purity of IEC isolations from murine and human intestinal sections. Western blot analysis was performed from primary IEC and leukocytes with 50 μg of total pooled protein using specific antibodies for CD3 and β-actin. (*A*) IEC from 5 germ-free and *E faecalis*-monoassociated WT as well as IL-10−/− mice. (*B*) IEC from 6 IBD and control patients.

stress responses are part of the inflammatory processes rather than a specific feature of IBD. We next sought to confirm the previous results that demonstrate increased grp-78 expression under inflammatory conditions. As shown in Figure 3*B*, the comparison of noninflamed vs inflamed regions of the same surgical specimen (UC patient) clearly revealed increased grp-78 expression levels in inflamed tissue compared with IEC from the noninflamed region. H&E staining of paraffin-embedded tissue sections confirmed the different grades of inflammation for the 2 tissue regions (Figure 3*B*). The absence of CD3+ cells confirmed the purity of IEC isolations from inflamed IL-10−/− mice and IBD patients (Figure 4).

IL-10 Triggers p38 MAPK Activation and grp-78 Expression Changes in IEC

To assess the molecular function of IL-10 with respect to grp-78 expression in IEC, we generated IL-10R reconstituted Mode-K cells. As shown in Figure 5, IL-10 triggered p38 MAPK and STAT3 phosphorylation in the presence but not in the absence of the IL-10R complex in Mode-K cells. The dose (Figure 5*A*)- and time-dependent analysis (Figure 5*B*) revealed IL-10-mediated p38 phosphorylation in IL10R reconstituted Mode-K cells after 3 hours of stimulation. Consistent with the induction of p38 phosphorylation, IL-10 triggered grp-78 protein degradation in IL-10R reconstituted Mode-K cells after 6 hours of stimulation at a concentration of 50 ng/mL (Figure 6*A*). Interestingly, TGF-β did not trigger grp-78 degradation (data not shown). In addition to the effects of IL-10 on grp-78 protein degradation, we next measured grp-78 mRNA levels using Light Cycler RT-PCR analysis. As shown in Figure 6*B*, IL-10 induced a time-dependent decrease in the steady state mRNA expression level starting after 12 hours and 24 hours of stimulation of IL-10R reconstituted Mode-K cells.

Most important for demonstrating the physiologic relevance of IL-10 signaling in primary IEC, phospho-p38 was present in *E faecalis*-monoassociated WT mice but was absent in IL-10−/− mice after 14 weeks of bacterial colonization (Figure 7*A*). Although phospho-STAT3 was slightly present in IEC from WT and IL-10−/− mice, the amount of phospho-STAT3 detected in IL-10−/− IEC was considerably higher (Figure 7*A*). Of note, IL-10R was

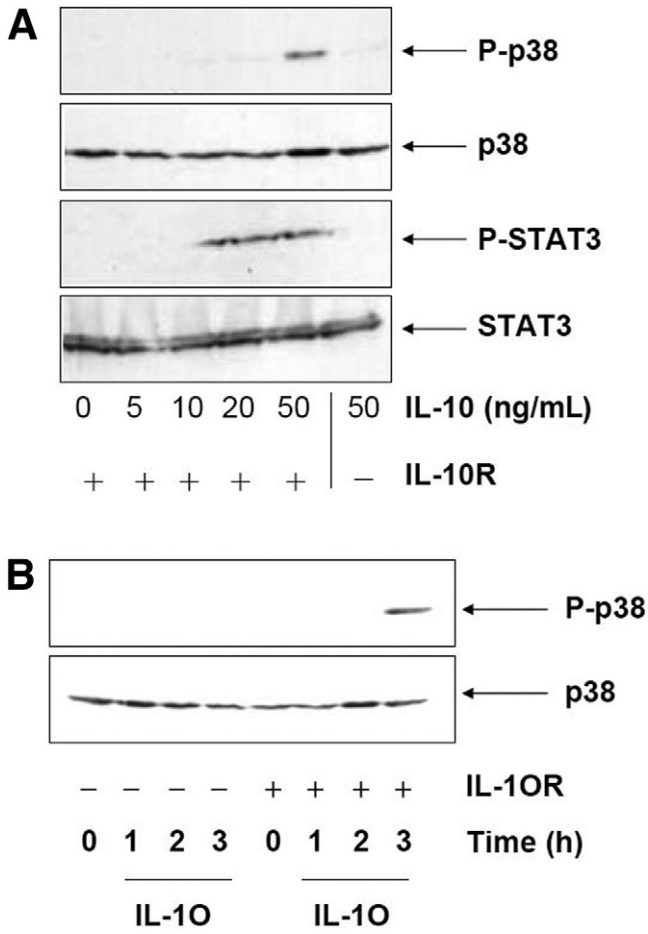


Figure 5. (*A* and *B*) IL-10 triggers p38 and STAT-3 phosphorylation in IL-10R reconstituted Mode-K cells. Mode-K cells were reconstituted with the murine IL-10R complex followed by stimulation with IL-10 at various concentrations (*A*) and times (*B*). Western blot analysis was performed with 20 μg of total protein using specific antibodies for phospho-p38, p38, phospho-STAT-3, and STAT3. Representative gels from at least 2 different experiments are shown.

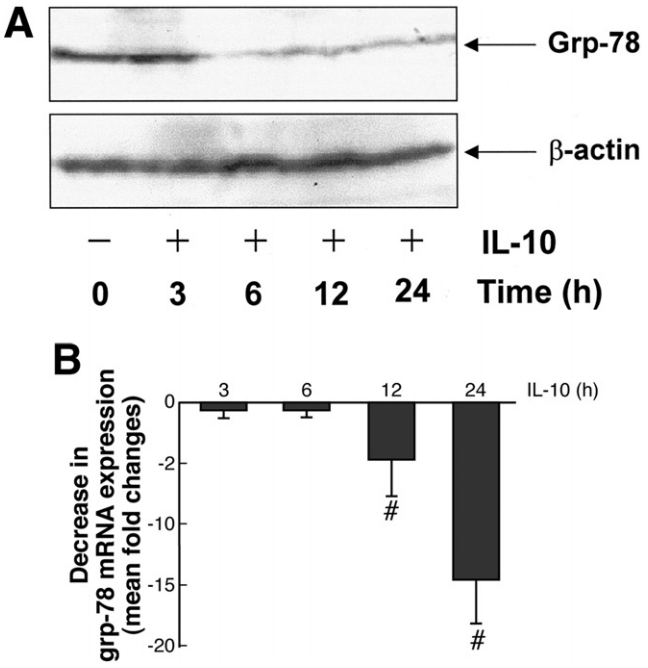


Figure 6. (*A* and *B*) IL-10 inhibits grp-78 mRNA and protein expression. Mode-K cells were reconstituted with the murine IL-10R complex followed by stimulation for 3, 6, 12, and 24 hours with IL-10 (50 ng/mL). (*A*) Western blot analysis was performed with 20 μg of total protein using specific antibodies for grp-78 and β-actin. Representative gels from at least 2 different experiments are shown. (*B*) Total RNA was extracted and reverse transcribed. Real-time PCR was performed using the Light Cycler system with specific primers for grp-78 and GAPDH. The expression changes (fold decrease) of the specific mRNA were calculated relative to unstimulated control cells, using the crossing point of the log-linear portion of the amplification curve after normalization with GAPDH. The data represent mean fold decrease ± SD from triplicate stimulations confirmed in at least 2 independent experiments. #Significant changes were indicated.

equally present in both WT and IL-10−/− IEC (Figure 7*A*). The pathophysiologic relevance of NF-κB RelA phosphorylation was confirmed in IL-10−/− IEC (Figure 7*B*), supporting our previously reported observations.[7] Interestingly, IL-10−/− but not WT IEC revealed increased expression levels of cleaved caspase-3 (Figure 7*B*), as well as decreased expression levels of E-cadherin (Figure 7*B*), suggesting that intestinal barrier functions are perturbed in IL-10−/− mice.

IL-10 Inhibits TNF-Induced NF-κB RelA Phosphorylation Through the Modulation of the ER Stress Response Protein grp-78

We next determined the molecular function of grp-78 in TNF-induced IEC activation using siRNA-mediated knockdown. First, Mode-K cells were transfected for 72 hours with control siRNA followed by stimulation with TNF for an additional 2 hours. Of note, TNF-induced RelA phosphorylation was transiently present after 1-hour stimulation of Mode-K cells (Figure 8*A*, lane 2). Second, Mode-K cells were transfected for 72 hours with grp-78-specific and control siRNA, followed by stimulation with TNF for 1 hour. As shown in Figure 8*B*, the grp-78 protein was almost completely depleted in the presence of grp-78-specific siRNA (lane 1 vs 2) but not in the presence of negative control siRNA (lane 1 vs 3). Interestingly and most important for the understanding of molecular mechanisms, TNF-induced RelA phosphorylation was inhibited after 1 hour of stimulation in the absence of grp-78 (Figure 8*B*, lane 2), supporting its proinflammatory mode of action.

In addition, the use of coimmunoprecipitation experiments revealed that grp-78 protein and phospho-RelA

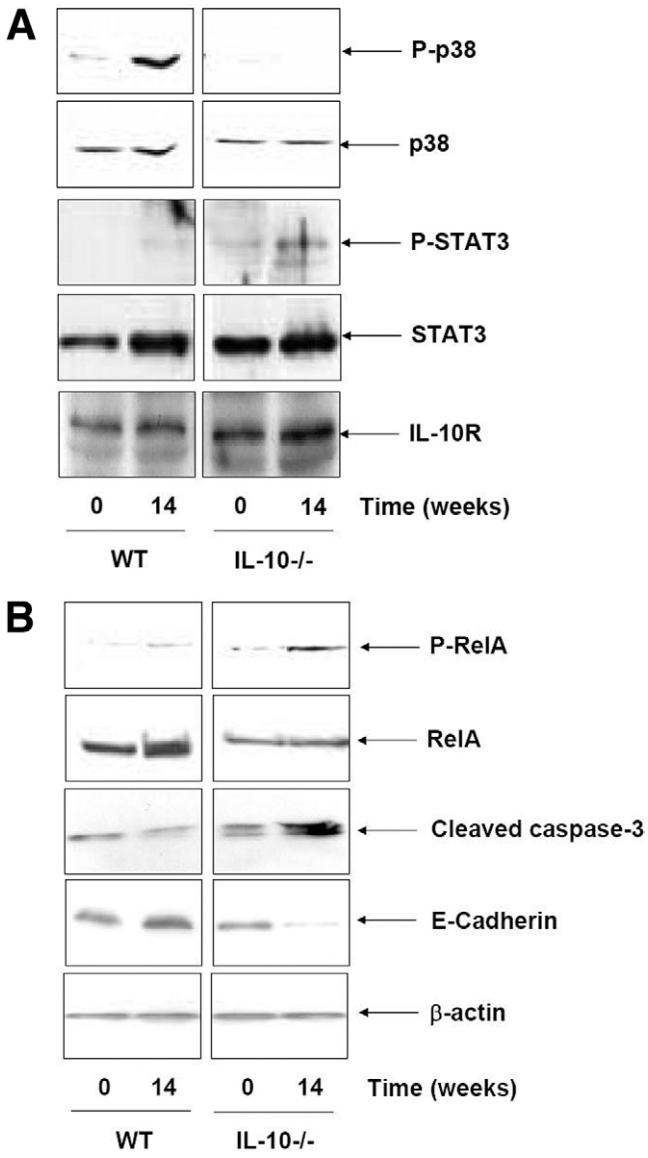


Figure 7. (*A* and *B*) Induction of p38 phosphorylation in primary IEC from WT but not IL-10−/− mice after the colonization with *E faecalis*. Germ-free WT and IL-10−/− mice were monoassociated for 14 weeks with *E faecalis*. Western blot analysis was performed with 50 μg total protein derived from pooled IEC samples from 5 germ-free and *E faecalis*-monoassociated mice, using immunoreactive (*A*) phospho-p38, p38, phospho-STAT3, STAT3, and IL-10R and (*B*) phosphor-RelA, RelA, cleaved caspase-3, E-cadherin, β-actin antibodies. Representative gels from at least 2 different immunoblots are shown.

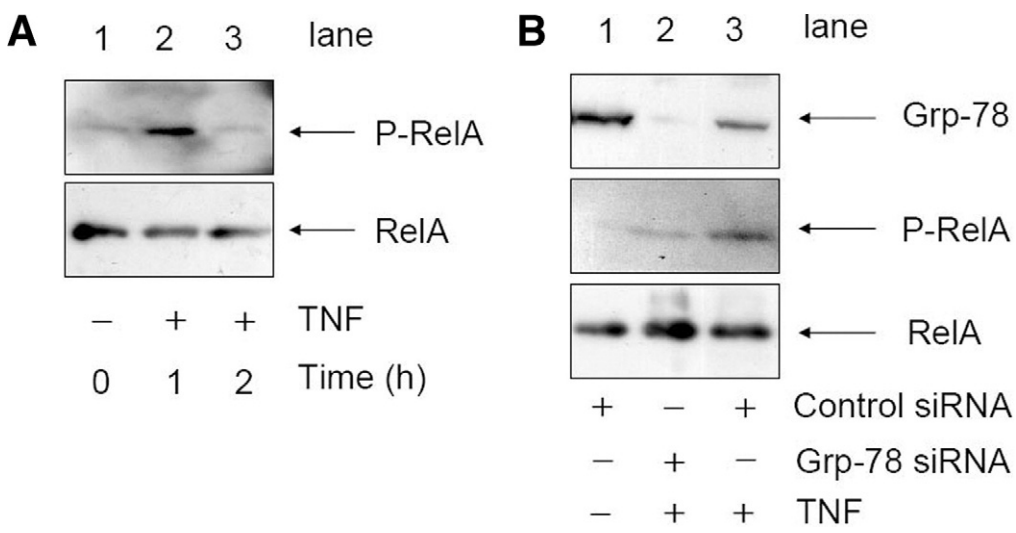


Figure 8. (*A* and *B*) Grp-78 knockdown inhibits TNF-induced RelA phosphorylation in Mode-K cells. (*A*) Mode-K cells were transfected for 72 hours with control siRNA followed by stimulation for 1 and 2 hours with TNF (10 ng/mL). (*B*) Mode-K cells were transfected with grp-78-specific as well as control siRNA for 78 hours followed by stimulation for 1 hour with TNF (10 ng/mL). Western blot analysis was performed with 20 μg of total protein using specific antibodies for phosphor-RelA, RelA, and grp-78. Representative gels from at least 2 different experiments are shown.

were recruited into the IKK complex after the stimulation of Mode-K cells with TNF (Figure 9*A*). Of note, the use of an overexpression plasmid for grp-78 (generous gift from Prof. Dr Rick Austin, McMaster University, Canada) demonstrated that cytoplasmic grp-78 associates with the IKK complex even in the absence of TNF stimulation (data not shown). To evaluate further the role of IL-10 in modulating TNF-induced RelA phosphorylation, we preincubated IL-10R reconstituted Mode-K cells for 24 hours with IL-10, followed by stimulation with TNF for 20 and 60 minutes. As shown in Figure 9*B*, IL-10 inhibited TNF-induced grp-78 recruitment into the IKK complex, and IL-10 blocked TNF-induced RelA phosphorylation.

IL-10-Mediated p38 Signaling Inhibits TNF-Induced ATF-6 Recruitment to the grp-78 Promoter Likely Through the Blockade of ATF-6 Nuclear Translocation

We next sought to characterize the molecular mechanism for TNF-induced ER stress responses and the inhibitory function of IL-10. As shown in Figure 10*A–C*, TNF induced grp-78 relocation from the ER lumen into the cytoplasmic compartment of IL-10R reconstituted Mode-K cells. Interestingly, the grp-78 Western blot analysis in fractionated cellular compartments clearly demonstrated the appearance of grp-78 in the cytoplasmic fraction after 1 hour of TNF stimulation (Figure 10*B*). The grp-78 expression levels seemed to remain constant in the ER compartment (Figure 10*B*), and TNF did not trigger significant grp-78 protein expression (Figure 10*A*), suggesting that the cellular redistribution of grp-78 at early stimulation time points seemed to be independent from grp-78 protein resynthesis. Creatine kinase was used to demonstrate the purity of the fractionated cellular compartments including mitochondria (MC), periplasmic ER, and cytoplasm (CP). In addition, we performed immunofluorescence analysis to confirm the fact that grp-78 relocates from the ER into the cytoplasm. As shown in Figure 10*C*, the nuclei were stained with DAPI (blue), and rhodamine-coupled grp-78 was shown alone as well as together with DAPI-stained nuclei (overlay picture, lower row). Indeed, the perinuclear staining of grp-78 (red) seemed to confirm its predominate ER localization (Figure 10*C*, control). TNF stimulation of IEC clearly induced the diffusion of grp-78 from the perinuclear ER region into the cytoplasm (Figure 10*C*, TNF).

We next characterized the effect of IL-10 on grp-78 expression in the absence and presence of TNF. As shown in Figure 11*A*, IL-10 strongly reduced the grp-78 mRNA expression levels in the absence of stimulation but even more in the presence of TNF stimulation. For these experiments, the IL-10R reconstituted Mode-K cells were pretreated with IL-10 for 24 hours. Where indicated, these cells were then stimulated with TNF for an additional 24 hours (Figure 11*A*) or 1 hour (Figure 11*B–D*).

To elucidate further the molecular mechanisms of these inhibitory effects of IL-10, we used ChIP analysis to determine the recruitment of transcription factors including ATF-6 and c-fos to the grp-78 promoter region. Consistent with the transcriptional and protein expression analysis, IL-10 completely inhibited histone 3 acetylation/phosphorylation of the grp-78 promoter as well as the recruitment of ATF-6 and c-fos (Figure 11*B*). Interestingly and most important for the understanding of the molecular mechanisms, we showed that the recruitment of the ER transmembrane protein ATF-6 was selectively blocked upon TNF stimulation in IL-10-pretreated

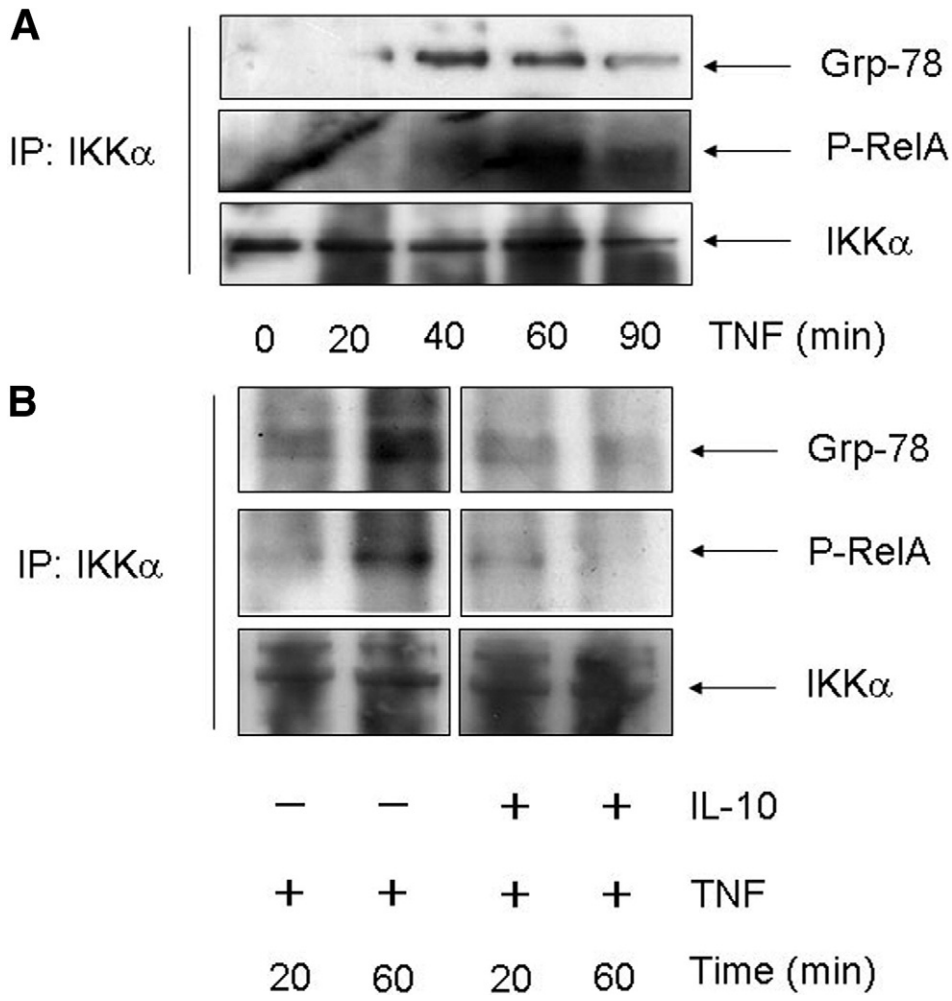


Figure 9. (*A* and *B*) IL-10 inhibits TNF-induced grp-78 recruitment and TNF-induced RelA phosphorylation in the IKK complex of IL-10R reconstituted Mode-K cells. (*A*) Mode-K cells were reconstituted with the murine IL-10R complex followed by the stimulation for 20, 40, 60, and 90 minutes with TNF (10 ng/mL). (*B*) IL-10R reconstituted cells were pretreated for 24 hours with IL-10 (10 ng/mL) followed by stimulation for 20 and 60 minutes with TNF. Coimmunoprecipitation experiments were performed using anti-IKKα antibodies followed by Western blot analysis with specific antibodies for p-RelA, RelA, IKKα, and grp-78. Representative gels from at least 2 different experiments are shown.

IL-10R reconstituted Mode-K cells (Figure 11*B*). In contrast, IL-10 failed to inhibit the recruitment of the ER-independent transcription factor c-fos to the grp-78 promoter after TNF stimulation.[18] Consistent with these observations, IL-10 also failed to inhibit TNF-induced acetylation/phosphorylation of histone 3, suggesting additional mechanisms for the specificity of the IL-10-mediated ATF-6 recruitment blockade.

In addition to the ChIP analysis, we performed immunofluorescence analysis to follow TNF-induced ATF-6 nuclear translocation in the absence and presence of IL-10. As shown in Figure 11*C*, rhodamine-coupled ATF-6 translocated from the perinuclear ER compartment into the nucleus upon TNF stimulation for 1 hour. Interestingly, the pretreatment of the cells for 24 hours with IL-10 completely blocked ATF-6 nuclear translocation after TNF stimulation (Figure 11*C*, IL-10 + TNF). ATF-6 diffusely remained in the cytoplasmic compartment in contrast to the perinuclear and compartmentalized staining in the control cells.

Finally, we performed additional ChIP analysis to validate and refine the question of whether IL-10 signals through the p38 cascade. As shown in Figure 11*D*, the pretreatment of the IL-10R reconstituted Mode-K cells with the pharmacologic p38 inhibitor SB203580 (1 hour) partially reversed the IL-10-mediated complete inhibition of ATF-6 recruitment to the grp-78 promoter upon TNF stimulation (1 hour). Consistent with the presence of phospho-p38 in WT but not IL-10−/− IEC, these results suggest that the IL-10-activated p38 signaling pathway contributes to the regulation of ER stress responses.

Discussion

Proteomic analysis in *E faecalis*-monoassociated IL-10−/− mice demonstrated that the expression of the

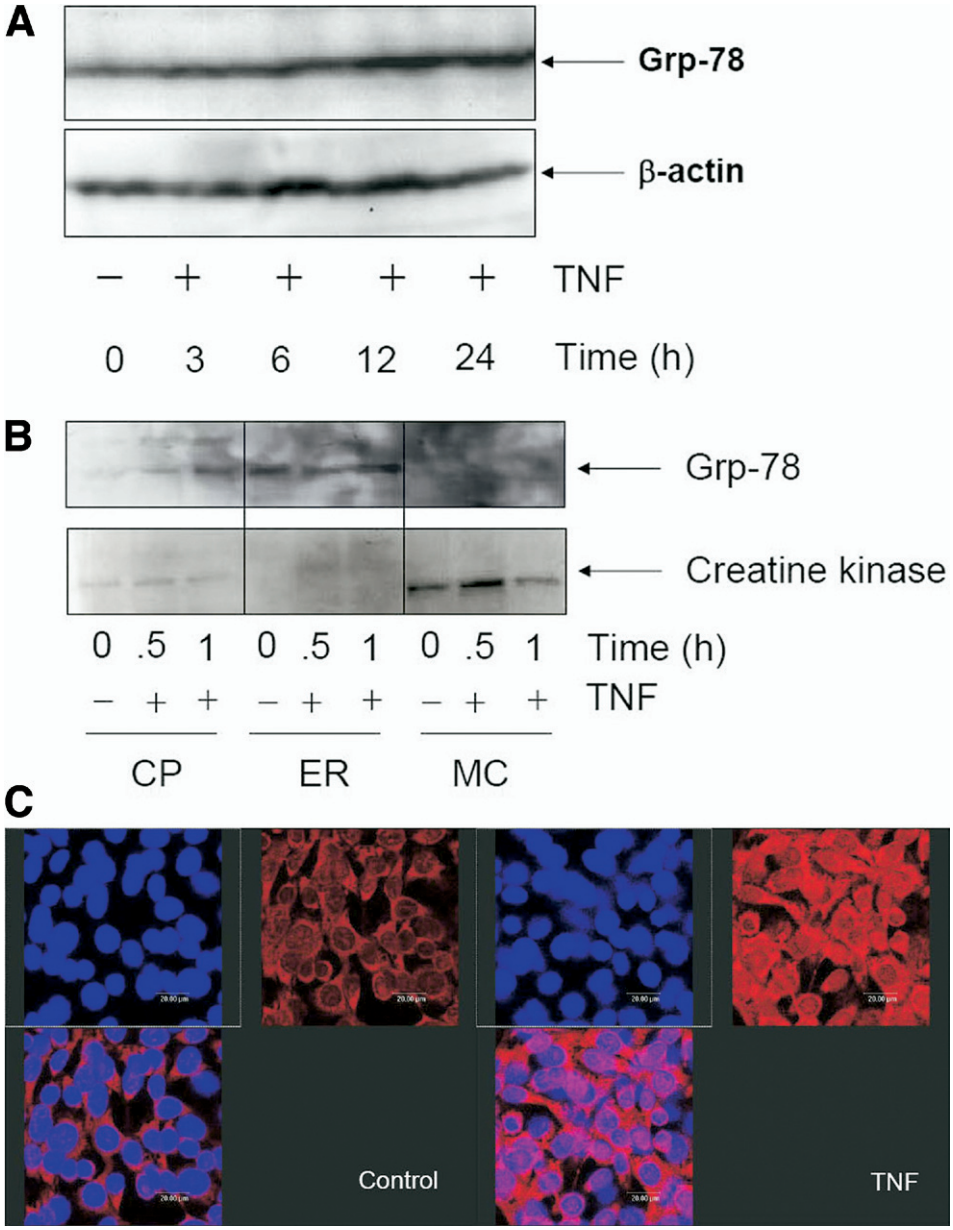
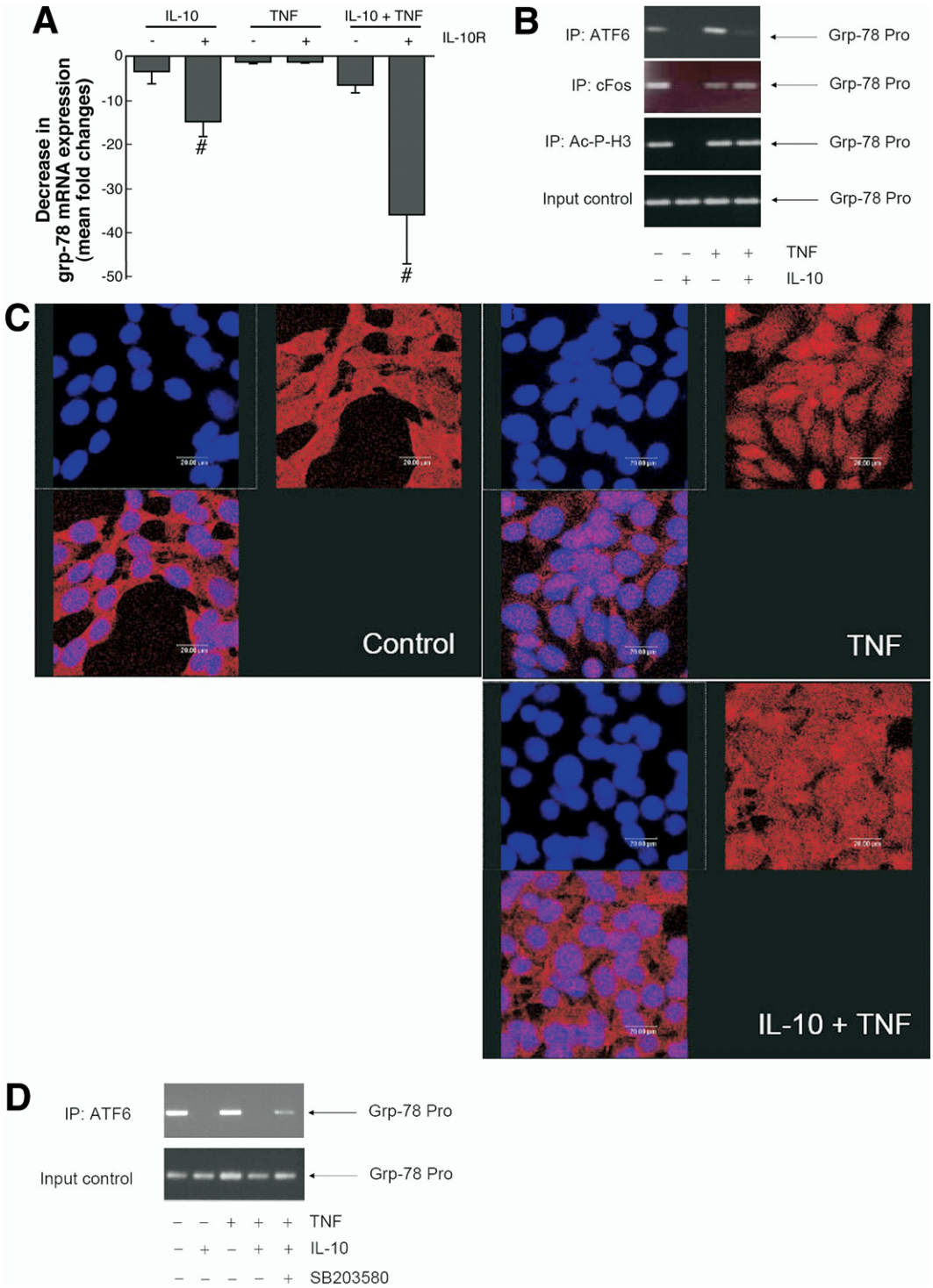


Figure 10. (*A–C*) TNF triggers relocation of grp-78 from the ER to the cytoplasmic compartment. IL-10R reconstituted Mode-K cells were stimulated with TNF (10 ng/mL) for various times. (*A*) Western blot analysis was performed with 20 μg of total protein using immunoreactive grp-78 and β-actin antibodies. (*B*) Cellular fractions from the mitochondrial (*MC*), *ER*, and cytoplasmic compartment (*CP*). Western blot analysis was performed with 20 μg of total acetone precipitated protein using immunoreactive grp-78, creatine kinase, and β-actin antibodies. (*C*) Immunofluorescence analysis was performed using DAPI for nuclear (*blue*) and rhodamine-coupled antibodies for grp-78-specific staining (*red*). Overlay pictures are shown in the *lower row*. The cells were stimulated with TNF for 1 hour. Representative gels from at least 2 different experiments are shown.

Figure 11. (*A–D*) IL-10-mediated p38 signaling inhibits TNF-induced ATF-6 recruitment to the grp-78 promoter. IL-10R reconstituted Mode-K cells were pretreated with IL-10 for 24 hours followed by stimulation with TNF (10 ng/mL) for 1 and 24 hours. (*A*) Where indicated, Mode-K cells were stimulated with TNF for 24 hours. Total RNA was extracted and reverse transcribed. Real-time PCR was performed using the Light Cycler system with specific primers for grp-78 and GAPDH. The expression changes (fold decrease) of the specific mRNA were calculated relative to unstimulated control cells, using the crossing point of the log-linear portion of the amplification curve after normalization with GAPDH. The data represent mean fold decrease ± SD from triplicate stimulations. (*B*) Where indicated, Mode-K cells were stimulated with TNF for 1 hour. ChIP analysis was performed using anti-ATF-6, anti-c-fos, and antiacetylated/phosphorylated H3 antibodies for immunoprecipitation followed by ATF-6 promoter-specific PCR as described in the Materials and Methods section. (*C*) Where indicated, Mode-K cells were stimulated with TNF for 1 hour. Immunofluorescence analysis was performed using DAPI for nuclear (*blue*) and rhodamine-coupled antibodies for grp-78-specific staining (*red*). Overlay pictures are shown in the *lower row*. (*D*) Where indicated, the cells were pretreated with the pharmacological p38 inhibitor SB203580 for 1 hour followed by 24 additional hours of stimulation with IL-10 (50 ng/mL). ChIP analysis was performed as described in the Materials and Methods section after stimulation of the cells with TNF for an additional 1 hour. Representative gels from at least 2 different experiments are shown.



ER stress response protein grp-78 was increased in IEC under conditions of chronic inflammation. Most important for the pathopysiologic relevance, we validated these findings in primary IEC from patients with CD and UC demonstrating increased grp-78 protein expression levels in inflamed but not in control tissues. Interestingly, IEC from sigmoid diverticulitis patients also revealed increased grp-78 protein expression levels, suggesting that ER stress responses are part of the inflammatory process rather than a specific feature of the IBD pathology. In addition, we showed for the first time that IL-10-mediated p38 signaling in the intestinal epithelium inhibited ER stress responses through mechanisms that inhibit nuclear recruitment of the ER transcription factor ATF-6

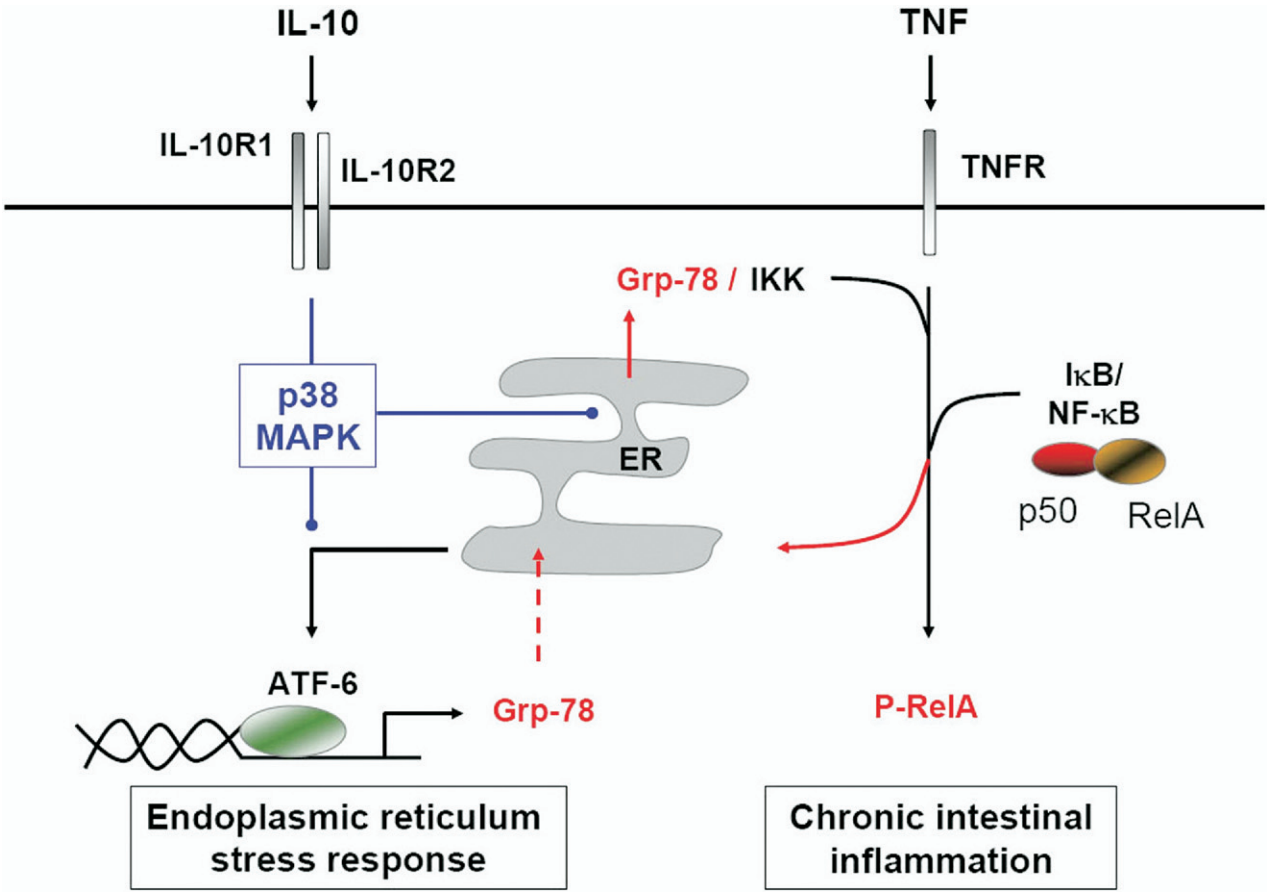


Figure 12. Schematic illustration for the proposed mechanisms of IL-10 on TNF-induced ER stress responses.

to the grp-78 promoter, suggesting that, in the absence of adequate host-derived control mechanisms, ER stress responses contribute to the pathogenesis of chronic intestinal inflammation. Considering our previous findings that protective TGF-β-mediated Smad signaling was present at the early but not at the late phase of bacterial colonization,[7] we suggest the possibility that TGF-β and IL-10 may both contribute to the maintenance of epithelial cell homeostatsis but differ in the timing and molecular mechanisms of their effects.

Adverse environmental and/or metabolic conditions trigger cellular stress responses including ER-specific mechanisms to ensure the transit of correctly folded proteins through the Golgi and cytoplasmic compartments.[19] Distinct signal transduction pathways direct ER stress responses toward the nucleus including the unfolded protein response (UPR), the ER-overload response (EOR), as well as the sterol regulatory element-binding protein (SREBP) pathway.[20] ER stress-induced changes in gene expression involve proteins of the ER molecular chaperone family grp, including grp-78 (also referred to as immunoglobulin heavy chain-binding protein, BiP). This family of proteins is critical to assist protein folding and assembly in the ER lumen. Grp-78 was identified as a prototypic ER stress marker and master regulator of UPR.[21] The accumulation of misfolded proteins in the ER because of environmental and/or metabolic stress conditions triggers grp-78 liberation from transmembrane ER signaling proteins to initiate grp-78 mRNA resynthesis. The grp-78 promoter contains 1 copy of the UPR response element (UPRE) and multiple copies of the ER stress response element (ERSE). ATF-6 is an ER transmembrane protein with an ER/perinuclear localization, and, most importantly for this study, is an important activator of the grp-78 promoter upon ER stress induction.[16,17]

The presence of phospho-p38 in IEC from *E faecalis*-monoassociated WT but not IL-10 deficient mice was associated with IL-10-mediated induction of p38 signaling in IL-10R reconstituted IEC, supporting the hypothesis that IL-10 may directly contribute to the maintenance of epithelial cell homeostasis. Interestingly, Kontoyiannis et al showed that IL-10-mediated p38 MAPK signaling modulates adenosine-uracil rich (ARE)-dependent TNF mRNA translation and most importantly attenuates intestinal pathology.[22] In addition, Lee and Chau showed that IL-10 induction of a p38 MAPK-dependent pathway affects heme oxygenase-mediated anti-inflammatory mechanisms in murine macrophages,[23] suggesting that the induction of p38 MAPK pathways

mediates protective mechanisms. Although IL-10 was shown to inhibit TNF-induced IKKβ activity in cultured human macrophages and HT-29 human epithelial cells,[24] the underlying molecular mechanisms by which IL-10 controls inflammation-mediated signal transduction in IEC remain poorly understood.

Our functional analysis demonstrated that IL-10 decreases the basal mRNA and protein expression levels of grp-78 in IL-10R reconstituted epithelial cell lines, suggesting a role for IL-10 in modulating ER stress responses. The more detailed molecular analysis using ChIP analysis and immunofluorescence microscopy revealed that IL-10 inhibited ATF-6 recruitment to the grp-78 promoter through mechanisms that inhibit ATF-6 nuclear translocation. Pharmacologic inhibition of the p38 MAPK cascade reversed the protective mechanisms of IL-10 at the level of ATF-6 recruitment to the grp-78 promoter, suggesting a direct mechanistic link between the p38 MAPK pathway and the protective function of IL-10. Although chromatin modifications of the grp promoter region including histone acetylation/phosphorylation have been shown to affect the transcription of ER stress response genes,[25] ChIP analyis revealed that IL-10 did not modulate histone 3 acetylation/phosphorylation at the grp-78 promoter region. These results are consistent with the observation that IL-10 selectively inhibited recruitment of the ER-associated transcription factor ATF-6 but not c-fos, supporting the hypothesis that IL-10 inhibits ATF-6 nuclear shuttling from the perinuclear region upon TNF-induced ER stress.

ER-nuclear signal transduction pathways not only trigger grp-78/BiP gene expression in the nucleus but also facilitate NF-κB activation through increased Ca^{2+} efflux and the production of reactive oxygen species (ROS).[26,27] In addition, the proinflammatory mediator TNF activates the IκB/NF-κB transcription factor system through a TNF receptor 2-mediated cascade by recruiting TNF receptor-associated factor 2.[28] The TNF-induced signal transduction converges with the TLR signaling cascade at various kinases including the NF-κB inducing kinase (NIK) or mitogen-activated protein kinase kinase 1 (MEKK1)/extracellular signal-regulated kinase (ERK)[29,30] that mediate the activation of the IκB kinase (IKK) complex followed by NF-κB RelA (Ser536) and IκBα (Ser32/34) phosphorylation.[31,32] At first glance, it appears a paradox that we find the ER-resident chaperone grp-78 to mediate cytoplasmic TNF signal transduction through recruiting grp-78 into the IKK complex. Immunofluorescence microscopy and cellular fractionation clearly refined and validated our findings that TNF triggers grp-78 cytoplasmic relocation from the ER compartment. These findings are consistent with limited published precedents in the literature, demonstrating that ER stress inducers trigger the redistribution of grp-78 from the ER lumen to the cytoplasm[33] or that the cytosolic ER interface as a transmembrane protein.[34] Because TNF triggers ROS-dependent EOR[35] independent of grp-78 resynthesis,[26] the appearance of grp-78 in the IKK complex may reflect TNF-induced ER stress response and redistribution of grp-78. Interestingly, IL-10 completely blocked TNF-induced RelA phosphorylation at the serine residue 536 in the IKK complex, suggesting that grp-78 directly affects TNF-induced NF-κB activation. Although the modulation of RelA serine 536 phosphorylation defines an IκBα-independent NF-κB pathway associated with changes in a distinct set of target genes,[36] the functional consequences of IL-10-mediated alterations of TNF-induced RelA phosphorylation will require additional studies.

Interestingly, the activation of NF-κB in response to ER stress was shown to be mediated through the ER-transmembrane kinase/ribonuclease IRE1 and TNF receptor-associated factor 2,[37] tightly connecting the cytoplasmic TNF signal transduction pathway with mechanisms of the ER stress response program. The induction of grp-78/BiP expression as a surrogate marker of ER stress responses was shown to be associated with increased transcriptional activation of the NF-κB-dependent gene ICAM-1 in the colonic mucosa of IRE1β-deficient (IRE1$\beta-/-$) mice when exposed to dextran sodium sulfate.[38] Although grp-78/Bip expression was increased under conditions of chronic inflammation, IRE1$\beta-/-$ mice revealed increased sensitivity to dextran sodium sulfate-induced colitis, suggesting protective functions of epithelial cell-specific IRE1β-dependent genes.[38] Interestingly, proteomic profiling of liver hepatocellular carcinoma revealed increased grp-78 expression, and, most important, grp-78 but not hsp-70 expression was associated with the disease progression.[39] In addition, HLA-B27 protein misfolding triggered UPR and grp-78/BiP expression in bone marrow-derived macrophages from HLA-B27 transgenic rats, supporting the hypothesis that ER stress responses and grp-78/BiP expression may contribute to development of chronic inflammatory processes.[40] It seems important to understand that, although the induction of grp-78/BiP expression may simply reflect stress-induced feedback mechanisms to prevent the continuous dissociation of grp-78/BiP from IRE1 and ER-stress signaling, there is accumulating evidence for the fact that grp-78 independently contributes to the initiation and/or perpetuation of chronic inflammatory processes.

Many additional stress conditions such as energy deficiency and glucose starvation, chemical toxicity, acidosis and hypoxia, oxidative stress, ER Ca^{2+} depletion, and inhibitors of glycosylation contribute to ER stress responses and the induction of the glucose-regulated protein family members including grp-78.[41] Interestingly, proteome analysis revealed a decreased expression level of the mitochondrial hydroxymethylglutaryl-CoA synthase in IEC from severely inflamed IL-10$-/-$ mice, indicating the possibility for reduced

cholesterol biosynthesis in the cell under conditions of chronic inflammation. In fact, the cleavage of SREBP from the ER membrane triggers nuclear transcription of the hydroxymethylglutaryl-CoA synthase gene through the interaction with the sterol regulatory element 1 to facilitate cholesterol biosynthesis.[42,43] These results suggest that changes in the metabolic function of IEC may contribute to ER stress responses in *E faecalis*-monoassociated IL-10−/− mice. In addition, grp-78/BiP shares approximately 60% homology with hsp70, and, like all hsp70 family members, binds ATP tightly. The depletion of cellular ATP inhibits protein folding in the ER and thereby contributes to ER stress responses.[44,45] It has been suggested that chronic intestinal inflammation represents an energy-deficiency disease with alterations in epithelial cells oxidative metabolism.[46,47] Our proteome analysis approach also identified decreased protein levels of creatine kinase and pyruvate kinase in primary IEC from severely inflamed IL-10−/− mice but not the WT controls. Both enzymes are critically involved in controlling the energy and ATP status of the cell, and their decline in steady state levels may support the concept that epithelial cells under conditions of inflammation become energy depleted.[48] It seems reasonable to assume that inflammatory mediators and metabolic changes in the intestinal environment contribute to ER stress responses.

In summary, we identified a novel anti-inflammatory mechanism for IL-10 with respect to the regulation of the ER stress response mediator grp-78 and its function on TNF-induced NF-κB signaling in IEC (Figure 12). The loss of regulation with respect to ER stress responses in the epithelium may contribute to the pathogenesis of chronic intestinal inflammation.

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Address requests for reprints to: Dirk Haller, PhD, Associate Professor, Experimental Nutritional Medicine, Technical University of Munich, Am Forum 5, 85350 Freising-Weihenstephan, Germany. e-mail: haller@wzw.tum.de; fax ++49 (0) 8161-712097.

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APPENDIX 6

Ruiz, PA, Braune, A, Hölzlwimmer, G, Quintanilla-Fend and Haller, D

"Quercetin inhibits TNF-induced NF-κB transcription factor recruitment to pro-inflammatory gene promoters in murine intestinal epithelial cells"

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[*In vivo* and *in vitro* experimental work by Ruiz, PA *(Tables 1-3 and Figures 1-5, 6B and 7)*; facilitation of quercetin bacterial metabolites by Braune, A; inmmunohistochemistry and histopathology by Hölzlwimmer, G and Quintanilla-Fend; writing by Haller, D and Ruiz, PA with editorial help of the co-authors]

Quercetin inhibits TNF-induced NF-κB transcription factor recruitment to pro-inflammatory gene promoters in murine intestinal epithelial cells

Ruiz PA [1], Braune A [2], Hölzlwimmer G [3], Quintanilla-Fend [3] and Haller D [1]*

(1)Else-Kroener-Fresenius-Center for Experimental Nutritional Medicine, Technical University of Munich, 85350 Freising-Weihenstephan, Germany (2)German Institute of Human Nutrition, Intestinal Microbiology, 14558 Nuthetal, Germany (3) Institute of Pathology, GSF Research Center, Munich-Neuherberg, Germany

Running title: Anti-inflammatory mechanisms of quercetin

ABSTRACT Flavonoids may play an important role for adjunct nutritional therapy of chronic intestinal inflammation. In this study, we characterized the molecular mechanisms by which quercetin and its enteric bacterial metabolites, taxifolin, alphitonin and 3, 4-dihydroxy-phenylacetic acid, inhibit tumor necrosis factor α (TNF)-induced pro-inflammatory gene expression in the murine small intestinal epithelial cell (IEC) line Mode-K as well as in heterozygous TNFΔARE/WT mice, a murine model of experimental ileitis. Quercetin inhibited TNF-induced interferon-γ-inducible protein 10 (IP-10) and macrophage inflammatory protein 2 (MIP-2) gene expression in Mode-K cells with effective inhibitory concentration of 40 and 44 μmol/L, respectively. Interestingly, taxifolin, alphitonin, and 3, 4-dihydroxy-phenylacetic acid did not inhibit TNF responses in IECs, suggesting that microbial transformation of quercetin completely abolished its anti-inflammatory effect. At the molecular level, quercetin inhibited Akt phosphorylation but did not inhibit TNF-induced RelA/I-κB phosphorylation and IκB degradation or TNF-induced NF-κB transcriptional activity. Most important for understanding the mechanism involved, chromatin immunoprecipitation (ChIP) analysis revealed inhibitory effects of quercetin on phospho-RelA recruitment to the IP-10 and MIP-2 gene promoters. In addition, and consistent with the lack of cAMP response element binding protein (CBP)/p300 recruitment and phosphorylation/acetylation of histone 3 at the promoter binding site, quercetin inhibited histone acetyl transferase activity. The oral application of quercetin to heterozygous TNFΔARE/WT mice (10 mg/day x kg body weight) significantly inhibited IP-10 and MIP-2 gene expression in primary ileal epithelial cells but did not affect tissue pathology. These studies support an anti-inflammatory effect of quercetin in epithelial cells through mechanisms that inhibit cofactor recruitment at the chromatin of pro-inflammatory genes.

The flavonol quercetin is the most common flavonoid in nature and is often linked to sugars such as rutin (quercetin-3-rutinoside) and quercitrin (quercetin-3-rhamnoside) (1,2). Quercetin has been reported to have anti-oxidant properties (3,4) associated with anti-thrombic, anti-hypertensive, anti-carcinogenic and anti-inflammatory effetcs (5-7). In addition, quercetin inhibits a broad-spectrum of protein kinases by its capability to compete with the binding of ATP at the nucleotide binding site (8). Interestingly, quercetin was the lead compound used to develop LY294002 and other inhibitors of phosphoinositide 3 (PI3) kinase (9), which is involved in the activation of a variety of downstream kinases including the protein kinase B/Akt (10). Rutin and quercitrin have been shown to exert intestinal anti-inflammatory effects in experimental models of rat colitis, the last being associated with loss of iNOS expression in dextran sodium sulphate (DSS)-induced colitis (11,12). Quercetin itself mediated anti-inflammatory effects in paw edema induced by carrageenan in rats (13). It has been suggested that the protective effects of quercetin may be attributed to its antioxidant and free radical scavenging properties thereby improving the colonic oxidative stress mechanisms under the inflammatory statuṣ(14). In addition, quercetin has been shown to inhibit both macrophage proliferation and activation *in vitro* by blocking the activation of LPS-induced NF-κB signaling (15). Although quercetin affects stress/cytokine-induced NF-κB and Akt signal transduction (16-18) and to some extent inhibit experimental colitis (15,19,20), the molecular mechanisms of this polyphenolic compound to inhibit epithelial cell activation under conditions of chronic intestinal inflammation is not yet well defined.

The presence of gastrointestinal infections, the genetic predisposition to dysregulated mucosal immune responses and environmental triggers in developed countries represent etiologic factors for the development of ulcerative colitis and Crohn's disease, the two distinct pathologies of inflammatory bowel disease (IBD) (21-23). Despite of the clinic development of specific biologic therapies in the last years (24) still little is known about the

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Abbreviations: EC_{50}, effective inhibitory concentration; IBD, inflammatory bowel diseases; IEC, intestinal epithelial cells; IKK, IκB kinase; IP-10, interferon-γ-inducible protein 10; MIP-2, macrophage inflammatory protein 2; NF-□B, nuclear factor κB; SEAP, secreted alkaline phosphatase; TNF, tumor necrosis factor α.

Correspondence*: Dirk Haller, Ph.D., Associate Professor, Else-Kroener-Fresenius-Center for Experimental Nutritional Medicine, Technical University of Munich, 85350 Freising-Weihenstephan, Germany; Email: haller@wzw.tum.de, Phone ++49-8161-71-2026, Fax ++49-8161-71-2097

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anti-inflammatory effects of dietary components. Increased activity of the NF-κB transcription factor system has been documented in the intestinal epithelium of animal models for experimental colitis (25) and IBD patients (26-28), therefore pharmacological blockade of the NF-κB signaling pathway may become particularly important in the treatment of chronic intestinal inflammation (29). IECs adapt to a constant changing environment by processing the combined biological information of luminal enteric bacteria/nutritional factors (30) as well as host-derived immune signals (31-33), therefore IECs become an excellent target cell type to assess the anti-inflammatory effects of dietary components on the host (34).

The treatment of a subset of IBD patients with monoclonal antibodies to tumor necrosis factor α (TNF) (infliximab) induced clinical remission of the inflammatory disease status (35) supporting the concept that TNF plays an important role in initiating and perpetuating NF-κB signaling and chronic intestinal inflammation (36,37). At the cellular level, TNF targets the TNF receptor 1 (TNFR1) to induce NF-κB RelA (Ser536) and IκBα (Ser32/34) phosphorylation, as well as IκBα ubiquitination and proteasomal degradation. Transcriptionally active NF-κB subunits such as RelA translocate to the nucleus to induce κB-dependent gene expression (38,39). Interestingly, the full activation of TNF-induced NF-κB activity and pro-inflammatory gene expression also requires additional mechanisms including Akt serine-threonine kinase activation (40).

We have previously shown that NF-κB signal transduction and gene expression of the IFN-γ-inducible protein 10 (IP-10) were persistently active in primary IECs under conditions of experimental colitis (25). Although flavonoids targeted the NF-κB and Akt signaling pathways to inhibit TNF-induced IP-10 gene expression in IECs (41), the molecular mechanisms for the inhibitory effects of quercetin remained unclear. In this study, we demonstrated that quercetin inhibits NF-κB binding to the pro-inflammatory IP-10 and MIP-2 gene promoters, blocking therefore further cofactor recruitment and histone acetyl transferase (HAT) activity at the chromatin of these promoters. Interestingly, quercetin metabolites accruing from bacteria-mediated degradation in the intestinal tract (42,43), did not inhibit TNF-induced gene expression, suggesting limited biological function along the intestinal tract. To further validate the physiological relevance of the inhibitory effects of quercetin with respect to the TNF-induced IP-10 and MIP-2 gene expression we orally administered quercetin (10 mg/day x kg body weight) to heterozygous TNFΔARE/WT mice. These mice lack the translational repression of TNF due to the absence of TNF adenosine-uracil rich elements in 3'-untranslated region of the TNF mRNA transcripts and as a consequence develop experimental ileitis (44,45).

MATERIALS AND METHODS

Cell culture and treatments - The mouse IEC line Mode-K (passage 10-30) was grown to confluency in 6-well tissue culture plates (Cell Star, Greiner bio-one, Frickenhausen, Germany) as previously described (30). Mode-K cells were stimulated with TNF (5 μg/L; R&D Systems, Heidelberg, Germany), in the absence or presence of quercetin, taxifolin (both from Roth, Karlsruhe, Germany), alphitonin (kindly provided by Dr. A. Braune, German Institute of Human Nutrition, Intestinal Microbiology) and 3,4-dihydroxy-phenylacetic acid (Sigma-Aldrich, Taufkirchen, Germany) at a final concentration of 100 μmol/L. The chemical structures of quercetin and its metabolite products are shown in Figure 1. Dose-response experiments were performed with quercetin in a concentration range of 1 – 200 μmol/L incubating with TNF for 24 hours. The effective inhibitory concentration of this compound (EC_{50}) was determined by calculating the inflection point of the inhibition curve. TNF-induced IP-10 and MIP-2 protein concentrations were blotted against the flavonoid concentration. Where indicated we used pharmacological inhibitors including the NF-κB inhibitor PDTC, the PI3K/Akt inhibitor LY291002 (both from Sigma-Aldrich, Taufkirchen, Germany) and the p38 MAPK inhibitor SB203580 (20 μmol/L; Calbiochem, Merck Biosciences, Schwalbach, Germany).

Oral application of quercetin to heterozygous TNFΔARE mice – Heterozygous TNFΔARE/WT and wild type (WT) mice at the age of 8 wk were orally fed with quercetin (10 mg/day x kg body weight) in the treatment group (Q, N = 11) and its dissolvent propylene glycol alone in the control group (C, N = 10) via crop gavage. All mice received a standard diet with very low nitrosamine contents, composed of fatty acids, minerals, amino acids, vitamins and crude nutrients, such as starch and crude protein (R/M-H, ssniff®, Soest, Germany). The mice were a generous gift from Dr. G. Kollias (Laboratory of Molecular Genetics, Hellenic Pasteur Institute, Athens, Greece). The animal use protocols were approved by the Bavarian Animal Care and Use Committee (AZ 55.2-1-54-2531-74-06). Mice were killed after 10 wk of treatment at the age of 18 wk by cervical dislocation and primary IECs were isolated from the ileum Sections of the distal ileum were fixed in 10% neutral buffered formalin. The fixed tissue was embedded in paraffin and histopathological analysis was performed as previously described, at the Institute of Pathology (GSF Research Center, Munich-Neuherberg, Germany) (46). In addition, we used the paraffin-embedded ileal tissue section to perform immunohistochemistry using anti-RelA antibodies (Santa Cruz, CA, USA) according to the protocol of the manufacturer.

Isolation of primary ileal epithelial cells - Primary IECs from the ileal epithelium of quercetin and propylene glycol fed WT and TNFΔARE/WT were purified as previously described (25). Cell purity was assessed by determining the absence of $CD3^+$ T-cell contamination. Trypan blue exclusion confirmed the presence of at least 80% viable cells after the 2 h isolation procedure. Primary IECs from the ileum were collected in sample buffer for subsequent RNA isolation.

RNA isolation and real-time Reverse-Transcription PCR - RNA from purified native IECs was extracted using Trizol Reagent (Invitrogen Life Technologies, Karlsruhe, Germany) according to the manufacturer's instructions. Extracted RNA was dissolved

in 20 μL water containing 0.1% diethyl-pyrocarbonate. Reverse transcription was performed from 1 μg total RNA. Real-time PCR was performed from 1 μL reverse transcribed cDNA in glass capillaries using a Light Cycler™ system (Roche Diagnostics, Mannheim, Germany) as previously described (25). Primer sequences were as follows: IP-10; sense 5′-TCCCTCTCGCAAGGAC-3′ and reverse 5′-TTGGCTAAACGCTTTCAT-3′; MIP-2; sense 5′-ATGAAGCTCTGCGTGT-3′ and reverse 5′-GGCTCACTGGGGTTAG-3′; GAPDH; sense 5′-ATCCCAGAGCTGAACG-3′ and reverse 5′-AAGT-CGCAGGAGACA-3′. The amplified product was detected by the presence of a SYBR green fluorescent signal. Melting curve analysis and gel electrophoresis was used to document the amplicon specificity. 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 $E^{\Delta Cp\ (control\ samples\ -\ treated\ samples)}$ and normalized for the expression of GAPDH. Samples from quercetin fed TNFΔARE/WT mice were measured as fold of control from propylene glycol fed TNFΔARE/WT mice.

Western blot analysis – Mode-K cells were pretreated with quercetin and its bacterial products (100 μmol/L) for 1 h followed by the stimulation with TNF (5 μg/L) for 0 – 180 min. Cells were lysed in 1X Laemmli buffer and 20-50 μg of protein was subjected to electrophoresis on 10% SDS-polyacrylamide (SDS-PAGE) gels. Anti-phospho-RelA (Ser536), anti-histone 3, anti-phospho-Akt (Ser473), Akt (all from Cell Signaling, Beverly, MA, USA), anti-RelA and anti-IκBα (all from Santa Cruz, CA, USA) were used to detect immunoreactive phospho-RelA, histone 3, phospho-Akt, Akt, RelA and IκBα respectively using the ECL Western blotting chemiluminescence detection kit (Amersham, Arlington Heights, IL, USA) as previously described (30).

Histone acetyl transferase (HAT) assay - Mode-K cells were pretreated with quercetin and its bacterial metabolites (100 μmol/L) for 1 h followed by the stimulation with TNF (5 μg/L) for additional 20 min. Nuclear extracts were prepared according to the manufacturer's instructions (Active Motif, Carlsbad, CA) using Tri(2-carboxyethyl) phosphine (TCEP) (Sigma-Aldrich, St. Louis, MS, USA) in the lysis buffer. Extracts (25 μg) were used to determine HAT activity by using HAT Activity Colorimetric Assay Kit (BioVision, Mountain View, CA, USA). The absorbance was read at 440 nm using a MultiScan spectrophotometer.

ELISA analysis - Mode-K cells were pretreated with quercetin and bacterial products (100 μmol/L) for 1 h followed by the stimulation with TNF (5 μg/L) for additional 24 h. Protein concentrations were determined in spent culture supernatants of IEC cultures. IP-10 and MIP-2 production was determined by mouse-specific ELISA assay kits, according to the manufacturer's instructions (R&D Systems, Heidelberg, Germany).

Reporter (SEAP) gene assay for NF-κB transcriptional activity - Mode-K cells were grown to 80 % confluency and then transfected with 2 μg of the NF-κB-inducible reporter plasmid pNiFty-SEAP (InvivoGen, San Diego, CA, USA) in the presence of 6μL FuGENE 6 Transfection Reagent (Roche Diagnostics, Mannheim, Germany). The pNiFty-SEAP reporter construct contains an engineered ELAM promoter with five NF-κB binding sites (GGGGACTTTCC) and the secreted alkaline phosphatase (SEAP) as reporter gene. Stable transfected cells were selected after the initial transfection (48 h) in the presence of the antibiotic zeocin (InvivoGen, San Diego, CA, USA). pNiFty-SEAP transfected Mode-K cells were pretreated for 1 h with quercetin and bacterial metabolites (100μmol/L) followed by the stimulation with TNF (5 μg/L) for additional 24 h. The secreted SEAP was measured according to the manufacturer's instructions (InvivoGen, San Diego, CA, USA) at 405 nm in a MultiScan spectrophotometer.

Chromatin immunoprecipitation (ChIP) analysis - After the treatment of Mode-K cells with quercetin and TNF, chromatin immunoprecipitation was performed by using the ChIP-IT kit from Active Motif (Carlsbad, CA, USA) as described by the manufacturer. Extracts were normalized according to their DNA concentration and immunoprecipitations were carried out using 5 μl anti-phospho-RelA (Ser536), anti-acetylated-phosphorylated H3 (Cell Signaling, Beverly, MA) (Santa Cruz, Europe) and anti-CBP/p300 antibodies (Biomol, Hamburg, Germany). DNA isolated from an aliquot of the total nuclear extract was used as a loading control for the PCR (input control). PCR was performed with total DNA and immunoprecipitated DNA using the following IP-10 promoter-specific primers 5′-AACAGCTCACGCTTTG-3′, 5′-GTCCTGATTGGCTGACT-3′ and MIP-2 promoter-specific primers 5′-GCCTATCGCCAATGAGC-3′, 5′-CAATTTTCTGAACCAAGGG-3′. The length of the amplified product was 186 bp and 185 bp respectively. The PCR products were subjected to electrophoresis on 2% agarose gels.

Statistical Analysis - Data are expressed as means ± SD of 9 independent experiments. Significant differences were determined on log-transformed data using One Way ANOVA followed by Tukey's test. Differences were considered significant at $P < 0.05$.

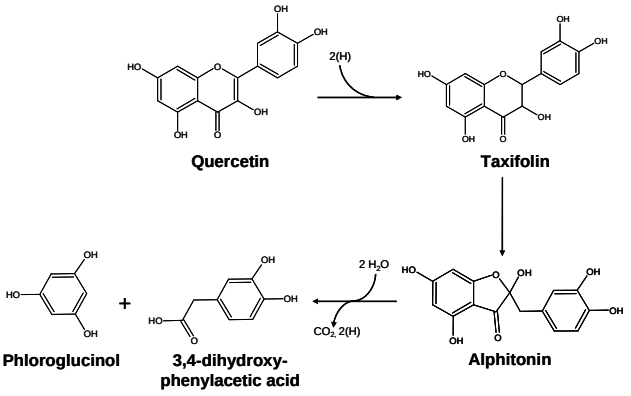


FIGURE 1. Chemical structures of quercetin and its bacterial metabolites.

RESULTS

Quercetin inhibits TNF-induced IP-10 and MIP-2 expression - Quercetin, but none of its bacterial metabolites, significantly inhibited MIP-2 and completely blocked IP-10 protein secretion after TNF stimulation in Mode-K cells (Table 1). Dose-response analysis for quercetin-mediated IP-10 (Figure 2 A) and MIP-2 (Figure 2 B) inhibition revealed effective concentrations (EC_{50}) of 40 and 44 μmol/L, respectively. Of note, quercetin did not induce significant cytotoxicity (less than 15 %). Interestingly, taxifolin, alphitonin and 3, 4-dihydroxy-phenylacetic acid did not inhibit TNF-induced IP-10 and MIP-2 protein production, suggesting that bacterial transformation of quercetin during the intestinal transit may reduce the anti-inflammatory mechanism of this polyphenolic compound.

TABLE 1

Differential effects of quercetin on TNF-induced IP-10 and MIP-2 expression in Mode-K cells[1]

	IP-10 (μg/L)	MIP-2 (ng/L)
CTRL	31.1 ± 3.3 [a]	64.3 ± 21.4 [a]
DMSO	20.3 ± 2.2 [a]	88.1 ± 152.6 [a]
TNF+DMSO	265.5 ± 23.0 [b]	1828.6 ± 142.7 [b]
TNF+Quercetin	58.2 ± 9.8 [a]	769.1 ± 39.3 [a]
TNF+Taxifolin	234.9 ± 33.3 [b]	1354.8 ± 153.6 [b]
TNF+Alphitonin	321.4 ± 37.2 [b]	1659.5 ± 325.3 [b]
TNF+3,4-dihydroxy-phenylacetic acid	307.7 ± 39.5 [b]	2019.1 ± 304.8 [b]

[1] Values are means ± SD, n=9. Means in a column without a common letter differ, P<0.01

Quercetin did not inhibit NF-κB RelA phosphorylation and NF-κB reporter gene activity - Since the pharmacological NF-κB (PDTC) and PI3 kinase (LY294002) inhibitors blocked TNF-induced IP-10 and MIP-2 production (Table 2), we next investigated the effects of quercetin on TNF-induced Akt and RelA phosphorylation (S536) as well as IκBα degradation in Mode-K cells.

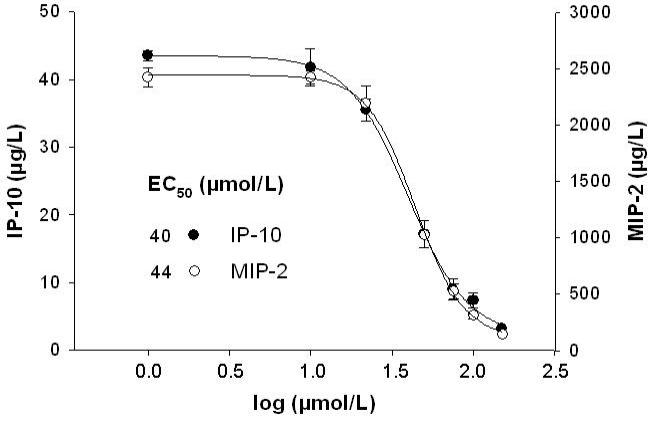


FIGURE 2. Effective inhibitory concentration of quercetin in Mode-K cells. TNF-induced IP-10 and MIP-2 protein concentrations were measured in the spent culture supernatant using ELISA analysis and blotted against the flavonoid concentration to determine the EC_{50}. Medium alone and medium with vehicle (DMSO) were use as controls. The results represent the mean of two independent experiments performed in duplicate 6-well cultures.

TABLE 2

Effect of inhibitors on TNF-induced IP-10 and MIP-2 expression in Mode-K cells[1]

	IP-10 (μg/L)	MIP-2 (ng/L)
CTRL	0.4 ± 0.4 [a]	138.1 ± 35.8 [a]
PDTC	0.9 ± 0.1 [a]	109.5 ± 74.3 [a]
LY294002	1.3 ± 0.1 [a]	66.7 ± 43.1 [a]
SB203580	0.2 ± 0.1 [a]	207.1 ± 25.8 [a]
TNF	133.1 ± 8.4 [b]	1488.1 ± 10.9 [b]
TNF+PDTC	0.9 ± 0.2 [a]	64.3 ± 12.4 [a]
TNF+LY294002	0.2 ± 0.1 [a]	142.9 ± 53.9 [a]
TNF+SB203580	82.6 ± 17.0 [b]	1342.9 ± 98.2 [b]

[1] Values are means ± SD, n=9. Means in a column without a common letter differ, P<0.01

Quercetin almost completely inhibited Akt phosphorylation in TNF treated Mode-K cells after 20 min of stimulation (Figure 3, lane 4). Consistent with lack of inhibitory functions on IP-10 and MIP-2 expression, the quercetin metabolites taxifolin, alphitonin and 3, 4-dihydroxy-phenylacetic acid did not affect the level of Akt phosphorylation (Figure 3, lane 5 - 7).

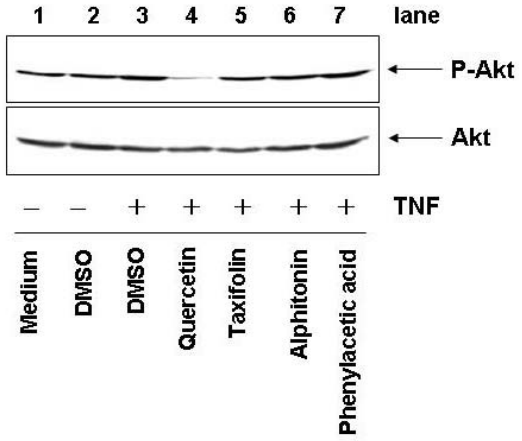
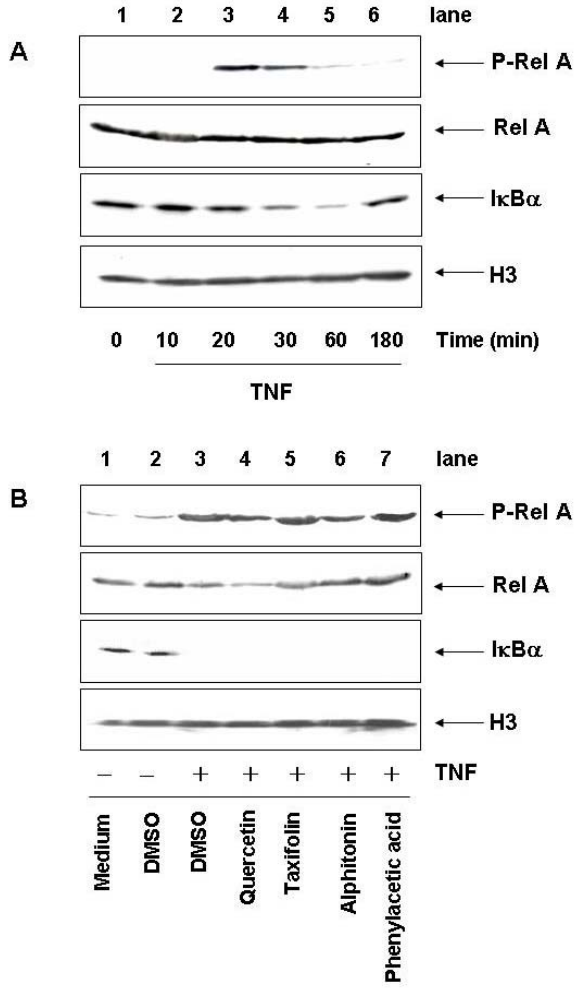


FIGURE 3. Quercetin inhibited Akt phosphorylation in Mode-K cells. Mode-K cells were stimulated with TNF in the presence of quercetin, taxifolin, alphitonin and 3, 4-dihydroxy-phenylacetic acid. Medium alone and medium with vehicle (DMSO) were use as controls. These results are representative of two independent experiments.

Time-response analysis revealed transient TNF-induced RelA phosphorylation after 20 min. (Figure 4 A, lane 3) followed by almost complete degradation of IκBα protein after 60 min. of stimulation (Figure 4 A, lane 5). Based on the kinetic analysis of signal-specific protein phosphorylation/degradation, we next measured TNF-

induced RelA phosphorylation (S536) after 20 min and IκBα degradation after 60 min of stimulation in the absence and presence of quercetin, taxifolin, alphitonin and 3, 4-dihydroxy-phenylacetic acid. Although quercetin blocked Akt phosphorylation as well as NF-κB-dependent IP-10 and MIP-2 expression, quercetin did not modulate TNF-induced RelA phosphorylation (Figure 4 B, lane 4 vs. 3) as well as IκBα degradation (Figure 4 B, lane 4 vs. 3). Consistent with our previous results, taxifolin, alphitonin and 3, 4-dihydroxy-phenylacetic acid did also not affect TNF-induced NF-κB/IκBα activation (Figure 4 B, lane 5 - 7).

FIGURE 4. Quercetin did not inhibit TNF-induced NF-



κB RelA phosphorylation and IκBα degradation in Mode-K cells. (A) Kinetic analysis for the TNF-induced activation of the NF-κB/IκB complex. Mode-K cells were stimulated with TNF for 10, 20, 30, 60 and 180 min. Medium alone and medium with vehicle (DMSO) were use as controls. (B) Mode-K cells were stimulated with TNF in the presence of quercetin, taxifolin, alphitonin and 3, 4-dihydroxy-phenylacetic acid. There results are representative of two independent experiments.

Quercetin did not modulate NF-κB transcriptional activity after stimulation of pNiFty-SEAP transfected Mode-K cells with TNF, but significantly inhibited HAT activity, suggesting that quercetin may specifically affect chromatin remodeling at native gene promoters (Table 3). Taxifolin, alphitonin and 3, 4-dihydroxy-phenylacetic acid did neither affect TNF-induced NF-κB reporter gene nor HAT activity (Table 3).

TABLE 3

Effect of quercetin and its metabolites on TNF-induced NF-κB reporter gene activity and histone acetyl transferase (HAT) activity in Mode-K cells[1]

	NF-κB reporter activity	Histone acetyl transferase (HAT) activity
CTRL	1.0 ± 0.2	% of TNF+DMSO
DMSO	0.8 ± 0.2 [a]	
TNF+DMSO	6.0 ± 0.6 [b]	100.0 ± 3.2 [a]
TNF+Quercetin	5.4 ± 0.2 [b]	61.1 ± 7.1 [b]
TNF+Taxifolin	6.4 ± 0.7 [b]	75.4 ± 4.1 [a]
TNF+Alphitonin	5.9 ± 0.4 [b]	85.7 ± 9.7 [a]
TNF+3,4-dihydroxy-phenylacetic acid	5.4 ± 0.9 [b]	88.8 ± 2.7 [a]

[1] Values are means ± SD, n=9. Means in a column without a common letter differ, P<0.05

Quercetin inhibits TNF-induced NF-κB and cofactor recruitment to the IP-10 and MIP-2 gene promoters –The treatment of Mode-K cells with quercetin inhibited TNF-induced phospho-RelA recruitment to the IP-10 (Figure 5A) and MIP-2 promoter as showed by ChIP analysis (Figure 5B). In addition, and consistent with the reduced HAT activity (Table 3), quercetin inhibited TNF-induced acetylation/phosphorylation of H3 and CBP/p300 binding at the IP-10 (Figure 5A) and MIP-2 (Figure 5B) gene promoters.

Quercetin inhibits IP-10 and MIP-2 gene expression in primary ileal IECs from inflamed TNFΔARE/WT mice – The histopathological analysis revealed moderate to severe inflammatory processes in the terminal ileum of quercetin fed TNFΔARE/WT mice (Histological score: 3.9 ± 0.7); this score did not differ from that in the propylene glycol fed control TNFΔARE/WT mice (Histological score: 4.4 ± 0.6). WT mice did not show histopathological changes in the terminal ileum (Histological score < 1) (Figure 6A).

Although quercetin feeding did not inhibit tissue inflammation, IP-10 and MIP-2 mRNA expression were significantly reduced in IECs from quercetin fed TNFΔARE/WT mice compared to control TNFΔARE/WT mice (Figure 6B). Consistent with the reduced IP-10 and MIP-2 gene expression in primary IECs, NF-κB nuclear staining was strongly reduced in quercetin fed TNFΔARE mice (Figure 6C).

DISCUSSION

In this study, we showed that the polyphenolic plant-derived flavonoid quercetin inhibits TNF-induced expression of the pro-inflammatory cytokines IP-10 and MIP-2 in primary IECs from TNFΔARE/WT mice as well as the epithelial cell line Mode-K. Consistent with its

inhibitory function on various protein kinases (8), quercetin inhibited Akt phosphorylation in Mode-K cells

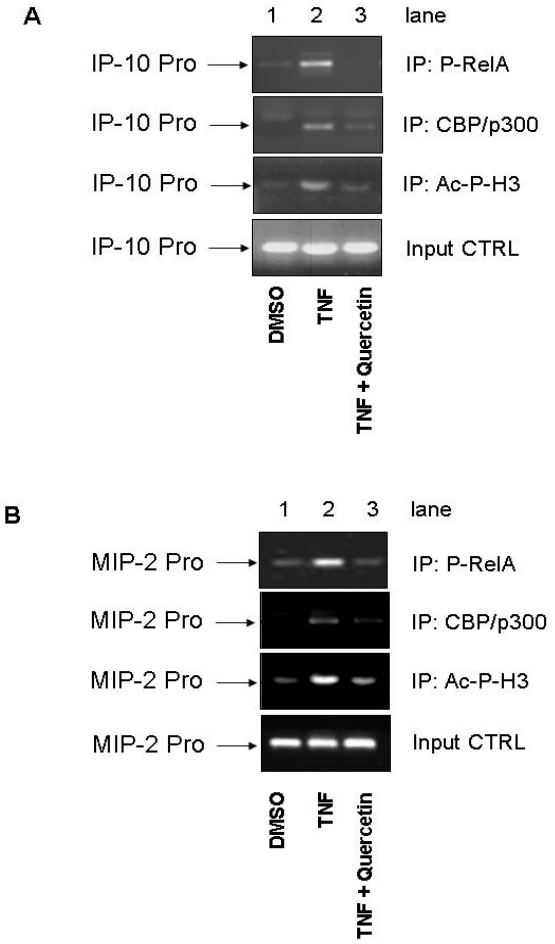


FIGURE 5 A and B. Quercetin inhibited TNF-induced NF-κB RelA and CBP/p300 binding to the IP-10 and MIP-2 gene promoters in Mode-K cells. Mode-K cells were stimulated with TNF in the absence and presence of quercetin. Medium with vehicle (DMSO) was used as control. ChIP analysis was performed using anti-phospho-RelA, anti-CBP/p300 and anti-acetylated/phosphorylated H3 antibodies for immunoprecipitation followed by IP-10 (A) and MIP-2 (B) promoter specific PCR as described in *Materials and Methods*. There results are representative of three independent experiments.

but did not inhibit TNF-induced NF-κB/IκB phosphorylation/degradation as well as NF-κB reporter gene activity. Interestingly and most important for understanding the mechanism involved, quercetin inhibited the recruitment of the NF-κB cofactor CBP/p300 to the IP-10 and MIP-2 gene promoters, suggesting that quercetin may target the TNF-induced transcriptional regulation at the chromatin. Indeed, we demonstrated that quercetin reduced total HAT activity and blocked TNF-induced acetylation/phosphorylation of H3 at the IP-10 and MIP-2 gene promoters. It seems likely that the inhibitory effect of quercetin on the PI3 kinase/Akt signaling cascade may directly affect the NF-κB-dependent gene expression by modulating CBP/p300 recruitment and/or HAT activity at the chromatin (Figure 7).

The transcriptional coactivators CBP and p300 play a central role in integrating various signal transduction pathways by coordinating the communication of transcription factors with the transcriptional apparatus to modulate stimulus-specific gene activity (51).

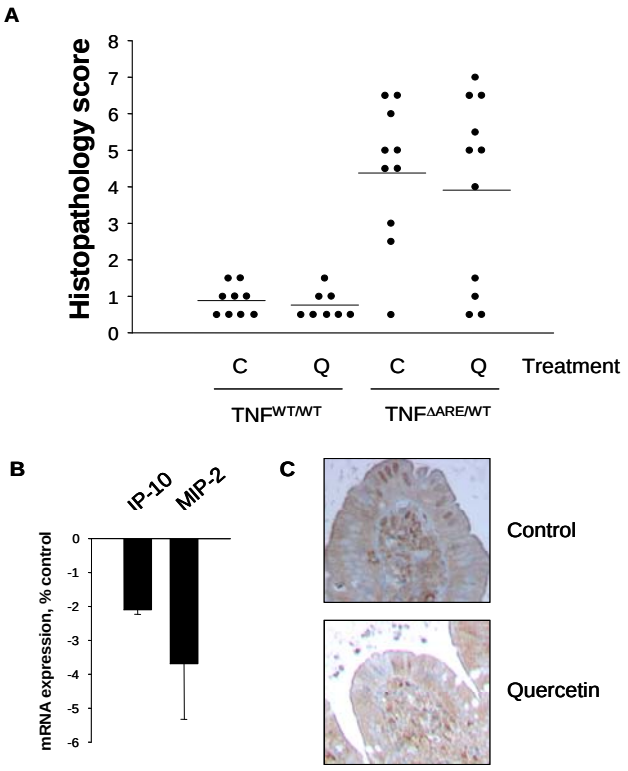


FIGURE 6 A - C. Quercetin inhibited IP-10 and MIP-2 gene expression in primary ileal epithelial cells but did not modulate tissue pathology. Heterozygous TNFΔARE/WT (11 mice) and WT mice (8 mice) were orally fed with quercetin for 10 wk. Feeding of propylene glycol alone was used as control (10 TNFΔARE/WT mice and 9 WT mice). The histopathological score of all mice was blindly assessed (A). IP-10 and MIP-2 gene expression from primary ileal epithelial cells was compared between quercetin and propylene glycol fed TNFΔARE/WT mice (B). Nuclear RelA staining shows representative nuclear RelA staining (C).

Interestingly, CBP/p300 exert its transcription-regulating properties by facilitating protein-protein interactions (51) as well as by its intrinsic HAT activity (52). The serine/threonine kinase Akt has been previously shown to interfere with the NF-κB signaling cascade at various levels including the induction of IκB kinase (IKK)β activity, NF-κB DNA binding activity as well as NF-κB transcriptional activity (30,53-55). It was recently demonstrated that TNF-induced nuclear Akt associates with CBP/p300 to phosphorylate the transcriptional coactivator at serine residue 1834 (56) and to repress CBP/p300 proteasomal degradation (57). The authors showed that p300 serine phosphorylation triggered histone acetylation and recruitment to the NF-κB-dependent gene promoter ICAM-1 (56). Quercetin did not block TNF-

induced RelA phosphorylation and IκBα degradation, suggesting that the inhibition of the PI3-kinase/Akt pathway did not affect cytoplasmic IKK activity. Interestingly, Mayo et al. showed in prostate cells that the reintroduction of PTEN, which is a lipid phosphatase responsible for the deactivation of PI3K/Akt signaling, results in the inhibition of TNF-induced NF-κB transcriptional activity by blocking the transactivation domain of the RelA/p65 subunit (58). Consistent with our findings, the authors showed that PTEN did not inhibit TNF-induced IKK activity, IκBα degradation and NF-κB RelA nuclear translocation but blocked transcriptional activation of NF-κB specific genes. These results strongly suggest that the inhibition of Akt phosphorylation by quercetin subsequently blocked TNF-induced phospho-RelA and CBP/p300 nuclear recruitment at the IP-10 and MIP-2 gene promoters through the modulation of histone 3 acetylation/phosphorylation.

Despite the significantly reduced NF-κB nuclear staining as well as IP-10 and MIP-2 mRNA expression in native IECs from quercetin-treated TNFΔARE/WT, these mice showed no differences in the severity of mucosal inflammation, indicating that the inhibition of IEC activation may not be sufficient to compensate for the pathologic mechanisms of TNF over-production in deeper layers of the mucosa. Recently, Comalada et al. showed that the aglycon quercetin, but not its glycoside form quercitrin (3-rhamnosyl-quercetin) inhibited macrophage activation/proliferation as well as iNOS expression. In contrast, only orally administration of quercitrin was able to inhibit DSS-induced colitis (15). This finding was further supported by the fact that fecal bacteria triggered the release of quercetin from its glycoside quercitrin. In addition, quercitrin showed protective effects in the intestinal epithelium of TNBS-treated mice but did not inhibit tissue inflammation (20).

These results clearly suggest that the mechanistically active anti-inflammatory structure quercetin was generated after cleavage of the glycoside residue during the colonic transit. Additional studies showed that the oral application of quercitrin to trinitrobenzene sulfonic acid (TNBS)-treated rats normalized hydroelectrolytic fluid transport in the intestinal epithelum but did not inhibit the development of TNBS-induced histopathology and myeloperoxidase activity in the colonic mucosa, suggesting that the protective effects of quercetin may vary among the various animal models of experimental colitis (20).

It seems important to understand that a major part of the ingested flavonoids are not metabolized by the gut. Several studies in the absorption and bioavailability of quercetin in humans show that although the absorption of quercetin aglycone has been reported to be of approximately 24%, the absorption of quercetin glycosides from onions was 52% (2,59). The dietary source of quercetin has also been reported to play an important role in the final concentration of this flavonol in plasma. Thus, the bioavailability of quercetin from both apples and pure quercetin rutinoside was only 30% compared with onions (60) with decreasing values when being ingested from black tea and wine (61,62). Flavonoids and their glycosides are partially degraded by strictly anaerobic colonic bacteria including the species *Clostridium scindens*, *Clostridium orbiscindens*, *Eubacterium desmolans* and *Eubacterium ramulus* (63,64). For example, *Eubacterium ramulus* is a flavonoid-degrading member of the normal flora whose unique carbon and energy source is quercetin-3-glucoside (isoquercetin) (42). The degradation of quercetin by these Gram-positive anaerobic bacteria results in the metabolite products taxifolin, alphitonin, 3,4-dihydroxy-phenylacetic acid and phloroglucinol (43). Since we demonstrated that the quercetin metabolites taxifolin, alphitonin and 3, 4-dihydroxy-phenylacetic acid did not inhibit TNF-induced pro-inflammatory gene expression in IECs, we sought to measure the effects of quercetin in the chronically inflamed small intestine using heterozygous TNFΔARE/WT mice as an animal model of experimental ileitis. Interestingly, the oral application of quercetin did not modulate the overall tissue damage in the ileum under conditions of chronic inflammation. On the other hand, the expression of the pro-inflammatory cytokines IP-10 and MIP-2 were significantly inhibited in primary ileal epithelial cells from quercetin-treated TNFΔARE/WT mice compared to the propylene glycol control group, supporting at least to some extend our mechanistic *in vitro* studies on the inhibitory effect of quercetin on TNF-induced IEC activation.

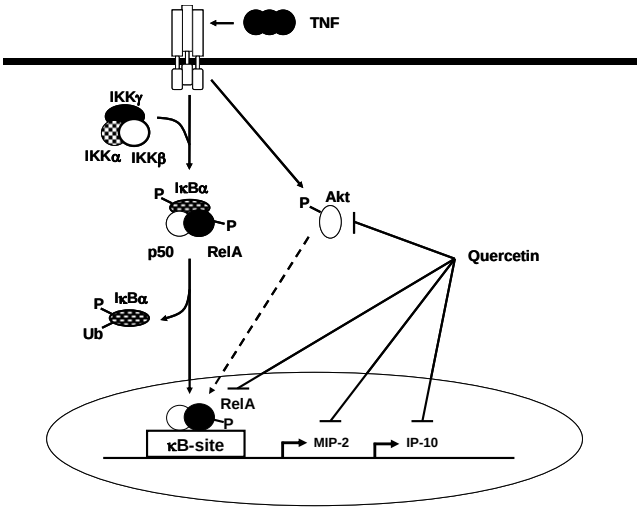


FIGURE 7. Schematic illustration for the inhibitory mechanism of quercetin. Inhibition of Akt phosphorylation was associated with the inhibition of TNF-induced recruitment of phospho-RelA to the pro-inflammatory gene promoters, abrogating thereby recruitment of CBP/p300 binding and histone acetylation/phosphorylation.

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CURRICULUM VITAE

Personal

Name:	Pedro Antonio Ruiz-Castro
Date of birth:	21 February 1973
Place of birth:	Zaragoza, Spain

University

Since May/2006	Technical University of Munich, Munich, Germany Research associated in Experimental Nutritional Medicine
Feb/2003-Mar/2006	Technical University of Munich, Munich, Germany Post-graduate PhD studies in Immunobiology of Nutrition
Sep/2001-Jan/2002	University of Zaragoza, Zaragoza, Spain Post-graduate Certificate Preliminary to PhD
Sep/1992-Aug/2001	University of Zaragoza, Zaragoza, Spain Graduated in Biochemistry

Internships

Jun/2005-Jul/2005	Rowett Research Institute, Aberdeen, Scotland, United Kingdom Internship as a trainee of the European Nutrigenomics Organisation (NuGO)
Feb/2002-Jan/2003	ProCorde GmbH, Martinsried, Germany Internship as a trainee of the European Commission's "Argo" program

Memberships

Since Feb/2005	Member of the German Society for Biochemistry and Molecular Biology (GBM)
Since Feb/2003	Member of the European Nutrigenomics Organisation (NuGO)

List of Scientific Publications

Ruiz, PA, Kim, SC, Sartor, RB and Haller, D "15-deoxy-delta12,14-prostaglandin J2-mediated ERK signaling inhibits gram-negative bacteria-induced RelA phosphorylation and interleukin-6 gene expression in intestinal epithelial cells through modulation of protein phosphatase 2A activity" J. Biol. Chem., 2004 Aug 20;279(34):36103-11.

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Ruiz, PA, Hoffmann, M, Szcesny, S, Blaut, M and Haller, D "Innate mechanisms for Bifidobacterium lactis to activate transient pro-inflammatory host responses in intestinal epithelial cells after the colonization of germfree rats" Immunology, 2005, 115:441-450

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Ruiz, PA, Braune, A, Hölzlwimmer, G, Quintanilla-Fend and Haller, D "Quercetin inhibits TNF-induced NF-κB transcription factor recruitment to pro-inflammatory gene promoters in murine intestinal epithelial cells" Submitted to J. Nutr.

Oral Presentations

Ruiz, PA and D. Haller "Molecular mechanisms for the inhibitory effects of flavonoids: significance of the nuclear factor κB in intestinal epithelial cells" 42nd Scientific Congress of the German Nutrition Society, March 2005, Kiel, Germany

Ruiz, PA, Shkoda, A, Kim, SC, Sartor, RB and Haller, D "IL-10 gene deficient mice lack TGF-beta-mediated Smad signaling and TLR2 protein degradation in intestinal epithelial cells after the colonization with colitogenic Enterococcus faecalis " 30th FEBS Congress - 9th IUBMB Conference, July 2005, Budapest, Hungary

Ruiz, PA, Braune, A and Haller, D "Molecular mechanisms of the inhibition of chronic intestinal inflammation through quercetin" 43nd Scientific Congress of the German Nutrition Society, March 2006, Stuttgart-Hohenheim, Germany

Selected Poster Presentations

Ruiz, PA, Kim, SC, Sartor, RB and Haller, D "15-deoxy-Δ12,14-prostaglandin J2-mediated ERK signaling inhibits Gram-negative bacteria-induced RelA phosphorylation and IL-6 gene expression in intestinal epithelial cells through modulation of protein phosphatase 2A activity"
8th Join Meeting of the Signal Transduction Society, November 2004, Weimar, Germany

Ruiz, PA, Shkoda, A, Kim, SC, Sartor, RB and Haller, D "IL-10 gene deficient mice lack TGF-beta-mediated Smad signaling and TLR2 protein degradation in intestinal epithelial cells after the colonization with colitogenic Enterococcus faecalis" International symposium. Inflammatory Bowel Disease, September 2005, Münster, Germany
Poster Presenting Award

Ruiz, PA, Braune, A and Haller, D "Quercetin inhibits TNF-induced transcription factor recruitment to the IP-10 gene promoter in intestinal epithelial cells through the modulation of histone acetyl transferase activity" Digestive Disease Week (DDW), May 2006, Los Angeles, USA