

From the Microbiology and Tumor Biology Center
Karolinska Institutet, Stockholm, Sweden

Pivotal Role of the Nuclear Receptor PPAR γ in Colon Epithelial cells

Emmelie Å Jansson



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To my beloved Family

Cover illustration

By Kalle Karlsson

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"Well," said Pooh, we keep looking for Home and not finding it, so I thought that if we looked for this Pit, we'd be sure not to find it, which would be a Good Thing, because then we might find something that we weren't looking for, which might be just what we were looking for, really.

I don't see much sense in that, said Rabbit.

No, said Pooh humbly, there isn't. But there was going to be when I began it. It's just that something happened to it along the way."

Winnie the Pooh

Abstract

When symbiosis between the residential intestinal flora and the epithelial cells of the gastrointestinal tract is broken one endpoint may be chronic inflammation, e.g. Inflammatory Bowel Disease (IBD). The disruption of the epithelial lining elicits activation of NF- κ B and secretion of potent inflammatory mediators. In the case of IBD, elevated levels of NF- κ B have been observed and interestingly several nuclear receptors have been reported to attenuate NF- κ B activity. One such receptor is PPAR γ . My thesis presents evidence of a possible molecular mechanism whereby commensal bacteria not only regulate the expression of PPAR γ , but also its ability to export NF- κ B from the nucleus, independently of I κ B, suggesting a novel pathway for nuclear export of NF- κ B.

In paper I we show that TLR4 signalling can increase expression levels of PPAR γ . Moreover we also show that germ free mice do not express PPAR γ indicating that bacteria can regulate its expression. Interestingly, patients with ulcerative colitis, one of the two main forms of IBD, display a defect in protein levels of PPAR γ in colonic epithelial cells compared to Crohn's disease patients and healthy controls.

In the second paper, we assess the possible anti-inflammatory effects of *Bacteroides thetaiotaomicron*, a common anaerobic commensal bacteria of the gastrointestinal tract, upon co-infection in CaCo-2 cells with the pathogenic *Salmonella enterica*. Immunohistochemistry in combination with biochemical and functional experiments revealed that PPAR γ , upon exposure to *B. thetaiotaomicron*, possess the ability to shuttle NF- κ B from the nucleus to the cytosol.

In the third manuscript we extend the observations presented in paper one and assess whether there is a relationship between high levels of NF- κ B and low PPAR γ levels in the colonic epithelium of IBD patients. No strict correlation was observed between low intestinal levels of PPAR γ and elevated NF- κ B levels. Individual variations of the absolute NF- κ B levels among IBD patients were however observed. In addition, we show that the apoptosis inducing ligand, TRAIL, is expressed at lower levels in inflamed colon mucosa, thereby linking it to chronic inflammation.

A DNA-binding protein may switch between an activator and a repressor, depending on co-factor assembly. This prompted us to assess whether such a factor could be identified for PPAR γ since opposing effects of its function in colonic tumor cells had been reported. One possible factor is the transcriptional co-factor β -catenin. In the final manuscript we examine the possible interplay between PPAR γ and β -catenin. Biochemical data show that β -catenin interacts with PPAR γ which results in elevated protein levels of PPAR γ . Functional data show that β -catenin and the known chemical agonist of Wnt signalling Lithium chloride, can activate a PPAR γ dependent reporter gene in a dose dependent manner. Hence, under conditions where abnormal levels of β -catenin is observed, e.g. in colon cancer, it is tempting to speculate that the altered β -catenin levels may change the net function of PPAR γ in favour of cell growth.

In summary, the data presented in this thesis, provide some aspects as to how homeostasis may be tightly regulated by a very limited set of factors and that these factors may be intimately connected to execute multiple functions under stringent control. The fine tuning of these functions can be guided by epigenetic factors, such as microbes and nutrients. The strategic tissue distribution of PPAR γ , and its pleiotropic functions serve as one example of an inflammatory gatekeeper that integrates gut homeostasis and metabolic control.

List of Publications

This thesis is based upon the following publications that will be referred to in the text by their Roman numerals:

- I Laurent Dubuquoy, **Emmelie Å Jansson**, Jean-Frédéric Colombel, Johan Auwerx, Sven Pettersson, Pierre Desreumaux (2003) Impaired expression of PPAR γ in Ulcerative Colitis. *The first two authors contributed equally* **Gastroenterology**. **124:1265-1276**
- II Denise Kelly, Jamie I. Campell, Timothy P. King, George Grant, **Emmelie A. Jansson**, Alistair G. Coutts, Sven Pettersson & Shaun Conway (2004) Commensal anaerobic gut bacteria attenuate inflammation by regulating nuclear-cytoplasmic shuttling of PPAR γ and RelA. **Nature Immunology**. **5(1): 104-12**
- III **Emmelie Å Jansson**, Ann-Kristin Spiik, Lars-Göran Axelsson, Fransesca Bresso, Robert Löfberg and Sven Pettersson (2004) Levels of NF- κ B in inflamed colonic mucosa from Inflammatory Bowel Disease patients are elevated in parallel with low levels of PPAR γ and TRAIL. *The first two authors contributed equally*. **Manuscript**
- IV **Emmelie Å Jansson**, Alexandra Are, Denise Kelly, Velmurugesan Arulampalam and Sven Pettersson. (2004) A functional link between Wnt-signalling/ β -catenin and the Peroxisome Proliferator Activated Receptor- γ . **Submitted**

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ABBREVIATIONS

AF-1, AF-2	activation function
APC	adenomatous polyposis coli
AR	androgen receptor
CD	Crohn's disease
DBD	DNA binding domain
DNA	deoxyribonucleic acid
DR-1	direct repeat-1
DR	dioxin receptor
ER	estrogen receptor
GALT	gut associated lymphoid system
GI	gastrointestinal tract
GR	glucocorticoid receptor
Grg	groucho related gene
IBD	inflammatory bowel disease
IEC	Intestinal epithelial cells
IL	interleukin
I κ B	inhibitory factor- κ B
IKK	I κ B kinase
LBD	ligand binding domain
N-CoR	nuclear receptor corepressor
NF- κ B	nuclear factor- κ B
NR	nuclear receptor
PAMP	pathogen associated molecular pattern
PG	prostaglandin
PPAR	peroxisome proliferator activated receptor
PPRE	PPAR response element
PRR	pattern recognition receptor
PXR	pregnane X receptor
RE	response element
RNA	ribonucleic acid
RXR	retinoic X receptor
Tcf	T cell factor
TLE	transducin like enhancer of split
TLR	toll like receptor
TNF- α	tumor necrosis factor- α
TRAIL	TNF related apoptosis inducing ligand
TZD	thiazolidenideones
UC	ulcerative colits

Introduction

This thesis will deal with events mainly occurring in our gastrointestinal tract. The scenario is the epithelial lining and the underlying mucosa and the story mainly entails the cells of the epithelium. Almost all of the actors in this molecular play can be described to be transcription factors, important deciders of cell fate, and include PPAR γ , NF- κ B and β -catenin. The supporting cast will be the bacteria of our intestinal flora. Will they be hero or villain?

The intestine is an interface with the outside world serving to both protect the body and absorb nutrients and the relationship between the mammalian host and its microbiota is to a large extent symbiotic, both parties benefit. However, as single entities, many species appear to expand their habitats if given the opportunity. This can result in opportunistic infections. Disturbance of this delicate balance can result in chronic inflammation detrimental to the host. Inflammatory bowel diseases are characterised by the recruitment of humoral and cellular components of the immune system to specific areas of the gut. Uncontrolled cytokine production is a result of activated epithelial cells, macrophages and B and T cells. This process is in part controlled by activated NF- κ B and the increased production of inflammatory mediators such as adhesion molecules, cytokines and chemokines. To avoid sustained inflammation, regulated activity of NF- κ B is essential and here new mechanisms involving the nuclear receptor PPAR γ are proposed.

For better and for worse-The intestinal ecosystem

Innate Immunity

The encounter with an invading microbe should result in its elimination as a result of actions taken by our body defenders. This is achieved through the coordinate action of the innate and adaptive immune system. Although both these systems rely on the recognition of molecular patterns on the invaders by specialised receptors on our immune cells, they use distinct mechanisms to achieve the final elimination of the pathogens that carry these patterns. The adaptive immune system consists of lymphoid cells, including the B and the T lymphocytes, which depend on somatic gene rearrangements and clonal expansion for the generation of an enormous repertoire of antigen recognising receptors present on these cells. By contrast, the innate branch of the immune system uses a set of essentially fixed receptors expressed by a broad range of cells including macrophages, neutrophils and epithelial cells (Medzhitov and Janeway, 1997a; Medzhitov and Janeway, 1997b).

The bacterial load at the mucosal surfaces in our body has resulted in a need to respond quickly to pathogenic invasion. Most of the invaders are identified and destroyed within minutes or hours by members of our innate immune system. However, if infection persists, the innate immune system calls for help and activates the adaptive immune system. The innate immune system, in contrast to the highly specific adaptive immune system, does not possess clonal specificity or memory (Medzhitov and Janeway, 1997b). As a tool in the identification of invaders, the innate immune system has developed a set of receptors recognising common and conserved structures on the microorganisms. These structures are dubbed the pathogen associated molecular patterns (PAMPs) and are molecules of the microbiota that have evolved to perform essential functions for microbial survival. Consequently they can not be subjected to a high rate of mutation. The PAMPs come in many

flavours, as examples one can mention a common component of gram negative bacteria, lipopolysaccharides (LPS), the mannans of yeast and the double-stranded RNAs of viruses (Brightbill and Modlin, 2000). A set of receptors on the host cells, the pattern recognition receptors (PRRs), can recognise these structures. The genes for these receptors are all encoded in the germline so in contrast to the receptors of the adaptive immune system, expression does not need time consuming gene rearrangement prior to mounting an immune response. Hence, the innate immune system is our first line of defence.

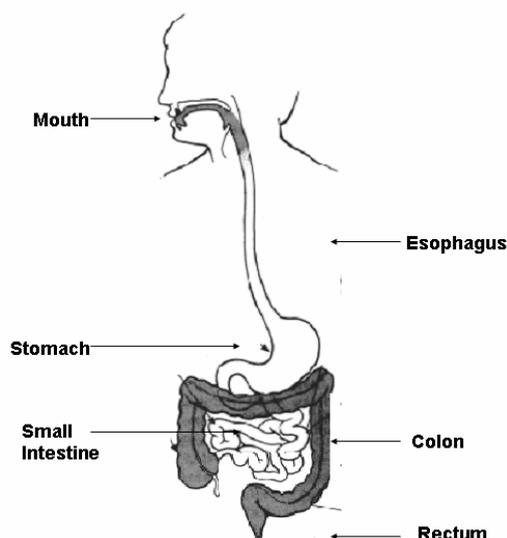


Fig 1. Outline of the gastrointestinal system

Toll like receptors

To illustrate PRRs the Toll like receptors (TLRs) are a good example. They are named after their homology to the *Drosophila* Toll receptors which are important in the innate response in flies. To date, 10 human TLRs have been cloned recognising a broad range of PAMPs (Underhill, 2003). TLRs are transmembrane receptors present on the cell membrane of various cell populations, indicative of recognition of extracellular PAMPs. The extracellular domain provides ligand specificity whereas the highly conserved cytoplasmic domain initiates a signalling cascade leading to NF- κ B activation and the induction of pro-inflammatory responses (Brightbill and Modlin, 2000). In the context of this thesis, it is the recognition of LPS, by TLR4, that is of importance. Binding of LPS to TLR4 does not seem to be direct, but rather involves accessory proteins such as MD-2, LBP and CD14 (Shimazu et al., 1999) (Wright et al., 1990). The TLRs are characterised by an extracellular domain containing leucine-rich repeats (LRR) and a cytoplasmic domain that share homology to the IL-1 receptor (TIR, Toll/interleukin-1receptor homology domain). A common signalling cascade following recognition of PAMPs by the TLRs is shared by all the known TLRs and the IL-1R (O'Neill, 2000). This pathway involves binding of the adaptor protein MyD88 to the TIR domain of TLR/IL-1R and subsequent association with a C-terminal death-domain of IL-1R-associated kinase (IRAK). Further signalling involves TRAF6 dependent activation of the I- κ B kinase (IKK) complex and activation of NF- κ B (Girardin et al., 2002). Support for the role of TLR4 in LPS signalling, is provided by the C3H/HeJ mouse strain. These mice have a naturally occurring dominant mutation in the *tlr4* gene that causes increased sensitivity to infection with gram-negative bacteria and hyporesponsiveness to LPS (Girardin et al., 2002).

The healthy gut acquires and maintains a hyporesponsive state towards the resident bacterial flora. Intestinal epithelial cells must be refractory to bacterial products and several mechanisms to achieve this have been proposed. Lack of co-receptors like CD14 and MD-2 on epithelial cells and intestinal macrophages (Abreu et al., 2001), TLR4 downregulation (Nomura et al., 2000) or expression of truncated MyD88 (Janssens et al., 2002) all lower LPS susceptibility. Another mechanism would be spatial separation of the TLRs from the PAMPs. Indeed, expression of TLR4 in the murine gut was recently shown to be restricted to the lower part of the crypts (Ortega-Cava et al., 2003), an area that has been considered a sterile, LPS-free site. The tissue destruction resulting from an invasive infection by enteric pathogens would allow for repositioning of TLR4 and engagement of the now proximal LPS. In addition, expression of TLR4 has been shown to be low in healthy individuals but upregulated in intestinal tissues from IBD patients (Cario and Podolsky, 2000) (Naik et al., 2001).

Gut microflora

Mammals are born without any microorganisms but extensive colonisation of the exposed body surfaces starts immediately after birth (Falk et al., 1998). In fact, the number of microbes associated with the mucosal surfaces, such as the respiratory-, genitourinary- and the gastrointestinal (GI) tracts, is estimated to outnumber the cells of our body. It has therefore been suggested that only 10% of our cells are of human origin. This gives that 90% of the cells in our body are of microbial origin (are we man or microbe?) (Savage, 1977). Of the mucosal surfaces, the gastrointestinal tract contains the largest number and complexity of microorganisms in our body. The lumen of the distal ileum and the entire colon contain high concentrations of bacteria, of which anaerobes, outnumber aerobes (Falk et al., 1998) (Sartor, 1997). In the distal ileum the concentration of bacteria has been estimated to be approximately 10^8 bacteria/ml of luminal contents, while in colon the number is increased and reach 10^{11} - 10^{12} (Sartor, 1997). *Bacteroides*, *Clostridium* and *Eubacterium* are some of the more than 400 species, but one must keep in mind that the microbial community in our GI tract is never static and varies with individual and environment (Falk et al., 1998) (Hooper et al., 2002).

To study the role of microbe-host interactions, the use of germ-free mice is of great importance. Born without resident microbes these mice represent a genetically defined and simplified *in vivo* system (Falk et al., 1998). Numerous studies in such mice have shown that the microflora influences maturation and turn over of the intestinal epithelial cells and in addition changes its gene expression profile (Hooper et al., 1998) (Hooper et al., 2002). As an example, the absence of a microflora reduces the rate of epithelial renewal in the small intestine (Savage et al., 1981) and result in underdeveloped lymphoid constituents of the mucosal immune system (McCracken and Lorenz, 2001).

An evolutionary consequence of the recruitment of the intestinal microorganisms is that it has relieved the host of the need to evolve a number of metabolic functions, for example degradation of otherwise non digested carbohydrates, fermentation (resulting in free fatty acids easily taken up by our cells) and synthesis of vitamins (Hooper et al., 2002) (Falk et al., 1998). In return the microbes gain access to a nutritional rich and protected environment. The intestinal ecosystem consists of the microflora, the epithelial cells and the gut associated immune system. Any ecosystem is in delicate balance, easily challenged by aberrant factors which can upset this balance, and this also applies to the intestinal ecosystem. If this balance is shifted, it can result in chronic diseases such as inflammatory bowel diseases and I will

return to this latter. Now, let us consider the two other members of the ecosystem, the epithelial cells and the immune cells.

Intestinal epithelial cells

The main functions of the epithelial cells of the gastrointestinal tract include absorption of nutrients and water whilst in addition they must provide a mechanical barrier to keep the microorganisms out (Kagnoff and Eckmann, 1997). The epithelial cells are an active barrier protecting the host by the production of a variety of innate antimicrobial defences (McCracken and Lorenz, 2001) (Podolsky, 1999). Antimicrobial peptides, intestinal peristaltic movements of food and infectious agents and the secretion of mucins are examples of such defensive mechanisms. The mucins form a mucus layer that covers the epithelial cells and aid in the defence by preventing attachment of microorganisms and facilitates their removal together with faeces (Hecht, 1999). Stimulation with TNF α and IL-1 or invasion with an invasive bacteria such as Salmonella results in expression and secretion of a number of cytokines with chemoattractant properties, such as IL-8, characterised by its ability to attract polymorphonuclear leukocytes (Eckmann et al., 1993). In addition, proinflammatory cytokines are also secreted, including TNF α , GM-CSF and IL-1, and this occurs rapidly after initial stimulation and peaks within a few hours. Thus, intestinal epithelial cells alert the innate immune system to the presence of pathogenic bacteria and signals provided by them are important for the onset of inflammatory responses in the period early after infection (Kagnoff and Eckmann, 1997) (Jung et al., 1995).

The intestinal epithelium lining the GI tract has a well defined architecture. A number of invaginations, called the crypts of Lieberkühns are the functional proliferative units and in the small intestine the crypts are surrounded by numerous villi (Marshman et al., 2002). This crypt-villi unit results in a greater surface area available for the absorption of nutrients. The epithelium is continuously and rapidly renewed by a process involving cell generation and migration from the stem cell population located at the bottom of the crypt. The cells then migrate upwards to the top of the villi where they preferentially die by apoptosis. Turnover time is considered to be 3-5 days (Hall et al., 1994) (Falk et al., 1998) and this plasticity of the system ensures that damaged and/or infected cells are dismissed and leave the body with the faeces, providing yet another defence mechanism against pathogenic bacteria. The intestinal epithelial lining consists mainly of four cell types; i: The columnar cells, which are the most abundant, called enterocytes in the small intestine and colonocytes in the colon. They are polarised cells with a basal nucleus and an apical brush border of microvilli bearing the glycocalyx. These cells have tight junctions and are functionally responsible for secretion and absorption (Wright, 2000); ii: the mucin secreting cells, called goblet cells because of their appearance; iii: the neuroendocrine cells and; iv: in the small intestine and ascending colon, the Paneth cells (Brittan and Wright, 2002) responsible for secretion of antimicrobial peptides such as defensins (Mallow et al., 1996).

A specialised type of epithelial cells is the M cell. It is an epithelial cell by all characteristics but executes important functions in antigen presentation to the gut associated lymphoid tissue (GALT). At the surface of lymphoid follicles (Peyer's patches in the small intestine and lymphoid follicles in the colon, particles which are taken up by the M cells are introduced to the intestinal immune system and elicit a mucosal immune response (Owen, 1999). In contrast to the normal epithelium, M cells have small microfolds (hence their name) on their surface instead of the brushborder microvilli, and are covered only by a thin mucus layer. This renders them more accessible to luminal flora and particles that reach the surface of M cells

are taken up and transported intact to be presented to the underlying immune cells (Owen, 1999). Hence, M cells are a way for the intestinal microflora to traverse the epithelial barrier.

The gut associated lymphoid tissue (GALT)

Now, the antigens have entered the mucosa. The principle effector sites in the intestine are the lamina propria and the intraepithelial spaces. The lamina propria is populated by resident cells of the immune system. More than 60% of the inhabitants in the lamina propria are macrophages, but antibody producing B cells and T cells can also be found there (McGhee, 1999). T lymphocytes in the lamina propria are primarily of the CD4⁺ subset of TCR α/β cells. In contrast, the intraepithelial T lymphocytes express CD8 and the γ/δ TCR and have cytolytic activity (Lefrancois, 1999) (James, 1999). The B lymphocytes in the lamina propria are responsible for the production and secretion of IgA. Secreted IgA, accounting for the largest population of the body's total pool of Igs, can bind luminal bacteria, neutralising them and facilitating the transport with the faeces out of the body (Robinson et al., 2001). A smart feature in the use of IgA is that they do not activate complement and can therefore be considered to be relatively non-inflammatory, executing its function without eliciting an inflammation that could get out of control (McCracken and Lorenz, 2001).

The molecular mediators

Wnt/ β -catenin signalling pathway

Of considerable importance in the development of the gastrointestinal tracts is the Wnt/ β -catenin pathway. The *Wnt* genes encode a large family of secreted glycoproteins that are important in controlling tissue patterning, cell fate, and cell proliferation in a variety of embryonic contexts. In the developing vertebrate embryo, the formation of the dorsal-ventral axis is dependent upon Wnt signalling and dysregulation of the pathway can cause developmental defects. Along with the various other tissues, the development of the gastrointestinal tract is suggested to be specified by Wnt signalling. The best understood of the Wnt signalling pathways is the Wnt/ β -catenin pathway, also known as the canonical pathway (Theodosiou and Tabin, 2003) (Giles et al., 2003). However, additional pathways have also been identified including the Wnt/Ca²⁺ pathway and the planar polarity pathway (for review see (Polakis, 2000) (Moon et al., 2002)).

Wnt signalling is initiated following Wnt ligand binding to a member of the Frizzled (Fz) family of transmembrane receptors (Giles et al., 2003). To date 19 members of the Wnt family have been identified in humans along with 10 members of the Frizzled family (Theodosiou and Tabin, 2003).

In the absence of Wnt ligands, the unstimulated cells regulate the cytoplasmic levels of β -catenin by a multi-component degradation complex. In this inhibitory complex, which consists of glycogen synthase kinase 3 β (GSK-3 β), axin and adenomatous polyposis coli (APC) (Theodosiou and Tabin, 2003), β -catenin is phosphorylated. This promotes subsequent ubiquitination and targeting to the proteasome for degradation. Upon binding of the Wnt ligand to its Frizzled receptor a cascade of events, not fully understood, activates Dishevelled which inhibits the constitutively active GSK-3 β , thus reducing the phosphorylation of β -catenin, APC and axin. The degradation complex is therefore dismissed and degradation of β -catenin is reduced. The stabilised β -catenin accumulates in the cytoplasm and can then subsequently enter the nucleus where it binds members of the T cell factor/lymphoid

enhancing factor (Tcf/LEF) family of transcription factors (Giles et al., 2003) (Theodosiou and Tabin, 2003) (Barker et al., 2000).

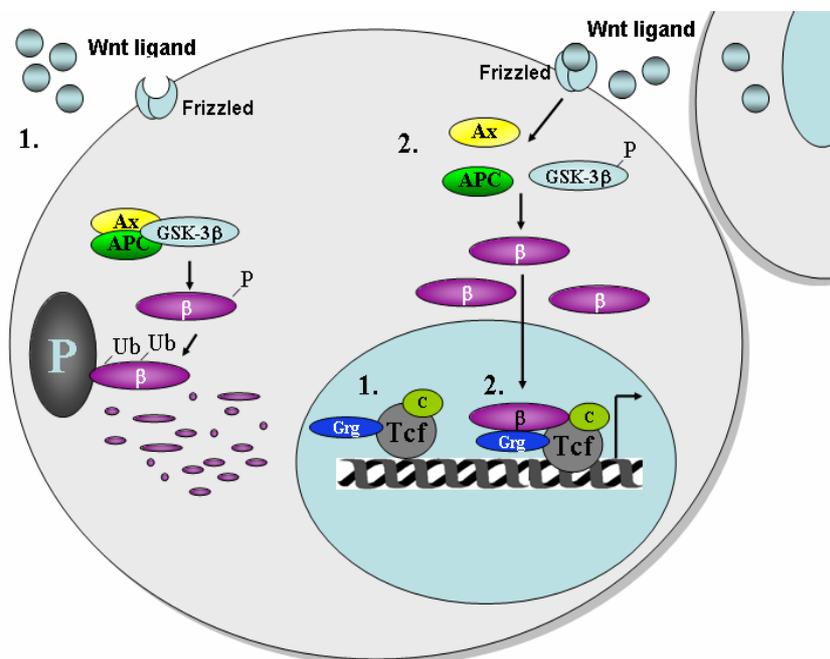


Fig 2. The Canonical Wnt pathway. In the absence of Wnt ligands, the cytoplasmic β -catenin (β) pool is bound by GSK-3 β , Axin (Ax) and APC and subsequently gets degraded by the proteasome (P). Wnt signalling induces stabilisation and nuclear import of β -catenin. In the nucleus, β -catenin displaces Groucho (Grg) from Tcf, allowing for transcription of target genes.

Axin, first identified as an inhibitor of Wnt signalling, appears to function as a scaffolding protein bringing GSK-3 β , APC and β -catenin together, hence stimulating phosphorylation and thus negatively regulating levels of cytosolic and nuclear β -catenin. The tumour suppressor gene APC encodes for a large (~300kDa) protein that interacts with several proteins in the cell, in addition to axin and β -catenin (Munemitsu et al., 1995). APC seems to be relatively ubiquitously expressed and in the epithelial cells of the gastrointestinal tract the expression is restricted to regions in which cell replication has ceased and terminal differentiation is established (Midgley et al., 1997).

β -catenin, the mammalian homologue of the *Drosophila* Armadillo protein, was first identified as a component of adherens junctions. There it binds directly to cadherins and by its association with α -catenin provides a link between the actin cytoskeleton and the cell-cell junctions (reviewed in (Kemler, 1993)). The cytoplasmic pool of β -catenin was identified later. The protein contains 12 tandemly arranged 42 residues long repeats, called the Arm repeats. These are responsible for the protein-protein interactions with cadherins, APC axin and Tcf (Huber et al., 1997). These arm repeats are sufficient for docking to the nuclear envelope and subsequent translocation, a process shown to be Ran-unassisted and occurring without a nuclear localisation signal (Henderson and Fagotto, 2002). Nuclear levels of β -catenin are dependent on both import and export and β -catenin is rapidly exported out of the nucleus. Recently, APC was shown to shuttle β -catenin in and out of the nucleus, providing the nuclear export signal and was suggested to act as a chaperone for β -catenin, directing it to its subcellular localisations (Henderson and Fagotto, 2002).

In the nucleus β -catenin binds and functions as a cofactor for the Tcf/LEF family of transcription factors. β -catenin can not bind DNA it self but its C-terminus contains a

transactivation domain that aids the otherwise transcriptionally dead Tcfs (Takemaru and Moon, 2000). In the absence of Wnt signal, Tcf/LEF binds a family of broadly expressed co-repressors, the groucho-related proteins also called TLE for transducin-like enhancer of split (Chen and Courey, 2000) (Cavallo et al., 1998). Consequently, in unstimulated cells the target genes of Wnt signalling are repressed. The target genes for the canonical Wnt signalling pathway includes several genes important for cellular proliferation such as c-myc and cyclin D1 (He et al., 1998) (Shtutman et al., 1999), revealing the importance of Wnt signalling in the development of cancers.

Wnts can be considered to be colon crypts architects, making the blueprint that directs the switch from proliferation to differentiation. The importance of the Wnt signalling pathway in intestinal development is demonstrated by the phenotype of the Tcf-4 knockout mice. In the intestinal epithelium, Tcf4 is the most prominently expressed Tcf family member (Barker et al., 1999) (Korinek et al., 1998). A recent gene disruption study shows that Tcf4 is necessary to maintain the proliferating compartment in the small intestinal crypts since these mice die shortly after birth. A single histopathological abnormality was observed; these mice were born with only non dividing, differentiated cells in their small intestinal epithelium (Korinek et al., 1998). Moreover, downregulation of the Wnt/ β -catenin pathway has been associated with the promotion of a more differentiated phenotype in colonic epithelial cells (Mariadason et al., 2001).

The nuclear factor- κ B (NF- κ B)

Colonisation of the gut by the intestinal microflora will result in major changes in epithelial gene expression and the pro-inflammatory transcription factor NF- κ B plays a central role in directing many of these changes in gene expression.

The NF- κ B family of transcription factors is a key player in controlling both innate and adaptive immunity. (reviewed in (Li and Verma, 2002)). The family include p65 (RelA), NF- κ B1 (p50;p105), NF- κ B2 (p52;p100), c-Rel and RelB (Verma et al., 1995) (Ghosh et al., 1998). All five members share the evolutionary conserved rel homology domain, a 300 amino acid long region that harbours the dimerization, nuclear localisation and DNA binding domains. The individual members form homo- and heterodimers (excluding RelB) with one another. The c-Rel, RelB and p65 proteins all have a transactivation domain in their C-terminus, which strongly activates transcription from NF- κ B sites in target gene promoters. The p50 and p52 proteins are generated by proteolytic cleavage of their precursors, p105 and p100 respectively (Ghosh et al., 1998) (Li and Verma, 2002). The most common transcriptional complex consists of a p65:p50 heterodimer and these subunits are expressed in a wide variety of cell types. Targeted disruption of all five members has revealed distinct roles of the NF- κ B proteins in innate and adaptive immunity, lymphocyte function and cell survival. Mice that lack the p65 subunit die during embryogenesis due to massive liver degeneration. Knocking out any of the other four subunits results in immunodeficient phenotypes at varying degrees, but without developmental defects and double knockouts have more severe phenotypes indicating redundancy between the members (Li and Verma, 2002).

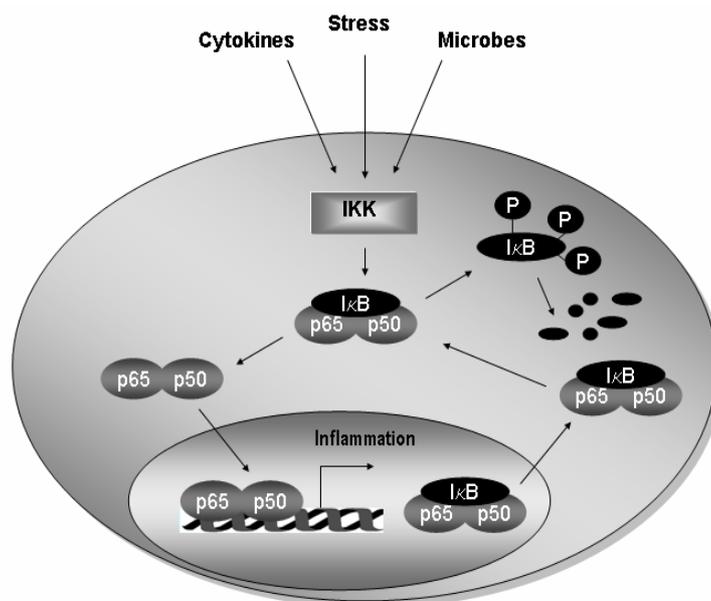


Fig 3. NF-κB activation. Various stimuli induce transcription of NF-κB (here represented by p65/p50) target genes by facilitating nuclear import of NF-κB and binding to cognate DNA sites. In unstimulated cells, p65/p50 is inhibited by I-κB.

In unstimulated cells the activation of NF-κB is controlled via interactions with the inhibitory proteins IκB α , β - or ϵ . These proteins are characterised by the presence of ankyrin repeats that mediate protein-protein interactions (Verma et al., 1995). Several stimuli are capable of eliciting NF-κB activation, including PRR signalling, stress signals and pro-inflammatory cytokines, just to mention a few. Upon activation, IκB become phosphorylated, ubiquitinated and subsequently degraded by the 26S proteasome. This results in the release of the NF-κB proteins allowing them to translocate into the nucleus and bind their cognate sites. The phosphorylation of IκB is mediated by a multiprotein complex called I-κB kinase (IKK). The IKK complex consists of two kinase subunits, IKK α and β , and one regulatory subunit, called NEMO or IKK γ . The IKK complex is a converging point for activation of NF-κB by a large number of stimuli (Li and Verma, 2002). Historically, it was considered that IκB retained NF-κB in the cytoplasm by masking its nuclear localisation signal. However, recent data implies a more regulated control of nuclear import and export seems to play an important role in the initial and sustained activation of NF-κB responses. In addition, accumulating evidence also demonstrates that activity of NF-κB is regulated by direct modification of the NF-κB proteins through phosphorylation and possible acetylation. For example, protein Kinase A mediated phosphorylation of p65 has been shown to be important for its transcriptional activity (Zhong et al., 1998).

Nuclear Receptors

Nuclear receptors (NR) comprise the largest evolutionary conserved family of ligand activated transcription factors, found in a variety of animal kingdoms from nematodes to man. The ligands for the NRs include small hydrophobic signalling molecules such as steroids, hormones, certain vitamins and metabolic intermediates. These ligands enter or are generated within the cell where they encounter and bind their cognate receptor leading to regulation of target gene expression (Laudet, 2002). The ligands, being lipophilic, easily pass through the

plasma membrane. In addition to the classical nuclear receptors with known ligands, a set of receptors have been identified that are referred to as “orphans” since no endogenous ligand have been found (Giguere, 1999) (Kastner et al., 1995). Many of these orphan receptors have now been adopted as their natural and synthetic ligands have been identified.

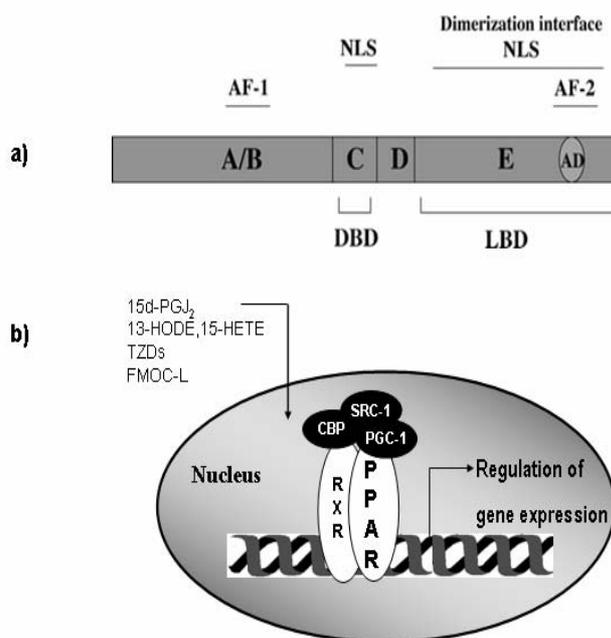
To date, 49 different nuclear receptors have been identified in the human genome, including receptors for fatty acids, retinoic acid, thyroid hormone, androgen and estrogen, vitamins A and D and many more (Francis et al., 2003). With a few exceptions, all NRs share a common modular structure with functionally different domains, including an less conserved amino-terminal domain (NTD), a central DNA binding domain (DBD) and a ligand binding domain (LBD) in the carboxy terminal (Laudet, 2002). Two transcriptional activation functions are present in most NRs, one ligand-independent located in the N-terminus (AF-1) and a ligand-dependent activation function called AF-2 placed in the highly structured ligand binding domain. The binding of a ligand results in a conformational change in the AF-2. In general, unliganded NRs form a complex with corepressors, such as NcoR and SMRT (Francis et al., 2003), which inhibit their transcriptional activity. This is often achieved through the recruitment of histone deacetylases (Glass and Rosenfeld, 2000). The conformational change induced upon ligand activation results in dissociation of these corepressors allowing the recruitment of coactivator complexes that facilitate target gene transcription (Glass and Rosenfeld, 2000). The specificity of the coregulator complex depends on a number of factors such as the ligand and the promoter and cellular context and is responsible for fine-tuning the physiological responses to a particular receptor-ligand interaction in a particular and/or variable environment. Nuclear receptors can bind their cognate response elements as monomers, homodimers or heterodimers with the retinoic X receptor (RXR). Most steroid hormone receptors such as ER and GR bind as homodimers whilst the nuclear receptors involved in metabolism (e.g. the PPARs, the Liver X Receptor and the Pregnane X Receptor) all form obligate heterodimers with RXR and binding of either ligand stabilises the interaction (Laudet, 2002).

Peroxisome Proliferator Activated Receptor γ (NR1C3)

The first peroxisome proliferator activated receptor (α) was identified in 1990 by Isseman and Green (Isseman and Green, 1990) as the receptor for the first lipid lowering drugs (Thorpe and Waring, 1962) (Hess et al., 1965). PPAR α ligands cause proliferation of the cell organelles peroxisomes in rodents and although this is not seen in humans the name has remained (Desvergne and Wahli, 1999). PPAR α was shown to transactivate a number of genes controlling fatty acid oxidation (Isseman and Green, 1990) and later PPAR β/δ and γ were cloned and identified as structural homologs controlling expression of other metabolic genes but not inducing peroxisome proliferation (Graves et al., 1992) (Dreyer et al., 1992) (Schmidt et al., 1992). All three members of the PPAR subfamily are activated by naturally occurring fatty acids or fatty acid derivatives and can be considered to be “metabolic nuclear receptors”, acting as metabolic sensors enabling the organism to adapt quickly to environmental changes by inducing the appropriate metabolic genes and pathways. The PPARs belong to the nuclear receptor family NR1C and of the PPARs, PPAR γ (NR1C3) is the most studied isoform (Laudet, 2002). This receptor participates in biological pathways of intense basic and clinical interest, such as insulin sensitivity, type 2 diabetes, atherosclerosis and cancer.

Fig4.a) Modular structure of the nuclear receptors.

The functional domains of NRs, including a less conserved, amino-terminal domain (NTD), a central DNA binding domain (DBD) and a ligand binding domain (LBD) in the carboxy terminal. Two transcriptional activation functions are present, one ligand-independent located in the N-terminus (AF-1) and a ligand-dependent activation function called AF-2 placed in the highly structured ligand binding domain.



b) RXR/PPAR γ DNA binding to a response gene.

PPARs bind DNA as a heterodimer with RXR and functional PPAR response elements are core motifs arranged as direct repeats spaced by one nucleotide (DR-1). The natural ligands include fatty acids and their derivatives such as 15d-PGJ₂, 13-HODE and 15-HETE. Synthetic PPAR γ ligands include the thiazolidinediones (TZDs) and FMOC-L-leucine.

Genetics

The human PPAR γ gene extends over more than 100 kb of the chromosome 3p25 and consists of nine exons (Fajas et al., 1997). Alternative promoter usage and splicing generates three different isoforms, PPAR γ 1, γ 2 and γ 3 (Auwerx, 1999). All PPAR γ mRNAs share the six 3' coding exons, but differ in their 5' region. (Zhu et al., 1995) (Fajas et al., 1997) PPAR γ 2 has a γ 2-specific exon encoding both the 5' untranslated region and 29 (30 in mice) additional N-terminal amino acids (reviewed in (Auwerx, 1999).

PPARs bind DNA as a heterodimers with RXR and functional PPAR response elements are core motifs arranged as direct repeats spaced by one nucleotide (DR-1) (Escher and Wahli, 2000). However, a recent report suggests that PPAR γ also binds DNA as a homodimer to a palindromic repeat spaced by 3 nucleotides (Pal3) when PPAR γ molecules outnumber RXR with a 30:1 ratio (Okuno et al., 2001). Since many cell types express more than one of the PPAR subtypes, the fine tuning of isoform specific targets are thought to occur through subtle sequence differences flanking the core element, the presence of specific coactivators and regulation of endogenous ligands (Rosen et al., 01).

Expression

PPAR γ 1 is highly expressed in adipose tissue, the colon and hematopoietic cells and in lower amounts in kidney, liver, muscle, pancreas and small intestine (Auboeuf et al., 1997) (Auwerx, 1999). PPAR γ 2 expression is confined to adipose tissue where it represents approximately 30 % of the PPAR γ population. Colon and macrophages are the only reported

tissues that express PPAR γ 3 (Fajas et al., 1998). Little is known about the regulation of PPAR γ protein levels but it has been suggested that protein levels are regulated by a ligand induced degradation of the protein via the ubiquitin-proteasome pathway (Hauser et al., 2000).

Activation of PPAR γ

The ligand binding pocket of PPAR γ is unusually large, approximately 1300 Å (Nolte et al., 1998; Uppenberg et al., 1998). This large ligand binding cavity is in part caused by the additional α -helix (H2') in the LBD of PPAR γ and results in a more accessible pocket which could explain the observed ligand promiscuity of PPAR γ (Auwerx, 1999). The natural ligands of PPAR γ are debated but are generally considered to include fatty acids and their derivatives such as 15-deoxy-delta12,14-prostaglandin J₂ (15d-PGJ₂) the major metabolite from prostaglandin D₂ derived from metabolism of arachidonic acid (Forman et al., 1995). 13-HODE and 15-HETE are other examples of eicosanoids activating PPAR γ (Rosen and Spiegelman, 2001). The most extensively studied of the synthetic PPAR γ ligands are the thiazolidinediones (TZDs), a class of drugs called the "glitazones" which are currently used in the clinical management of type II diabetes (Hulin, 1996) (Lehmann et al., 1995). Additional synthetic ligands include the FMOC-L-leucine (Rocchi et al., 2001) and certain non-steroidal anti-inflammatory molecules (Lehmann et al., 1997). However, ligands are not the only way to modulate activation of PPAR γ . Phosphorylation of serine 82 of γ 1 or 112 of γ 2 by members of the mitogen-activated protein (MAP) kinase family recruits corepressors such as SMRT, which reduce receptor activity in most but not all studies (Zhang et al., 1996) (Hu et al., 1996).

Target genes induced include those involved in growth regulatory pathways, lipid transport and storage, colon differentiation and immune modulation. Several crucial genes for adipogenesis is regulated by PPAR γ including, lipoprotein lipase, acyl coenzymeA synthase and fatty acid transport protein (for review see (Gelman and Auwerx, 1999) (Francis et al., 2003).

Similar to other nuclear receptors, the activity of PPAR γ is controlled by interactions with various co-factors. In an inactive state PPAR γ is complexed with co-repressors such as NCoR. Upon ligand binding conformation changes lead to release of the repressors and activated PPAR γ can bind co-activators. Many co-activators have been identified including SRC-1, TIF-2 and CBP/p300 (Gelman et al., 1999). In addition, a new nuclear receptor co-activator was identified and named PGC-1 for PPAR γ coactivator-1 (Puigserver et al., 1998). As an explanation to the diverse biological actions of ligand activation of PPAR γ , ligand type specific interactions have been suggested. Koderá et al showed that the endogenous ligand 15d-PGJ₂ induced a different set of co-activator interactions compared to the synthetic ligand Troglitazone (Koderá et al., 2000).

PPAR γ and control of adipogenesis and Glucose homeostasis

Adipose tissue is composed of adipocytes which store energy in the form of triglycerides and release it as free fatty acids. Together with muscle, adipose tissue is the major regulator of energy homeostasis in the body. PPAR γ is highly expressed in adipose tissue and is required for its development. Several lines of evidence support that PPAR γ is the master regulator of differentiation and energy storage by adipocytes (Auwerx et al., 1996) (Debril et al., 2001). Mice with a targeted deletion of the PPAR γ gene die in utero due to a placental defect (Barak

et al., 1999), but heterozygote mice are resistant to obesity and are more insulin sensitive (Miles et al., 2000) (Kubota et al., 1999). Patients with loss-of function mutations have lower body-mass index, greater insulin sensitivity and improved lipid profile (Deeb et al., 1998) (Altshuler et al., 2000). In contrast, gain of function mutations, for example the rare pro115ala mutation affecting the phosphorylation by MAP kinase on serine 114 renders the carriers of this mutation obese and insulin resistant (Ristow et al., 1998).

PPAR γ has also major effects on glucose homeostasis, a process that on a whole-body level involves several tissues such as fat, muscle and pancreas. Adipose tissue is required for proper glucose homeostasis, absence leads to severe insulin resistance. Classical PPAR γ agonists induce not only adipose differentiation, but also improve glucose control (reviewed in (Francis et al., 2003).

PPAR γ in colon

In both humans and rodents the levels of PPAR γ in the colon are nearly equivalent to that found in adipocyte tissue (Fajas et al., 1997). The expression has been reported to be higher in the distal parts of the colon than in the more proximal parts and the small intestine. The expression pattern in the colon mucosa is consistent with the proposed role of PPAR γ in differentiation since expression is primarily localised to the postmitotic, differentiated epithelial cells facing the lumen (Lefebvre et al., 1999). In addition, colon cancer cell lines and tumours and polyps derived from colorectal cancers often contain high levels of PPAR γ (DuBois et al., 1998) (Sarraf et al., 1998) and its expression have been shown to increase upon and induce differentiation of colon cells (DuBois et al., 1998) (Lefebvre et al., 1999). Several target genes of PPAR γ has been suggested in the colon, almost all involved in cellular differentiation (Gupta et al., 2001).

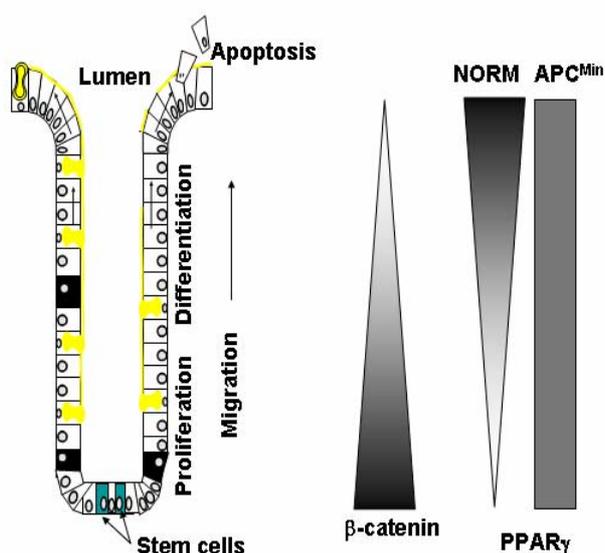


Fig 5. Expression of β -catenin and PPAR γ in the colon epithelium.

Schematic outline of a colon crypt. Stem cells are located at the bottom of the crypt and migrate up through zones of proliferation to their final destination as terminally differentiated cells on the top where they preferentially die by apoptosis. Also shown are expression levels of β -catenin and PPAR γ . Expression of PPAR γ is shown for both wild type and the APC^{Min} mice.

PPAR γ in immunecells

Macrophages express PPAR γ and it has been shown that expression of PPAR γ increases from bone-marrow derived resting monocytes to activated macrophages ((Ricote et al., 1998b). Moreover, studies in human and murine atherosclerosis (AS) lesions demonstrate that PPAR γ is highly expressed in macrophage foam cells (Ricote et al., 1998a) (Nagy et al., 1998) suggesting a role in development of AS. Recent reports also show that dendritic cells

express PPAR γ and that ligand activation influence maturation. Furthermore, PPAR γ is expressed in both B and T cells of the adaptive immune system (reviewed in (Daynes and Jones, 2002)).

In sickness and in Health

Inflammatory Bowel Disease

The two main forms of the inflammatory bowel diseases (IBD), Ulcerative Colitis (UC) and Crohn's disease (CD) are characterised by chronic inflammation of the gastrointestinal tract with episodes of flare ups followed by periods of spontaneous or treatment-induced remission (Carty and Rampton, 2003) (Podolsky, 2002). Both diseases appear in the young population, the onset often occur during the early twenties (Järnerot, 1992). During the active periods of their disease patients suffer from diarrhoea, abdominal pain and blood in the faeces. There might also be weight loss and fatigue and in some cases anemia as a cause of the intestinal bleeding (Järnerot, 1992). Although these diseases share many clinical and pathological features, they also have markedly different characteristics (Bouma and Strober, 2003). In Ulcerative Colitis, the inflammation is limited to the colon and invariably involves the rectum. In cases where it does not extend in to the colon it is referred to as proctitis (Järnerot, 1992). In contrast, Crohn's disease can affect any part of the gastrointestinal tract, from the mouth to the anus (Carty and Rampton, 2003) (Järnerot, 1992) but most commonly the terminal ileum, cecum and colon (Bouma and Strober, 2003). Crohn's disease is characterised by the presence of normal bowel segments between affected areas, known as "skip lesions", in contrast to the continuous inflammation seen in ulcerative colitis (Bouma and Strober, 2003). The histology differs between the two diseases. Crohn's disease is a transmural inflammation that affects all layers of the bowel wall with dense infiltrations of macrophages and lymphocytes and the presence of granulomas is common. In contrast, the inflammation in ulcerative colitis only affects superficial layers of the mucosa with infiltration of granulocytes and lymphocytes. Ulcerations and crypt abscesses are often seen (Bouma and Strober, 2003). In, addition, extra-intestinal manifestations involving inflammation in the eyes, skin, liver and bile-ducts (Carty and Rampton, 2003) (Järnerot, 1992) can also be seen in some IBD patients. Also on the cellular level there are differences between the two forms of IBD. It has been suggested that the inflammation in Crohn's disease is mediated by a T helper 1 driven immune response with increased secretion of interleukin-12 (IL-12) and interferon- γ (IFN- γ), whereas ulcerative colitis is the result of mainly a T helper 2 response associated with increased production of IL-4, IL-5 and IL-13 (Strober et al., 2002) (Carty and Rampton, 2003).

Epidemiological and linkage studies suggest that genetic factors are an important factor in determining the susceptibility to IBD. Collectively, these findings lend support to the theory that susceptibility is inherited and that the genetic contribution is more important in Crohn's disease than in ulcerative colitis, but in addition reveal the complexity of IBD (Podolsky, 2002). Most notable is the recent identification of a susceptibility locus (IBD1) located on chromosome 16 associated to Crohn's disease. This locus was shown to contain the gene for NOD2/CARD15, a member of the NOD/Apaf-1 family (Pena, 2003).

The conventional treatment of IBD involve corticosteroids and aminosalicylates or surgical removal of inflamed parts of the intestine. In severe cases of ulcerative colitis a complete removal of the colon might be necessary (Järnerot, 1992). Both Crohn's disease and ulcerative colitis have a prevalence of 10-200 cases per 100 000 individuals in North America and Europe and disease incidence is the highest in developed and urbanised (and westernised)

countries. There is an increasing incidence of Crohn's disease whereas no clear trend is seen for ulcerative colitis (Bouma and Strober, 2003).

Although the aetiology behind IBD remain unclear, its pathology is gradually being revealed and is doubtless multifactorial (Sartor, 1995). The present hypothesis is that IBD is a result of an inappropriate and exaggerated mucosal immune response directed against constituents of the mucosal microflora normally present in the intestinal milieu (Sartor, 1995) (Sartor, 1997) (Bouma and Strober, 2003). The interaction of environmental factors with genetic susceptibility leads to inflammation in the gut mucosa (Carty and Rampton, 2003) (Sartor, 1997). The observation that, in general, experimental colitis does not develop when mice are kept in a germ-free environment strongly supports that the presence of the normal mucosal microflora is required to initiate and maintain the inflammatory reaction (Bouma and Strober, 2003) Consistent with this idea is the fact that various strategies to decrease the luminal bacterial contents, such as broad spectrum antibiotics, results in attenuation of inflammation in Crohn's disease (Sutherland et al., 1991). Increasing evidence suggests that aberrant expression of cytokines is one of the contributing factors for initiation and perpetuation of IBD (Mitsuyama et al., 2001) (Papadakis and Targan, 2000). Several studies have disclosed that levels of pro-inflammatory cytokines such as IL-1, IL-6, IL-8 and TNF α are elevated in patients with IBD. These cytokines recruit inflammatory cells and mount an immune response at the site of lesions (Mitsuyama et al., 2001). In line with this, a new cohort of therapies have been evolved that targets the inflammatory processes via inhibition of pro-inflammatory and augmentation of anti-inflammatory cytokines. Several studies in animal models have shown reduction of inflammation after cytokine treatment and based on these observations cytokine-targeted therapy for IBD have been initiated, including using anti-tumour necrosis factor (TNF)- α antibody or IL-10. In addition, targeting of transcription factors, such as NF- κ B, STATs and p38 MAPK, important for the production of the inflammatory cytokines have also been evaluated for therapeutic interventions (reviewed in (Mitsuyama et al., 2001).

NF- κ B and inflammation

The critical role of NF- κ B in acute and chronic inflammation is well established and most likely mediated by its ability to activate genes connected to the inflammatory process. NF- κ B activation has been implicated in diverse diseases such as rheumatoid arthritis, IBD, multiple sclerosis and asthma. Several studies have reported elevated NF- κ B levels in intestinal mucosa of IBD patients. For example Neurath et al described increased p65 activity in isolated mononuclear cells from the lamina propria of patients with Crohn's disease (Neurath et al., 1998) and Rogler et al demonstrated that NF- κ B is activated in macrophages and epithelial cells of inflamed intestinal mucosa of IBD patients (Rogler et al., 1998). Moreover, Ellis and co-workers observed elevated NF- κ B levels within large mononuclear cells in all layers of inflamed areas in Crohn's disease patients (Ellis et al., 1998) and Schreiber et al. showed that nuclear levels of p65 are increased in lamina propria biopsy specimens from Crohn's patients compared to normal controls and ulcerative colitis patients (Schreiber et al., 1998). In addition, IBD patients treated with steroids display lower NF- κ B levels in their mucosa (Ardite et al., 1998) and steroid resistant patients have high levels of NF- κ B in their intestinal epithelial cells (Bantel et al., 2002).

Several modes of action to inhibit NF- κ B activity has been proposed, including inhibition of I κ B degradation, glucocorticoid-mediated inhibition of NF- κ B and antisense treatment directed against subunits of NF- κ B. Indeed, local administration of antisense molecules

directed against p65 abrogated signs of experimental colitis and colitis in IL-10 knock out mice (Neurath et al., 1996).

PPAR γ and Inflammation

As mentioned earlier, activated macrophages express high levels of PPAR γ in mice (Ricote et al., 1998b) and in humans (Jiang et al., 1998) and PPAR γ was shown to be a negative regulator of macrophage activation. Th2 derived IL-4 can induce expression of PPAR γ via upregulation of 12/15-lipoxygenase that in turn generates endogenous PPAR γ ligands from linoleic and arachidonic acid (Huang et al., 1999). It has also been shown that activation of PPAR γ inhibits production of proinflammatory cytokines, such as TNF α , IL-1 β and IL-6 release from both monocytes/macrophages (Ricote et al., 1998b) (Jiang et al., 1998) and epithelial cells (Daynes and Jones, 2002). This feature of PPAR γ has been suggested to be mediated largely through the ability to trans-repress the activity of several transcription factors including NF- κ B and AP-1.

Immune cells of the adaptive immune system express PPAR γ . PPAR γ has been shown to inhibit expression of IL-2 after T cell activation via physical trans-repression of NF-AT and NF- κ B (reviewed in (Daynes and Jones, 2002) suggesting that PPAR γ could have a suppressive effect on the development of immune responses. Expression of PPAR γ is low in resting T cells and increases after T-cell activation (Harris and Phipps, 2001). As in macrophages, IL-4 upregulate expression of PPAR γ in T cells. IL-4 is an important cytokine for the development of a Th2 response and it has been shown that Th2 cells express high levels of PPAR γ (Yang, 2002), so it seems that PPAR γ can induce a Th2 skewed immune response. In addition, PPAR γ can cause apoptosis in T cells (Harris and Phipps, 2001). Although it is known that PPARs are expressed in B cells their functions in B-cell physiology is not fully elucidated. In the original report locating PPAR γ in B cells it was described that PPAR γ ligands have an antiproliferative and cytotoxic effect on B cells (Padilla et al., 2002).

The expression of PPAR γ in the colon together with the increasing evidence of PPAR γ mediated inhibition of inflammation have lead to an interest in PPAR γ function in intestinal inflammation. Ligands for PPAR γ have been shown to inhibit induction of pro-inflammatory cytokines in colonic epithelial cells through an NF- κ B-dependent mechanism (Su, 1999). Treatment with TZDs attenuates colitis induced by either oral administration of dextran sodium sulphate (Su, 1999) (Tanaka et al., 2001) or by intra rectal administration of TNBS (Tanaka et al., 2001) (Desreumaux et al., 2001). Ligand activation of the obligate heterodimer of PPAR γ , RXR, also attenuated colitis in the latter study (Desreumaux et al., 2001)). Mice heterozygous for the PPAR γ gene show increased susceptibility to develop chemically induced colitis adding further support of a role of PPAR γ in intestinal inflammation (Desreumaux et al., 2001) (Nakajima et al., 2001). A pilot study in humans have recently been performed which provide promise to the role of PPAR γ in therapeutic treatment of colitis (Lewis et al., 2001).

Studies of the anti-inflammatory actions of PPAR γ have been difficult since receptor independent effects of ligand stimulation have been reported (Chawla et al., 2001) (Patel et al., 2002). For example 15d-PGJ2 has been shown to inhibit multiple steps in the NF- κ B signalling pathway, both PPAR γ dependent and independent (Straus et al., 2000).

Colon Cancer

Colon cancer is one of the most common malignancies among populations in the westernised countries and one of the leading causes of death due to cancer worldwide (Greenlee et al., 2000). The incidence is considered to vary with environmental and dietary factors but is generally high in developed countries (Parkin et al., 1999). Colorectal tumours are thought to arise through a series of genetic “hits” in a set of tumour-suppressor genes and oncogenes (the so called “multi-hit” model) resulting in a gradual histological changes, the adenoma-carcinoma sequence. In general, an intestinal cell requires two essential features to develop into a tumour: it needs to acquire both a selective advantage to allow for initial clonal expansion and genetic instability to permit additional genetic hits responsible for tumour progression and malignant transformation (Fodde, 2002). Inactivation of APC seems to provide both these features.

Wnt signalling and Cancer

The *APC* gene can be considered to be the gene for colorectal carcinomas. Initially identified by positional cloning of the FAP (familial adenomatous polyposis) locus (Grodin et al., 1991) (Kinzler et al., 1991), germ-line mutations in this gene has been shown to be responsible for the development of this hereditary colorectal cancer (Fodde, 2002). As well as sporadic colorectal tumours (Nagase and Nakamura, 1993). Inactivation of APC seems to underlie both initiation of tumour development and the promotion in the intestine (FC). Almost all of the mutations found in the *APC* gene are insertions, deletions or nonsense mutations causing a stop codon that results in production of a truncated protein (Iwamoto et al., 2000) that is not able to bind axin and or β -catenin. In the digestive epithelium, β -catenin is membranous expressed along the crypt-villus axis, but nuclear accumulation is specifically found in the epithelial cells located at the bottom of the crypt corresponding to the proliferative compartment (van de Wetering et al., 2002) (Batlle et al., 2002) In contrast, APC is expressed in regions of the crypt where cell division has ceased and terminal differentiation is established (Midgley et al., 1997) (Deka et al., 1999). Target genes such as c-myc and cyclinD1 seem highly relevant in tumour formation because of their role in proliferation and differentiation (Fodde, 2002).

A role for APC in intestinal carcinogenesis has been further corroborated by the development of the *APC*^{Min} mouse model. These mice carry a nonsense mutation at codon 850 of the murine homolog of the *APC* gene, analogous to that seen in FAP patients and serve as a good model of FAP neoplasia (Su et al., 1992) (Moser et al., 1990). Homozygote mice die in utero but heterozygote animals are viable but develop multiple intestinal neoplasia (MIN) at an early age and seldom survive beyond 3 month of age (Gould and Dove, 1997) (Giles et al., 2003).

PPAR γ and Cancer

Whereas the importance of the Wnt/ β -catenin pathway and APC in carcinogenesis seems clear, the role played by PPAR γ in tumour formation is still uncertain and highly debated. Early studies suggested that PPAR γ controlled adipocyte differentiation since ectopic expression of PPAR γ caused both fibroblasts (Tontonoz et al., 1994) and muscle (Hu et al., 1995) cells to undergo adipocyte differentiation. Current evidence suggests that PPAR γ can induce differentiation pathways beyond adipocytes. For example, ligand activation inhibits the proliferation of epithelial cells derived from breast, prostate, stomach and lung (Gupta 16-

19). Consistent with expression pattern of PPAR γ in the colon epithelial lining, exposure of cultured human colon cancer cells to PPAR γ ligand induces growth inhibition and cellular differentiation (Sarraf et al., 1998) (DuBois et al., 1998). However, while the effect of PPAR γ ligand stimulation *in vitro* seems clear, whether or not PPAR γ ligand exerts anti-neoplastic effects *in vivo* remains controversial. Troglitazone has been shown to inhibit tumour growth in a xenograft model of colon cancer (Sarraf et al., 1998) and to reduce the formation of aberrant crypt foci (ACF) secondary to azoxymethane treatment (Tanaka et al., 2001) (Osawa et al., 2003). In stark contrast to these results, two studies in APC^{Min} mice showed that PPAR γ ligands failed to suppress polyp formation, on the contrary, it lead to a small but significant increase in colon polyp formation (Saez et al., 1998) (Lefebvre et al., 1998). The increase was seen only in the number of colon polyps consistent with the expression of PPAR γ being low in small intestine. It is worth mentioning that no increase in polyp formation could be seen in wild type mice.

Aims of the Study

The purpose of the studies presented in this thesis has been to elucidate important functions of the nuclear receptor PPAR γ in the colon epithelium. The specific aims have been:

- i. To determine how intestinal bacteria and their signalling effect PPAR γ regulation during inflammatory bowel disease and to evaluate expression of this nuclear receptor in these diseases in parallel with known factors important for the inflammation
- ii. To identify potential molecular mechanisms for the observed contribution of non-pathogenic intestinal bacteria to immune homeostasis
- iii. To identify novel interaction factors for PPAR γ that could shed light on the proposed role of PPAR γ in colon carcinogenesis

Results

Impaired expression of Peroxisome Proliferator Activated Receptor- γ in ulcerative colitis (Paper I)

The intestinal epithelial cell responds to a bacterial infection, in part, via signalling through the TLR4 receptor and exerts its role by controlling expression of many pro-inflammatory molecules. In order to avoid a sustained response a negative feed back loop could be a resolution. Recent *in vitro* studies have shown that PPAR γ can inhibit NF- κ B activation and as a result suppress the production of cytokines and ligand stimulation of PPAR γ can attenuate experimental colitis when administered *in vivo* (Su, 1999) (Desreumaux et al., 2001). Supporting a role for PPAR γ in intestinal homeostasis is the fact that PPAR γ +/- heterozygous mice exhibit increased susceptibility to develop induced colitis (Desreumaux et al., 2001). It has been suggested that the commensal flora (CF) possesses the ability to attenuate activation of the innate immune system. We speculated that the elevated levels of NF- κ B that has been reported in IBD patients may be explained by the lack of inhibitory signals resulting in an uncontrolled inflammation. Our hypothesis was that the anti-inflammatory capacity of PPAR γ lies in its ability to impose a negative feedback loop on NF- κ B activation, possibly via TLR4.

We first set out determine if TLR4 could regulate levels of PPAR γ . In the colon carcinoma cell line CaCo-2 stimulated with LPS for 24 hours, PPAR γ mRNA expression increased approximately 3-fold as monitored by RNase protection assay (RPA). These data were confirmed using a constitutively active construct of TLR4, dubbed mCD4/toll. This is a chimeric construct in which the extracellular portion of the mouse CD4 receptor has been fused to the transmembrane and intracellular parts of human TLR4 (Medzhitov et al., 1997). Transient transfections with this construct resulted in increased PPAR γ mRNA similar to the wt-TLR4 results. The fact that the increase is seen after stimulation for 24 hours implies that the regulation might be posttranscriptional rather than direct. The elevation of PPAR γ levels also resulted in increased transcriptional activity of PPAR γ since co-transfections with a PPRE and mCD4/toll resulted in a modest increase in reporter gene activity. This increase could be suppressed by the introduction of a dominant negative IKK β . We continued to examine the bacterial influence on PPAR γ *in vivo*. For this purpose we analysed PPAR γ expression by immunohistochemistry (IHC) performed in colon biopsies from germ free (GF) mice and mice colonised with commensal flora (CF) or humanised flora (HF). PPAR γ expression was barely detectable in colonic tissue from GF mice but colonisation with CF or HF dramatically increased expression selectively at the mucosal surface. Additionally, IHC performed in colonic specimens from TLR4-mutant mice displayed virtually undetectable expression of PPAR γ .

In patients with CD but not UC, the site of chronic inflammation is coupled to accumulation of intra-abdominal fat (Berliner et al., 1982). The lack of mesenteric fat suggested to us that levels of the adipocyte regulator PPAR γ could be different in UC versus CD patients. By the use of RT-PCR and RPA we quantified levels of mRNA in colonic biopsy samples from UC and CD patients. We found that levels of PPAR γ mRNA were reduced in both patient groups compared to controls but that in UC patients the expression was severely impaired. Western blot analysis confirmed that also protein levels of PPAR γ were reduced in UC. We next set out to determine the cellular source of the reduced expression. Again using IHC we could show that as previously reported (Mansen et al., 1996) expression was mostly confined to

epithelial cells facing the lumen in controls and CD patients and, in contrast, biopsy samples taken from UC patients showed no detectable PPAR γ in the epithelial layer. These data were confirmed by western blot analysis of purified epithelial cells. Interestingly, when PPAR γ expression was assessed in peripheral blood monocytes extracted from control and IBD patients we detected almost comparable levels of PPAR γ in all groups. In addition, no previously unknown mutations could be found in the PPAR γ promoter or gene explaining the impaired expression. Hence, epigenetic events may be involved in deregulating PPAR γ in colon epithelial cells.

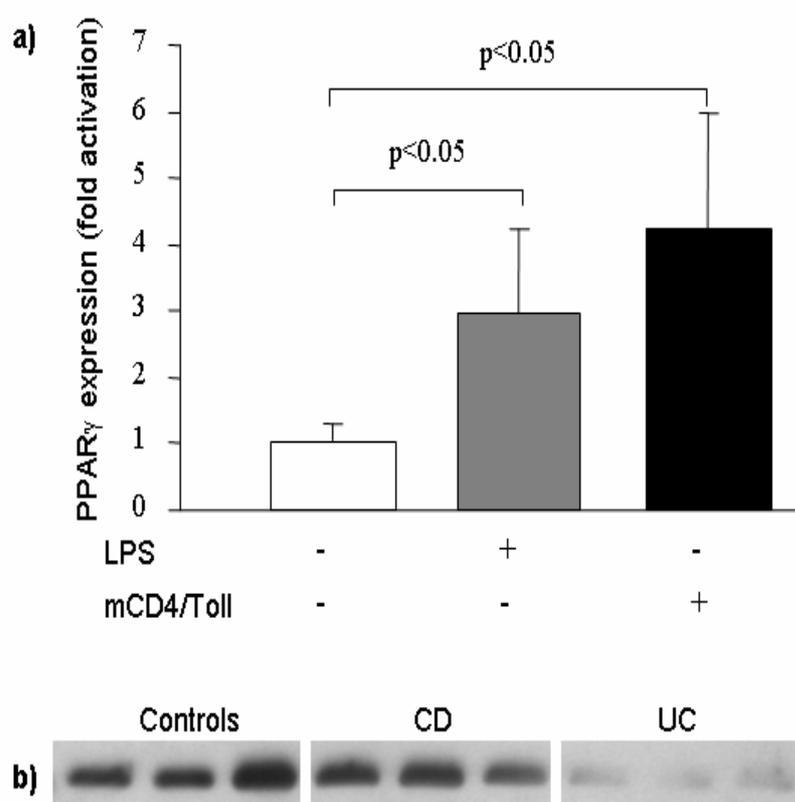


Figure 4. a) Modulation of PPAR expression. Increased expression of PPAR γ mRNA analysed by RPA and compared to an internal standard after stimulation of Caco-2 cells with LPS (50 ng.ml $^{-1}$) or transfection with the constitutively active TLR-4 (mCD4/Toll). **b) Impaired PPAR γ protein levels in UC patients.** PPAR γ protein levels were determined by Western blot analysis. Protein extracts were prepared from whole non-inflamed colonic mucosa of patients with UC, CD and biopsies obtained from healthy control patients. Immunodetection of PPAR γ protein (approximately 57 kDa) in the colon of three representative patients with UC, CD and controls.

In conclusion, in paper I we show that the intestinal flora, via TLR4, can regulate levels of PPAR γ . Moreover, we show that the expression of PPAR γ is severely impaired in the epithelial lining from patients with the inflammatory bowel disease Ulcerative colitis.

Commensal anaerobic gut bacteria attenuate inflammation by regulating nuclear-cytoplasmic shuttling of PPAR γ -RelA (Paper II)

We continued our investigation on the proposed role of commensal bacteria in controlling gut homeostasis. Considerable clinical and experimental evidence links immune responses directed against the normal intestinal microflora to the pathogenesis of inflammatory bowel diseases (Sartor, 1997). Recently, non pathogenic gut bacteria have been implicated to participate in this process (Neish et al., 2000). The hypothesis behind this study is that commensal bacteria influence inflammation by modulating activation of the innate immune response mounted by epithelial cells.

To investigate this we examined the effects of *Bacteroides thetaiotaomicron*, a prevalent anaerobic commensal bacterium of the gastrointestinal tract, on the acute inflammation triggered by the pathogenic *Salmonella enterica*. Using cDNA microarray technology we identified several inflammatory genes that were upregulated by infection CaCo-2 cells with *S. enterica* and modulated by co-infection with *B. thetaiotaomicron*. Relevant inflammatory genes include TNF, IL-8, cyclooxygenase-2 (COX-2) and MIP-1 α . The results were confirmed by Northern blot analysis and real-time PCR. This attenuation by *B. thetaiotaomicron* was specific as a related aerotolerant strain *B. vulgatus* did not have the same ability. In addition, although the same effect could be seen after exposure of CaCo-2 cells to *E.coli*, phorbol 12-myristate 13-acetate (PMA), TNF and LPS the upregulation of IL-8 in CaCo-2 after IL-1 α and β was not affected by exposure to *B. thetaiotaomicron* indicating that the attenuation is not a universal event. The physiological relevance in inflammation *in vitro* and *in vivo* was established by measuring polymorphonuclear leukocyte (PMN) recruitment. *In vitro* this was measured by transepithelial migration through a CaCo-2 monolayer which confirmed that *B. thetaiotaomicron* can attenuate inflammation. *In vivo* we used “minimal flora” rats and measured PMN migration as well as performed IHC in colon of rats infected with *S. enterica* and *B. thetaiotaomicron* to confirm these results. Immunostaining of the NF- κ B subunit RelA in Caco-2 cells 2 hours after infection with *S. enterica* revealed nuclear accumulation of RelA. EMSA and super shift analyses showed that RelA was the prominent nuclear subunit. 30 and 60 minutes after co-infection with *S. enterica* and *B. thetaiotaomicron* the nuclear content of RelA was similar however, after 2 hours almost all RelB was found in the cytoplasmic compartment. Subsequent EMSA analysis showed that the effect of *B. thetaiotaomicron* seemed highly specific to RelA since another transcription factor, AP-1, was unaffected 2 hours after infection.

Live bacteria, possibly through bacteria-epithelia cell contact, were shown to be essential to mediate the attenuation since heat inactivated *B. thetaiotaomicron*, culture supernatant and conditioned medium from CaCo-2 cells infected with *B. thetaiotaomicron* were unable to repeat the attenuation.

The mechanism seems to be independent of I κ B because the kinetics of phosphorylation and degradation of I κ B was not changed after *S. enterica* infection in the presence or absence of *B. thetaiotaomicron*. We postulated that increased nuclear clearance of activated NF- κ B could be a possible explanation. In order to analyse if the cellular export of NF- κ B after infection with *B. thetaiotaomicron* occurred via Crm-1 (exportin 1), the only known pathway so far for NF- κ B/I κ B export (Huang et al., 2000), we inhibited this pathway with the inhibitor leptomycin B (LMB) (Kudo et al., 1999). However, LMB treatment did not influence nuclear export of NF- κ B or IL-8 expression induced by *B. thetaiotaomicron*.

The notion that PPAR γ can attenuate inflammation in a number of in vivo models (Su, 1999) (Nakajima et al., 2001) (Katayama et al., 2003) and the fact that PPAR γ and NF- κ B has been shown to form complex in solution suggested to us that this receptor could be involved in the mechanism of *B. thetaiotaomicron* induced cellular localisation of RelA. As with RelA, *S. enterica* exposure in CaCo-2 cells induced nuclear accumulation whereas co-infection with *B. thetaiotaomicron* induced nuclear export of PPAR γ . Furthermore and similar to RelA shuttling this export was not inhibited by LMB treatment. Co-immunoprecipitation experiments showed that RelA and PPAR γ can be found in the same protein complex after exposure of CaCo-2 cells to *S. enterica* and *B. thetaiotaomicron*. A direct interaction between these proteins were shown using in vitro translated proteins. However, a dominant negative form of PPAR γ , mutated in the AF-2 domain known to interact with co-factors, did not form protein complex with RelA. This dominant negative PPAR γ could not, in contrast to wild type PPAR γ , aid in the repression of se induced activation of a NF- κ B-luciferase reporter construct by *B. thetaiotaomicron*.

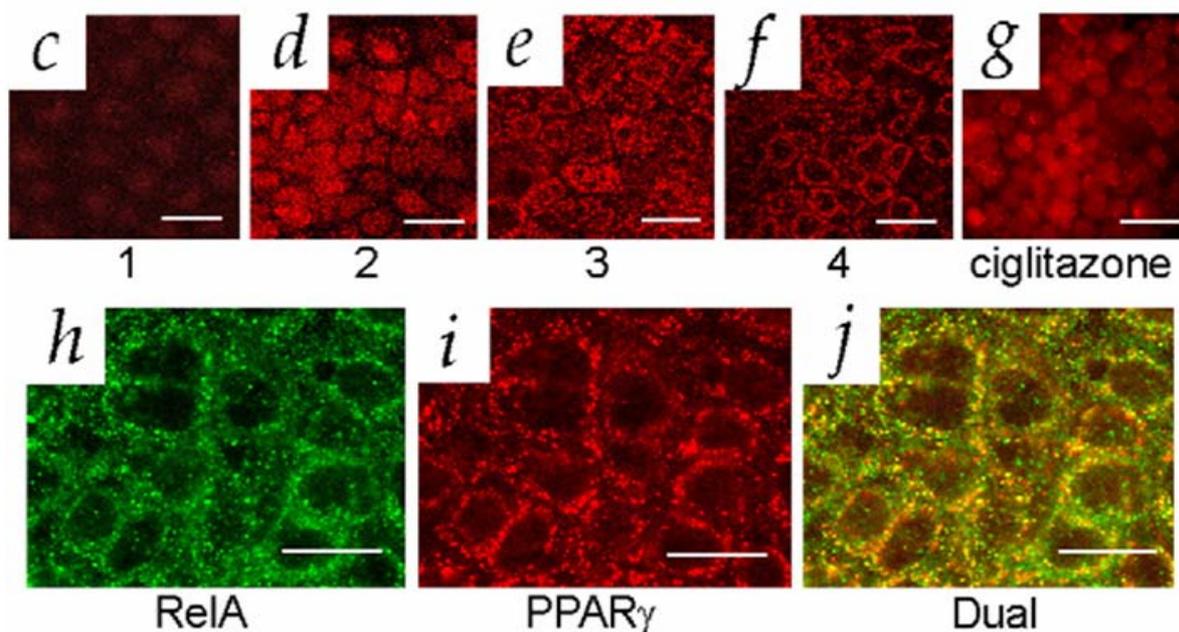


Fig 7. c-g) CaCo2 Cells infected with: 1) No bacteria, 2) *S. Enterica*, 3) *B. Thetaotaimicron*, 4) Co-infection with both *S. Enterica* and *B. Thetaotaimicron*. **g)** Stimulation with syntehtic ligand, ciglitazone, and stained with anti-PPAR γ **h-j)** Co-infection with both *S. Enterica* and *B. Thetaotaimicron*, Stained for PPAR γ and RelA as indicated

In order to further investigate the importance of PPAR γ in the nuclear export of RelA we took advantage of chimeric fluorescent constructs of wild-type and dominant negative PPAR γ linked to a cyan fluorescent protein (CFP) and RelA linked to yellow fluorescent protein (YFP). These results supported the co-localisation of the two proteins and the nuclear export induced after exposure of *S. enterica* infected CaCo-2 cells to *B. thetaiotaomicron*. Again, the dominant negative PPAR γ was impaired in nuclear export. RNA interference directed against PPAR γ reduced the levels of transfected PPAR γ -CFP but did not affect levels of RelA-YFP. Cells transfected with RNAi and exposed to the co-culture of *S. enterica* and *B.*

thetaitoamicon showed nuclear accumulation of RelA at 2 hours which was in contrast to the non-RNAi-transfected surrounding cells which showed mostly cytoplasmic RelA staining.

The study in paper II mainly entails the identification of a cellular mechanism to explain the observed ability of commensal intestinal bacteria to attenuate immune activation by pathogenic bacteria. We propose a model where commensal flora hinders a sustained NF- κ B response via PPAR γ linked nuclear export.

Levels of NF- κ B in inflamed mucosa from inflammatory bowel disease patients in parallel with low levels of PPAR γ and TRAIL (Paper III)

NF- κ B has been shown to be upregulated in intestinal biopsies from patients suffering from both Crohn's disease and ulcerative colitis (Schreiber et al., 1998) (Neurath et al., 1996) but previous studies have not been in agreement on the cellular source and levels of this elevation. Since we showed in paper I that levels of PPAR γ are impaired in colonic epithelial cells from UC patients this suggested to us that decreased levels of PPAR γ could correlate with exaggerated NF- κ B levels. Moreover, in normal intestinal mucosa, cells that reach the top of the crypt/villus are eliminated by apoptosis (Hall et al., 1994) and members of the death receptor family have been implicated in this process. It has been shown that the number of apoptotic cells is increased in the epithelium in UC patients suggesting that epithelial damage ultimately lead to decreased epithelial barrier function, allowing invasion of pathogenic bacteria. We speculated that levels of the apoptosis inducing ligand TNF related apoptosis inducing ligand (TRAIL) was differentially regulated in IBD.

19 patients with CD and 25 patients with UC were enrolled in our study. EMSA analysis showed, in line with previous studies (Schreiber et al., 1998) (Neurath et al., 1996), elevated levels of NF- κ B in inflamed colon mucosa. To circumvent epigenetic events influencing the activation of NF- κ B we compared inflamed areas with non-inflamed areas from the same individual. In contrast to a prior study, we detected prominent levels of NF- κ B in biopsies from UC compared to CD, both in inflamed tissue and non-inflamed. We next wanted to shed some light on the cellular source of the elevated levels since earlier studies have not been consistent. NF- κ B was here detected in both cells of the epithelium as well as cells scattered throughout the lamina propria. These cells, as judged by morphological appearance, resembled macrophages.

In agreement with our previous finding (paper I) we could only detect PPAR γ expression using IHC in approximately 1/10 of the colonic biopsies from UC patients whereas in CD patient material almost all stained positive with the PPAR γ antibody. Both inflamed and non-inflamed areas showed the same staining pattern. However, low level of PPAR γ did not correlate with increased level of NF- κ B. Thus, the negative feedback loop proposed in paper I is not as straightforward. It might still be there but require additional steps. Evaluation of TRAIL expression showed no difference in levels and cellular source between UC and CD colonic specimens, however, the levels were decreased in the inflamed areas of both diseases implying that a pathogenic regulation of TRAIL expression might exist.

Our studies corroborate the previous notion that NF- κ B is elevated in inflamed colon mucosa of IBD patients. However, we show that the regulation of NF- κ B is not a direct target of

PPAR γ since the levels of these proteins do not correlate. In addition we link the apoptosis inducing ligand TRAIL to chronic inflammation.

A functional link between Wnt signalling/ β -catenin and the Peroxisome Proliferator Activated Receptor- γ (Paper IV)

PPAR γ and β -catenin are both expressed in the colonic epithelium although at inverse expression patterns reflecting their apparent opposing effects on cell fate. The role of oncogenic activation of β -catenin in colon cancer seems firmly proven but the outcome of PPAR γ stimulation *in vivo* is on the other hand controversial. The report of altered expression pattern of PPAR γ in the intestinal epithelial cells in the APC^{Min} mice, where expression was not only confined to differentiated cells on the luminal surface (Saez et al., 1998) as in the wild type, suggested to us that aberrant expression of either β -catenin or PPAR γ could be responsible for the reported discrepancy in PPAR γ 's role in tumour transformation. We set out to determine if these two proteins interacted, both physically and functionally.

Histological samples from the colon of APC^{Min} mice were assessed by IHC using a PPAR γ specific antibody. Colonic tissue from the APC^{Min} mice stained more prominently than tissue from wild type mice. In addition, splenocytes from both mouse strains were also immunostained with PPAR γ antibody and we could demonstrate a 25 % increase in number of intensely stained cells in the APC^{Min} mice. We next analysed if PPAR γ and β -catenin could interact physically. Co-immunoprecipitation experiments performed in a colon carcinoma cell line (SW480) showed that β -catenin was co-precipitated in a protein complex with PPAR γ . Transient transfections with a FLAG tagged PPAR γ construct and subsequent immunoprecipitation with a FLAG antibody confirmed these results. Having established an interaction between these two proteins we moved forward to study if this interaction could stabilise the PPAR γ protein. Western blot analysis after transfection with a phosphorylation deficient β -catenin in the SW480 cell line revealed an increase in levels of PPAR γ protein. The mutation in Ser33 of β -catenin stabilises cytoplasmic and subsequent nuclear levels of β -catenin by making β -catenin degradation resistant (Hsu et al., 1998). These data were also confirmed in another epithelial cell line, HEK293 which contain low levels of PPAR γ and wild type APC. RPA analysis and co-transfection with luciferase reporters of the γ 1 and γ 2 promoters showed that the increased PPAR γ protein levels after over expression of β -catenin were not mediated on a transcriptional level. We next assessed whether over expression of β -catenin influenced transcriptional activity of PPAR γ . Co-transfections of β -catenin and a luciferase linked PPAR-response element (PPRE) showed a clear dose dependent elevation of reporter gene activity compared to the PPRE alone. This elevation could be repressed by a dominant negative form of PPAR γ (Gurnell, 2000). EMSA with a radio labelled PPRE showed increased DNA binding of PPAR γ following increased β -catenin levels. LiCl is an established agonist to mimic Wnt signalling via its ability to repress GSK-3, thus stabilising protein levels of β -catenin (Stambolic et al., 1996). Using transient transfections, IHC and western blot we could illustrate that LiCl induced levels of β -catenin both functionally activate transcriptional activity of PPAR γ and elevated PPAR γ protein levels.

Working as downstream repressors of Wnt signalling, the Groucho/TLE co-repressors bind members of the Tcf family and repress transcription of Wnt target genes (Chen and Courey, 2000). We wished to examine if the β -catenin mediated activation of a PPRE driven luciferase could be regulated by these repressors. Co-transfections in HEK293 demonstrated that both

Grg4/TLE4 and the shorter AES/Grg5 repressed the PPRE driven reporter gene when activated by β -catenin, but interestingly not after activation with the PPAR γ synthetic ligand Rosiglitazone, implicating different complex formation after the two stimulations.

The main finding of paper IV is the identification of a physical and functional interaction between PPAR γ and the important protein of the canonical Wnt signalling pathway, namely β -catenin.

General Discussion

The importance of context in transcription has recently gained greater prominence. Cell and promoter specific factors, as well as kinetics and packaging of DNA result in a temporal and spatial control over gene expression. One example illustrating the functions that can be mediated by an individual factor is the nuclear receptor PPAR γ . What fascinates me with a protein like PPAR γ is its pleiotropic functions, as one might expect from a genome that “only” encodes for 30 000 genes and not the earlier proposed 100 000 (Venter et al., 2001). So, fewer workers have to do more work. But how is specificity maintained? The expression of PPAR γ in several tissues in combination with tissue specific co-workers and highly regulated temporal and spatial expression offers a possible molecular explanation. Looking through the literature of the PPAR field one gets the feeling that a considerate disagreement exists. Often an effect seen in a specific cell type/species can not be repeated or is even opposed by studies in another cell type/species. The discrepancy between the effect of PPAR γ ligand stimulation observed in different cell types could maybe be explained by a recent study by Gupta et al. (Gupta et al., 2003) where they identify a previously unknown mutation in the ligand binding domain of PPAR γ . This mutation seems to make colon cell lines harbouring the mutation resistant to ligand induced growth inhibition and differentiation. However, there was no deficiency of this mutant to induce adipocyte differentiation of fibroblasts and the authors suggest that the codon affected by the mutation is important for cell-lineage specific co-factor binding.

The presence of a metabolic receptor like PPAR γ in the intestinal milieu is not surprising. To use the same protein in control over nutritional metabolism and gut homeostasis is a way to integrate these two systems. In fact, it might give the organism a possibility to respond swiftly to changes in the bacterial flora with metabolic adjustments assuring a tight control over whole body homeostasis. So what we eat influences the bacterial flora which in turn influences what we can metabolize and the circle is closed. In this context, PPAR γ can be considered a sensor of environmental changes, influencing both development and maintenance of the intestinal tissues. It is fascinating that the body has evolved endogenous substances that can mediate control over inflammation via nuclear receptors. In situations where an organism is less capable to cope with an infection the homeostasis is protected by down regulation of the inflammation. For example during stress, glucocorticoids are released from the adrenal cortex, which then activates the glucocorticoid receptor that can inhibit activation of the pro-inflammatory NF- κ B. The suggested negative cross-talk between transcription factors with opposing functions (NF- κ B versus GR and PPAR α/γ) provides an organism with primary control mechanisms to maintain homeostasis when a host defence is activated. The signals that activate NF- κ B, such as bacterial infection, can at the same time activate anti-inflammatory pathways, in the context of this thesis mediated via a nuclear receptor, PPAR γ . The evolution of bacteria proceeded mammal development with eons of time and it is therefore not surprising that they have learned to control us rather than the opposite. The symbiosis has proven beneficial for us both. That luminal bacteria can exert anti-inflammatory actions has been reported earlier (Schesser et al., 1998). Control of inflammation can only be beneficial since a sustained immune response may result in chronic inflammatory disorders and increased clearance of bacteria. That this regulation is performed via TLR4 seems logical since these receptors recognise not only intruding bacteria and signals via NF- κ B to alert their presence, but also assures (via PPAR γ) that the activation is dampened. The data showing that induction of PPAR γ is not immediate and rather peaks after

approximately 24 hours suggests that the activation of the immune response gets a head start which is then followed by the cue to slow down.

In the same issue as we published paper I, a connected study was published. In line with our results Katayama et al. showed that experimentally induced colitis reduce expression of PPAR γ . Taken together, the fact that PPAR γ is impaired in colitis is consistent with the observation that PPAR γ ligands only attenuate colitis when administered before the onset of inflammation (Katayama et al., 2003). One can speculate that when colitis is full blown, the ligand stimulation of PPAR γ can do no good since levels of the receptor is reduced, making an anti-inflammatory effect impossible. However, there exists a discrepancy between the results in our study and the one by Katayama and colleagues, namely, the cellular location of the reduced PPAR γ expression. As mentioned in the results section, we find PPAR γ almost exclusively in the epithelial cells, supported by previous published data (Lefebvre et al., 1998). In contrast, Katayama et al. show a dramatic decrease in PPAR γ in lamina propria lymphocytes and macrophages isolated from DSS-treated mice, but not in epithelial cells. Maybe species differences and differences in experimental design can explain this controversy. The data showing that administration of PPAR γ ligands attenuates only inflammation in the superficial layers would indicate that the epithelial cells are the important mediators. Additional experiments are clearly warranted to solve this issue

The finding that the reduced expression is confined to the epithelial cells and not observed in PBMCs suggests that epigenetic events control the stabilisation of the PPAR γ protein. As mentioned in the introduction, ligand stimulation of PPAR γ is linked to subsequent degradation in adipocytes (Hauser et al., 2000). Such a mechanism could be present in colon epithelial cells. Aberrant expression of an endogenous ligand might be exclusively present in UC patients, or cellular co-factors present to stabilise PPAR γ in the differentiated cells of the crypt epithelium might be perturbed. The imbalance in expression of TLR4 and PPAR γ (paper I) implies that although levels of TLR4 are upregulated signalling to PPAR γ is impaired leading to reduced levels of PPAR γ .

That levels of PPAR γ in the colon are different in UC versus CD is interesting and provides further evidence that although they share many symptoms, the two diseases are different regarding aetiology. Patients suffering from Ulcerative colitis have an increased risk of developing colorectal cancers (Järnerot, 1992). The reduced level of PPAR γ that we observe in the epithelial lining in the colon mucosa of these patients makes it tempting to speculate that the absence of a proposed tumour suppressor gene might be a causative factor. It makes one wonder, will therapeutic treatment of these patients with PPAR γ ligands stabilise PPAR γ levels or does the absence of the protein result in that there is no effect of ligand stimulation?

Interestingly, both pathogenic *Salmonella* and non-pathogenic *Bacteroides* induced increased expression of PPAR γ protein but the cytosolic localization was quite distinct; nuclear in the context of *Salmonella* and cytoplasmic in the context of *Bacteroides* (paper II, figure 4b). These data are consistent with the observation in paper I that signalling via the TLR4 can upregulate levels of PPAR γ . PPAR γ has been considered a strict nuclear receptor, i.e. only expressed in the nuclear compartment. Results from both paper I and II contradict this and we detect PPAR γ expression in the cytoplasm. In order for the mechanism that we suggest in paper II to be correct, the cytoplasmic localisation is essential. Immunohistochemistry stainings in colon mucosa clearly demonstrates cytoplasmic localisation of PPAR γ . The data presented in paper II provides evidence of a novel mechanism for how PPAR γ can exert anti-

inflammatory actions and provide yet another link between gut bacteria and homeostasis. In addition to their important role in metabolism, the resident commensal bacteria assure their indispensable presence by communicating with the epithelial cells. Unfortunately, the delicate balance sometimes gets disturbed with the detrimental result being an inflammatory condition.

Recently, a set of nuclear receptors have been shown to interact with β -catenin, i.e. the androgen receptor (AR), raising the intriguing possibility that β -catenin function as a novel cofactor for these transcription factors. No nuclear localisation signal has been identified in β -catenin and the ins and outs of this protein are suggested to be controlled in part by the interaction with APC. However, nuclear shuttling of β -catenin has also been shown to occur in the absence of APC (Eleftheriou et al., 2001) and it is tempting to speculate of a mechanism involving nuclear receptors e.g. AR or PPAR γ . The association of β -catenin with these receptors would enhance nuclear import and stimulate transcription of cognate response genes. The inverse expression of PPAR γ and β -catenin in colon mucosa and their apparent role in differentiation of the epithelium suggested to us that they might interact. Indeed, in colon carcinoma cell lines they did (paper IV). A difference from our studies regarding PPAR γ and the studies involving AR is the ligand dependency reported in the latter studies (Truica et al., 2000) (Pawlowski et al., 2002). The presence of unidentified intracellular endogenous ligands might explain this feature of PPAR γ .

The reported controversy of the consequence of PPAR γ stimulation on tumour formation could possibly be explained by the interaction of PPAR γ and β -catenin. As Girnun et al recently showed, stimulation with PPAR γ ligands only affects the outcome in a cell with wild type APC (Girnun et al., 2002). PPAR γ activation would then only be beneficial before such mutations, before nuclear levels of β -catenin reaches a threshold, potentially overriding the normal PPAR γ function. The aberrant interaction of β -catenin with PPAR γ enhances nuclear localisation of the two proteins in terminally differentiated epithelial cells facing the lumen. In these cells, which normally contain low levels of β -catenin in their nuclear compartment, increases levels of β -catenin would activate target genes resulting in the re-entry of the cells into the cell cycle and aberrant proliferation. Again, the fact that polyps have been shown to stem from the cells in the top of the villi supports this. Also interesting is the observation that PPAR γ can stimulate terminal differentiation and apoptosis in almost all tissues examined. As far as I know, the debate about PPAR γ is focused on its role in colon tissue and it is in colon that the tumour suppressor APC plays an important role, although it is expressed in a variety of tissues. Maybe the collision of these two pathways is the detrimental event leading to tumour formation? In support of this, APC^{Min} mice develop mammary tumours, another tissue that express Tcf-4 (Barker et al., 1999). Another interesting inlay in the debate is the paper from Wang et al where they show that the magnitude of synthetic PPAR γ ligand concentrations affect the outcome of stimulation. Concentrations in the μ M range, which are used in most studies, result in apoptosis. In contrast, nM concentrations lead to enhanced cell survival (Wang et al., 2002).

It is not a novel finding that NF- κ B activation is upregulated in the intestinal mucosa of inflammatory bowel disease patients. The functional outcome of this is not difficult to foresee, an exaggerated and aberrant proinflammatory cytokine production resulting in uncontrolled and sustained inflammation. However, the cell types responsible for this are not certain. We detected elevated levels of p65 in both epithelial cells and cells scattered throughout the lamina propria. Consistent with the important role that epithelial cells play in

defence against microorganisms, they will probably be likely targets for dysregulated gene expression. In being the outermost layer towards a luminal milieu inhabited with numerous bacteria, any change in bacterial contents or increased contact via adhesion of bacteria, these cells would be the first to react. If mechanisms to down regulate the initial indispensable activation of NF- κ B is missing, a sustained activation of NF- κ B can go out of control resulting in chronic inflammation. In paper I we show that levels of the suggested anti-inflammatory PPAR γ is impaired in colon mucosa of ulcerative colitis patients. The switch to turn the inflammation off is missing. However, life is never simple. We corroborate the findings of reduced PPAR γ expression in the epithelial cells but also show (paper III) that there is not a direct correlation between levels of NF- κ B and PPAR γ . The absence of PPAR γ does not automatically results in elevated levels of NF- κ B. How come? The negative feed back loop might still be there but not work in a direct manner. The kinetics can be completely different than what we are looking at. Another mechanism might be that additional nuclear receptors are present to inhibit activation of NF- κ B, revealing a functional redundancy between the nuclear receptors in the intestinal tract. It will therefore be of great interest to study the expression of NRs such as RXR in colonic mucosa. Ligand activation of RXR has in resemblance of the stimulation of PPAR γ the ability to attenuate experimental colonic inflammation (Desreumaux et al., 2001). Maybe a direct coupling between the two players is hindered by their apparent opposing role in apoptosis and a possible control of epithelial development since NF- κ B mainly is involved in a proliferative response while PPAR γ is considered to mediate differentiation.

Chronic inflammation of the intestinal mucosa has been show to increase permeability of the epithelial lining (Den Hond et al., 1998) (Schmitz et al., 1999). This might be a consequence of the inflammation since polymorpho nuclear cells (leukocyte) transmigration across the epithelium has *per se* been suggested to increase permeability. On the other hand, there are speculations that the increase in permeability is a causative event and preludes the inflammation since luminal bacteria then get the opportunity to cross the epithelial border and activated the underlying immune system (Sartor et al., 1997). During the course of inflammation, an increased damage of the intestinal epithelial lining can be observed. To explain this several mechanisms have been proposed including activation of apoptosis by members of the TNF family (Strater et al., 1997) (Strater and Moller, 2000). We could demonstrate a differential expression of TRAIL in inflamed versus non-inflamed mucosa from the same individual (paper III). Since IBD is associated with an increased risk to develop colorectal cancer it is intriguing that levels are lower in epithelial cells of inflamed tissue. Could a reduced expression of TRAIL result in lower number of apoptotic cells in inflammation-damaged areas, ultimately resulting in survival of cells that should go into apoptosis? Thus, tumour formation might be the outcome of this. The model of intestinal carcinogenesis describing that tumour-transformation proceeds in a top-down pattern on the crypts (Shih et al., 2001) lends some support to this. Cells that would normally die as they reach their final destination in the colon crypts stay alive bearing genetically and epigenetically caused damage.

It is tempting to ask the question, do levels of NF- κ B correlate with the severity of disease? Are target genes downstream NF- κ B indispensable for maintaining homeostasis? And is the down regulation of the anti-inflammatory function of PPAR γ the physiological response needed to allow initiation of an immune defence against invading bacteria? What seems to be a delayed activation of PPAR γ by bacterial signaling could be a primary down regulation of PPAR γ followed by subsequent elevation of the protein to assure that the inflammation is

controlled. Is it that the mechanism perturbed in UC patients? Well, there are many questions to be answered in the future.

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