

**Peptidoglycan Recognition Protein 3:  
A novel Microbial Pattern Recognition Receptor  
Mediating Immune Regulation Depending on  
Microbial and Dietary Factors**

**DISSERTATION**

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*My lovely kids*

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## ABBREVIATIONS

<b><i>B. subtilis</i></b>	<i>Bacillus subtilis</i>
<b>BCFA</b>	Branched-chain fatty acid
<b>C15</b>	13-methylmyristic acid
<b>C17</b>	15-methylpalmitic acid
<b>Caco2</b>	Human colon adenocarcinoma cell line
<b>COX-2</b>	Cyclooxygenase-2
<b>DC</b>	Dendritic cell
<b>DHA</b>	Docosahexaenoic acid
<b>EMSA</b>	Electrophoretic mobility shift assay
<b>EPA</b>	Eicosapentaenoic
<b>FAE</b>	Follicle-associated epithelium
<b>FFA</b>	Free fatty acid
<b>FOS</b>	Fructo-oligosaccharides
<b>GALT</b>	Gut-associated lymphoid tissue
<b>GI</b>	Gastrointestinal
<b>IFN-<math>\gamma</math></b>	Interferon-gamma
<b>IKK</b>	I $\kappa$ B kinase
<b>IL-</b>	Interleukin
<b>IRAK</b>	IL-1 receptor-associated kinase
<b>LCFA</b>	Long-chain fatty acid
<b>LGG</b>	<i>Lactobacillus rhamnosus GG</i>
<b>LPS</b>	lipopolysaccharides
<b><i>M. luteus</i></b>	<i>Micrococcus luteus</i>
<b>MDP</b>	Muramyl dipeptide
<b>MyD88</b>	Myeloid differentiation 88
<b>NIK</b>	NF- $\kappa$ B inducing kinase
<b>NODs</b>	Nucleotide-binding oligomerization domain-containing proteins
<b>PAMP</b>	Pathogen-associated molecular pattern
<b>PGlyRP</b>	Peptidoglycan recognition protein (human)
<b>PGN</b>	peptidoglycan
<b>PGRP</b>	Peptidoglycan recognition protein (general)
<b>PMNs</b>	polymorphonuclear leukocytes
<b>PPAR</b>	Peroxisome proliferator activated receptor
<b>PPRE</b>	PPAR-response element
<b>PRM</b>	Pattern recognition molecule
<b>PRRs</b>	Pattern-recognition receptors
<b>PUFA</b>	Poly-unsaturated fatty acid
<b>RNAi</b>	RNA interference
<b><i>S. aureus</i></b>	<i>Staphylococcus aureus</i>
<b>SCFA</b>	Short-chain fatty acid
<b>siNEG</b>	Negative control siRNA sequence
<b>siRNA</b>	Small interfering RNA
<b>TFs</b>	Transcription factors
<b>TLRs</b>	Toll-like receptor family
<b>TNF-<math>\alpha</math></b>	Tumor necrosis factor- $\alpha$
<b>TRAF6</b>	Receptor-associated kinase 6

## SUMMARY

Intestinal microorganisms are recognized by Toll-like receptors (TLRs), nucleotide-binding oligomerization domain-containing proteins (NODs) and peptidoglycan recognition proteins (PGRPs). PGRPs are a novel family of microbial pattern recognition receptors that recognize bacterial peptidoglycan and function in antibacterial innate immunity. The mammalian PGlyRP family consists of four members: PGlyRP1, 2, 3 and 4. Many studies have focused on the role of TLRs and NODs in immune response, whereas the expression and immunological role of PGlyRPs had gained less attention.

Also, many studies have focused on the intestinal immune modulation by dietary fatty acids (FFA), prebiotic products and probiotic bacteria, which are also able to produce short- and branched-chain fatty acids (SCFA and BCFA). The mechanisms of this immunomodulation are still not fully understood.

Hypothesising that nutrition and intestinal microbes may be involved in immunomodulation through PGlyRPs, the present study aimed to investigate the role of bacterial and dietary FFA and prebiotic products in the inflammation in intestinal Caco-2 cells, and to elucidate the impact of PGlyRP3 in the immune response and inflammation in these intestinal cells.

PCR and Western blot demonstrated the expression of only PGlyRP3 in Caco-2 cells. This expression was enhanced by the PPAR $\gamma$  agonist GW1929 and other PPAR $\gamma$  ligands (bacterial and dietary FFAs). This result was supported by the observed physical binding of PPAR $\gamma$  to the PGlyRP3 promoter, as demonstrated by EMSA. Overexpression / silencing of PGlyRP3 decreased / increased gene expression of proinflammatory cytokines (IL-12, IL-8, TNF- $\alpha$ ). FFAs that induced PGlyRP3 inhibited expression of these cytokines. Silencing of the PGlyRP3 gene caused the same FFAs to increase the cytokine gene expression.

The oligosaccharides  $\alpha$ 3-sialyllactose and fructo-oligosaccharides (Raftilose p95) increased the expression of PGlyRP3, reduced the expression of IL-12 p35, IL-8 and TNF- $\alpha$ , and reduced IL-12 release. Both oligosaccharides induced PPAR $\gamma$  expression. PPAR $\gamma$  antagonists abolished the oligosaccharide-induced inhibition of cytokines' expression.



Different peptidoglycans (PGNs) increased significantly the expression of IL-12p35, IL-8 and TNF- $\alpha$  and the secretion of IL-12, although they enhanced PGlyRP3 expression. PGlyRP3 overexpression, together with the inhibition of MyD88, which is a central adaptor protein of the TLR pathway, significantly reduced the expression of proinflammatory cytokines.

A negative regulation of the NF- $\kappa$ B pathway, including up-regulation of I $\kappa$ B $\alpha$  and down regulation of NF- $\kappa$ B and COX-2, was involved in the anti-inflammatory effects of PGlyRP3.

It is concluded that PGlyRP3 has an anti-inflammatory effect. Oligosaccharides and FFAs mediated their anti-inflammatory effect depending on PPAR $\gamma$  and PGlyRP3. Microbial PGNs act proinflammatory due to TLR pathway activation, prevailing PGlyRP3's anti-inflammatory action. When PGlyRP3 expression is increased, the balance may be shifted towards lower levels of inflammation.

The results of this study imply that nutrition (lipophilic ligands of PPAR, prebiotic oligosaccharides) and microbial products (PGNs and FFA) are able to modulate immunity in intestinal Caco-2 cells by modulation of PGlyRP3 expression.

## ZUSAMMENFASSUNG

Intestinale Mikroorganismen werden von Toll-Like Rezeptoren (TLRs), Nucleotide-binding Oligomerization Domain-containing Proteinen (NOD) und Peptidoglycan Recognition Proteinen (PGRPs) erkannt. PGRPs gehören zur Familie der Pattern Recognition Rezeptoren, welche bakterielle Peptidoglykane erkennen und eine Rolle bei der Antwort des angeborenen Immunsystems auf Bakterien spielen. Die humane PGlyRP Familie besteht aus vier Mitgliedern: PGlyRP1, 2, 3 und 4. Anders als TLRs und NODs ist über die Expression und immunologische Funktion der PGlyRPs im Darm kaum etwas bekannt.

In zahlreiche Studien konnten immunmodulierende Eigenschaften von Nahrungsfettsäuren, prebiotische Produkte und probiotische Bakterien, die kurzkettige (SCFA) and verzweigt-kettige Fettsäuren (BCFA) synthetisieren können, gezeigt werden. Die dabei zugrunde liegenden Mechanismen sind aber nur zum Teil aufgeklärt.

Der Fokus der vorliegenden Arbeit lag auf der durch PGlyRP3 vermittelten immunmodulierenden Wirkung von Nahrungsfaktoren und intestinalen Mikroorganismen. Die Rolle bakterieller und Nahrungsfettsäuren und prebiotischer Produkten auf Entzündungsprozesse wurde in den intestinalen Caco-2-Zellen untersucht. Weiterhin wurde der Einfluss des PGlyRP3 auf die Immunantwort und Entzündungsprozesse in diesen intestinalen Zellen aufgeklärt.

In der intestinalen Zelllinie (Caco-2) konnte mittels PCR und Western-Blot die Expression des PGlyRP3-Gens gezeigt werden. Diese Expression konnte durch PPAR $\gamma$  Agonist GW1929 und Liganden (bakterielle und Nahrungsfettsäuren) induziert werden. Dieses Ergebnis wurde durch die beobachtete physikalischen Bindung zwischen PGlyRP3 Promoter und PPAR $\gamma$  unterstützt, wie EMSA zeigte. Durch Überexpression / Silencing von PGlyRP3 wurde die Expression der proinflammatorischen Zytokine IL-8, IL-12 und TNF- $\alpha$  unterreguliert / erhöht. FFA, welche PGlyRP3 induzierten, inhibierten die getesteten Zytokine. Das Silencing von PGlyRP3 wiederum führte zu einer FFA-vermittelten Aktivierung der Zytokinexpression.

Die Oligosaccharide  $\alpha$ -3-Sialyllactose und Fructooligosaccharid (Raftilose p95) erhöhten die Expression von PGlyRP3 und inhibierten die Expression von IL-12p35, IL-8 und TNF- $\alpha$  und die Sekretion von IL-12. Beide Oligosaccharide induzierten darüber hinaus die

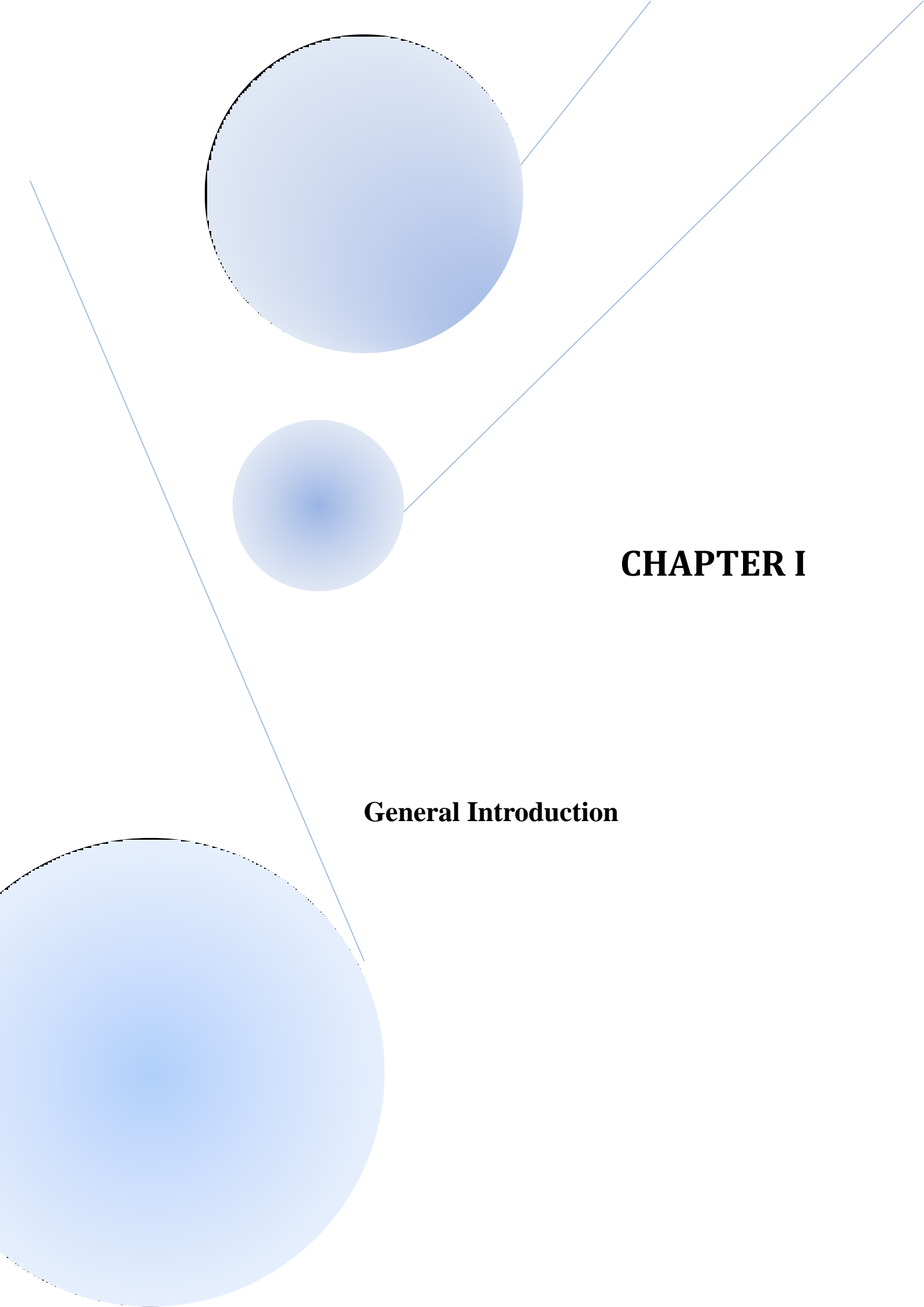
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Expression von PPAR $\gamma$ , während PPAR $\gamma$  Antagonisten die Oligosaccharid-induzierte Inhibierung der Zytokinexpression aufhoben.

Obwohl die untersuchten Peptidoglykane (PGNs) die Expression von PGlyRP3 erhöhten, stieg die Genexpression von IL-12 p35, IL-8 und TNF- $\alpha$  sowie die Sekretion von IL-12. Die Überexpression von PGlyRP3 und die Hemmung von MyD88 – ein zentrales Adaptormolekül des TLR-Signalweges – führten zu einer signifikant geringeren Expression proinflammatorischer Zytokine.

Eine negative Regulation des NF- $\kappa$ B Signalwegs, gezeigt an der Aktivierung von I $\kappa$ B $\alpha$  sowie der Hemmung von NF- $\kappa$ B und COX-2, ist am anti-inflammatorischen Effekt von PGlyRP3 beteiligt.

Die vorliegenden Daten deuten darauf hin, dass PGlyRP3 anti-inflammatorische Effekte aufweist. Der Oligosaccharid-vermittelte anti-inflammatorische Effekt ist von PPAR $\gamma$  und PGlyRP3 abhängig. Mikrobielle PGNs wirken über den TLR Signalweg proinflammatorisch und überdecken dadurch die anti-inflammatorische Wirkung von PGlyRP3. Ist die Expression von PGlyRP3 erhöht, verschiebt sich das Gleichgewicht in Richtung einer geringeren Entzündungsaktivität. Die Ergebnisse dieser Studie lassen den Schluss zu, dass Ernährungsfaktoren (lipophilic ligands, Prebiotika, Oligosaccharide) und mikrobielle Produkte (PGNs, Fettsäuren) über PGlyRP3 eine immunmodulierende Wirkung aufweisen.



# **CHAPTER I**

## **General Introduction**

## **Hypothesis and aim of the work**

The human gut microbiota may contain pathogenic, benign and beneficial microbial genera. The former can lead to intestinal disorders that can be acute (e.g. gastroenteritis) or chronic (e.g. inflammatory bowel disease). Attempts have been made to influence the balance of the gut flora in favor of beneficial microorganisms. This is achieved by adding such microbial strains to an appropriate food vehicle (probiotic bacteria). Probiotics have been defined as non-pathogenic bacteria that, upon ingestion in certain amounts, exert beneficial effects for the host (6). Another attempt was the prebiotics. These are defined as non-digestible food ingredients (as oligosaccharides) that selectively stimulate the growth and activity of beneficial bacteria in the colon leading to beneficial health effects (6). Both bacteria and prebiotics interact with the host enterocytes in various ways, including their pathogen-associated molecular patterns (PAMPs) as lipopolysaccharides and peptidoglycan, and fermentation products as short-chain and branched-chain fatty acids (SCFAs and BCFAs). However, the pathways of interaction between enterocytes and those products are not sufficiently investigated.

Enterocytes are able to recognize bacterial PAMPs by 3 receptor families: Toll-like receptors (TLRs), nucleotide binding oligomerization domains receptors (NODs) and peptidoglycan recognition proteins (PGlyRPs). The PGlyRPs comprise a novel 4-member-family in mammals that specifically recognize peptidoglycans. The gene expression pattern, functions and intracellular pathways of PGlyRPs are not studied in enterocytes. On the other side, numerous studies are available in the literature concerning the host-pathogen relationship and focussing on TLRs and NODs. Most of these studies were performed with the emphasis to unravel pathways underlying immunological reactions of the host. However, no studies were performed from

the metabolic point of view. In the present work, it is hypothesised that bacterial products, such as some fatty acids, and prebiotic compounds, such as fructo-oligosaccharides, may exert an immunological response through pathways not based on direct ligand binding. It is suggested that this may be mediated by transcription factors sensitive to these products, which affect the expression level of pattern recognition receptors (PGlyRPs), which in turn exert their immunological effect. Therefore, it was the aim of this work to:

1. Examine which of the *PGlyRP* genes are expressed in the human colon adenocarcinoma cell line (Caco-2) that has been shown to be a useful *in vitro* model for the intestinal epithelium.
2. Investigate whether a physical binding exists between the promoter region of the expressed PGlyRP and a candidate transcription factor (PPAR $\gamma$ ).
3. Study the effect of the bacterial products (fatty acids and peptidoglycans) as ligands of this transcription factor,
4. Examine whether certain prebiotic oligosaccharides modulate the *PGlyRP* gene expression, and,
5. Study the immunological consequences of such interaction.

## **Review of Literature**

### **The intestinal environment**

The intestine is an important contact surface between the external environment and the body. The intestinal barrier function is ascertained mainly by intestinal epithelial cells connected by tight junctions (1). These cells import luminal nutrients and play a crucial active role in immunity by producing antimicrobial peptides and proinflammatory cytokines under the influence of pattern-recognition receptors triggered by microbes. *In vivo* experiments by using mouse models showed that any dysfunction in intestinal epithelial cells increases the susceptibility to intestinal inflammation (2).

The intestinal lumen contents play an important role in the intestinal homeostasis. The active interaction between microbiota and dietary compounds present in the intestinal lumen from one side and the intestinal tissue, and the immune system from the other side determine the intestinal immune homeostasis. The human colon is colonized by  $10^{11}$ - $10^{12}$  bacteria per gram of content. Intestinal bacteria could be beneficial for the sake of host's health, but also may be harmful and exert harmful effects such as the initiation of gut inflammation. Their composition changes along the intestinal tract. The total bacterial content is increasing from the stomach ( $10^2$ ) to the colon ( $10^{14}$ ). A number of studies have focused on the role of the intestinal flora in modulating the immune system (3). Comparisons with germ-free animals suggested that low basal inflammation is associated with the maturation of immune system and the commensal microbiota. Definitely, the lymphoid tissues are underdeveloped, in the absence of flora (4).

## **Intestinal bacteria produce fatty acids**

The microbes that colonize adult intestines function as a metabolic organ that complements the human metabolic apparatus. The metabolism of carbohydrates and amino acids in the human large intestine due to fermentative activity of the gut microbiota yields fatty acids. Short-chain fatty acids (SCFA) are the major end products of this fermentation, and include primarily lactate, acetate, propionate and butyrate, although formate, valerate, caproate are also produced. Some branched-chain fatty acids (BCFA) as isobutyrate, 2-methylbutyrate and isovalerate may also result, but in lesser amount than SCFA (5). In the human intestine fatty acids are formed by the fermentation of poly or oligosaccharide, protein, peptide and glycoprotein precursors by intestinal microorganisms (6).

Fatty acid formation by intestinal bacteria is regulated by many different environmental, host, dietary and microbiological factors. Readily-digestible carbohydrates and the period that digestive material spends in the colon affect types and amounts of SCFA produced (7). It was also found that long time exposure of food ingredients in the large intestine can have great effects on bacterial metabolism, leading to protein and amino acid fermentation into SCFA, which, in turn, increase SCFA in colon (8). SCFA concentrations are highest in the proximal large intestine, mainly because of greater carbohydrate availability. Studies using intestinal material obtained from human sudden death victims found that acetate, propionate, butyrate values were similar in different regions of the large intestine (9). However, subsequent studies showed that bacterial fermentation products vary in different regions of the colon both qualitatively and quantitatively (10).



## **Dietary fatty acids are known to modulate immune responses in the intestine**

It has been reported that intestinal epithelial cells produce various inflammatory mediators by different intra-luminal stimulants, and participate in the development of intestinal inflammation (11, 12). A close relationship between fatty acids and intestinal inflammation has also been reported. Tanaka *et al.* (13) showed that both oleic and capric acids (C10) enhanced IL-8 production in the intestinal Caco-2 cells after incubation for 3 and 6 hrs, whereas decosahexaenoic and eicosapentaenoic acids did not. Andoh *et al.* (14) found that in intestine-407 cells octanoic acid (C8) increased IL-1 $\beta$ -induced IL-8 secretion in dose-dependent manner, whereas capric acid micelles (C10) did not, contrasting the results of Tanaka *et al.* (13). Mariadason *et al.* (15) have shown that SCFA maximally induced a number of endpoint read-out analyses including IL-8 secretion in undifferentiated Caco-2 cells, but had a different effect in differentiated Caco-2 cells. On the other hand, an anti-inflammatory effect of the n-3 long-chain fatty acids (LCFA) has been reported in patients with the inflammatory Crohn's disease (16). It has also been suggested that n-3 PUFAs are beneficial in ameliorating chronic inflammatory diseases (17). A low-fat diet has often been used as a therapy of different gastrointestinal diseases. Linoleic acid, but not oleic acid, caused a marked increase in the secretion IL-8 by human intestinal smooth muscle cell from Crohn's disease patients. Treatment of normal cells with Linoleic acid did not induce translocation of NF- $\kappa$ B nor I $\kappa$ B- $\alpha$ , and treatment of cells from Crohn's disease patients did not induce further translocation. Linoleic acid stimulated IL-8 production only in cells of Crohn's disease but not in normal cells. Thus, linoleic acid increased the production of IL-8 only in situations where NF- $\kappa$ B was activated (18).

## **The interaction between intestinal epithelial cells and microbes**

Pathogen-associated molecular patterns (PAMPs) or microbe-associated molecular patterns (MAMPs) are common motifs found in microorganisms but not in eukaryotes. Bacterial PAMPs include lipopolysaccharide (LPS) from Gram-negative bacteria, peptidoglycans (PGNs) from both Gram-positive and Gram-negative bacteria, and lipopeptides from various bacteria. The innate immune system consists of specific receptors which known as pattern recognition molecules (PRMs) discriminate between microorganisms and self by recognize PAMPs (19).

Peptidoglycan recognition is a key event in the innate immune response in mammals. PGNs are essential constituents of the cell walls of Gram-positive and Gram-negative bacteria. The minimal essential structure of PGN to induce cell-mediated immunity was chemically synthesized and designated muramyl dipeptide (MDP). Nucleotide binding oligomerization domains NOD1 and 2, proteins were demonstrated to be intracellular receptors for MDP (20, 21). In fact, two families of proteins mediate peptidoglycan recognition; these are the PGlyRP and Nod proteins. Whereas the Nod proteins Nod1 and Nod2 are cytoplasmic proteins, mammalian PGlyRP likely detect peptidoglycan in different cellular compartments owing to the fact that they are membrane-bound, stored in vesicles, or secreted proteins. Nod1 and Nod2 are involved in innate immune defence through pathways that are likely to be independent of TLR signalling. Nod1 is an upstream activator of NF- $\kappa$ B. Nod1 pathway activation leads to proinflammatory cytokines production, such as IL-8. Nod1 was first described as an intracellular receptor for LPS. Afterwards, PGN exclusively from Gram-negative bacteria was demonstrated to be the real agonist of Nod1 (22, 23). It is now clear that Nod1 is a sensor for both Gram-

positive and Gram-negative bacteria that have mesodiaminopimelic acid (DAP)-type peptidoglycan in their wall, Nod2 is able to induce NF- $\kappa$ B activation as well as Nod1. However, unlike Nod1, Nod2 is able to sense L-lysine (LYS)-type peptidoglycans in Gram-positive and Gram-negative bacteria cell walls (24). After recognition of PGN fragments via Nod2, NF- $\kappa$ B activation leads to production of cytokines such as IL-1, IL-6 and TNF- $\alpha$  (25).

It should be mentioned that PGN is also recognized by a membrane-bound PRM, the Toll-like receptor (TLR2) (26), although an intact glycan chain of PGN is required for TLR2 to recognize PGN, therefore TLR2 could not recognize free MDP (27).

### **Peptidoglycan Recognition proteins**

There is a novel family of pattern recognition molecules (PRMs) called Peptidoglycan recognition proteins (PGRPs). These PGRPs are highly conserved from insects to mammals (28, 29). PGRPs were first identified in the silk worm *Bombyx mori*, which identified as a protein that found in the hemolymph and binds to Gram-positive bacteria this in turn activates an immune response (30). Thereafter different PGRPs were identified in insects, and were classified according to their length into two classes, short PGRPs (PGRP-S) and long PGRPs (PGRP-L) (31, 32).

Insect PGRPs bind directly to PGN, each with separate favourites for Lys-PGN or DAP-PGN results in activation of two different immune signalling pathways (Toll and Imd). All PGRPs contain a conserved PGRP domain region, which has homology to bacteriophage T7 lysozyme and bacterial type two N-acetylmuramoyl-L-alanine amidase and are predicted to have amidase activity. In *Drosophila*, there are 13 PGRPs, which are expressed in different organs,

such as the fat body and gut. and up-regulated by PGN (33), suggesting critical roles in host defense against microbial infections in insects. *Drosophila* PGRP-SA is required for the activation of the Toll receptor pathway by Gram-positive bacteria, which results in the induction of anti-bacterial peptides and the generation of an effective immunity to Gram-positive bacteria (34). *Drosophila* PGRP-LC participates in the induction of anti-bacterial peptides in response to Gram-negative and Gram-positive bacteria (35). PGRP-LE is involved, in addition to its anti-bacterial defense, in the activation of a prophenoloxidase cascade in response to the diaminopimelic acid (DAP)-type PGN from most of Gram-negative and some Gram-positive bacteria (36). In general the insect, PGRPs with amidase activity have anti-inflammatory role and reduce the inflammatory effect of peptidoglycan (37- 39). Thus, insect PGRPs play important roles in innate immune responses to bacteria.

In mammals, PGRPs from human (40), mouse (41), rat (42), cow (43) and camel (44) have been described. Recently, four human PGRPs designated PGRP-S (PGlyRP1) (41), PGRP-L (PGlyRP2), PGRP-I $\alpha$  (PGlyRP3) and PGRP-I $\beta$  (PGlyRP4) (40) were identified. Liu *et al.* (45) described the functional similarity of PGRPs from mammals to that in insects. They reported that a mouse recombinant PGRP, probably PGRP-S bound to *Staphylococcus aureus* PGN and some Gram-positive bacteria and exhibited a bacteriostatic activity against Gram-positive bacteria, but not against Gram-negative bacteria such as *Escherichia coli*. Dziarski *et al.* (2003) (46) described that PGRP-S mRNA was predominantly expressed by polymorphonuclear leukocytes (PMNs) in mice and humans, and that PGRP-S inhibited phagocytosis after the infection with Gram-positive bacteria, as well as inflammatory cytokine production in response to PGN in macrophages. PGRP-S-deficient mice were more susceptible than wild-type mice upon intraperitoneal infection with

bacteria. They also reported the defective intracellular killing of bacteria in PMNs from PGRP-S knockdown mice.

The family members show selective and differential expression; PGRP-S (PGlyRP1) is highly expressed in bone marrow, PGRP-L (PGlyRP2) in the liver, and PGRP-I $\alpha$  and PGRP-I $\beta$  (PGlyRP3 and 4) in the oesophagus (40). Their distinct expression patterns suggest that each may play a unique role in host defence. Consistent with the presence of a PGRP domain, each human PGlyRP is able to bind *Staphylococcus aureus* PGN and whole cells of *Bacillus subtilis* and *Micrococcus luteus* with high affinity (40). Concerning PGlyRP3, the crystal structure of the C-terminal PGN binding site has been exhaustively characterized (47). Both murine (48) and human (49) PGRP-L exhibited lytic activity towards PGN. Even though all four members have the PGlyRP domain, only one mammalian PGlyRP, PGlyRP-L, has been reported to have amidase activity. It is an MurNAc-L-Ala amidase, which allows this enzyme to cleave PGN between the sugar moiety and the peptide moiety (48). All mammalian PGlyRPs have been shown to be proteins that bind directly to PGN as well as live bacteria. Their mRNA expression increased significantly by synthetic PAMPs. Suppression of the expression of TLR2, TLR4, NOD1, NOD2 RNA interference specifically inhibited the upregulation of PGlyRPs mRNA expression induced by different synthetic PAMPs (26).

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The page features a decorative graphic consisting of three blue circles of varying sizes and two thin, light blue lines that intersect to form a large 'V' shape. One circle is at the top, a smaller one is in the middle, and a large one is at the bottom left. The lines intersect at the top right and extend towards the bottom left and top right corners.

## CHAPTER II

**PPAR $\gamma$ -dependent peptidoglycan recognition protein 3 (PGlyRP3) expression regulates proinflammatory cytokines by microbial and dietary fatty acids**

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**PPAR $\gamma$ -dependent peptidoglycan recognition protein 3  
(PGlyRP3) expression regulates proinflammatory cytokines  
by microbial and dietary fatty acids**

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**Key words:** PGlyRP3, fatty acids, inflammation, PPAR, Caco-2.

## **ABSTRACT**

PGlyRPs recognize bacterial peptidoglycan and function in antibacterial innate immunity. Focusing on the interference between nutrition and recognition pattern proteins, free fatty acids (FFA) of dietary and bacterial sources may exert their immunological response through modulating the expression level of the PGlyRPs in enterocytes. PGlyRP3 was the only PGlyRPs' member expressed in Caco-2 cells. *In silico* analysis showed that the promoter of PGlyRP3 has some PPRE regions that, as tested by EMSA, bind physically to the PPAR $\gamma$ -RXR $\alpha$  complex. *PGlyRP3* gene expression was induced by PPAR $\gamma$  ligands including GW1929 and some FFA. Overexpression of PGlyRP3 in Caco-2 cells down regulated the expression of the inflammatory cytokines IL-8, IL-12 and TNF- $\alpha$ , while its silencing increased the expression of these cytokines. FFA that induced the PGlyRP3 inhibited the tested cytokines. Silencing of *PGlyRP3* gene caused the same FFA to increase the cytokine gene expression. A negative regulation of NF- $\kappa$ B pathway, including up-regulation of I $\kappa$ B- $\alpha$  and down regulation of NF- $\kappa$ B and COX-2, is involved in the anti-inflammatory effects of PGlyRP3. In conclusion, PPAR $\gamma$  mediates a modulation of *PGlyRP3* gene expression, which is involved in inhibiting inflammation through negative regulation of NF- $\kappa$ B pathway.

### **INTRODUCTION**

“Inflammation is the body’s immediate response to damage of its tissues and cells by noxious stimuli such as chemicals or physical injury and pathogens” (1). The human colon is colonized by  $10^{11}$ - $10^{12}$  bacteria per  $\text{cm}^3$ . Intestinal bacteria may be beneficial provide the host health or harmful exert adverse effects such as the gut inflammation and the activation of precursors of toxic food components. Metabolization of carbohydrates and amino acid due to fermentative activity of the gut microflora yields short chain fatty acids (SCFA) as acetic, propionic, butyric, valeric, and caproic acids, and some branched-chain fatty acids (BCFA) as isobutyric and iso-valeric acids (2, 3). These have been associated with reduced risk of some diseases, including the irritable bowel syndrome, inflammatory bowel disease and cancer (4).

The interaction between intestinal epithelial cells and microbes is partly mediated by members of the Toll-like receptor family (TLRs), the family of nucleotide-binding oligomerization domain-containing proteins (NODs) and the peptidoglycan recognition protein family (PGlyRPs). TLRs play a essential role in the detection of invading microorganisms and the induction of immune response and inflammatory signalling pathway to defend the host (5, 6). NODs and PGlyRPs also recognize bacteria, and interact with pro-inflammatory signalling pathways (7).

Dietary FFAs are known to modulate immune responses. It has been suggested that n-3-PUFAs are beneficial in ameliorating chronic inflammatory diseases (8). Some reports have focused on the interference between nutrition and recognition pattern proteins, especially TLRs and NODs. Lauric acid (C12:0) dose dependently activated NF- $\kappa$ B and induced IL-8 expression. Conversely, decosahexaenoic acid (DHA), an n-3-PUFA, inhibited NF- $\kappa$ B activation and IL-8 expression. These effects were inhibited by dominant

negative forms of NOD1 and NOD2, but not TLR2, TLR4 or TLR5 (9). In another study, the increasing levels of nutritional FFA in obesity were associated with activation of TLR4 signalling in adipocytes and macrophages (10). The capacity of FFA to induce such inflammatory signalling in adipose cells and macrophages is blunted in the absence of TLR4 (10). FFA can activate TLR4 signalling and subsequent NF- $\kappa$ B-activation to regulate adipokine and chemokine secretion from adipocytes (11). Thus, nutritional components may initiate some immunological events through interaction with NODs and TLRs. However, the underlying mechanisms for the effects of dietary or microbial FFA remain unclear. In addition, no information is existing about PGlyRPs, the third pattern recognition receptor family, regarding their modulation by FFA. PGlyRPs are innate immunity proteins that are conserved from insects to mammals (12). They recognize bacterial peptidoglycan and function in antibacterial innate immunity. Mammals have four PGlyRPs named PGlyRP1, 2, 3, and 4. There are only two known functions of mammalian PGlyRPs. PGlyRP1, which is expressed in PMN's granules, as well as PGlyRP3 and 4, which are found in many organs, have direct bactericidal effect. PGlyRP2, which is an N-acetylmuramoyl-L-alanine amidase, it is highly expressed in the liver and secreted into blood, that hydrolyzes peptidoglycan (13). PGlyRPs, NODs and TLRs, represent three different types of pattern-recognition receptors, playing interdependent *in vivo* roles in local inflammation (13).

The effects of FFA are mediated either directly due to their specific binding to various nuclear receptors leading to changes in the activity of these transcription factors, or indirectly as the result of changes in the abundance of regulatory transcription factors (14). Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear hormone receptors super-family of ligand-activated transcription factors that are related to retinoid, steroid, and thyroid hormone receptors. So far, three different PPAR subtypes have been identified

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and are commonly designated as PPAR $\alpha$ , PPAR $\beta$  and PPAR $\gamma$ . The PPAR $\gamma$  plays a pivotal role in the regulation of lipid metabolism and control of inflammation (15), since PPAR $\gamma$  is associated with anti-inflammatory responses (16). PPARs are activated by different FFA and their metabolites, indicating that PPARs act as fatty acid sensors (17). PPAR $\gamma$  has been shown to regulate cell activation, differentiation, proliferation, and/or apoptosis (16) and ameliorate inflammation in Bowel disease (18).

Many studies have focused on the inflammatory pathways that are downstream of the recognition patterns. However, less focus was put on its interactions with fatty acid metabolism. We hypothesise that FFA of nutritional and bacterial sources exert an immunological response, which could be mediated by transcription factors of lipophilic ligands, thus affecting the expression level of some recognition patterns. We therefore examined which of the *PGlyRP* genes are expressed in the human colon adenocarcinoma cell line (Caco-2) that has been shown to be a useful *in vitro* model for small intestinal epithelium (19), and determined the expression of PGlyRP3. We further investigated whether a physical binding exists between the promoter region of PGlyRP3 and lipid transcription factors as PPARs. Additionally, we studied the effect of the lipophilic ligands of these transcription factors on *PGlyRP3* gene expression, and the immunological consequences of such an effect.

## **MATERIAL AND METHODS**

### ***Cell culture***

Caco-2 cells were obtained from the American Type Culture Collection Company (ATCC). Cells between passages 10-50 were grown in 75 cm<sup>2</sup> culture flasks at 37°C and 5% CO<sub>2</sub> using Minimum Essential Medium (MEM + Glutamax, Invitrogen, Karlsruhe, Germany) supplemented with 20% fetal calf serum, 100 IU penicillin/ml and 1% non-essential amino acids. For different experiments, cells were cultured in 6 well plates until reaching 7 days post confluence. Cells were fasted for 2 hours in Minimum Essential Medium (MEM + Glutamax) without any supplementations before different incubations. For over-expression and RNA-silencing experiments, cells were cultured in 24-well plates until reaching 80-100% confluence.

### ***In silico analysis***

The promoter region of the human *PGlyRP3* gene has been inspected for the presence of transcription factors of lipophilic ligands. A 2-kB region of the promoter sequence upstream to the transcriptional coding sequence of the human *PGlyRP3* gene was taken from the Ensembl database ([www.ensembl.org](http://www.ensembl.org)). For the prediction of putative transcription factor binding sites, the *in silico* analysis of the *PGlyRP3* promoter using the TRANSFAC Professional Database ([www.biobase.de/pages/products/transfac.html](http://www.biobase.de/pages/products/transfac.html)) was performed. A core similarity of 0.85 or greater and a matrix similarity of 0.90 or greater were defined as a cut-off for consideration of potential query sequence matches.



### ***Electron mobility shift assay (EMSA)***

The nuclear protein was extracted from Caco-2 cells and transfected HeLa cells using the kit from Active Motif (Rixensart, Belgium). To transfect HeLa cells with PPAR $\gamma$ , cells were seeded to a confluency of 50-70%. One hundred ng PPAR $\gamma$  plasmid DNA was added. The transfection method was similar to that of PGlyRP3 transfection (see below). EMSA was performed using LightShift chemiluminescent EMSA kit (Pierce). DNA-oligonucleotides including the predicted putative binding sites for PPAR and RXR were ordered as single stranded 3'-end labelled with biotin or non-labelled from MWG Biotech (Ebersberg, Germany) (supplementary data, table 1, the underlined letters indicate the putative PPRE sequence). The double strand probes were prepared by annealing 3'-end biotin- labelled and non-labelled oligonucleotides. The DNA-protein binding assay was performed at room temperature for 30 min in a final volume of 20  $\mu$ l containing 1x binding buffer, 2.5% glycerol, 0.1 mM EDTA, 1  $\mu$ g of poly (dI-dC), 10.fmol of double-stranded biotinylated probe, and 10  $\mu$ g of nuclear extract. For the competition assay, 100x (2 pmol) of non-labelled oligonucleotide were included in the binding reaction (Fig. 2, lane 3). For supershift EMSA, 2  $\mu$ l of PPAR $\gamma$  monoclonal antibody (Fig. 2, lane 4) or 2  $\mu$ l of RXR $\alpha$  polyclonal antibody (Fig. 2, lane 5) (Santa Cruz Biotechnology Heidelberg, Germany) were preincubated in the binding reaction for 30 min before the probe was added. The DNA-protein complexes were separated by 4% PAGE in 0.5 $\times$  TBE at 200 V in Ice for 2 h. DNA-protein complexes in gel were transferred to Hybond N+ nylon membrane (Amersham Biosciences) by electroblotting with 0.5 $\times$  TBE at 350 mA for 1.5 h. DNA-protein complexes were fixed to the membrane by UV cross-linker and detected by incubation of the membrane with the substrate solution and then exposure to radiographic film.

### ***Preparation of fatty acids and incubations***

Palmitic (C 16:0), oleic (C 18:1), linoleic (C 18:2), eicosapentaen acid (C 20:3) (EPA), 13-methylmyristic acid (C 15), 15-methylpalmitic acid (C 17), phytanic acid were purchased from Sigma. Stock solutions were prepared by dissolving the FFA in 5% bovine serum albumin (BSA) to a final concentration of 3 mM after incubation in ultrasonic bath.

Fasted cells were incubated for 24 hours with one of the different FFA at a concentration of 100  $\mu$ mol/l. Control incubations were supplied with the vehicle only (final concentration of BSA is 0.05%). For examining the effect of PPAR $\gamma$ , the PPAR $\gamma$  agonist GW1929 (Sigma) was dissolved in dimethylsulphoxide (DMSO, final culture concentration 0.1%) and added to make a final concentration of 10  $\mu$ mol/l. Control culture were supplied with 0.1 % DMSO.

### ***RNA isolation, cDNA synthesis, and real time PCR***

Total RNA was isolated from Caco-2 cells using RNeasy kit (Qiagen, Hilden, Germany). To assure absence of genomic DNA, all RNA samples were treated with DNase-1, and multiple exons-spanning primers were used for PCR amplification. For reverse transcription, 1  $\mu$ g of the total RNA was reverse transcribed to first strand complementary DNA in 25  $\mu$ l reactions using the cDNA synthesis Taqman kit (Applied biosystem). RT-PCR analysis of PGlyRP genes expression in addition to  $\beta$ -actin as a household gene was performed using specific primers for each gene (see table 1; for PGlyRP1, 2 and 4, we used primers as reported by Liu et al (20). PCR products were electrophoresed on agarose gel and their bands were visualised by ethidium bromide staining.

### ***Quantitative real time PCR analysis***

All quantitative RT-PCR analyses were performed on an Applied Biosystems ABI Prism 7000 system using cDNA samples corresponding to 20 ng RNA. Quantitative analysis of *PGlyRP3* gene expression was undertaken by using TaqMan gene expression assay in a 30  $\mu$ l reaction with universal PCR master mix and TaqMan MGB probe (No. Hs 01074825\_ml, Applied Biosystems, Cambridge, UK). To normalize expression data,  $\beta$ -actin was used as an internal control gene. Data are presented as fold difference from control, considering the control values as 1.

Quantitative RT-PCR analyses of all other studied genes were performed using SYBR Green (Power SYBR green Mastermix, Applied Biosystems). All studied genes were quantified within the same cDNA samples. The thermal cycling program was 10 min at 95°C for enzyme activation and denaturation for 15 s at 95°C, 60 s annealing at 60°C. A dissociation curve was performed for each product to assure the absence of primer dimers or unspecific products. Primers used in the present study are listed in table 1 (supplementary data). To normalize expression data,  $\beta$ -actin was used as an internal control gene. Data are presented as fold difference from control, considering the control values as 1.

### ***Transfection of Caco-2 cells with PGlyRP3 plasmid***

RNA was isolated from human oesophagus (approved by the ethics committee of the Christian-Albrechts-University of Kiel “protocol A 120/05”) and using the designed primers (supplementary data, table 1), full-length cDNAs coding for *PGlyRP3* was cloned. We designed oligonucleotide primers after testing the sequences for restriction enzymes (*Xho*1 and *Eco*R1) that neither cut within the *PGlyRP3* nor the vector sequences. The PCR products

were ligated into the pCAGGS vector (T4-Ligase Rapid-Kit(Fermentas, Karlsruhe, Germany), and clones with the proper full-length PGlyRP3 insert were selected and identified by restriction digestion and sequencing the inserts. The cloned PGlyRP3 was identical to the genomic sequences.

Caco-2 cells were seeded in a 24-well plate at a density of  $4 \times 10^4$  cells per well in 1 ml of culture medium and transfections performed at 60–80% cell confluence. Prior to transfection, TurboFect (Fermentas) and MEM (Invitrogen) were mixed and incubated at room temperature for 20 mins. 100 ng DNA was added in a ratio of 3:1 (TurboFect in  $\mu$ l: DNA in ng) as recommended by the manufacturer. Control samples were treated similarly and transfected with the empty vector (pCAGGS). After 48 and 72 hrs post transfection, RNA was isolated and reverse transcribed to cDNA. *PGlyRP3* gene overexpression was confirmed using quantitative RT-PCR and Western blot (supplementary data, Fig. S1).

### ***Small interfering (si) RNA experiments***

Two siRNA sequences targeting PGlyRP3 (Si Select si RNA no. s41580 and s41581) and a non-targeting negative control sequence (SC 4390843) were purchased from (Ambion, Applied Biosystems). Caco-2 cells were reverse transfected with a mixture of the two siRNA using siPORT NeoFX Transfection Reagent (Ambion, Applied Biosystems). siRNA was prepared in Opti-MEM serum-free medium (Invitrogen) by mixing 2  $\mu$ l of the transfection reagent with 5 nM siRNA, respectively, at room temperature for 10 min. Caco-2 cells ( $4 \times 10^4$  cells per well) were transferred to a 24-well plate containing siRNA transfection reagent complexes, allowing transfection to occur during initial cell adherence. The medium was replaced after 24 hrs by a culture medium containing 20% FCS (allowing cell recovery) and incubation continued for additional 24 and 48

hrs, then RNA was isolated and reverse transcribed to cDNA. *PGlyRP3* gene knock-down was confirmed by quantitative RT-PCR and Western blot (supplementary data, Fig. S1).

### ***Protein extraction and Western blot analysis***

The cell lysates were loaded into 12% SDS-PAGE and blotted onto PVDF transfer membrane. Membranes were blocked with a mixture of 2.5% BSA and 2.5% skimmed milk and incubated with mouse monoclonal anti-human PGlyRP3 (1:200, AXXORA, Lörr, Germany), rabbit polyclonal anti-human NF- $\kappa$ B (p65) (1:200, Panomics, Affymetrix), rabbit polyclonal anti-human GAPDH (1:500, Santa Cruz Biotechnology) or rabbit polyclonal anti-human Histone3 (H3) (1:2000, abcam). Immunoreactive bands were detected with HRP-conjugated secondary antibody.

### ***Interleukin 12 protein assay***

The human total IL-12 was detected using an ELISA kit ( Mabtech AB, Hamburg, Germany) in the supernatant obtained from Caco-2 cell cultures after each treatment according to the manufacturer's instructions.

### ***TransAm assay for NF- $\kappa$ B nuclear localization***

Caco-2 cells were transfected with PGlyRP3 plasmid or siRNA for 48 hrs or were incubated with 10  $\mu$ mol/l GW1929, 100  $\mu$ mol/l oleic or 100  $\mu$ mol/l palmitic acids for 24 hrs. Thereafter, nuclear extracts were prepared using a nuclear extraction kit (Panomics, Affymetrix, Heidelberg, Germany) according to manufacturer's instructions. Nuclear localization of NF- $\kappa$ B was quantified

using Transcription Factor ELISA kit (TransAm) to detect activated p65 subunit of NF- $\kappa$ B as illustrated in (Panomics, Affymetrix) user manual.

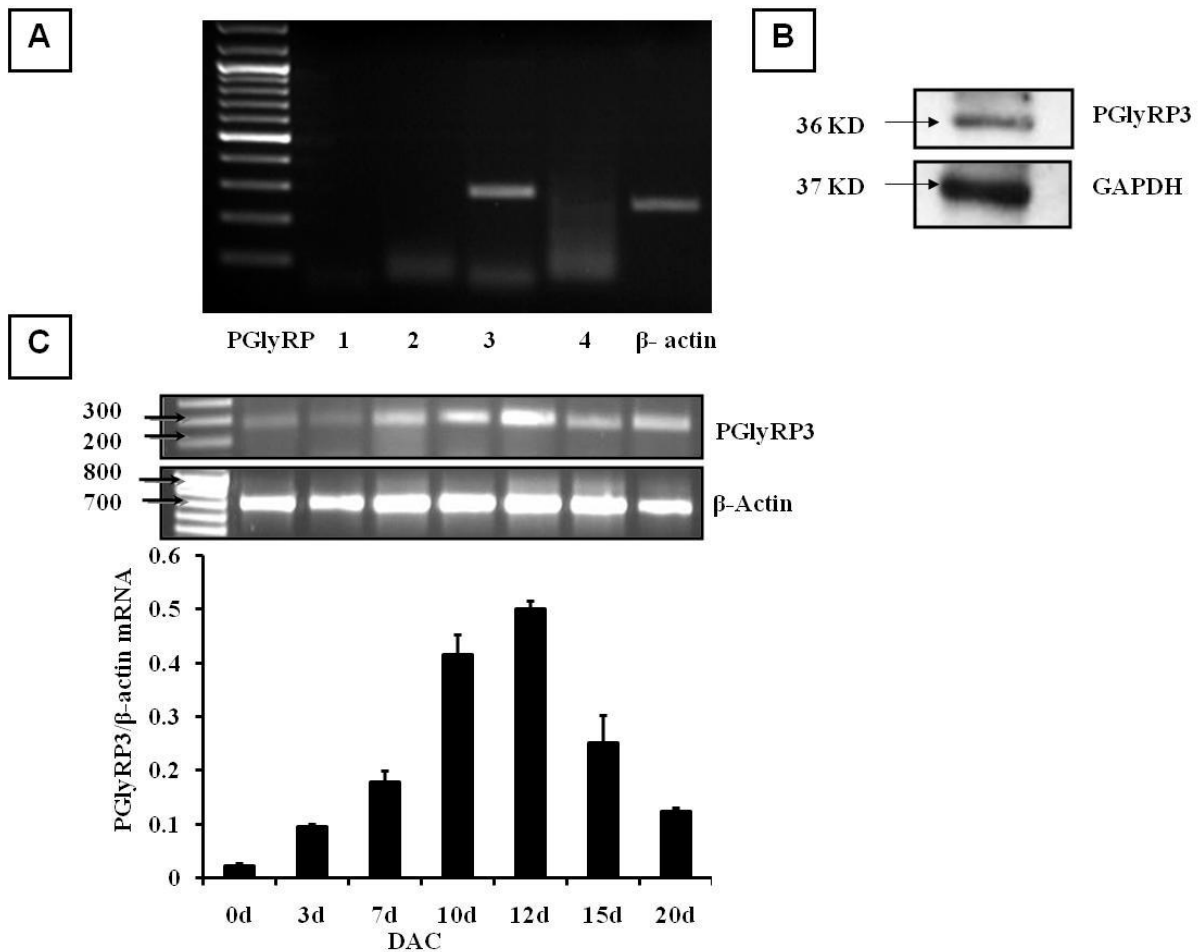
### ***Statistical analyses***

All samples were measured in duplicates. Values were expressed as mean  $\pm$  S.E.M. with  $n \geq 4$  in all experiments and p indicated in the Results section. Group statistical comparisons were performed by one-way analysis of variances (ANOVA) followed by Mann-Whitney (Wilcoxon) multi-range analysis as a post-hoc test. A statistical difference was considered significant if  $p < 0.05$ .

## **RESULTS**

### ***Detection of PGlyRP3 gene and protein in Caco-2 cells***

We first sought to determine which PGlyRP is expressed in Caco-2 cells. For this purpose, RNA was isolated from Caco-2 cells and processed for RT-PCR amplification using primers of PGlyRPs listed in table 1 (supplementary data). RT-PCR analysis of the 4 *PGlyRP* genes revealed that only PGlyRP3 is expressed in Caco-2 cells (Fig. 1A). Since it is known that the expression level of some genes differs during the differentiation of Caco-2 cells in culture, we isolated RNA from Caco-2 cells at different stages after reaching confluence. Normal and quantitative RT-PCR of those samples indicate that *PGlyRP3* gene expression varies during Caco-2 cell differentiation, reaching a peak on the twelfth day and decreasing thereafter (Fig. 1C). The expression of PGlyRP3 was also confirmed by Western blot analysis (Fig. 1B).



**Figure 1.** Detection of *PGlyRP3* gene expression in Caco-2 cells: A) Only *PGlyRP3* gene is expressed by Caco-2 cells. RNA was isolated from Caco-2 cells and analysed by RT-PCR for the expression of the 4 *PGlyRPs* types. B) Detection of *PGlyRP3* protein in Caco-2 cells by Western blot: Caco-2 cells were lysed and equal protein amounts were loaded. The membranes were incubated with mouse anti-*PGlyRP3* serum (1:200), and immunoreactive bands were detected with anti-mouse secondary antibody. C) *PGlyRP3* gene expression level varies during Caco-2 cell differentiation. RT-PCR was applied using mRNA isolated from Caco-2 cells during the mentioned days after confluence (DAC). Quantitative RT-PCR analysis for the expression of *PGlyRP3* in Caco-2 cells during its differentiation was performed using *PGlyRP3* TaqMan MGB probe. Data was normalized to the expression of  $\beta$ -actin household gene. The shown data (mean  $\pm$  SEM) are averages of 3 separate experiments performed in duplicates.

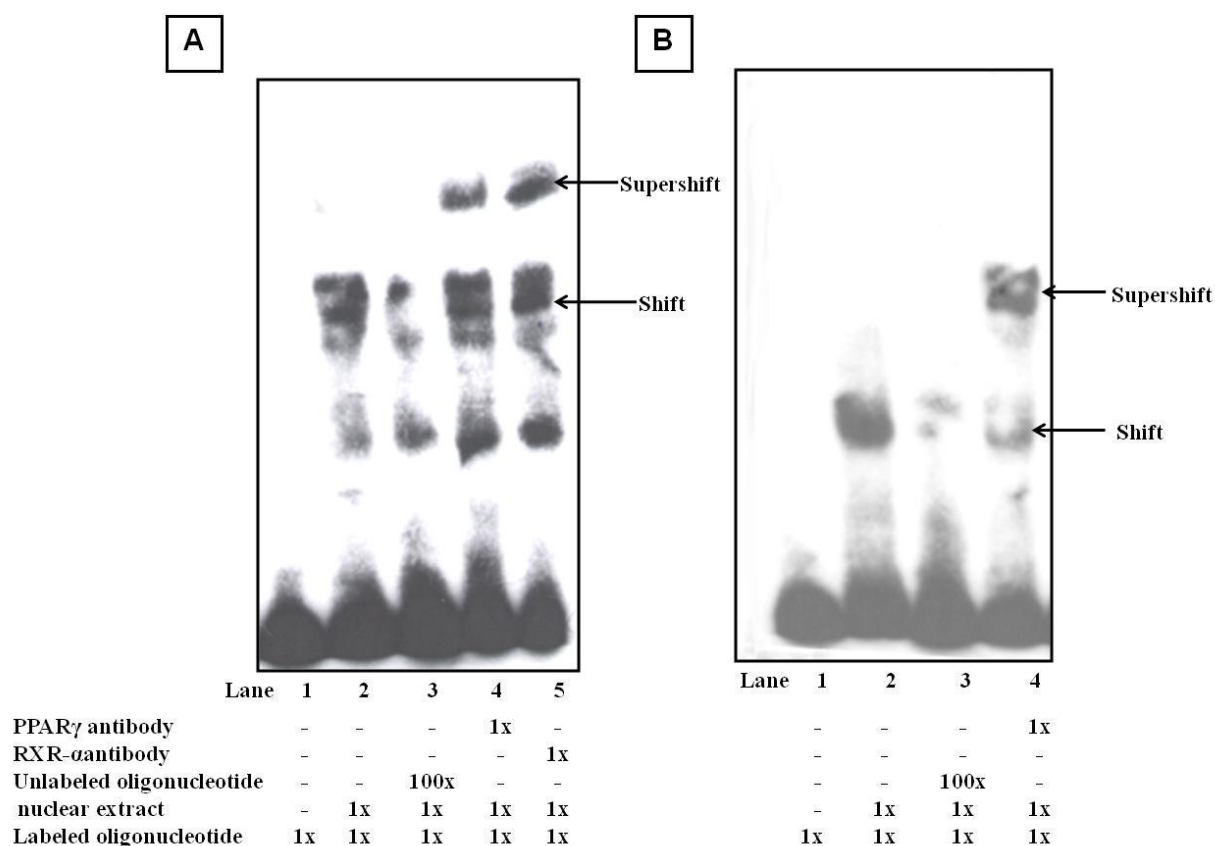
### ***PPAR $\gamma$ and PPAR $\gamma$ -RXR $\alpha$ complex bind to PGlyRP3 promoter***

We have inspected the promoter region of the human *PGlyRP3* gene for the presence of response elements of transcription factors binding lipophilic ligands. The *in silico* analysis using TRANSFAC data demonstrated the presence of several putative binding sites for PPAR $\gamma$  and PPAR $\gamma$ -RXR $\alpha$  complex (PPRE) on the *PGlyRP3* gene promoter at different sites (for example at -1359, -801, -309 and -178). One of these putative binding sequences (-1359) was chosen to test the physical binding ability of PPAR $\gamma$  and PPAR $\gamma$ -RXR $\alpha$  complex to the human *PGlyRP3* gene. To determine whether the PPAR $\gamma$  or PPAR $\gamma$ -RXR $\alpha$  complex are capable of binding the -1359 region of the *PGlyRP3* promoter, we performed an electrophoretic mobility shift assay (EMSA) using nuclear extracts obtained from Caco-2 (Fig. 2A).

As shown in Fig. 2A, lane 2, the biotin labelled double-stranded probe, when incubated with nuclear extract from differentiated Caco-2 cells forms DNA-protein complexes (shift). Incubation of the Caco-2 nuclear extract and biotin labelled double-stranded probe with the antibody against PPAR $\gamma$  or RXR $\alpha$  supershifted one of these complexes to higher molecular weight position (Fig. 2A, lanes 4 and 5, respectively). This indicated that one of the above complexes (lane 2) was formed between the PPRE in the *PGlyRP3* promoter and the PPAR $\gamma$ /RXR $\alpha$ . However, there were some nonspecific complexes formed between PPRE sequence and other transcription factors. It was previously shown that the PPRE sequence is able to bind in vitro the PPARs, HNF4 $\alpha$ , PPAR $\gamma$  / $\beta$ / $\alpha$ -RXR $\alpha$  complexes, and RXRs (21- 23). Similarly, DNA-protein complex (shift) was formed when biotin labelled double-stranded probe was incubated with nuclear extracts prepared from transfected Hela cells with PPAR $\gamma$  plasmid (Fig. 2B, lane 2). Incubation of nuclear extracts of these cells and biotin labelled double-stranded probe with antibody against PPAR $\gamma$



supershifted the complex (Fig. 2B, lane 4). This experiment confirms the binding that occurred in Caco-2 cells.



**Figure 2.** PPAR $\gamma$  and RXR $\alpha$  bind to PGlyRP3 promoter: EMSA was applied to detect the *in vitro* binding of nuclear PPAR $\gamma$  protein to PGlyRP3 promoter sequence. A) Nuclear protein extracts from Caco-2 cells were mixed with PGlyRP3 double strand labelled probe in the presence (lane 3) or absence of a 100x unlabelled probe. The 30bp-probe was extracted from the PGlyRP3 promoter region and has a putative binding ability to the mentioned nuclear proteins. Mobility retardations (supershift) in the presence of an antibody against PPAR $\gamma$  and RXR $\alpha$  are shown in lanes 4 and 5, respectively. B) Nuclear protein extract from HeLa cells transfected with PPAR $\gamma$  plasmid was mixed with PGlyRP3 double strand probe. A supershift in the presence of an antibody against PPAR $\gamma$  is shown in lane 4.

### ***PGlyRP3 gene expression is regulated by a PPAR $\gamma$ agonist and different FFA***

Then we investigated the regulation of *PGlyRP3* gene expression by a specific PPAR $\gamma$  agonist in Caco-2 cells (GW1929) for 24 hrs. *PGlyRP3* gene expression was significantly enhanced by treatment with GW1929 ( $1.89 \pm 0.26$ ) ( $P < 0.003$ ) (Fig. 3A). Western blot analysis showed an increase of PGlyRP3 protein after treatment with GW1929 for 24 hrs (Fig. 3A, lower panel).

Next we examined the regulation of *PGlyRP3* gene expression in Caco-2 by different FFA. Caco-2 cells were incubated for 24 hrs with 100  $\mu$ M of either palmitic acid (16:0), a saturated FFA; oleic acid (18:1), a monounsaturated FFA; linoleic acid (18:2), an omega-6 polyunsaturated FFA; eicosapentaenoic (EPA); an omega-3 polyunsaturated FFA; 13-methylmyristic acid (C15) and 15-methylpalmitic acid (C17), branched-chain fatty acids (BCFA) and phytanic acid, a short-chain fatty acid (SCFA). Differential effects of different FFAs on PGlyRP3 protein and gene expressions were observed (Fig. 3B). *PGlyRP3* gene expression increased to  $1.91 \pm 0.21$  by palmitic,  $3.13 \pm 0.63$  by oleic,  $2.19 \pm 0.36$  by EPA,  $2.50 \pm 0.44$  by C15, and  $1.89 \pm 0.25$ -folds by phytanic acid compared with control experiments ( $p < 0.05$ ). On the other hand, PGlyRP3 mRNA levels were not affected by linoleic acid or by C17 ( $1.09 \pm 0.09$  and  $1.20 \pm 0.16$ -folds, respectively ( $p > 0.05$ ) compared with control experiments.

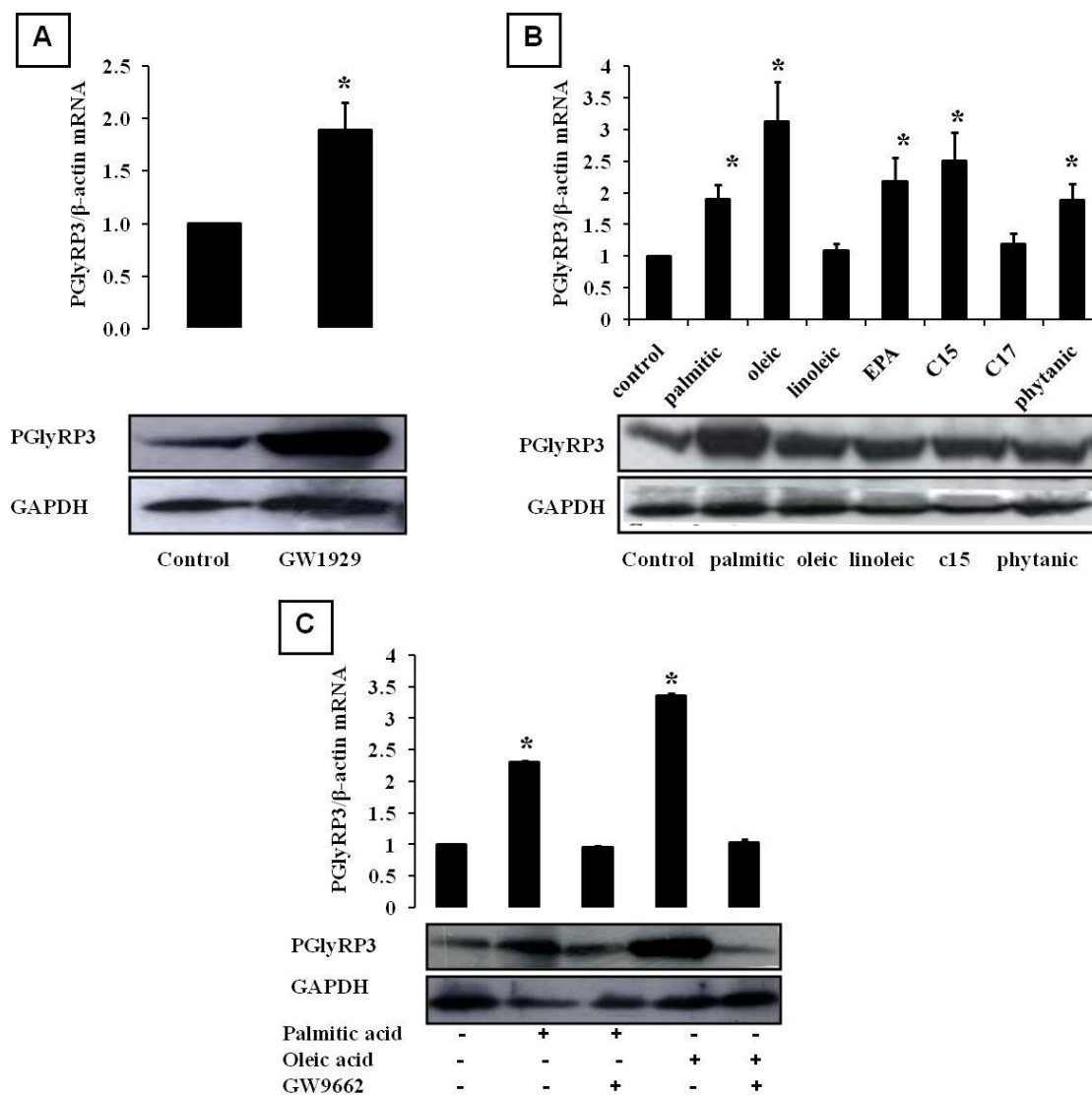
### ***FFA regulate PGlyRP3 gene expression through PPAR $\gamma$***

To prove this, we investigated the PGlyRP3 expression in Caco-2 cells treated with palmitic or oleic acids in the presence or absence of 10  $\mu$ mol/l of the PPAR $\gamma$  antagonist GW9662 (Fig. 3C).

The FFA-induced expression (Fig. 3C, upper panel) of PGlyRP3 (2.30±0.03 and 3.36±0.03-folds for palmitic and oleic acids, respectively, compared to the control without FFA) was abolished by the co-incubation with the PPAR $\gamma$  antagonist GW9662 (0.96±0.02 and 1.04±0.04-folds, respectively,  $p<0.05$ ). This effect was also remarkable for PGlyRP3 protein (Fig. 3C, lower panel).

### ***FFA modulate the gene expression of the cytokines IL-8, IL-12p35 and TNF- $\alpha$***

We measured the mRNA level of interleukins 8, 12p35 (IL-8, IL-12p35) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) in Caco-2 cells treated with different FFAs at concentration of 100  $\mu$ M for 24 hrs (Fig. 4). Linoleic, oleic, C15, EPA and phytanic acids significantly ( $p<0.05$ ) inhibited *IL-8* to 0.20±0.02, 0.35±0.05, 0.55±0.02, 0.65±0.01 and 0.64±0.02, respectively; *IL-12p35* to 0.61±0.03, 0.59±0.04, 0.68±0.02, 0.70±0.02 and 0.54±0.02, respectively; and *TNF- $\alpha$*  genes expression to 0.78±0.06, 0.64±0.04, 0.73±0.04, 0.72±0.02 and 0.54±0.07, respectively. Palmitic acid significantly induced the gene expressions of *IL-8* to 1.60±0.12, *IL-12p35* to 1.48±0.18 and *TNF- $\alpha$*  to 2.38±0.45. C17 acid inhibited *IL-8* gene expression to 0.72±0.05, ( $p<0.05$ ), while it has no effect on *IL-12p35* and *TNF- $\alpha$*  gene expressions (0.95±0.1 and 1.19±0.16, respectively).

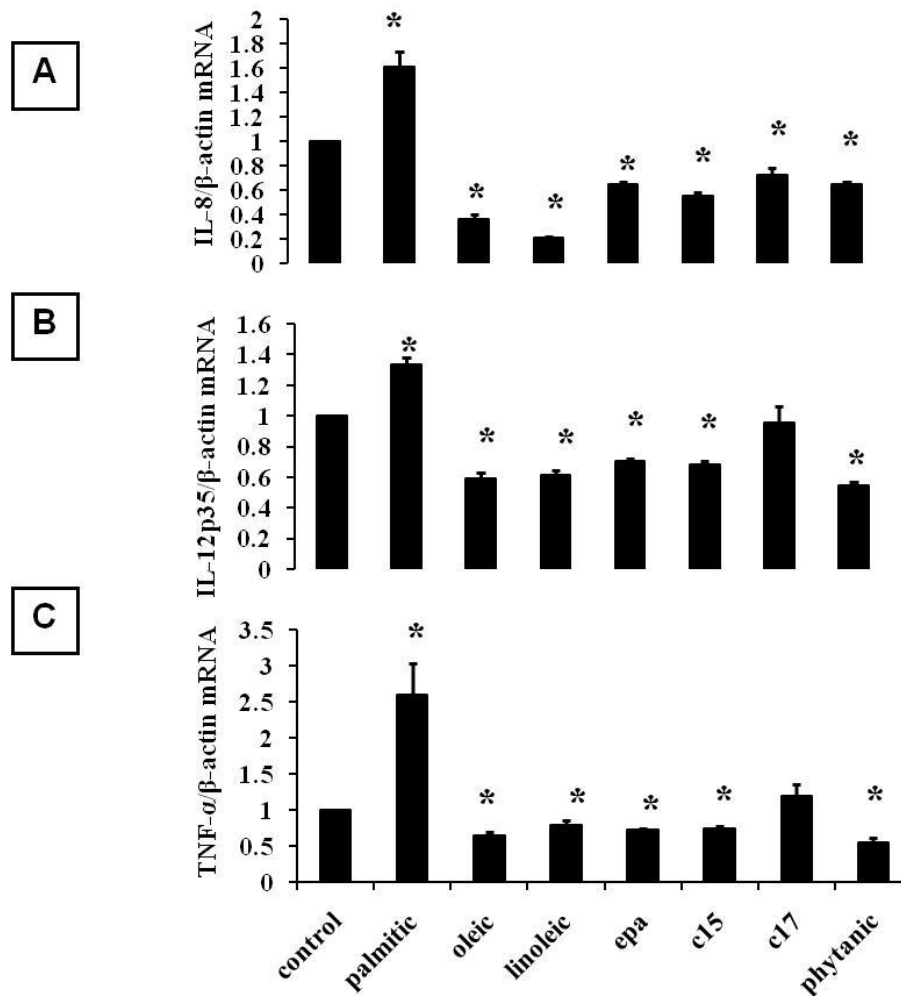


**Figure 3.** PPAR $\gamma$  and FFA regulate *PGlyRP3* gene expression in Caco-2 cells: Caco-2 cells were treated with PPAR $\gamma$  agonist GW1929, antagonist GW9662 or different FFA. After incubation for 24 hr, the expression of *PGlyRP3* gene was assessed by quantitative RT-PCR. Results are expressed as relative to  $\beta$ -actin mRNA as internal standard. The shown data are averages of 6 separate experiments performed in duplicates. Western blot analysis was applied to samples of the same treatments. (A) The PPAR $\gamma$  agonist GW1929 (10 $\mu$ M) induces the *PGlyRP3* gene (upper panel) and protein (lower panel) expressions in Caco-2 cells after culture for 24 hrs. (B) FFA (100  $\mu$ M) differentially regulate *PGlyRP3* gene (upper panel) and protein (lower panel) expressions. (C) The PPAR $\gamma$  antagonist GW9662 (10 $\mu$ M) inhibits the palmitic and oleic acid-induced *PGlyRP3* gene expressions (upper panel) and protein (lower panel). Statistical analysis: ANOVA; \* denotes  $p < 0.05$  in comparison to the control cultures.

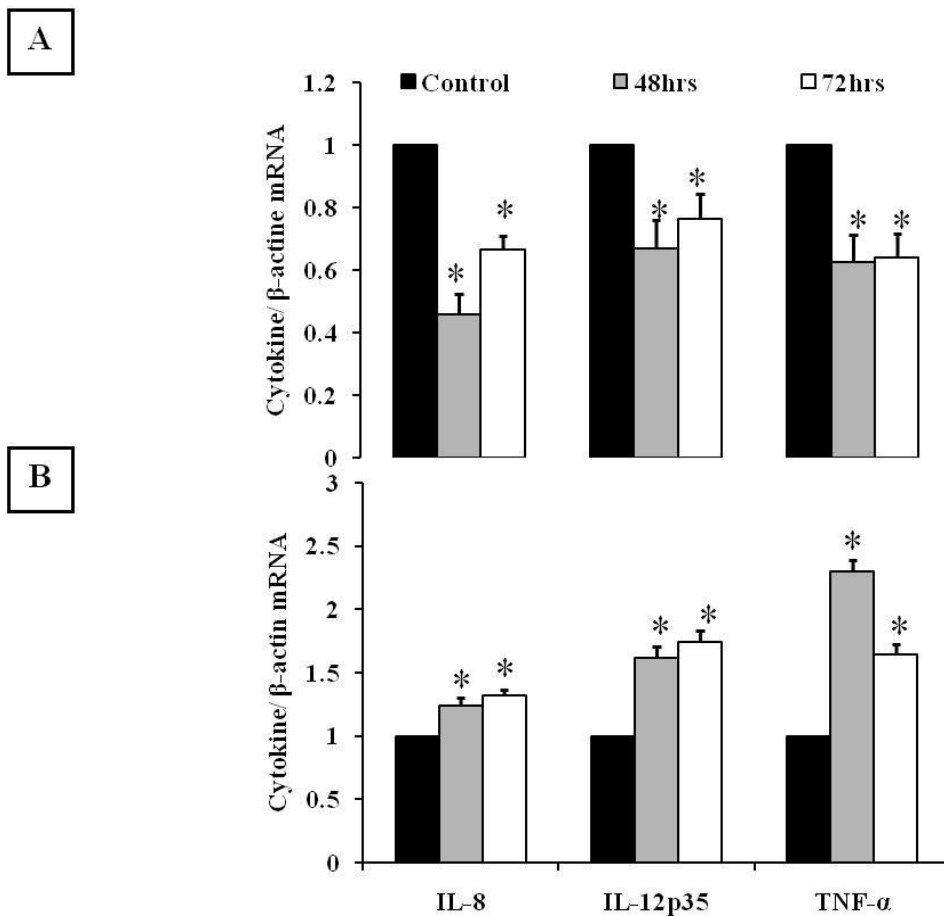
### ***PGlyRP3 down-regulates inflammatory cytokine expression in Caco-2 cells***

We next tried to evaluate whether PGlyRP3 was involved in the inflammatory response. Caco-2 cells were transfected with PGlyRP3 plasmid or pCAGGS empty control vector for 48 and 72 hrs. *PGlyRP3* gene overexpression was confirmed after 48 and 72 hrs post-transfection by quantitative RT-PCR and Western blotting (supplementary data, Fig. S1). *PGlyRP3* gene overexpression resulted in significant inhibition of the gene expression of *IL-8* to  $0.45\pm 0.06$ ,  $0.66\pm 0.04$ , *IL-12p35* to  $0.66\pm 0.09$ ,  $0.76\pm 0.07$  and *TNF- $\alpha$*  to  $0.62\pm 0.09$ ,  $0.63\pm 0.08$ , 48 and 72 hrs post-transfection, respectively in Caco-2 cells, when compared with control cells that were transfected with the empty vector only ( $p < 0.005$ ) (Fig. 5A).

Two siRNA sequences specifically targeting PGlyRP3 were mixed and transiently transfected into Caco-2 cells. The *PGlyRP3* gene knockdown was investigated 48 and 72h post-transfection by quantitative RT-PCR and Western blotting (supplementary data, Fig. S1). Specificity of gene silencing was confirmed, since transfection of a non-targeting, control siRNA sequence (siNEG) did not affect PGlyRP3 expression. The *IL-8*, *IL-12p35* and *TNF- $\alpha$*  gene expressions were significantly increased in the siPGlyRP3 cells (Fig. 5B).  $1.24\pm 0.05$ ,  $1.61\pm 0.11$  and  $2.30\pm 0.16$ -folds, respectively, after 48 h post silencing and  $1.32\pm 0.08$ ,  $1.75\pm 0.31$  and  $1.65\pm 0.16$ , respectively, after 72 h. In parallel experiments, siNEG sequence had no effect on *IL-8*, *IL-12p35* and *TNF- $\alpha$*  gene expression.



**Figure 4.** Differential effects of FFA on cytokine gene expressions: Caco-2 cells were incubated with 100  $\mu$ M of different FFA. After 24 hrs of incubation, mRNA expression of IL-8 (A), IL-12p35 (B) and TNF- $\alpha$ (C) were analyzed by quantitative RT-PCR which was performed using SYBR Green. Data are expressed as mean  $\pm$  SEM of n=4 experiments, \* denotes significant difference from the non-treated control value.



**Figure 5.** PGlyRP3 down regulates inflammatory cytokines expression in Caco-2 cells: Caco-2 cells were transfected with pCAGGS empty control vector or PGlyRP3 overexpression plasmid for 48 and 72 hrs (A) , or control negative siRNA or PGlyRP3 siRNA for 48 and 72 hrs (B). mRNA expression of IL-8, IL-12p35, TNF- $\alpha$  were analyzed by quantitative RT-PCR which was performed using SYBR Green. Data is expressed as relative mRNA accumulation corrected to  $\beta$ -actin mRNA as internal standard. The shown data is averages of 6 separate experiments performed in duplicates. Statistical analysis: \* denotes  $p < 0.05$  in comparison to the corresponding control.

### ***FFA that upregulate PGlyRP3 inhibit cytokine gene expression***

The assumption that FFA regulate IL-8, IL-12p35 and TNF- $\alpha$  expression through PGlyRP3 was confirmed by incubating Caco-2 cells for 24 hrs with 100  $\mu$ M of oleic or palmitic acids after PGlyRP3-silencing and over-expression. Oleic acid which has been shown to induce the *PGlyRP3* gene expression (Fig.

3B) and at the same time inhibit *IL-8*, *IL-12p35* and *TNF- $\alpha$*  gene expressions (Fig. 4), significantly increased the gene expression of the tested cytokines (Fig. 6A, left panel) and also protein expression of IL-12 (Fig. 6A, right panel) when added to the PGlyRP3-silenced Caco-2 cells. On the other side, incubation of PGlyRP3-overexpressing cells with this FFA enhanced the anti-inflammatory effect through inhibiting the gene expression of the tested cytokines (Fig. 6A, left panel) and also the protein secretion of IL-12 (Fig. 6A, right panel). In contrast, all cytokine gene expressions (Fig. 6B, left panel) and IL-12 protein secretion (Fig. 6B, right panel) were significantly increased in both PGlyRP3-silenced and PGlyRP3-over-expressing Caco-2 cells after incubation with palmitic acid, which has been previously shown to induce both PGlyRP3 and cytokines expression. Thus, palmitic acid enhanced the cytokine gene expression independent of the anti-inflammatory role of PGlyRP3.

### ***PGlyRP3 anti-inflammatory role includes the negative regulation of NF- $\kappa$ B pathway***

PPAR ligands have been reported to exert anti-inflammatory activities in different cells types by antagonizing the transcriptional activity of NF- $\kappa$ B through mechanisms that involve the induction of I $\kappa$ B- $\alpha$  expression and decay of COX-2 expression (24, 25). Therefore, we assumed that PGlyRP3 exerts its anti-inflammatory actions through a negative regulation of NF- $\kappa$ B pathway.

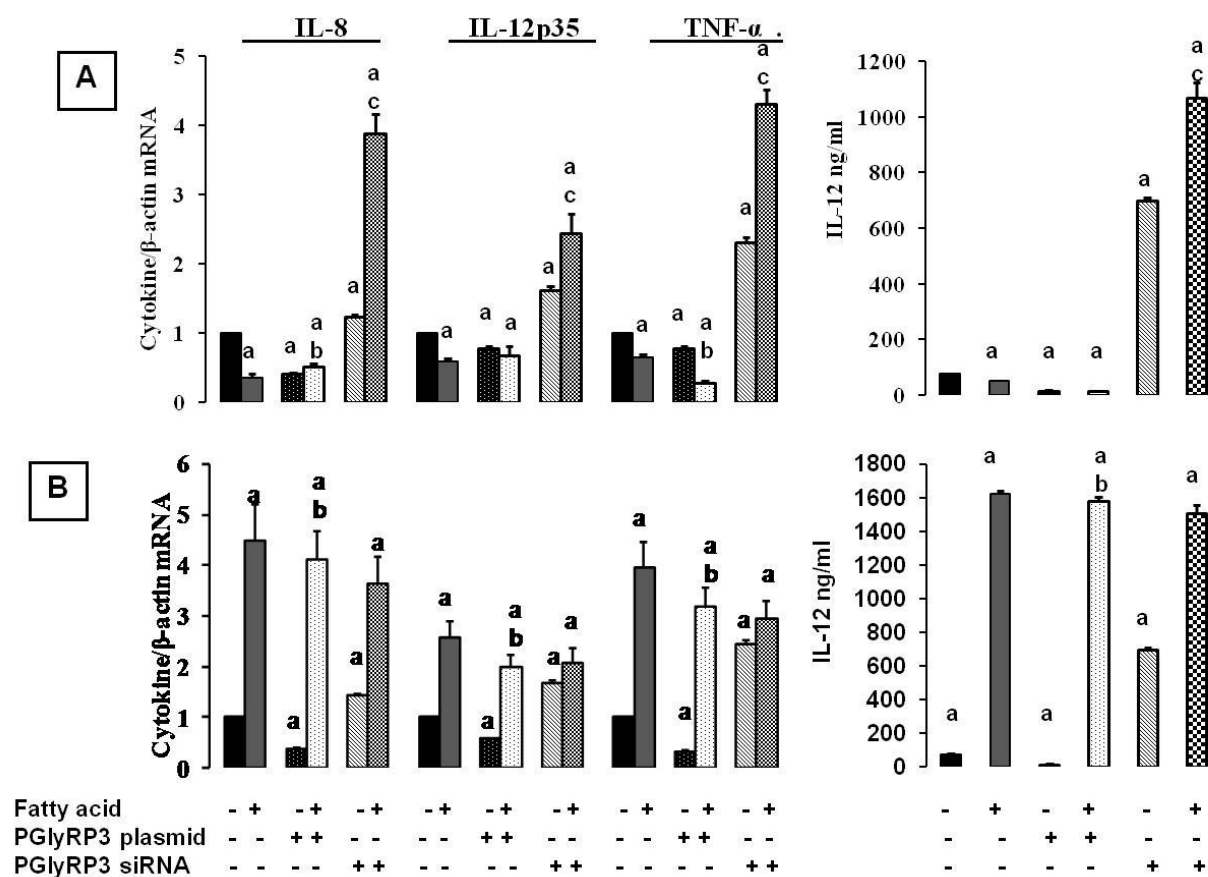
Incubation of Caco-2 cells with the PPAR $\gamma$  ligands (GW1929, or oleic acid) induced the I $\kappa$ B- $\alpha$  and inhibited NF- $\kappa$ B and COX-2 gene expressions (supplementary data, Fig. S3). In the same line, PGlyRP3 over-expression caused an increased expression of I $\kappa$ B- $\alpha$  and a decreased expression of NF- $\kappa$ B and COX-2 genes (supplementary data, Fig. S3). On the other side, knocking down the PGlyRP3 and palmitic acid suppressed the gene expression of I $\kappa$ B- $\alpha$



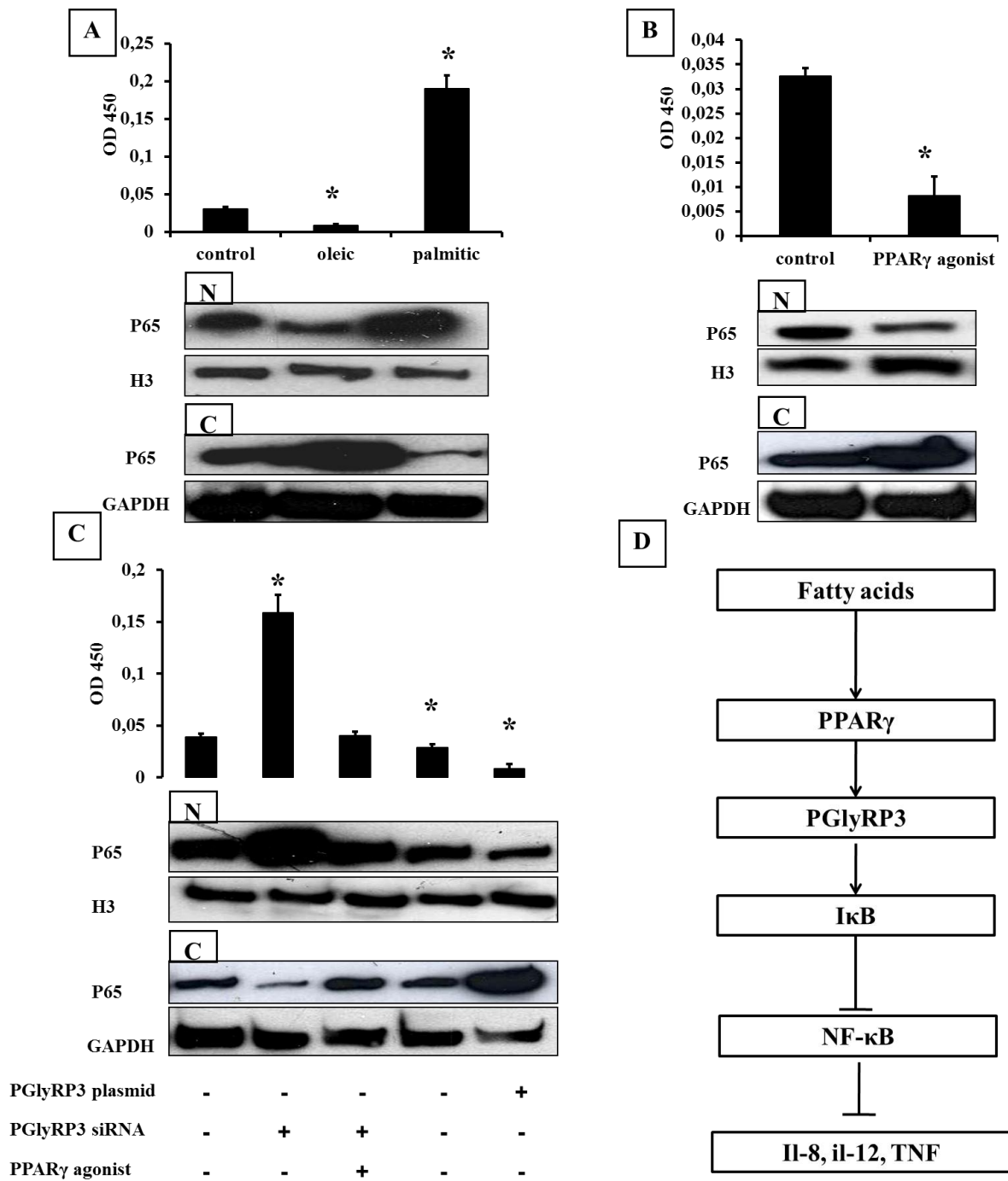
and induce the gene expression of NF- $\kappa$ B and COX-2 (supplementary data, Fig. S3). However, incubation of PGlyRP3-silenced cells with PPAR $\gamma$  agonist restored the I $\kappa$ B- $\alpha$ , NF- $\kappa$ B and COX-2 expression levels to the control value (supplementary data, Fig. S3). This indicates that PPAR $\gamma$ 's anti-inflammatory role is mediated via PGlyRP3-dependent and independent pathways.

To further examine the NF- $\kappa$ B signalling pathway in Caco-2 cells, we investigated the effect of PGlyRP3 on the activation state of NF- $\kappa$ B in Caco-2 cells by TransAm technique and Western blotting. Since NF- $\kappa$ B activation is accompanied by its translocation to the nucleus, we compared levels of NF- $\kappa$ B found in nuclear extracts from Caco-2 cells transfected with PGlyRP3 plasmid or PGlyRP3-silencing plasmid. Furthermore, we determined the NF- $\kappa$ B activation after incubation of Caco-2 cells with PPAR $\gamma$  agonist, oleic or palmitic acids. We found that PGlyRP3-silencing or palmitic acid-treatment induced the translocation of NF- $\kappa$ B into the nucleus (Fig. 7A,B,C), while PGlyRP3 over-expression, PPAR $\gamma$  agonist and oleic acid suppressed the NF- $\kappa$ B translocation.

Taken together, these results indicate that PGlyRP3 up-regulates the expression of I $\kappa$ B- $\alpha$  gene, down-regulates NF- $\kappa$ B and COX-2 genes expressions and inhibits the NF- $\kappa$ B nuclear localization. The present results suggest that the anti-inflammatory role of PGlyRP3 is exerted through the NF- $\kappa$ B pathway (Fig. 7D).



**Figure 6.** FFA regulate cytokine gene expression through PGlyRP3: Caco-2 cells were transfected with PGlyRP3 expression plasmid or with PGlyRP3 siRNA for 24 hrs and treated with oleic (A) or palmitic acids (B). After 24 hrs incubation, mRNA expression of IL-8, IL-12p35, TNF- $\alpha$  were analyzed by quantitative PCR analysis which was performed using SYBR Green and IL-12 secretion was analyzed by ELISA (A and B right panel). The shown data are averages of 6 separate experiments performed in duplicates. Statistical analysis: a, significantly different from the control non-treated; b, significantly different from the corresponding PGlyRP3 over-expression values; c, significantly different from the corresponding PGlyRP3 silencing values.



**Figure 7.** A negative regulation of NF- $\kappa$ B pathway is involved in the anti-inflammatory PGlyRP3 role: Caco-2 cells were incubated with oleic or palmitic acids (A), GW1929 (B) or transfected with PGlyRP3 plasmid or siRNA (C). Nuclear and cytoplasmic proteins were extracted from these cells. NF- $\kappa$ B (p65) activation was assayed by TransAm (A, B and C upper panel). Western blot analysis was applied to Caco-2 nuclear (N) and cytoplasmic (C) extracts of the same treatments (A, B and C lower panel). Data is presented as mean  $\pm$  SEM of n = 4 experiments performed in duplicates. Statistical analysis: \*, significantly different from the control. (D) Proposed sequence of events that lead to suppression of inflammation by PPAR $\gamma$ -induced PGlyRP3 expression.

## DISCUSSION

This study was designed to investigate the immunomodulatory role of FFA through the regulation of PGlyRPs. We focused on PGlyRP3 because of the observation that Caco-2 cells express only the *PGlyRP3* gene (Fig. 1). Previous studies have shown that *PGlyRP3* gene is widely expressed in human tissues including skin, eyes, salivary glands, throat, tongue, esophagus, stomach and intestine (20). Our results demonstrate that PGlyRP3 modulate inflammatory cytokine production in enterocytes through PPAR $\gamma$  and its lipophilic ligands, which enhance induction of PGlyRP3 in Caco-2. The results also show an anti-inflammatory role of *PGlyRP3* gene in the intestinal cells.

The present results demonstrate the regulation of *PGlyRP3* gene expression in Caco-2 cells by PPAR $\gamma$ -RXR $\alpha$  complex. First, *in silico* analysis demonstrated that there is a PPAR $\gamma$ -responsive element (PPRE) in the distal region of the *PGlyRP3* gene promoter. This was verified by EMSA showing that PPAR $\gamma$  and PPAR $\gamma$ -RXR $\alpha$  complex bind to the distal PGlyRP3 promoter region (Fig. 2). Second, this PPAR $\gamma$ -responsive element on the *PGlyRP3* gene promoter is functional: PGlyRP3 expression was enhanced by GW1929 (PPAR $\gamma$  agonist) (Fig. 3A) and different FFAs (Fig. 3B), which are ligands of PPAR $\gamma$ . Third, the effect of FFAs on *PGlyRP3* gene expressions was abolished by treatment with the PPAR $\gamma$  antagonist GW9662 (Fig. 3C). Taken together, the present results prove the modulation of *PGlyRP3* gene expression by PPAR $\gamma$ -RXR $\alpha$  complex. This modulation represents a novel regulatory pathway of the *PGlyRP3* gene.

It is known that up-regulation of PGlyRP mRNAs expression induced by bacterial cell surface components is mediated through NF- $\kappa$ B. Suppression of NF- $\kappa$ B activation prevented the induction of *PGlyRPs* gene expression induced by PAMPs (7, 26). On the other hand, it was also reported that ligand-activated

PPAR $\gamma$  down-regulates NF- $\kappa$ B transcription (27) and TLR4 protein and mRNA expressions (28). We therefore have expected that activation of PPAR $\gamma$  will downregulate NF- $\kappa$ B, which in turn down-regulates *PGlyRP3* gene expression. The present results demonstrate that *PGlyRP3* is, in contrast to our expectation, up-regulated by a PPAR $\gamma$  agonist and some PPAR $\gamma$  ligands. This up-regulation seems to be due to the direct binding between PPAR $\gamma$  and its PPRE in the promoter region of *PGlyRP3* gene, rather than being mediated through NF- $\kappa$ B.

The induction of *PGlyRP3* gene expression and the dietary FFA that induce *PGlyRP3* gene expression modulate the expression and production of some inflammatory cytokines in Caco-2 cells. The induction of *PGlyRP3* gene expression, by FFA (Fig. 4, 6) or by over-expression (Fig. 5A, 6), inhibits, while suppression of its expression, by silencing, induces IL-8, IL-12p35 and TNF- $\alpha$  expression (Fig. 5B, 6). These results suggest that mammalian *PGlyRP3* has anti-inflammatory properties.

The modulation of cytokines by FFA was also reported. The anti-inflammatory effects of dietary oleic acid (29) and n-3-PUFAs (30) has been suggested to be mediated through the inhibition of TLR-induced signaling pathways, and the possibility that both the beneficial and detrimental effects of different dietary FFA may in part be mediated through the modulation of TLRs (31) or NODs (32). Activation of TLR or NODs synergistically initiates a signaling pathway leading to the activation of NF- $\kappa$ B (33, 34). In fact, activation of TLR and NOD pathways lead to activation of inflammatory pathways. NF- $\kappa$ B turns on many cytokine and chemokine genes (31), which mostly contain binding motifs for NF- $\kappa$ B in their promoter regions (35). Thus, palmitate increased, whereas poly-unsaturated FFA inhibited *IL-8* gene expression and secretion through mechanisms involving activation of NF- $\kappa$ B in colonic epithelial cells (36). The present results demonstrate that *PGlyRP3* exerts a direct anti-inflammatory role, by inhibiting the studied pro-

inflammatory cytokines through a mechanism including the suppression of NF- $\kappa$ B pathway (Fig. 7).

On the other side, the activation of PPAR $\gamma$  by certain polyunsaturated FFA (37), phytanate (31) and butyrate (38) has been reported. It is also known that PPAR $\gamma$  agonists inhibit the production of inflammatory cytokines (39, 40). This is confirmed in our present study in which the PPAR $\gamma$  agonist inhibited *IL-8*, *IL-12p35* and *TNF- $\alpha$*  genes expression in Caco-2 cells (supplementary data, Fig. S4). In addition, Clare's group found that Cis-9, trans-11-conjugated linoleic acid but not trans-vaccenic acid reduced the gene expression of *TNF- $\alpha$* , *IL-12p35* and *IL-6* and the concentration of *IL-12p10* in Caco-2 cells via NF- $\kappa$ B and/or PPAR $\gamma$ -dependent mechanism (41). It was showed that PPAR ligands exert their anti-inflammatory activities in different cells by antagonizing the transcriptional activity of NF- $\kappa$ B by the induction of I $\kappa$ B- $\alpha$  expression (23). In addition, treatment with the PPAR $\gamma$  ligands promotes the decay of COX-2 expression. Induction of I $\kappa$ B- $\alpha$  and the subsequent inhibition of NF- $\kappa$ B have been reported to mediate the inhibition of adhesion molecules and COX-2 expression (25). However, the mechanism by which PPAR $\gamma$  up-regulates the I $\kappa$ B- $\alpha$  induction is not fully understood. Our study demonstrates that PPAR $\gamma$  exerts its anti-inflammatory effect due to a direct binding to and modulation of the expression and activity of the *PGlyRP3* gene, which in turn activates I $\kappa$ B- $\alpha$  and inhibits the NF- $\kappa$ B translocation into the nucleus. In this context, it could be shown that FFA of food or microbial origin could modulate the immunological *PGlyRP3* gene through PPAR $\gamma$ . The induction of *PGlyRP3* gene was sufficient to up-regulate I $\kappa$ B- $\alpha$  and down-regulate the transcription of NF- $\kappa$ B, COX-2 and different cytokines.

In summary, our results suggest that PGlyRP3 protein is involved in inhibiting inflammation and possibly immunotolerance, and is modulated by

non-microbial as well as by microbial lipophilic ligands of PPAR $\gamma$ . These results demonstrate a differential modulation of IL-8, IL-12 and TNF- $\alpha$  expression by PPAR $\gamma$  and its lipophilic ligands (FFA) in Caco-2 cells mediated through binding of PPAR $\gamma$  to *PGlyRP3* gene promoter, which leads to PGlyRP3 transcription.

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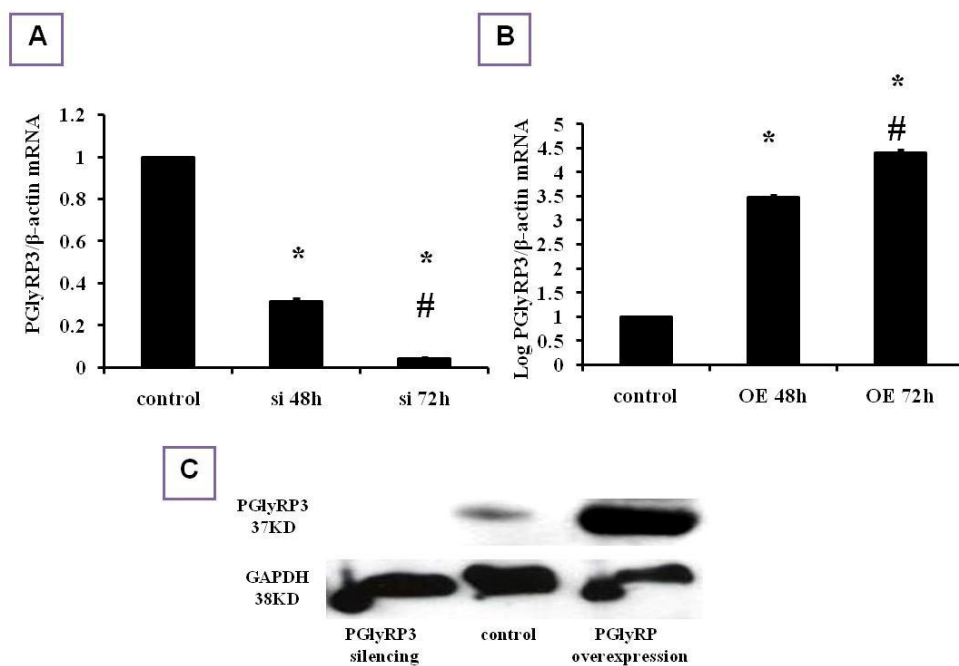


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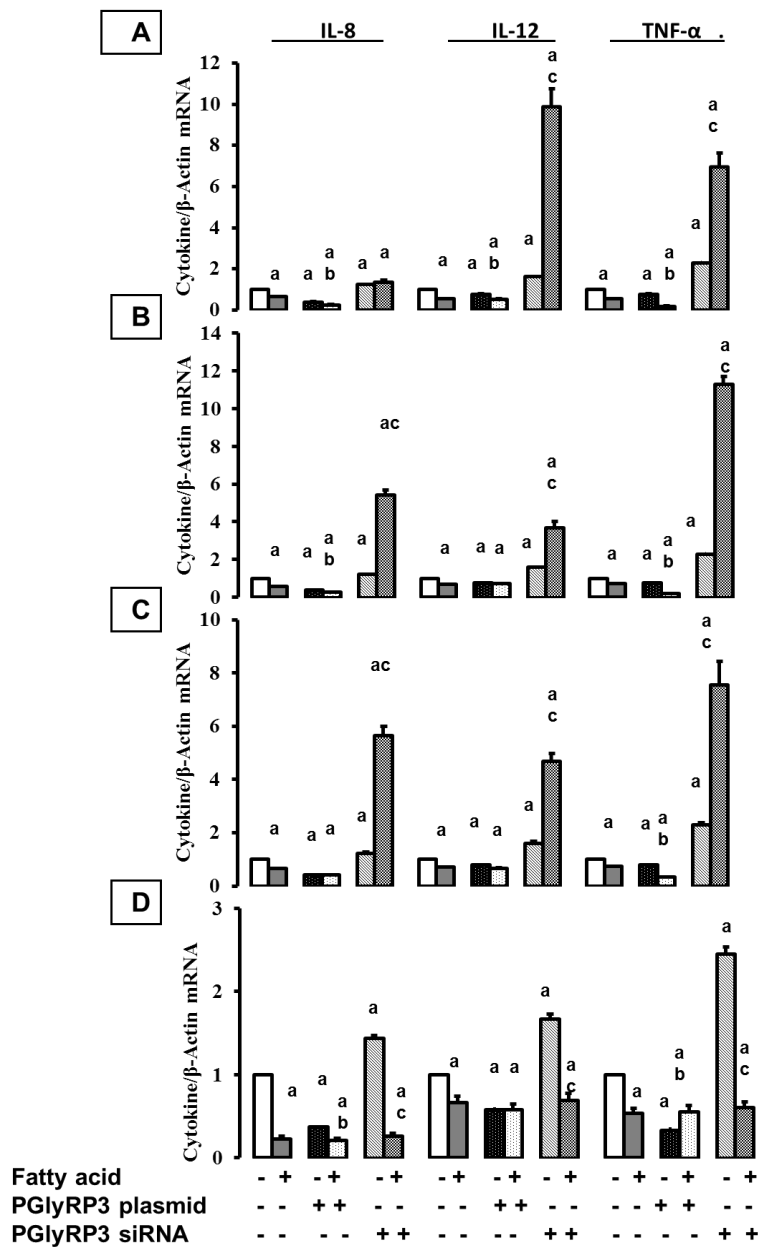
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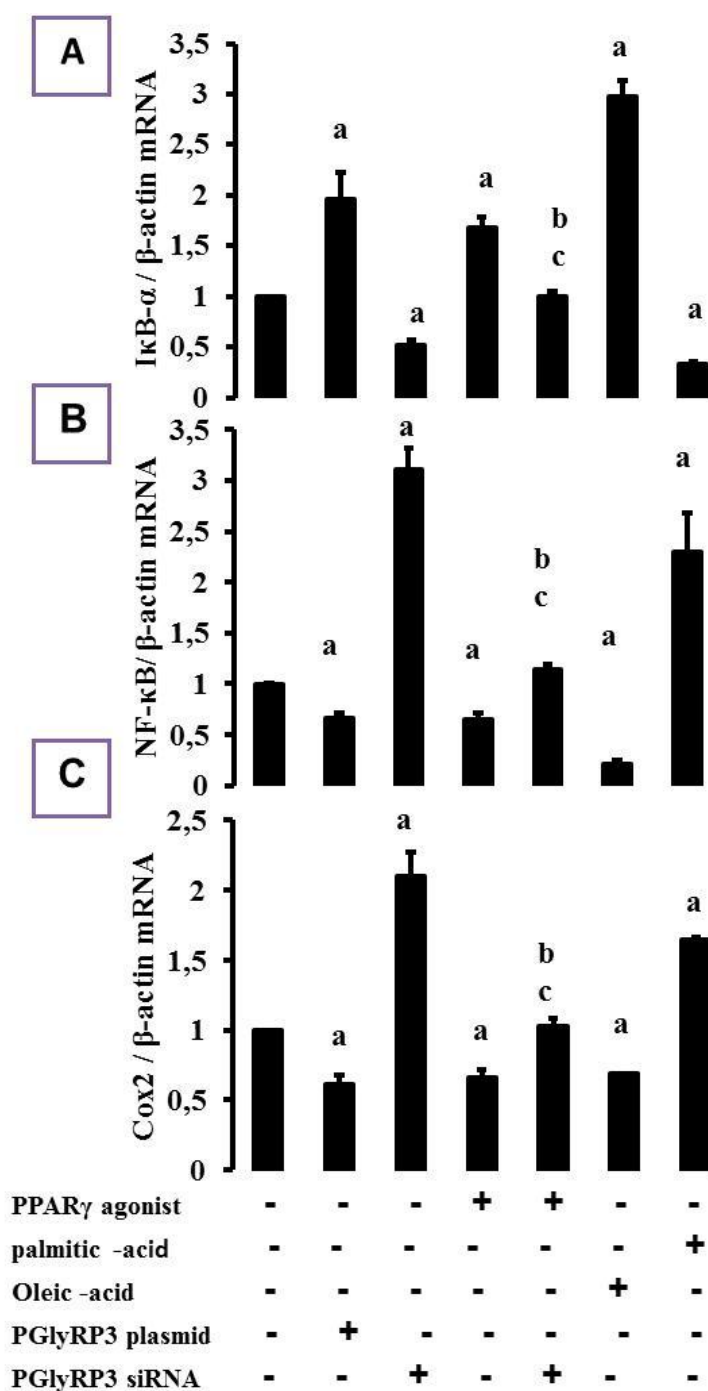
## SUPPLEMENTARY DATA



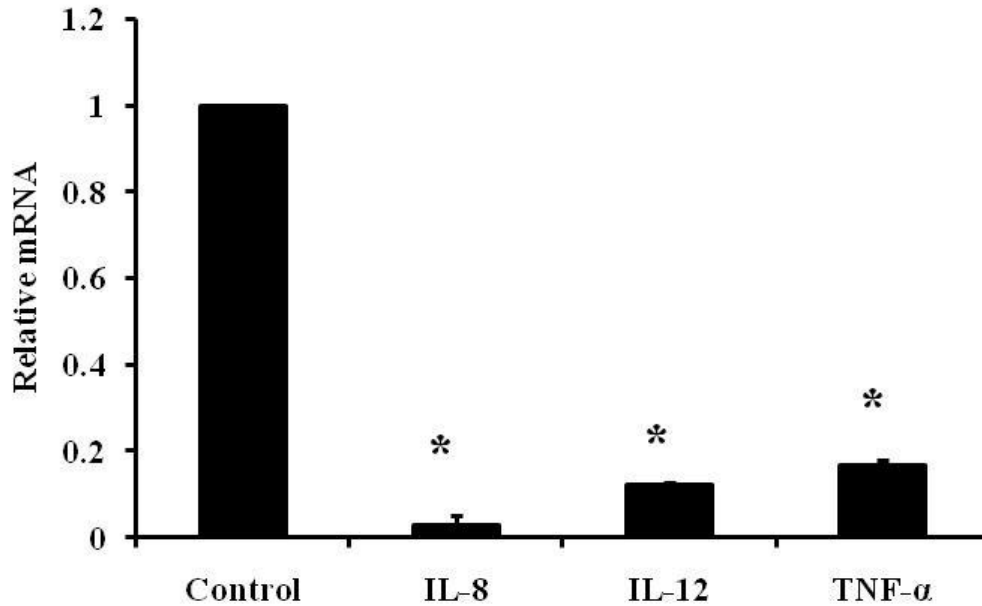
**FIG. S1.** Evidence of PGlyRP3 silencing / over-expression in Caco-2 cells: Cells were transfected with a mixture of 2 siRNA, silencing negative, empty vector or PGlyRP3 plasmid. RNA was isolated after either 48 or 72 hrs. A and B) Quantitative PCR analysis of *PGlyRP3* gene expression after silencing (si) and overexpression (OE). Statistical analysis: \* denotes difference from control; # denotes difference from the 48 hr-value. C) Western blot analysis of PGlyRP3 protein expression after silencing and over-expression.



**Fig. S2.** Fatty acids regulate cytokine gene expression through PGlyRP3: Caco-2 cells were transfected with PGlyRP3 expression plasmid or with PGlyRP3 siRNA for 24 hrs and treated with one of the following fatty acids: (A) phytanic, (B) c15, (C) EPA, (D) linoleic acids. After 24 hrs incubation, mRNA expression of IL-8, IL-12p35, TNF-α were analyzed by quantitative RT-PCR analysis which was performed using SYBR Green. Results are expressed as relative mRNA to β-actin mRNA as internal standard. The shown data are averages of 6 separate experiments performed in duplicates. Statistical analysis: a, significantly different from the control non-treated; b, significantly different from the corresponding PGlyRP3 over-expression values; c, significantly different from the corresponding PGlyRP3 silencing values.



**Fig. S3.** A negative regulation of NF- $\kappa$ B pathway is involved in the anti-inflammatory PGlyRP3 role: Caco-2 cells were incubated with GW1929, oleic or palmitic acids, or transfected with PGlyRP3 plasmid or siRNA. mRNA was analysed for the gene expression of (A) IkB- $\alpha$ , (B) NF- $\kappa$ B and (C) COX-2 quantitatively by RT-PCR. Data is presented as mean  $\pm$  SEM of n = 4 experiments performed in duplicates. Statistical analysis: a, significantly different from the non-treated control; b, significantly higher than the PPAR $\gamma$  value; c, significantly lower than the silencing value.



**Fig. S4.** PPAR $\gamma$  inhibit cytokines expression: Caco-2 cells were cultured with or without 10 $\mu$ M of the PPAR $\gamma$  agonist GW 1929 for 24 hrs. After incubation, total RNA was extracted and reverse transcribed to cDNA, then the expression of IL-8, IL-12p35 and TNF- $\alpha$  were analyzed by quantitative RT-PCR using SYBR Green assays. Results are expressed as relative to  $\beta$ -Actin mRNA as internal standard. The shown data are averages of 4 separate experiments performed in duplicates. Statistical analysis: \* denotes  $p < 0.003$  in comparison to the control cultures.

Table 1. Nucleotide sequences of studied genes for RT-PCR

Gene	Accession No.	Sense primer	Antisense primer	Product size (bp)
PGlyRP3	NM_052891	5'-ACCCTCAACATCCAGTGATGC-3'	5'-ATCCAACCCCTTCATACACGC-3'	273
IL-8	NM_000584	5'- GTGCAGTTTTGCCAAGGAGTG -3'	5'- ACTTCTCCACAACCCTCTGC -3'	215
IL-12p35	NM_000882	5'- TTGTGGCTACCCTGGTCCT-3'	5'- AGAGTTTGTCTGGCCTTCTGG -3'	151
TNF- $\alpha$	NM_000594	5'- TCAACCTCCTCTCTGCCATC -3'	5'- CCAAAGTAGACCTGCCCAGA -3'	187
I $\kappa$ B- $\alpha$	NM_020529	5'- CTCCATCCTGAAGGCTACCA -3'	5'- CCCCACACTTCAACAGGAGT -3'	203
NF- $\kappa$ B1	NM_003998	5'- ACTGTGAGGATGGGATCTGC -3'	5'- GCACCAAGAGTCCAGGATTA -3'	165
COX-2	NM_000963	5'- CCACCCGCAGTACAGAAAGT -3'	5'- CAGGATACAGCTCCACAGCA -3'	196
$\beta$ - Actin	NM_001101	5'- GATATCGCTGCGCTCGTC-3'	5'-TCCATATCGTCCCAGTTGG-3'	239
<i>Oligonucleotide sequences for EMSA</i>				
PGlyRP3	NM_052891	5'-TCTCAT <b>AGGTCA</b> TTGTGAGACTACTTAGTG-3'	5'-ACTAAGTAGTCTCACAA <b>TGACCT</b> ATGAGA-3'	
<i>Oligonucleotide sequences for PGlyRP3 gene cloning</i>				
EcoRIPGlyRP3		5'-CTGGAATTCATGGGGACGCTGCCATGG-3'		1044
XhoI-PGlyRP3			5'-CAGCTCGAGTCAGTGCTTCAAATGAGG-3'	



A decorative graphic on the left side of the page consists of three overlapping light blue circles of varying sizes. Two thin, light blue lines extend from the top right and bottom left towards the circles, creating a sense of movement and design.

## **CHAPTER III**

**Prebiotic oligosaccharides reduce  
pro-inflammatory cytokines in  
intestinal cells depending on  
PPAR $\gamma$  and peptidoglycan  
recognition protein 3 (PGlyRP3)**

*Journal of Nutrition, in press*

**Prebiotic oligosaccharides reduce pro-inflammatory cytokines in intestinal cells depending on PPAR $\gamma$  and peptidoglycan recognition protein 3 (PGlyRP3).**

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3. Clinical Research Centre Kiel, Kiel, Germany.

**Key words:** PGlyRP3, Prebiotic, Oligosaccharide, Cytokine, inflammation, PPAR $\gamma$ , Caco-2.

## **ABSTRACT**

Prebiotic oligosaccharides modulate the intestinal microbiota and beneficially affect the human body through reducing the intestinal inflammation. This immunomodulatory effect was assumed to be bacterial in origin. However, some observations suggest that oligosaccharides may exert an anti-inflammatory effect *per se*. We hypothesize that oligosaccharides affect the intestinal immunity via activation of PGlyRP3, which reduces the expression of pro-inflammatory cytokines.

The results showed that the oligosaccharides  $\alpha$  3-sialyllactose and the fructo-oligosaccharide (Raftilose p95) have anti-inflammatory potency through reduction of IL-12 secretion in Caco-2 cells and gene expression of *IL-12 p35*, *IL-8* and *TNF- $\alpha$* . They also reduced the gene expression and nuclear translocation of NF-kB. Both oligosaccharides induced dose and time-dependently the production of PGlyRP3, whose silencing abolished the anti-inflammatory role of both oligosaccharides. Incubation of Caco-2 cells with both oligosaccharides induced PPAR $\gamma$ . PPAR $\gamma$  antagonism inhibited the oligosaccharide-induced PGlyRP3 production and the anti-inflammatory effect.

We conclude that oligosaccharides may exert an anti-inflammatory effect by inducing the nuclear receptor PPAR $\gamma$ , which regulates the anti-inflammatory PGlyRP3.

### **INTRODUCTION**

Prebiotics are non-digestible food components that beneficially affect the human body through modulation of the intestinal microbiota by selectively stimulating the growth and/or activity of some bacterial species in the colon (1). They are complex carbohydrates with the fructooligosaccharides (FOS) and the manooligosaccharides (MOS), which are the most frequently encountered commercial forms. These oligosaccharides contain three-to-ten monosaccharide residues covalently linked through glycosidic bonds. Neutral oligosaccharides contain no charged carbohydrate residues, while acidic oligosaccharides contain one or more negatively charged COOH-containing residues such as N-acetylneuraminic acid (sialic acid) (2). Oligosaccharides are present at a high concentration in human milk. About 80 different oligosaccharides with well defined chemical structures have been isolated from human milk (3). In bovine milk, oligosaccharides are present in smaller amounts. In bovine colostrum the concentrations are considerably higher than in bovine milk. The most abundant acidic oligosaccharides in bovine colostrum are 3-sialyllactose, sialyllactose amine, 6-sialyllactose and disialyllactose (4). Also in plants (vegetables, cereals, roots, and fruits) prebiotic oligosaccharides were found, where they serve as storage carbohydrates. They consist of mixtures of fructose moieties linked by  $\beta$ -(2 $\rightarrow$ 1)-glycosidic bonds with a terminal glucose unit. These are neither digestible nor absorbed in the stomach and small intestine, and reach the colon, where they are fermented by the microbiota (5). Raftilose P95 is composed of 95% FOS, it is the prebiotic inulin enriched with oligofructose (5).

The immune system highly depends on a satisfactory supply of nutrients to function (6). Few studies have considered the immunomodulatory effects of oligosaccharides in humans and experimental animal models. The daily administration of different oligosaccharides significantly reduced damage

associated with Crohn's disease, ulcerative colitis and experimental colitis in rats (7-10). It was also reported that the acidic oligosaccharide increased the secretion of the interferon-gamma in CD3<sup>+</sup> CD4<sup>+</sup> and CD3<sup>+</sup> CD8<sup>+</sup> cells and the IL-13 secretion in CD3<sup>+</sup> CD8<sup>+</sup> cells (11). Moreover, human and plant milk-derived oligosaccharides regulate the secretion of some in cord blood derived T cells *in vitro* (11). An immunomodulatory effect of bovine colostrum has been reported on the gut-associated lymphoid tissue (GALT), which responded by producing both Th1 pro-inflammatory cytokines (IL-2, IFN- $\gamma$  and IL-12) and Th2 anti-inflammatory cytokines (IL-4 and IL10). This balance between Th1 and Th2 cytokines production protected the weaned piglets from both allergic and infectious diseases (12). It was found that, the FOS supplementation affect the mucosal dendritic cell (DC) function by increasing the faecal bifidobacteria concentrations which, increased the percentage of *lamina propria* DC releasing IL-10 and decreased Crohn's disease activity. This *in vivo* immunomodulatory effect was reported to be dependent on the bifidogenic effect of FOS acting via TLRs or other pattern recognition receptors (9, 13). However, the anti-inflammatory events such as triggering IL-10 production also occur *in vitro* (9). This suggests that oligosaccharides render this effect *per se* (9). In addition, there is evidence that oligosaccharides can interact with the immune system not only via the intestinal flora but also by direct interaction with immune cells (14)

PGlyRPs are a novel family of pattern recognition molecules (PRMs) that are highly conserved from insects to mammals (15, 16). Mammals have four PGlyRPs named PGlyRP1, 2, 3, and 4 (15,16). The family members show selective and differential expression; PGlyRP1 is highly expressed in bone marrow, PGlyRP2 in the liver, and PGlyRP3 and 4 in the oesophagus and elementary canal (17). Their distinct expression patterns suggest that each may play a unique role in host defence. Our previous study (18) showed that the only PGlyRP3 is expressed in Caco-2 cells. So far, there is no information available

whether the immunomodulation of oligosaccharides takes place through recognition pattern receptors such as PGlyRPs. Our previous results showed that PGlyRP3 mediates anti-inflammatory effects through NF- $\kappa$ B pathway suppression (18). We hypothesize that oligosaccharides can initiate an anti-inflammatory response even in the absence of intestinal bacteria, through a direct activation of PGlyRP3. To test this hypothesis, we exposed the intestinal Caco-2 cells *in vitro* to 2 different oligosaccharides and quantified the effect on PGlyRP3 expression and tried to elucidate the role of this pattern recognition receptor.

## MATERIAL AND METHODS

### *Cell culture*

Caco-2 cells were obtained from the American Type Culture Collection Company (ATCC). Cells between passages 10-50 were grown in 75 cm<sup>2</sup> culture flasks at 37°C and 5% CO<sub>2</sub> using Minimum Essential Medium (MEM + GlutaMAX<sup>TM</sup>, Invitrogen, Karlsruhe, Germany) supplemented with 20% fetal calf serum, 100 IU penicillin/ml and 1% non-essential amino acids. For investigating the effects of oligosaccharides, cells were cultured in 6 well plates until reaching 7 days post confluency. Cells were fasted for 2 hours in Minimum Essential Medium (MEM + GlutaMAX<sup>TM</sup>) without any supplementations before the experimental incubations. For over-expression and RNA-silencing experiments, cells were cultured in 24-well plates until reaching 80-100% confluence.

### ***Preparation of oligosaccharides and incubation regimes***

$\alpha$ 3-sialyllactose was provided by GALAB Technologies (Geesthacht, Germany), while Raftilose P95 was obtained from Orafiti (Tienen, Belgium). Stock solutions were prepared by dissolving oligosaccharides in MEM + GlutaMAX<sup>TM</sup>. Fasted cells were incubated for 24 h with different concentrations of  $\alpha$ 3-sialyllactose or Raftilose P95 or for 3, 6, 12, 24 hours with a concentration of 50 mg/L or 50 g/L of  $\alpha$ 3-sialyllactose and Raftilose P95, respectively. Control incubations were supplemented with the MEM + GlutaMAX<sup>TM</sup>.

### ***RNA isolation, cDNA synthesis, and quantitative real time PCR analysis***

All quantitative RT-PCR analyses were performed on an Applied Biosystems ABI Prism 7000 system using cDNA samples corresponding to 20 ng RNA. Quantitative analysis of *PGlyRP3* gene expression was undertaken by using TaqMan gene expression assay in a 30  $\mu$ l reaction volume with universal PCR master mix and TaqMan MGB probe (No. Hs 01074825\_ml, Applied Biosystems, Cambridge, UK). Analyses of all other studied genes were performed using SYBR Green (Power SYBR green Mastermix, Applied Biosystems). All studied genes were quantified within the same cDNA samples. A dissociation curve was performed for each product to assure the absence of primer dimers or unspecific products. Primers used in the present study are listed in Table 1.

To normalize expression data,  $\beta$ -actin was used as an internal control gene. Data are presented as fold difference from control, considering the control values as 1.

## Chapter III

**Table 1.** Nucleotide sequences of primers for the genes studied by using RT-PCR.

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NF- $\kappa$ B1	NM_003998	5'-ACTGTGAGGATGGGATCTGC-3'	5'-GCACCAAGAGTCCAGGATTA-3'	165
PPAR $\gamma$	NM_013124	5'-TGGGAGATCCTCTGTTGAC-3'	5'-GGAGCAGAAATGCTGGAGAA-3'	214
$\beta$ -Actin	NM_001101	5'-GATATCGCTGCGCTCGTC-3'	5'-TCCATATCGTCCCAGTTGG-3'	239

*Oligonucleotide sequences for PGlyRP3 gene cloning*

EcoRIPGlyRP3		5'-CTGGAATTCATGGGGACGCTGCCATGG-3'		1044
XhoI-PGlyRP3			5'-CAGCTCGAGTCAGTGCTTGAATGAGG-3'	

### *Transfection of Caco-2 cells with PGlyRP3 overexpression plasmid*

RNA was isolated from human oesophagus (approved by the ethics committee of the Christian-Albrechts-University of Kiel “protocol A 120/05”) and using the designed primers (table 1), full-length cDNAs coding for PGlyRP3 was cloned. We designed oligonucleotide primers after testing the sequences for restriction enzymes (XhoI and EcoRI) that neither cut within the PGlyRP3 nor the vector sequences. The PCR products were ligated into the pCAGGS vector (T4-Ligase Rapid-Kit, Fermentas, Karlsruhe, Germany), and clones with the proper full-length PGlyRP3 insert were selected and identified by restriction digestion and sequencing the inserts. The cloned PGlyRP3 was identical to the genomic sequences.

Caco-2 cells were seeded in a 24-well plate at a density of  $4 \times 10^4$  cells per well in 1 ml of culture medium and transfections performed at 60–80% cell confluence. Prior to transfection, TurboFect (Fermentas) and MEM (Invitrogen)



were mixed and incubated at room temperature for 20 min. 100 ng DNA was added in a ratio of 3:1 (TurboFect in  $\mu$ l: DNA in ng) as recommended by the manufacturer. Control samples were treated similarly and transfected with the empty vector (pCAAGS). After 48 and 72 h post transfection, RNA was isolated and transferred into cDNA. *PGlyRP3* gene over-expression was confirmed using quantitative RT-PCR and Western blot.

### ***Small interfering (si) RNA experiments***

Two siRNA sequences targeting *PGlyRP3* (SiSelect siRNA no. s41580 and s41581) and a non-targeting negative control sequence (SC 4390843) were purchased from Ambion (Applied Biosystems). Caco-2 cells were reverse transfected with siRNA using siPORT NeoFX Transfection Reagent (Ambion, Applied Biosystems). siRNA was prepared in Opti-MEM serum-free medium (Invitrogen) by mixing 2  $\mu$ l of the transfection reagent with 5 nM siRNA, respectively, at room temperature for 10 min. Caco-2 cells ( $4 \times 10^4$  cells per well) were transferred to a 24-well plate containing siRNA transfection reagent complexes, allowing transfection to occur during initial cell adherence. Medium was replaced after 24 h by medium containing 20% FCS (allowing cell recovery) and incubation continued for an additional 24 and 48 h then RNA was isolated and transferred into cDNA. *PGlyRP3* gene knock-down was confirmed by quantitative RT-PCR and Western blot.

### ***TransAm assay for NF- $\kappa$ B nuclear localization***

Caco-2 cells were incubated with 50 mg/L of  $\alpha$ 3-sialyllactose or 50 g/L of Raftilose p95 for 6 h. Nuclear extracts were prepared using a nuclear extraction kit (Panomics, Affymetrix, Heidelberg, Germany) according to manufacturer's

instructions. Nuclear localization of NF- $\kappa$ B was quantified using Transcription Factor ELISA kit (TransAm) to detect activated p65 subunit of NF- $\kappa$ B as illustrated in the kit user manual (Panomics, Affymetrix).

### ***Protein extraction and Western blot analysis***

The cell lysates were subjected to 12% SDS-PAGE and blotted onto PVDF transfer membranes. Membranes were blocked with a mixture of 2.5% BSA and 2.5% skimmed milk and incubated with mouse monoclonal anti-human PGlyRP3 (1:200, AXXORA, Lörr, Germany) and rabbit polyclonal anti-human GAPDH (1:500, Santa Cruz Biotechnology, Heidelberg, Germany). Immunoreactive bands were detected with HRP-conjugated secondary antibody.

### ***Interleukin 12 protein assay***

The human total IL-12 in the supernatant obtained from Caco-2 cell cultures after each treatment was detected using an ELISA kit (Mabtech AB, Hamburg, Germany) according to the manufacturer's instructions.

### ***Statistical analyses***

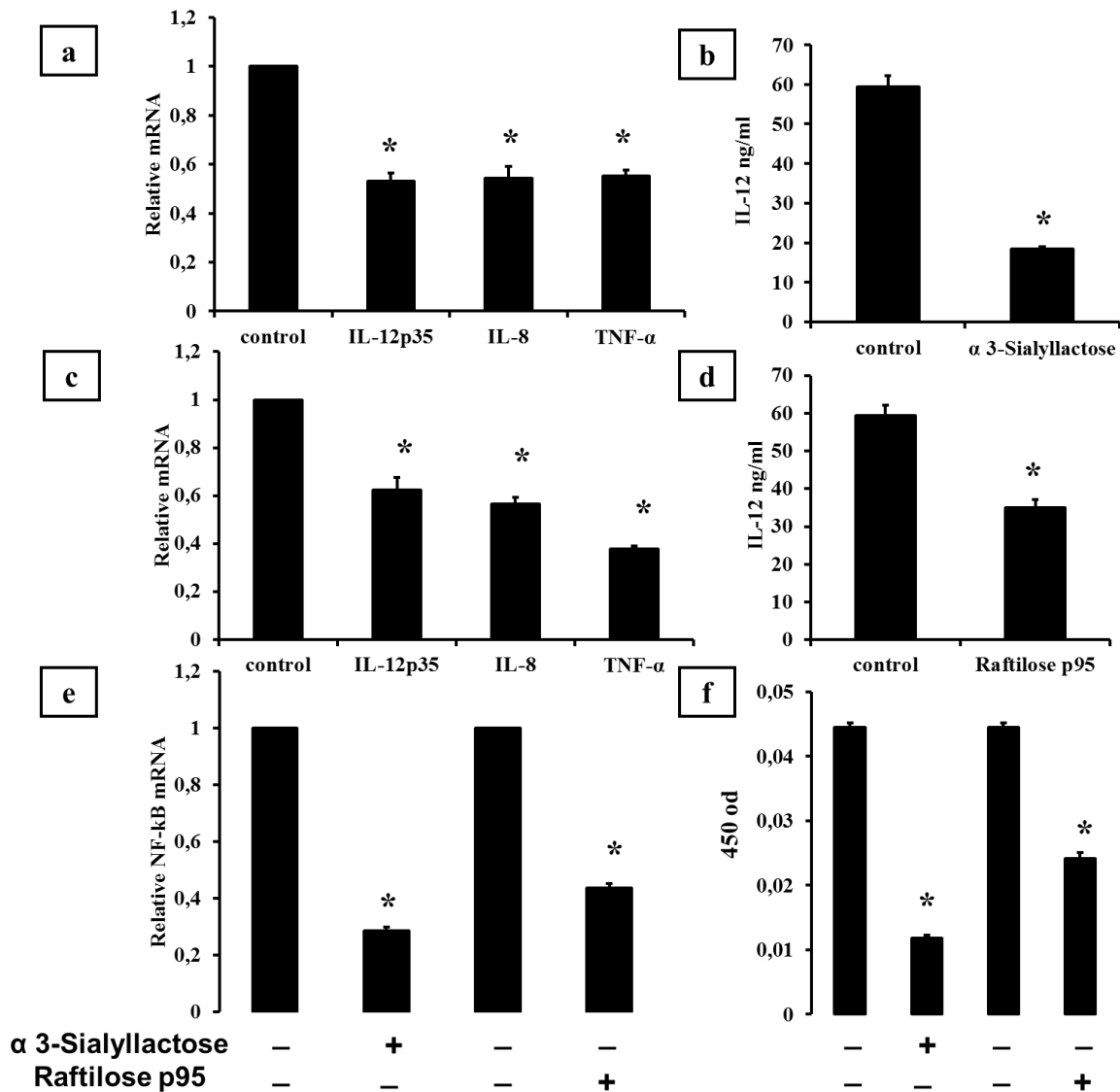
All samples were measured in duplicates. Values were expressed as mean  $\pm$  SEM. with  $n \geq 4$  in all experiments and the p value was indicated in the Results section. Group statistical comparisons were performed by one-way analysis of variances followed by Mann-Whitney multi-range analysis as a post-hoc test. For comparisons in dose and time curves, data was analyzed by ANOVA with repeated measures. Paired comparisons were performed by t-test, when application of ANOVA was not necessary. All analyses were performed

using Statgraphics plus software and differences among means were considered significant at P values < 0.05.

## **RESULTS**

### *Inhibition of pro-inflammatory cytokines by oligosaccharides*

We measured the mRNA level of interleukins IL-12p35, IL-8 and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) by quantitative RT-PCR and IL-12 protein secretion by ELISA in Caco-2 cells treated with 50 mg/L of  $\alpha$ 3-sialyllactose or 50 g/L of Raftilose p95 for 6 h. Both oligosaccharides suppressed significantly (P<0.05) IL-12p35 to 53.0 $\pm$ 1.3% and 62.0 $\pm$ 5.0%; IL-8 to 54.0 $\pm$ 1.5% and 56.5 $\pm$ 2.7% and TNF- $\alpha$  expression to 55.0 $\pm$ 1.0% and 38.0 $\pm$ 1.1% of the control, respectively (Fig. 1a, c). ELISA measurement of IL-12 revealed also that both oligosaccharides inhibited the protein secretion of IL-12 significantly (P<0.05) (Fig. 1b, d).  $\alpha$ 3-sialyllactose reduced this secretion from 59.4.0 $\pm$ 2.7 to 18.4 $\pm$ 0.6 ng/ml, while Raftilose p95 decreased the secretion of IL-12 from 59.4.0 $\pm$ 2.7 to 35.0 $\pm$ 2.1 ng/ml.



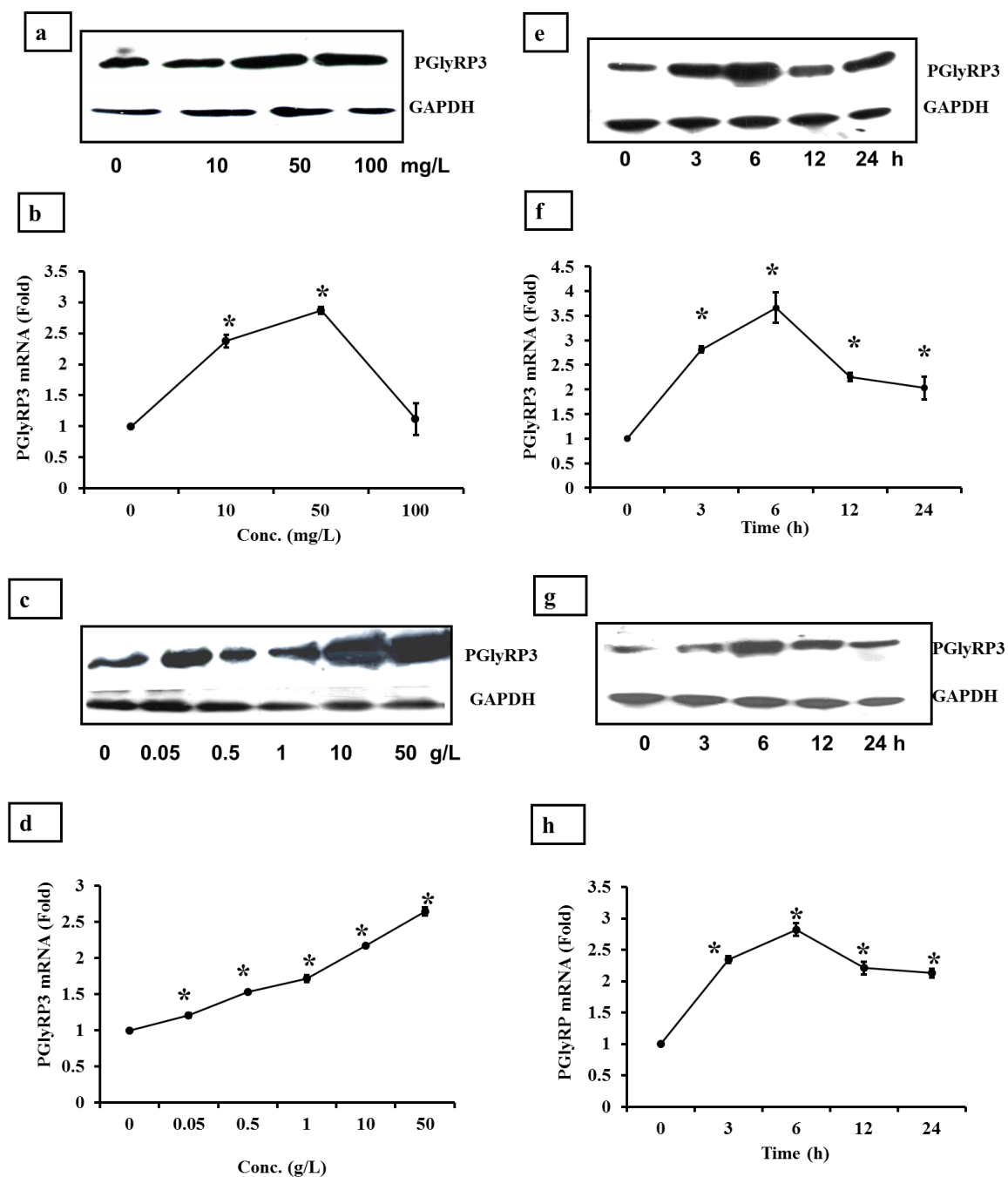
**Figure 1. Inhibition of pro-inflammatory cytokine expression and NF-kB in intestinal Caco-2 cells by oligosaccharides:** Caco-2 cells were incubated with 50 mg/L of  $\alpha$ 3-sialyllactose (a, b) or 50 g/L of Raftilose p95 (c, d). After 6h incubation, total RNA was extracted and supernatants were collected. mRNA expression of *IL-12p35*, *IL-8* and *TNF- $\alpha$*  were analyzed by quantitative RT-PCR using SYBR Green (a, c). IL-12 protein secretion was measured by ELISA (b, d). (e) Relative NF-kB1 mRNA expression after treatment with the same oligosaccharides. (f) The NF-kB part P65 was estimated in nuclear extracts obtained from the treated cells at 450 OD. PCR results are expressed as relative to  $\beta$ -actin mRNA. Data are expressed as mean  $\pm$  SEM of n=4 experiments. (\*) denotes significant difference from the non-treated control value (t-test, p<0.05).

### ***Dose and time-dependence of induction of PGlyRP3 by oligosaccharides***

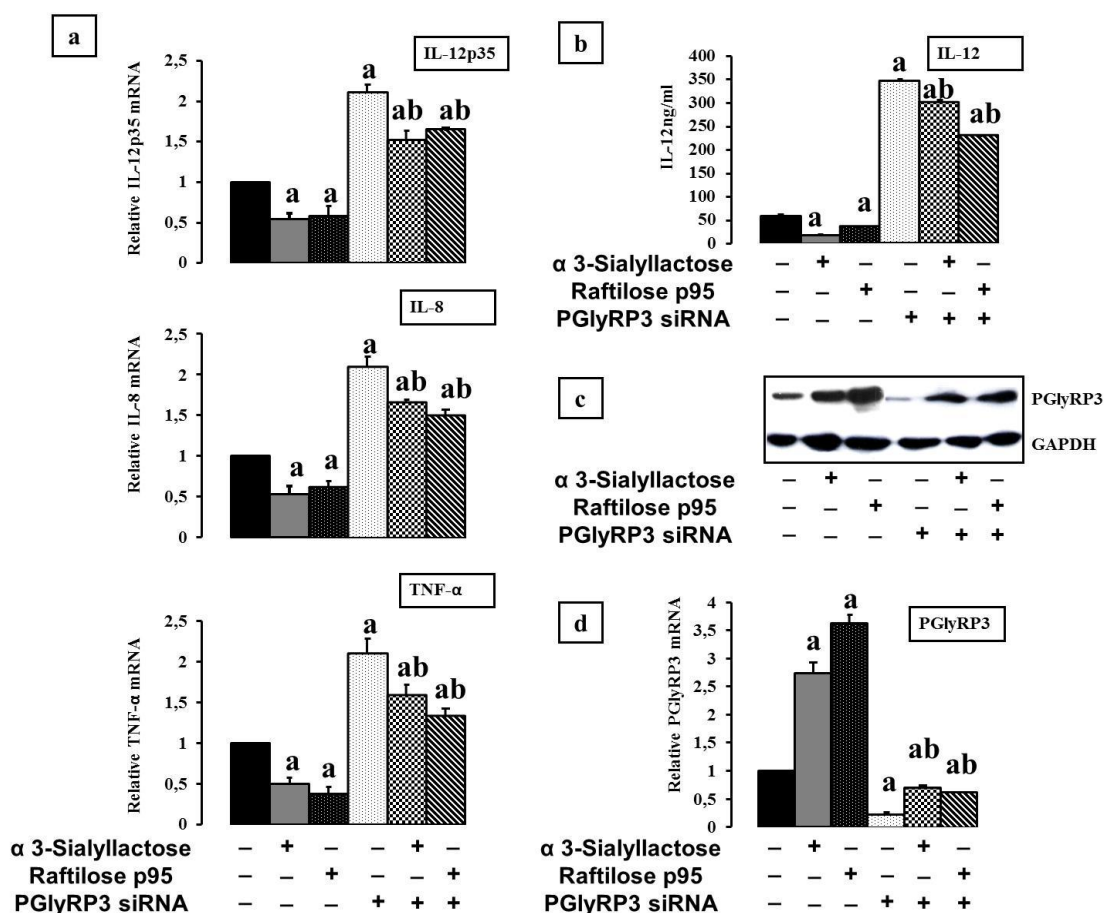
Caco-2 Cells incubation for 24 h with various concentrations of  $\alpha$ 3-sialyllactose or Raftilose p95 resulted in a dose-dependent induction of PGlyRP3 transcripts and proteins (Fig. 2). Increased expression of PGlyRP3 mRNA (Fig. 2a) and increased amounts of protein (Fig. 2b) were observed when 50 mg/L of  $\alpha$ 3-sialyllactose was applied, while the relative PGlyRP3 mRNA levels decreased when the concentration of  $\alpha$ 3-sialyllactose was higher than 50 mg/L. The incubation with Raftilose p95 resulted in a dose-dependent induction of PGlyRP3 mRNA (Fig. 2c) and protein (Fig. 2d) in the range of 0.05 to 50 g/L. Both  $\alpha$ 3-sialyllactose and Raftilose p95 time-dependently influenced *PGlyRP3* gene expression in Caco-2. The expression of PGlyRP3 mRNA and protein were induced by  $\alpha$ 3-sialyllactose (Fig. 2e and f) or Raftilose p95 (Fig. 2g and h) within the first 3h, and reached a peak after 6 h.

### ***Dependence of the anti-inflammatory effect of oligosaccharides on PGlyRP3 gene expression***

Incubation of Caco-2 cells for 6 h with 50 mg/L of  $\alpha$ 3-sialyllactose or 50 g/L of Raftilose p95 inhibited cytokine gene expression (Fig.3). PGlyRP3-silencing significantly increased the gene expression of *IL-12p35*, *IL-8* and *TNF- $\alpha$*  genes (Fig. 3a) as well as IL-12 protein secretion (Fig. 3b). The inhibitory effect of oligosaccharides was associated with increased *PGlyRP3* gene and protein expressions (Fig. 3c and d). Despite PGlyRP3 silencing, treatment with the oligosaccharides could significantly reduce the pro-inflammatory cytokines, when compared with that in PGlyRP3-silenced cells. This indicates that PGlyRP3 is not the only way of oligosaccharides to exert their anti-inflammatory action, and that both oligosaccharides and PGlyRP3 can reduce the cytokines independently from each other.



**Figure. 2. Dose and time-dependence of induction of PGlyRP3 in the intestinal Caco-2 cells by oligosaccharides:** Caco-2 cells were cultured with different concentrations of  $\alpha$ 3-sialyllactose (a, b), or Raftilose p95 (c, d) for 24 h, or cultured with 50 mg/L of  $\alpha$ 3-sialyllactose(e, f) or 50 g/L Raftilose p95 (g, h) for 0, 3, 6, 12 or 24 h. After incubation, total RNA was extracted and transcribed to cDNA, and then the expression of PGlyRP3 was assessed by quantitative RT-PCR using TaqMan gene expression assays. Results are expressed as relative to  $\beta$ -actin mRNA as internal standard. Immunoblots represent PGlyRP3 protein of the same experiments. The shown data are averages of separate experiments performed in duplicates. \* denotes  $p < 0.05$  in comparison to the control cultures.



**Figure 3. Dependence of the anti-inflammatory effect of oligosaccharides on PGlyRP3 expression:** Caco-2 cells were transfected with 5 nM PGlyRP3 siRNA. After 24h the cells were treated with 50 mg/L of  $\alpha$ 3-sialyllactose or 50 g/L of Raftilose p95. After 6h incubation, total RNA was extracted and supernatants were collected. a) mRNA expression of IL-12p35, IL-8 and TNF- $\alpha$  were analyzed by quantitative RT-PCR analysis. b) Protein secretion of IL-12 was measured by ELISA. c) PGlyRP3 protein was assayed by Western blotting. d) mRNA expression of PGlyRP3 was analyzed by quantitative RT-PCR analysis using Taqman. The shown data are averages of 4 separate experiments performed in duplicates. Statistical analysis: a, significantly different from the control; b, significantly different from the corresponding PGlyRP3 silencing values (ANOVA followed by Mann-Whitney test).

### ***PPAR $\gamma$ regulates PGlyRP3 expression and triggers the oligosaccharide-induced anti-inflammatory effects***

#### **I) Oligosaccharides induce PPAR $\gamma$ expression**

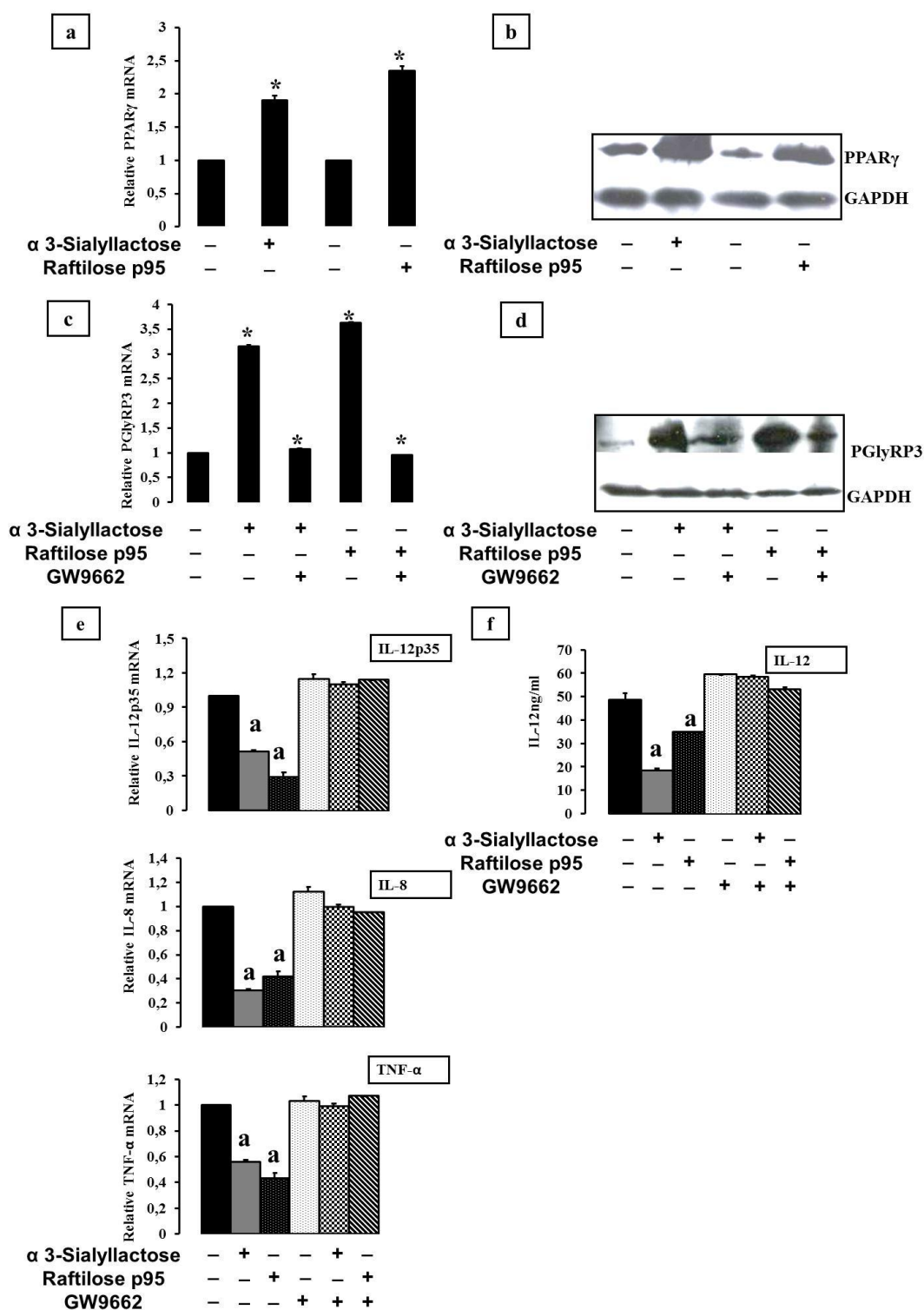
In Caco-2 cells incubated with 50 mg/L of  $\alpha$ 3-sialyllactose or 50 g/L of Raftilose p95 for 6 h, the expression of PPAR $\gamma$  was increased to  $200\pm 6.6\%$  and  $235\pm 7.0\%$  for  $\alpha$  3-Sialyllactose and Raftilose p95, respectively, compared to the control without oligosaccharide (Fig. 4a). This effect was confirmed on the protein level, as assessed by Western blot analysis (Fig. 4b).

#### **II) PPAR $\gamma$ antagonism abolished oligosaccharide-induced PGlyRP expression**

In Caco-2 cells treated with 10  $\mu$ mol/L of the PPAR $\gamma$  antagonist (GW9662) for 24h and then with 50 mg/L of  $\alpha$ 3-sialyllactose or 50 g/L of Raftilose p95 for 6h, the oligosaccharides induced the gene expression of *PGlyRP3* to  $315\pm 2.7\%$  and  $362\pm 2.0\%$  for  $\alpha$ 3-sialyllactose and Raftilose p95, respectively,

compared to the control without oligosaccharides (Fig. 4c). This induction was abolished by the incubation with the PPAR $\gamma$  antagonist GW9662 ( $107\pm 1.3$  and  $95\pm 1.1\%$ , respectively,  $p < 0.05$ ). This effect was also observed on the *PGlyRP3* protein level (Fig. 4d).





**Figure 4. Oligosaccharides regulate PGlyRP3 expression and trigger anti-inflammatory effects through PPAR $\gamma$ .** Caco-2 cells were cultured with and without 50 mg/L of  $\alpha$  3- Sialyllactose or 50 g/L of Raftilose p95 for 6 h. After incubations, total RNA was extracted and transcribed to cDNA, and then the expression of PPAR $\gamma$  was assessed qPCR using Sybr-green assay (a) and Western blot (b). Oligosaccharides-treated cells were incubated in the

presence or absence of the PPAR $\gamma$  inhibitor GW 9662. PGlyRP3 mRNA level was then assessed by qPCR using Taqman (c), PGlyRP3 protein was detected by Western blot (d), mRNA level of *IL-12p35*, *IL-8* and *TNF- $\alpha$*  genes was assessed by qPCR using Sybr-green (e), and IL-12 secretion was determined by ELISA in the supernatant (f). All qPCR results are expressed in relation to  $\beta$ -actin mRNA as internal standard. The shown data are averages of 6 separate experiments performed in duplicates. Statistical analyses: \* denotes  $p < 0.05$  in comparison to the control cultures (t-test). a, significantly different from the control; b, significantly different from the corresponding PGlyRP3 silencing values (ANOVA followed by Mann-Whitney test).

#### IV) PPAR $\gamma$ antagonism abolished the oligosaccharide-induced inhibition of cytokines expression

In Caco-2 cells treated with 10  $\mu$ mol/L of GW9662 for 24h and then with 50 mg/L of  $\alpha$ 3-sialyllactose or 50 g/L of Raftilose p95 for 6 h the inhibition of gene expressions of *IL-12p35*, *IL-8* and *TNF- $\alpha$*  (Fig. 4e) and the secretion of IL-12 (Fig. 4f) by oligosaccharides was abolished by the incubation with the PPAR $\gamma$  antagonist GW9662.

### DISCUSSION

This study was designed to investigate whether prebiotic oligosaccharides from plant and milk sources exert a direct anti-inflammatory effect in enterocytes and whether this is mediated by PGlyRPs. In the present study, we focused on PGlyRP3 because our previous results demonstrated that Caco-2 cells express only PGlyRP3 (18). The present results demonstrate that the anti-inflammatory role of  $\alpha$ 3-sialyllactose and Raftilose p95 in intestinal cells depends on the regulation of PPAR $\gamma$  and accordingly, PGlyRP3.

Both applied oligosaccharides inhibited the expression of pro-inflammatory cytokines and the nuclear translocation of NF-kB (Fig 1). The modulation of cytokines by oligosaccharides was previously observed in *in vivo*

studies and *in vitro* in lymphocytes (10, 12, 13). Also, our previous results demonstrated that PGlyRP3 exerts a direct anti-inflammatory role by inhibiting IL-8, IL-12 and TNF- $\alpha$  pro-inflammatory cytokines through a mechanism including the suppression of NF- $\kappa$ B pathway (18). In the present study, we found that *PGlyRP3* expression was enhanced by both applied oligosaccharides and this induction was dose- and time- dependent (Fig. 2). The induction of *PGlyRP3* gene expression by oligosaccharides, similar to its overexpression, inhibited *IL-8*, *IL-12* and *TNF- $\alpha$*  expression. On the other hand, the anti-inflammatory effect of oligosaccharides was masked by PGlyRP3 silencing (Fig 3a, b). Accordingly, this anti-inflammatory effect by oligosaccharides showed a dependence on *PGlyRP3* expression (Fig 3c). Taken together, the results suggest that the *in vitro* anti-inflammatory effect of the oligosaccharides  $\alpha$ 3-sialyllactose and Raftilose p95 are triggered through induction of the anti-inflammatory PGlyRP3 pathway.

The modulation of cytokines by oligosaccharides through bifidobacteria was previously reported (9-12). Oligosaccharides were also reported to initiate some immunological events through interaction with TLRs, such as increasing TLR2 and/or TLR4 expression following daily FOS supplementation (9). It is known that oligosaccharides are fermented in the colon by intestinal microbial metabolic activity to short-chain fatty acids (SCFA) (19-22), which may have anti-inflammatory effect through activation of transcription factors. However, in the absence of microbiota and fatty acids in *in vitro* experiments, the immunomodulatory effect of oligosaccharides should be due to a direct signaling initiated by the oligosaccharides themselves (Fig. 1, 3, and 4). This was shown by Lindsay et al (9) in their dendritic cells (DC) model and by using enterocytes. This effect of oligosaccharides is suggested to be mediated through interaction with other recognition molecules than TLRs, since activation of TLR2 and 4 leads mostly to inflammatory response and does not explain, for

example, the increase of IL-10 in the DC-model (9). However, this recognition molecule may interact with some transcription factors as PPAR $\gamma$  and render its immunomodulatory effect. PPAR $\gamma$  is activated by certain polyunsaturated fatty acids (23), phytanic acid (24) and the short-chain butyric acid (25). In addition, PPAR $\gamma$  was reported to inhibit the production of inflammatory cytokines in different cell types by interfering with TLR4-dependant signaling pathway (26-28). Our previous results demonstrated that PGlyRP3 is up-regulated by a PPAR $\gamma$  agonist and some free fatty acids, which are also PPAR $\gamma$  ligands. This up-regulation was due to a direct binding between PPAR $\gamma$  and its PPAR response elements PPRE on the promoter region of the *PGlyRP3* gene. Thus, PPAR $\gamma$  also exerts its anti-inflammatory effect due to a direct binding to and modulation of the expression and activity of the *PGlyRP3* gene, which in turn activates I $\kappa$ B- $\alpha$  and inhibits the NF- $\kappa$ B translocation into the nucleus and down-regulate different pro-inflammatory cytokines (18). The applied oligosaccharides induced the expression of PPAR $\gamma$  (Fig 4) and induced an inhibition of pro-inflammatory cytokines. The oligosaccharides-induced suppression of pro-inflammatory cytokine expressions was abolished by the PPAR $\gamma$  antagonist GW9662 (Fig. 4). However, knocking down the PGlyRP3 itself, increased the same pro-inflammatory cytokines with and without treatment with oligosaccharide (Fig. 3). These results suggest that the oligosaccharide mediated anti-inflammatory effect depends principally on PPAR $\gamma$ , which activation leads to PGlyRP3 induction. It seems that PPAR $\gamma$  has several ways to exert its anti-inflammatory role, and that activation of PGlyRP3 is one of these ways.

In summary, our results suggest that prebiotic oligosaccharides may have a direct anti-inflammatory effect. This effect seems to be PPAR $\gamma$  dependent and at least in part depend on PGlyRP3 expression. Furthermore, the results support

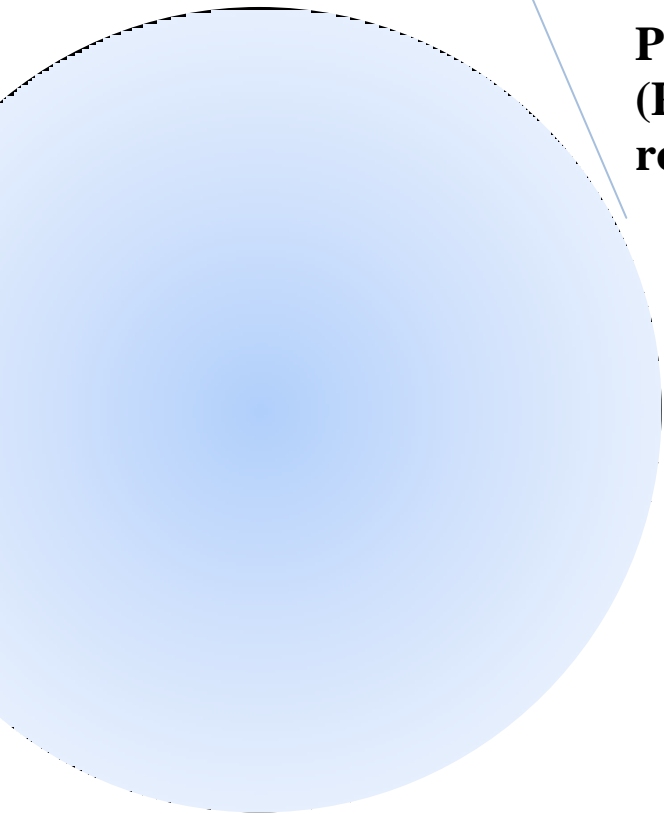
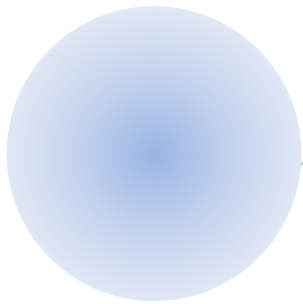
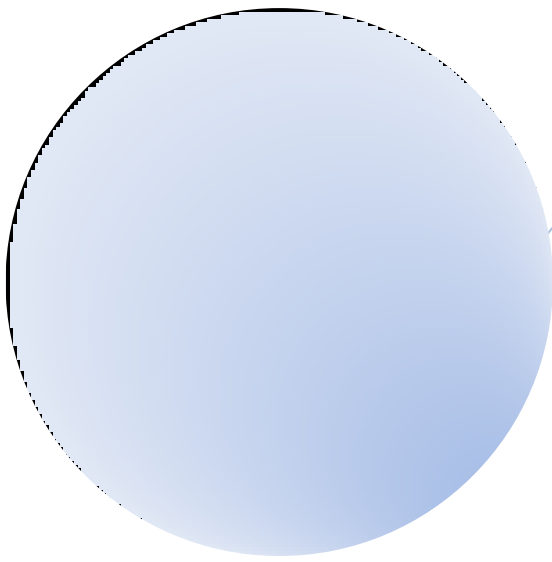
the idea that nutrients like dietary oligosaccharides may modulate the inflammatory state.

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## **CHAPTER IV**

**Peptidoglycan recognition protein 3  
(PglyRP3) has an anti-inflammatory  
role in intestinal epithelial cells**



**Peptidoglycan recognition protein 3 (PglyRP3) has an anti-inflammatory role in intestinal epithelial cells**

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**Key words:** Peptidoglycan (PGN), PGlyRP3, inflammation, Caco-2

## **ABSTRACT**

Intestinal epithelial cells produce cytokines in response to bacterial peptidoglycan (PGN), which is detected by several classes of pattern-recognition receptors (PRRs) as peptidoglycan recognition proteins (PGlyRPs), Toll-like receptor 2 (TLR2) and NOD receptors. All types of PGlyRPs recognize bacterial peptidoglycan and function in antibacterial innate immunity. In this study, we investigated the role of PGlyRP3 in the response of intestinal epithelial cells (Caco-2) to PGN from pathogenic (*Staphylococcus aureus*), opportunistic pathogenic (*Micrococcus luteus*) and non-pathogenic (*Bacillus subtilis* and *Lactobacillus rhamnosus GG*) bacteria. All PGNs induced the proinflammatory cytokines IL-12p35, IL-8 and TNF- $\alpha$  and, time-dependently, PGlyRP3, at both the transcription and protein levels. In this context, no difference was observed between the effects of PGN obtained from different bacterial sources. The inflammatory response to PGN is mediated via the TLR2 pathway, since blocking this pathway by inhibiting MyD88 reduced the expression of proinflammatory cytokines. In addition, PGlyRP3 overexpression suppressed, while PGlyRP3 knocking down enhanced the expression of PGN-induced inflammatory cytokines. It is concluded that PGN stimulates inflammatory responses in the intestinal epithelia through activation of the TLR pathway. PGlyRP3 is also stimulated by PGN and has, in contrast to activation of the TLR pathway, an anti-inflammatory effect.

## INTRODUCTION

The innate immune system improves the early recognition of any invading microorganisms. Vertebrates and invertebrates have evolved pattern-recognition receptors (PRRs) that sense the presence of pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide, nonmethylated CpG sequences, peptidoglycan (PGN), bacterial DNA and lipopolysaccharide (LPS), which are products of microbial metabolism and not produced by the host (1, 2). Bacteria stimulate the innate immune system of the host and the release of inflammatory molecules such as cytokines and chemokines (3, 4), which are the major cause of the various signs and symptoms that occur during bacterial infections, including fever, inflammation, and acute phase responses (5, 6). LPS is a well-known activator of the innate immune system in infections caused by Gram-negative bacteria (5), while infections with Gram-positive bacteria are sensed by PGN, the major component of the bacterial cell wall of Gram-positive bacteria (5, 6).

PGN had been largely neglected for its capacity to induce immune responses. However, this has been changed recently with the discovery of several classes of PRRs required for PGN detection such as peptidoglycan recognition proteins (PGRPs), Toll-like receptor 2 (TLR2) and nucleotide-binding oligomerization domain proteins (NODs) (7, 8). It was found that peptidoglycan from Gram-positive bacterial cell walls induce inflammation in rats and rabbits (9, 10). The immunomodulatory properties of peptidoglycan had been identified towards different *in vitro* models, for example: monocytes, macrophages (11, 12), neutrophils (13) and respiratory epithelial cells (14).

Toll-like receptor 2 (TLR2) has been shown to recognize PGN, leading to the activation of the transcription factor NF- $\kappa$ B (15, 17), and immune stimulation (7). “This activation requires the signal transduction molecules

myeloid differentiation protein (MyD88), IL-1 receptor-associated kinase (IRAK), tumor necrosis factor (TNF) receptor-associated kinase 6 (TRAF6), NF- $\kappa$ B inducing kinase (NIK), and I $\kappa$ B kinase (IKK)” (18- 20).

NOD1 and NOD2 are intracellular PRRs which, recognize the soluble PGN fragments in the cytoplasm (21, 22) and are involved also in innate immune defense responses through pathways that are independent of TLR (23, 24). NOD1 is an upstream activator of NF- $\kappa$ B (25, 26). However, NOD2 was a susceptibility gene included in the Crohn's disease etiology , an inflammatory bowel disease controlled by both genetic and environmental factors (27, 28).

PGRPs are a member of PRRs, they are highly conserved from insects to mammals. They recognize with high affinity bacterial peptidoglycan, and function in antibacterial immunity. Insects have many PGRPs with different functions, such as induction of antimicrobial peptide gene expression through activation of the TLR, IMD, and prophenoloxidase cascade pathways, induction of phagocytosis, hydrolysis of peptidoglycan, and down regulation of immune responses (22). The mammalian PGRP family is composed of four members. PGlyRP1, 3 and 4 are directly bactericidal (29), whereas PGlyRP2 has been reported to have amidase activity and hydrolyzes peptidoglycan (30, 31). In contrast to the insect PGRPs, little is known about the stimulatory role of mammalian PGRPs in intracellular signaling pathways. Nevertheless, few studies were conducted regarding the inflammatory role of PGRPs (32), while it is known that activation of TLR2 and NODs activates inflammatory pathways.

We have recently detected the expression of PGlyRP3 in enterocytes (33). Whether it plays an inflammation promoting role in these cells is still unknown. The aim of our study was to investigate the response of PGlyRP3 in intestinal epithelial cells (Caco-2) to peptidoglycans isolated from different bacterial sources. For this purpose, we used PGN from non-pathogenic (*Lactobacillus rhamnosus GG (LGG)* and *Bacillus subtilis*), opportunistic

pathogenic (*Micrococcus luteus*) and pathogenic bacteria (*Staphylococcus aureus*). We identified an anti-inflammatory role of PGlyRP3, which is independent of TLR2.

## **MATERIAL AND METHODS**

### ***Cell culture***

Caco-2 cells were obtained from the American Type Culture Collection Company (ATCC). Cells between passages 10-50 were grown in 75 cm<sup>2</sup> culture flasks at 37°C and 5% CO<sub>2</sub> using Minimum Essential Medium (MEM + Glutamax, Invitrogen, Karlsruhe, Germany) supplemented with 20% fetal calf serum, 100 IU penicillin/ml and 1% non-essential amino acids. For different experiments, cells were cultured in 6 well plates until reaching 7 days post confluence. Cells were fasted for 2 hours in Minimum Essential Medium (MEM + Glutamax) without any supplementations before experimental intervention. For over-expression and RNA-silencing experiments, cells were cultured in 24-well plates until reaching 80-100% confluence.

### ***Preparation of PGN and incubations***

PGN from *B. subtilis*, *M. luteus* and *S. aureus* were purchased from (Sigma) PGN from *LGG* was prepared as previously described (34). Briefly, *LGG* was grown in deMan Rogosa Sharp (MRS) liquid medium for 48 h at 37 °C without shaking. The culture was quickly chilled in ice bath. Bacteria were harvested by centrifugation and washed several times with water. They were placed in a boiling water bath for 20 min followed by sonication. The suspension was centrifuged at 1 000 r/min for 15 min to sediment unbroken bacteria and the supernatant containing the cell walls was decanted. After

centrifugation at 10.000×g for 10 min, the pelleted cell walls were incubated in 2% sodium dodecyl sulphate (SDS) for 30 min at 60 °C. Covalently bound proteins were digested with 0.02% trypsin in 0.1 mol/l Tris-HCl buffer (pH 7.5) for 20 h at 37 °C after being washed four times with water and twice with dehydrated alcohol. The walls were washed several times with water, then lyophilized and resuspended in 10% trichloroacetic acid and stirred for 20 min at 60° C . PGN was concentrated by centrifugation (10 000×g, 20 min) and washed extensively with water. Stock solutions were prepared by dissolving PGN in MEM at a concentration of 1 mg/ml.

Caco-2 cells were incubated for 3, 6, 12, 24 hours with one of the different PGN at a concentration of 10 µg/ml.

### ***Incubation with Myeloid differentiation 88 (MyD88) inhibitor***

The MyD88 inhibitory peptide set was purchased from InvivoGene (France). Caco-2 cells were seeded in a 24-well plate. MyD88 inhibitory peptide or the control peptide (100 µM) were applied for 24 hrs according to the manufacturer's instructions.

### ***Transfection of Caco-2 cells with PGlyRP3 plasmid***

RNA was isolated from human oesophagus (approved by the ethics committee of the Christian-Albrechts-University of Kiel “protocol A 120/05”) and using the designed primers (table 1), full-length cDNAs coding for PGlyRP3 was amplified. We designed oligonucleotide primers after testing the sequences for restriction enzymes (Xho1 and EcoR1) that neither cut within the PGlyRP3 nor the vector sequences. The PCR products were ligated into the pCAGGS vector (T4-Ligase Rapid-Kit (Fermentas, Karlsruhe, Germany), and clones with the proper full-length PGlyRP3 insert were selected and identified

by restriction digestion and sequencing the inserts. The cloned PGlyRP3 was identical to the sequence predicted from the genomic sequences.

Caco-2 cells were seeded in a 24-well plate at a density of  $4 \times 10^4$  cells per well in 1 ml of culture medium and transfections performed at 60–80% cell confluence. Prior to transfection, TurboFect (Fermentas) and MEM (Invitrogen) were mixed and incubated at room temperature for 20 min. 100 ng DNA was added in a ratio of 3:1 (TurboFect in  $\mu\text{l}$ : DNA in ng) as recommended by the manufacturer. Control samples were treated similarly and transfected with the empty vector (pCAAGS). After 72 hrs post transfection, RNA was isolated and reverse transcribed into cDNA. *PGlyRP3* gene over-expression was confirmed using quantitative RT-PCR and Western blot.

### ***Small interfering RNA experiments***

Two siRNA sequences targeting PGlyRP3 (Si Select si RNA no. s41580 and s41581) and a non-targeting negative control sequence (SC 4390843) were purchased from Ambion (Applied Biosystems). Caco-2 cells were reverse transfected with siRNA using siPORT NeoFX Transfection Reagent (Ambion, Applied Biosystems). siRNA was prepared in Opti-MEM serum-free medium (Invitrogen) by mixing 2  $\mu\text{l}$  of the transfection reagent with 5 nM siRNA, respectively, at room temperature for 10 min. Caco-2 cells ( $4 \times 10^4$  cells per well) were transferred to a 24-well plate containing siRNA transfection reagent complexes, allowing transfection to occur during initial cell adherence. Medium was replaced after 24 hrs by medium containing 20% FCS (allowing cell recovery) and incubation continued for an additional 48 hrs then RNA was isolated and transferred into cDNA. *PGlyRP3* gene knock-down was confirmed by quantitative RT-PCR and Western blot.

## ***RNA Isolation, cDNA Synthesis, and Quantitative real time PCR analysis***

Total RNA was isolated from Caco-2 cells using RNeasy kit (Qiagen, Hilden, Germany). To assure absence of genomic DNA, all RNA samples were treated with DNase-1, and multiple exon-spanning primers were used for PCR amplification. For reverse transcription, 1 µg of the total RNA was reverse transcribed to first strand complementary DNA in 25 µl reactions using the cDNA synthesis Taqman kit (Applied Biosystems).

All quantitative RT-PCR analyses were performed on an Applied Biosystems ABI Prism 7000 system using cDNA samples corresponding to 20 ng RNA. Quantitative analysis of *PglyRP3* gene expression was undertaken by using TaqMan gene expression assay in a 30 µl reaction with universal PCR master mix and TaqMan MGB probe (No. Hs 01074825\_ml, Applied Biosystems, Cambridge, UK). To normalize expression data,  $\beta$ -actin was used as an internal control gene. Data are presented as fold difference from control, considering the control values as 1.

Quantitative RT-PCR analyses of all other studied genes were performed using SYBR Green (Power SYBR green Mastermix, Applied Biosystems). All studied genes were quantified within the same cDNA samples. The thermal cycling program was 10 min at 95°C for enzyme activation and denaturation for 15 s at 95°C, 60 s annealing at 60°C. A dissociation curve was performed for each product to assure the absence of primer dimers or unspecific products. Primers used in the present study are listed in Table 1.

To normalize expression data,  $\beta$ -actin was used as an internal control gene. Data are presented as fold difference of control, considering the control values as 1.



**Table 1.** Nucleotide sequences of studied genes for RT-PCR and cloning.

Gene	Accession No.	Sense primer	Antisense primer	Product size (bp)
PGlyRP3	NM_052891	5'-ACCCTCAACATCCAGTGATGC-3'	5'-ATCCAACCCCTTCATACACGC-3'	273
IL-8	NM_000584	5'-GTGCAGTTTTGCCAAGGAGTG-3'	5'-ACTTCTCCACAACCCCTCTGC-3'	215
IL-12p35	NM_000882	5'-TTGTGGCTACCCTGGTCT-3'	5'-AGAGTTTGTCTGGCCTTCTGG-3'	151
TNF- $\alpha$	NM_000594	5'-TCAACCTCCTCTCTGCCATC-3'	5'-CCAAAGTAGACCTGCCCAGA-3'	187
$\beta$ - Actin	NM_001101	5'-GATATCGCTGCGCTCGTC-3'	5'-TCCATATCGTCCCAGTTGG-3'	239

*Oligonucleotide sequences for PGlyRP3 gene cloning*

EcoRIPGlyRP3		5'-CTGGAATTCATGGGGACGCTGCCATGG-3'		1044
XhoI-PGlyRP3			5'-CAGCTCGAGTCAGTGCTTCAAATGAGG-3'	

***Protein Extraction and Western blot analysis***

The cell lysates were loaded into 12% SDS-PAGE and blotted onto PVDF transfer membranes. Membranes were blocked with a mixture of 2.5% BSA and 2.5% skimmed milk and incubated with mouse monoclonal anti-human PGlyRP3 (1:200, AXXORA, Lörr, Germany) and rabbit polyclonal anti-human GAPDH (1:500, Santa Cruz Biotechnology, Heidelberg, Germany). Immunoreactive bands were detected with horsereddish peroxidase(HRP)-conjugated secondary antibodies.

### ***Interleukin 12 protein assay***

The human total IL-12 was detected using an ELISA kit (Mabtech AB, Hamburg, Germany) in the supernatant obtained from Caco-2 cell cultures after each treatment according to the manufacturer's instructions.

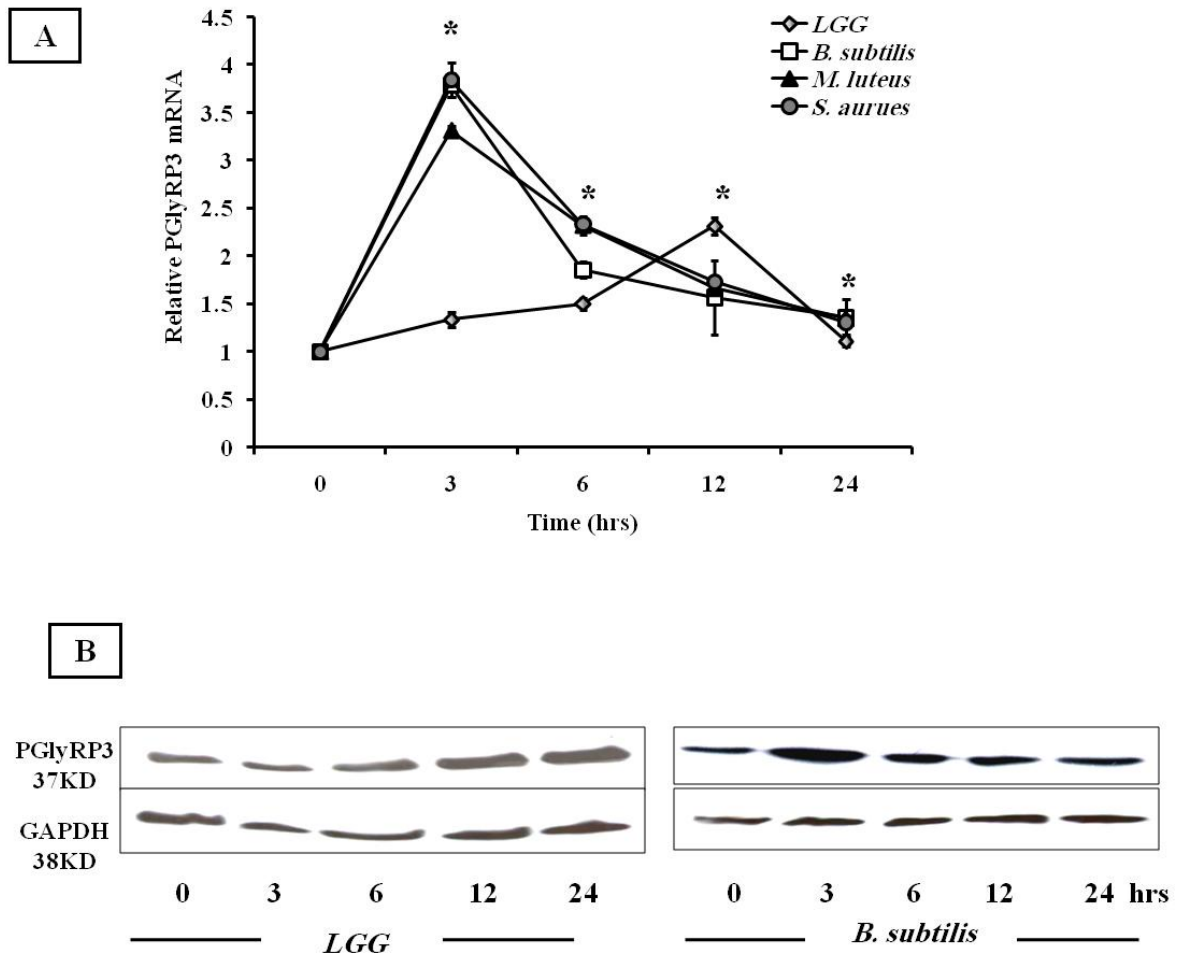
### ***Statistical analyses***

All samples were measured in duplicates. Values were expressed as mean  $\pm$  S.E.M. with  $n \geq 4$  in all experiments and p indicated in the Results section. Data were analyzed by paired t test or one way analysis of variance with repeated measures. All analyses were performed using Statgraphics plus software and differences among means were considered significant at P values  $< 0.05$ .

## **RESULTS**

### ***Different PGN induced PGlyRP3 expression***

*PGlyRP3* gene expression was significantly induced after incubation of Caco-2 cells with 10  $\mu\text{g/ml}$  PGN from *B. subtilis*, *M. luteus*, and *S. aureus* ( $p < 0.05$ ) for 3 hrs. This induction was reduced thereafter (Fig. 1A). After incubation of Caco-2 cells with PGN from *LGG*, a peak of *PGlyRP3* gene expression was achieved after 12 hrs (Fig. 1A). Therefore, we chose the 3 hrs for *B. subtilis*, *M. luteus*, and *S. aureus* and 12 hrs for *LGG* as incubation time for the next experiments. Both *B. subtilis* and *LGG* PGNs induced *PGlyRP3* protein in Caco-2 cells along with its gene expression in a time-dependent course (Fig. 1B).



**Figure 1. Time dependence of induction of PGlyRP3 by PGN :**

A) Quantitative PCR analysis for the expression of PGlyRP3 using cDNAs from Caco-2 cells treated with PGN of different bacterial sources: *B. Subtilis*, *LGG*, *M. luteus* and *S. aureus*. PCR was performed using PGlyRP3 TaqMan MGB probe. Data were normalized to the expression of  $\beta$ -actin. The data (mean  $\pm$  SEM) are averages of 6 separate experiments performed in duplicates. \* denotes significant differences from the untreated control value. B) PGlyRP3 protein production analysed by Western blotting. Cells were incubated for the indicated periods with 10  $\mu$ g/ml of PGN from *B. Subtilis* and *LGG*. Protein was then detected.

### ***PGN induce inflammatory cytokine***

Incubation of Caco-2 cells with 10  $\mu$ g/ml *B. subtilis*, *M. luteus*, *S. aureus* and *LGG* significantly ( $p < 0.05$ ) induced interleukin 12p35 (IL-12p35), interleukin 8 (IL-8) and tumor necrosis factor alpha (TNF- $\alpha$ ) mRNA levels (Fig.

2A). IL-12 protein concentration was detected by IL-12 ELISA in supernatants obtained from Caco-2 cell cultures after treatment with different PGNs. The protein concentrations of IL-12 were affected in the same manner like the mRNA levels (Fig. 2B).

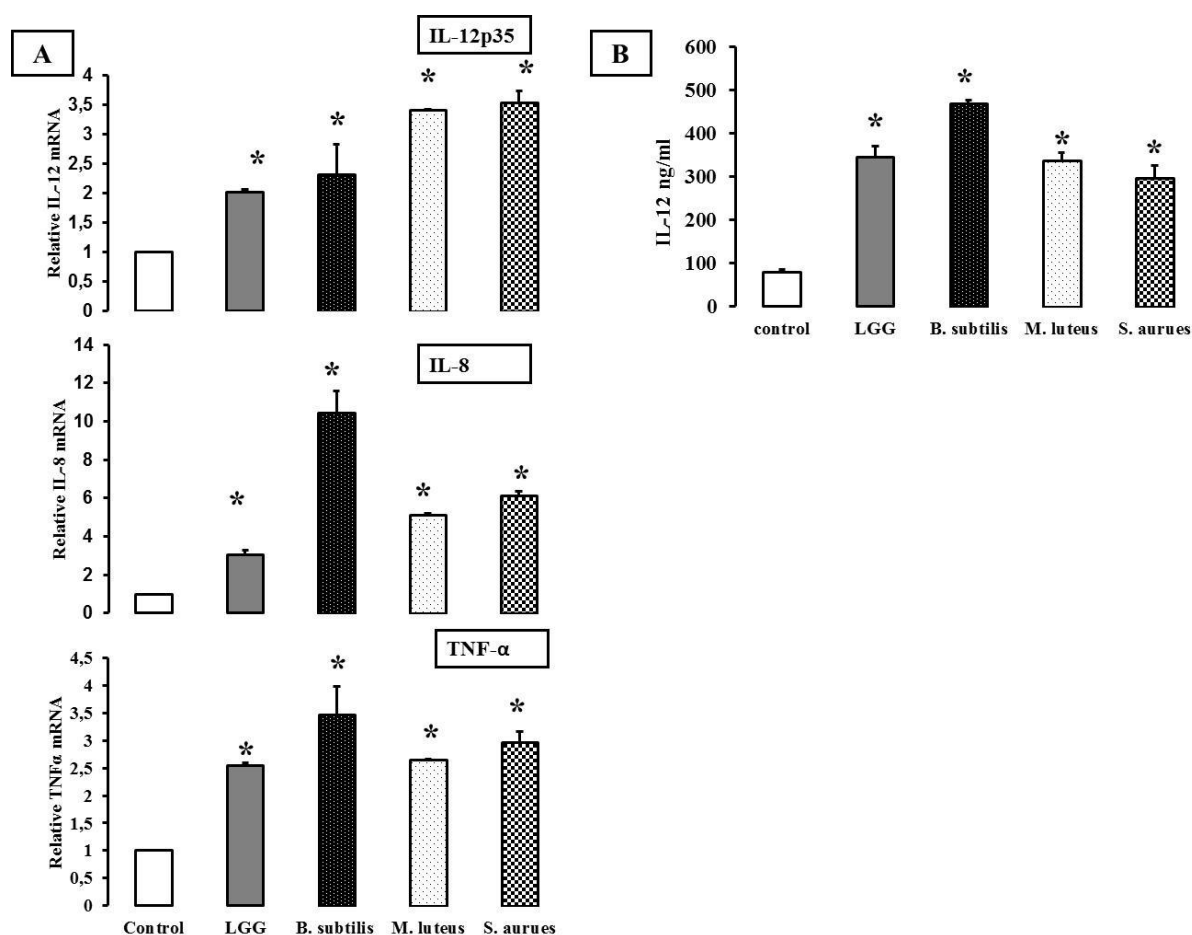


Figure 2. PGN induces inflammatory cytokine expression: Caco-2 cells were treated with 10  $\mu$ g/ml of different bacterial source PGN. A) The mRNA expression of IL-12p35, IL-8 and TNF- $\alpha$  was assessed by quantitative RT-PCR using SYBR Green. Results are related to  $\beta$ -actin mRNA as internal standard. B) IL-12 protein secretion was assessed by ELISA. Data are expressed as mean  $\pm$  SEM of n=6 experiments, measured in duplicates, \* denotes significant difference from the untreated control value.

### ***PglyRP3 suppresses the PGN-induced inflammatory cytokines***

Caco-2 cells were transfected with a plasmid carrying the PglyRP3 or siRNA against the same gene for 48 hrs. Cells were then incubated with 10

$\mu\text{g/ml}$  PGN from *B. subtilis*, *M. luteus*, *S. aureus* for 3 hrs or PGN from *LGG* for 12 hrs. The results showed that PGlyRP3 overexpression reduced the *LGG* PGN-induced IL-12p35, IL-8 and TNF- $\alpha$  mRNA levels significantly to 1.12, 1.30 and 1.46-folds compared to 2.01, 3.03 and 2.54-folds for the control. Similarly, PGlyRP3 overexpression reduced the *B. subtilis* PGN-induced IL-12p35, IL-8 and TNF- $\alpha$  mRNA levels significantly to 1.34, 6.90 and 1.68-folds compared to 2.31, 10.44 and 3.40-folds for the controls. PGlyRP3 overexpression reduced the *M. luteus* PGN-induced IL-12p35, IL-8 and TNF- $\alpha$  mRNA levels significantly to 1.90, 3.10 and 1.4-folds compared to 3.40, 5.14 and 2.65-folds for the controls, and *S. aureus* PGN-induced IL-12p35, IL-8 and TNF- $\alpha$  mRNA levels significantly to 1.2, 2.29 and 1.4-folds compared to 3.53, 6.14 and 2.97-folds for the controls (Fig. 3A). PGlyRP3 overexpression itself reduced the cytokine gene expression. IL-12p35, IL-8 and TNF- $\alpha$  mRNAs in PGlyRP3-overexpressing cells were 0.72, 0.59 and 0.49-folds respectively, compared to control cells, which were transfected with empty vector. PGlyRP3 overexpression also reduced the PGN-induced IL-12 protein secretion to 197.14, 314.72, 214.37 and 209.25 ng/ml compared to 345.29, 469.56, 389.53 and 337.04 ng/ml for PGNs isolated from *LGG*, *B. subtilis*, *M. luteus* and *S. aureus*, respectively (Fig. 3B). These results indicate a modulation of the PGN mediated inflammatory effect by PGlyRP3.

We found also that PGlyRP3 knocking increased significantly the *IL-12p35*, *IL-8* and *TNF- $\alpha$*  gene expression to 1.94, 2.10 and 1.39-folds, respectively, compared with the control value of 1. Addition of *LGG*, *B. subtilis*, *M. luteus* or *S. aureus* PGNs additively and severely increased the gene expression of *IL-12p35* to 2.90, 13.70, 9.12 and 14.4-folds, *IL-8* to 3.33, 21.68, 20.26 and 23.05-folds, and *TNF- $\alpha$*  to 3.25, 11.75, 4.28 and 7.3-folds, respectively (Fig. 3A), and the IL-12 protein expression to 1181.58, 13331.4, 1328.16 and 1266.99 ng/ml,

compared to 201.27 ng/ml for the group of PGlyRP3 silencing alone, and 345.29, 469.56, 389.53 and 337.04 ng/ml for the PGN groups, respectively (Fig. 3B).

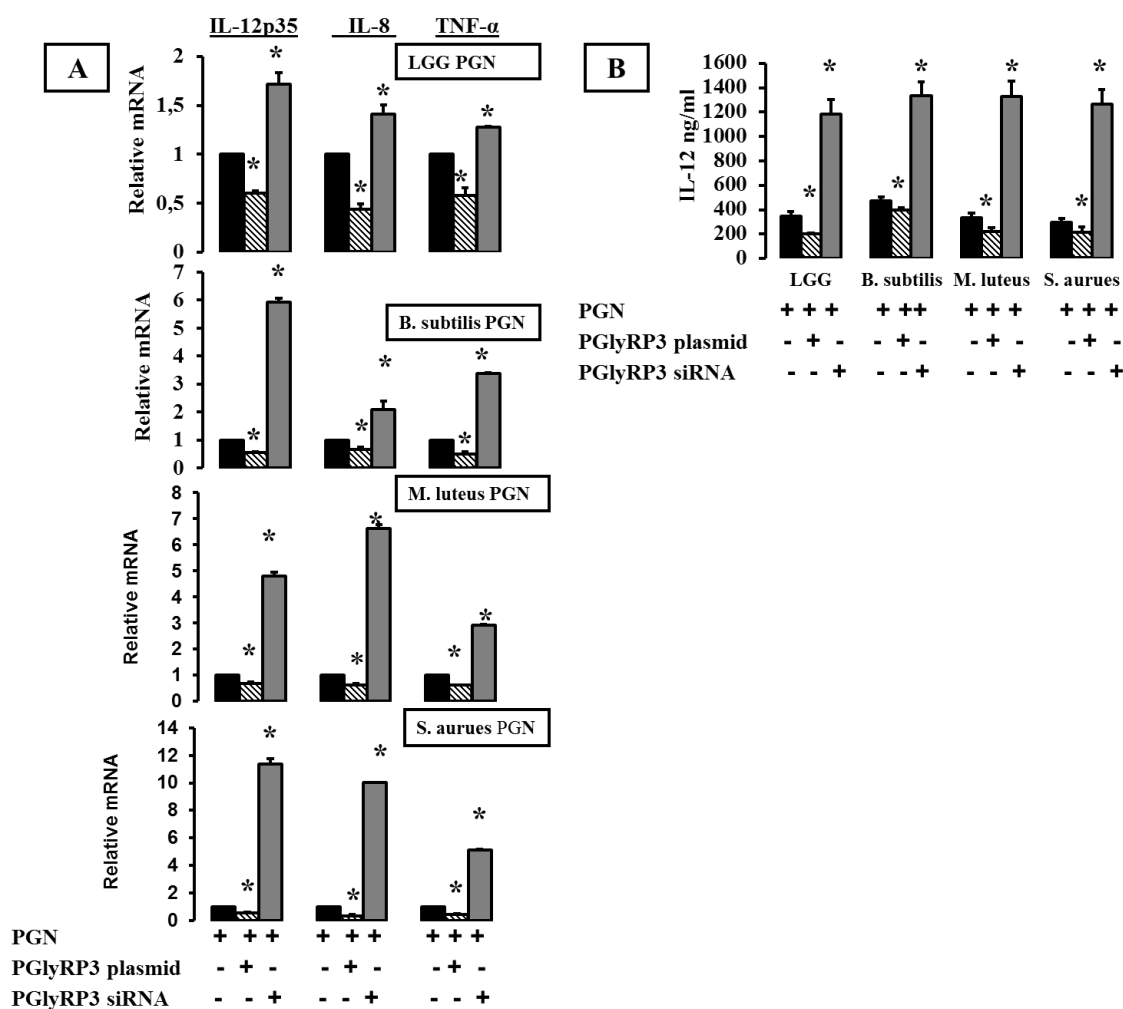


Figure 3. PGlyRP3 overexpression reduces the PGN-induced inflammatory cytokine: Caco-2 cells were transfected with PGlyRP3 plasmid or siRNA for 48 hrs and then treated with one of the different PGN for additional 24 hrs. After the incubation, mRNA expression of IL-12p35, IL-8 and TNF- $\alpha$  were analyzed by quantitative PCR using SYBR Green (A). The protein secretion of IL-12 was analyzed by ELISA (B). The control values (untreated Caco-2 cells or Caco-2 cells treated with empty plasmid or negative siRNA) are mentioned in **Table 2**. Data here are only compared to the PGN values. The shown data are averages of 6 separate experiments performed in duplicates. Statistical analysis: ANOVA \* = significantly different from the control Caco-2 cells treated with 10 $\mu$ g/ml PGN.

***TLR but not PGlyRP3 pathway is involved in the inflammatory response to PGNs***

PGN from *LGG*, *B. subtilis*, *M. luteus*, or *S. aureus* increased the mRNA expression of IL-12p35 to 3.03, 2.31, 5.14 and 6.14-folds; IL-8 to 1.8, 10.44, 3.40 and 3.53-folds and TNF- $\alpha$  to 2.54, 3.40, 2.65 and 2.97-folds, respectively; and IL-12 secretion to 345.29, 469.56, 337.04 and 297.14 ng/ml respectively, compared to untreated controls 94,26 ng/ml (Fig. 2). As shown in figure 4, the mRNA level of all tested cytokines and IL-12 protein secretion were significantly reduced ( $p < 0.05$ ) after incubation of MyD88 inhibitory peptide-treated Caco-2 cells with different PGNs.

These results confirm that microbial PGNs act proinflammatory due to TLR pathway prevailing the anti-inflammatory PGlyRP3's action. When PGlyRP3 expression is increased, the balance may be shifted towards lower inflammation.

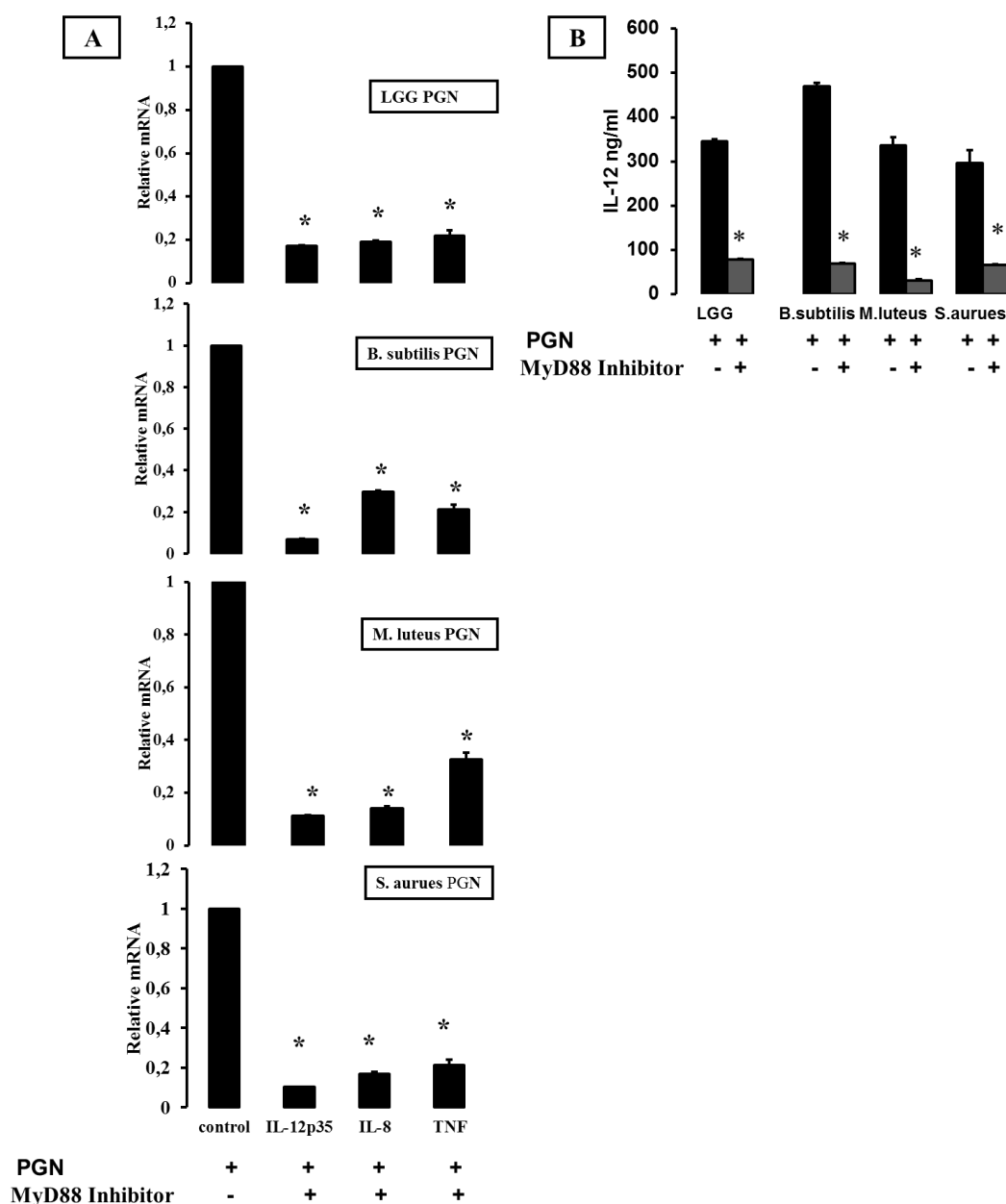


Figure 4. Neutralization of the TLR-pathway by MyD88 inhibitor suppressed the PGN-induced inflammatory cytokine expression in Caco-2 cells: Caco-2 cells were pre-treated with 100  $\mu$ M MyD88 inhibitory peptide or control peptide for 24 hrs then treated with 10  $\mu$ g/ml PGN of different bacterial sources. A) mRNA expression of IL-12p35, IL-8 and TNF- $\alpha$  were analyzed by quantitative RT-PCR using SYBR Green. Results are expressed as relative mRNA to  $\beta$ -actin mRNA as internal standard. B) The secretion of IL-12 was analyzed by ELISA. The shown data is averages of 6 separate experiments performed in duplicates. Statistical analysis: \* denotes  $p < 0.05$  in comparison to the PGN group which is considered here as a control group. Other control values (untreated Caco-2 cells or Caco-2 cells treated with control peptide) are mentioned in **Table 2**.



Furthermore, as shown in Figure 5, IL-12p35, IL-8 and TNF- $\alpha$  mRNA level and IL-12 protein secretion were significantly decreased after PGlyRP3 overexpression, while PGlyRP3 knocking down induced significantly ( $p < 0.05$ ) all tested cytokine mRNA level and IL-12 secretion. These results confirm the anti-inflammatory role of PGlyRP3.

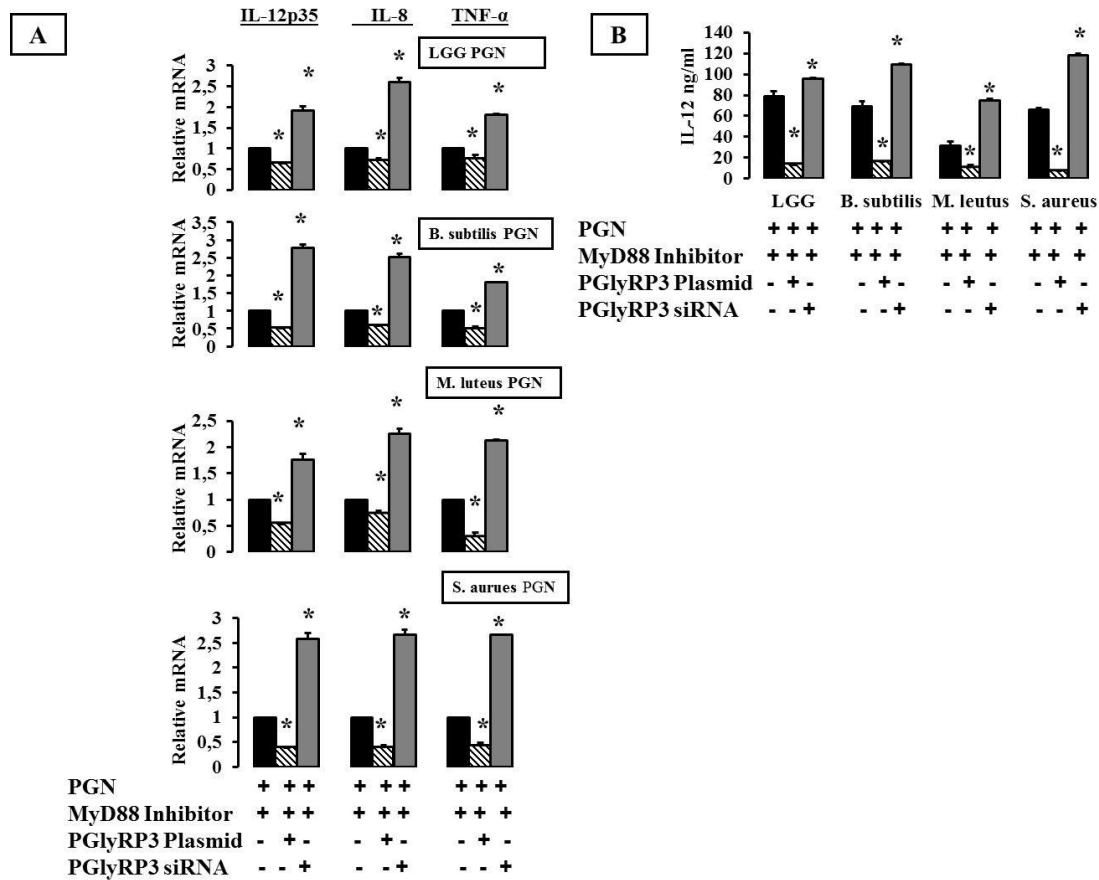


Figure 5. PGlyRP3 reduces the inflammatory cytokine expression despite the PGNs: Caco-2 cells were transfected with PGlyRP3 plasmid or siRNA and pre-treated with MyD88 inhibitory peptide then treated with 10  $\mu\text{g/ml}$  PGN from different bacterial sources. After the incubation time, mRNA expression of IL-12p35, IL-8 and TNF- $\alpha$  were analyzed by quantitative PCR analyses, which were performed using SYBR Green (A). IL-12 protein was detected using ELISA assay (B). Data is compared to PGN alone or with values of MyD88 inhibitor groups. The control values of both groups (untreated Caco-2 cells or Caco-2 cells treated with empty plasmid, negative siRNA or control peptide) are mentioned in **Table 2**. The shown data are averages of 6 separate experiments performed in duplicates. Statistical analysis: \*= significantly different from the control Caco-2 cells treated with 10  $\mu\text{g/ml}$  PGN and 100  $\mu\text{M}$  MyD88 inhibitory peptide.

## DISCUSSION

PGlyRP3 protein is expressed in tissues that come in contact with the environment (skin epidermis, sebaceous glands, tongue, esophagus, stomach and intestinal tract) (35). Our previous studies showed that it is also expressed in intestinal epithelial cells Caco-2 cells (33). In this study we examined the effect of PGN from both non-pathogenic (*B. subtilis* and *LGG*), opportunistic pathogenic (*M. luteus*) and pathogenic bacteria (*S. aureus*) on PGlyRP3 expression in Caco-2 cells. It is known that PGlyRP1, 3 and 4 are bactericidal both *in vitro* and *in vivo* against pathogenic and non-pathogenic Gram-positive bacteria. *B. subtilis*, *Lactobacillus LGG* (non-pathogenic bacteria) and *S. aureus* (pathogen bacterium) are equally sensitive to killing by different PGlyRPs (29).

The present results showed that both pathogenic, opportunistic pathogenic and non-pathogenic bacterial PGN time-dependently induced PGlyRP3 expression (Fig. 1). No difference was observed regarding the source of PGN. It is known that all mammalian PGlyRPs are able to bind peptidoglycan with high affinity, while they did not bind to other cell wall components (36). It is also known that human PGlyRP3 directly binds to bacterial PGN by two PGRP domains, each has one binding site for PGN and hydrophobic domains on the opposite side of the molecule from the PGN binding groove, which play a role in PGlyRP's interaction with bacterial PGN (37, 38). The induction of PGlyRP protein production by PGN in Caco-2 cells is in agreement with the results of Ping *et al.* (39).

Our results agree with previous studies that PGN from different bacterial sources induced cytokine and chemokine expression and release (40- 42). There was no difference between PGN from different sources. All of them were inflammatory in effect. This also agrees with previous reports that pathogenic or non-pathogenic bacteria induced the secretion of inflammatory cytokines such as IL-1 $\beta$ , IL-6 and IL-8 in intestinal epithelial Caco-2 cells (43, 44).

TLR2 has been shown to be a receptor recognizing PGN, and its activation in response to PGN induces the production of proinflammatory cytokines and chemokines (45, 46). MyD88 is an adaptor protein, which has an important role in the intracellular signalling stimulated by IL-1R and TLRs (47, 48). With the exception of TLR3, all known TLRs depend entirely or partly on MyD88 for intracellular signalling (49). TLR2 utilizes the adaptor proteins MyD88 and MyD88-adaptor like (Mal) to activate IL-1 receptor-associated kinase. Then, the activated IL-1 receptor-associated kinase dissociates the MyD88/Mal complex from the receptor, followed by association with TNF-associated factor 6 (TRAF6). This triggers the activation of the Rel family transcription factor NF- $\kappa$ B, which is required for transactivation of gene expression (45, 46). Our results showed that PGN from different bacterial strains utilizes MyD88 for TLR-pathway activation, and therefore, IL-12, IL-8 and TNF- $\alpha$  expression were significantly reduced by the inhibition of MyD88. We suggest that PGN utilizes TLR-mediated signalling pathways to induce a significant inflammatory response. After stopping the TLR-pathway by inhibiting MyD88, PGlyRP3 overexpression reduced the PGN-induced inflammatory cytokines and chemokine expression (Fig. 4 and 5), while its silencing increased severely the PGN-induced cytokines.

In conclusion the present results suggest that PGN from pathogenic, opportunistic pathogenic and non-pathogenic bacterial sources have inflammatory roles mediated through activation of the TLR-pathway. There were no differences between PGN obtained from these different bacterial sources in exerting this inflammatory responses. In contrast to TLRs and NODs, PGlyRP3 seems to have an anti-inflammatory role in intestinal epithelial cells. The inflammatory effect of PGN through the TLR-pathway could be reduced by increasing PGlyRP3 expression and even could be turned into anti-

inflammatory effects, if the TLR pathway is impeded. This may open perspectives in prevention and treatment of the inflammatory bowel disease.

**Table 2.** Appropriate control values of various experiments.

	mRNA	mRNA	mRNA	Protein
	IL-12p35	IL-8	TNF- $\alpha$	IL-12
Control	1,00 $\pm$ 0,00	1,00 $\pm$ 0,00	1,00 $\pm$ 0,00	94,26 $\pm$ 1,92
LGG PGN	2,02 $\pm$ 0,02	3,04 $\pm$ 0,25	2,54 $\pm$ 0,04	345,29 $\pm$ 4,84
B. subtilis PGN	2,31 $\pm$ 0,07	10,44 $\pm$ 1,11	3,42 $\pm$ 0,65	469,56 $\pm$ 7,74
M. luteus PGN	3,40 $\pm$ 0,16	5,14 $\pm$ 0,06	2,65 $\pm$ 0,02	389,53 $\pm$ 8,03
S. aureus PGN	3,52 $\pm$ 0,35	6,14 $\pm$ 0,21	2,97 $\pm$ 0,47	337,04 $\pm$ 8,55
control(empty vector)	1,00 $\pm$ 0,00	1,00 $\pm$ 0,00	1,00 $\pm$ 0,00	79,99 $\pm$ 4,84
LGG PGN +PGlyRP3 plasmid	1,12 $\pm$ 0,02	1,31 $\pm$ 0,05	1,46 $\pm$ 0,07	197,37 $\pm$ 4,84
B. Subtilis PGN +PGlyRP3 plasmid	1,34 $\pm$ 0,05	6,87 $\pm$ 0,25	1,68 $\pm$ 0,03	314,72 $\pm$ 9,37
M. leutus PGN +PGlyRP3 plasmid	1,89 $\pm$ 0,09	3,10 $\pm$ 0,24	1,44 $\pm$ 0,01	214,37 $\pm$ 4,08
S. aureus PGN +PGlyRP3 plasmid	1,26 $\pm$ 0,03	2,29 $\pm$ 0,17	1,42 $\pm$ 0,09	209,25 $\pm$ 3,61
PGlyRP3 plasmid	0,72 $\pm$ 0,22	0,59 $\pm$ 0,05	0,49 $\pm$ 0,07	14,56 $\pm$ 2,21
control(NegativesiRNA)	1,00 $\pm$ 0,00	1,00 $\pm$ 0,00	1,00 $\pm$ 0,00	115,16 $\pm$ 2,84
LGG PGN +PGlyRP3 siRNA	2,90 $\pm$ 0,14	3,33 $\pm$ 0,11	3,25 $\pm$ 0,03	1181,58 $\pm$ 201,27
B. subtilis PGN +PGlyRP3 siRNA	13,70 $\pm$ 0,52	21,68 $\pm$ 0,39	11,75 $\pm$ 0,29	1331,42 $\pm$ 114,10
M. leutus PGN +PGlyRP3 siRNA	9,12 $\pm$ 0,37	20,62 $\pm$ 0,45	4,28 $\pm$ 0,12	1328,16 $\pm$ 127,44
S. aureu PGN s+PGlyRP3 siRNA	14,40 $\pm$ 0,77	23,05 $\pm$ 0,43	7,31 $\pm$ 0,32	1266,99 $\pm$ 110,37
PGlyRP3 siRNA	1,94 $\pm$ 0,62	2,10 $\pm$ 0,24	1,39 $\pm$ 0,02	201,27 $\pm$ 3,80
control negative peptide	1,00 $\pm$ 0,04	1,05 $\pm$ 0,02	1,02 $\pm$ 0,04	102,24 $\pm$ 4,73
MyD88 inhibitor	1,02 $\pm$ 0,00	1,00 $\pm$ 0,00	1,04 $\pm$ 0,00	104,89 $\pm$ 3,73
MyD88 inhibitor+LGG PGN	0,52 $\pm$ 0,00	0,38 $\pm$ 0,00	0,56 $\pm$ 0,02	79,04 $\pm$ 2,21
MyD88 inhibitor+B. subtilis PGN	0,72 $\pm$ 0,00	0,68 $\pm$ 0,00	0,71 $\pm$ 0,01	69,09 $\pm$ 1,49
MyD88 inhibitor+M. leutus PGN	0,58 $\pm$ 0,00	0,47 $\pm$ 0,00	0,86 $\pm$ 0,03	31,73 $\pm$ 0,82
MyD88 inhibitor+S. aureu PGN	0,62 $\pm$ 0,00	0,59 $\pm$ 0,00	0,63 $\pm$ 0,00	65,81 $\pm$ 2,79
MyD88 inhibitor+PGlyRP3 plasmid	0,44 $\pm$ 0,00	0,48 $\pm$ 0,00	0,46 $\pm$ 0,00	19,72 $\pm$ 2,68
MyD88 inhibitor+LGG PGN+PGlyRP3 plasmid	0,37 $\pm$ 0,00	0,47 $\pm$ 0,00	0,42 $\pm$ 0,00	13,36 $\pm$ 0,35
MyD88 inhibitor+B. Subtilis PGN+PGlyRP3 plasmid	0,41 $\pm$ 0,00	0,38 $\pm$ 0,00	0,36 $\pm$ 0,00	16,04 $\pm$ 2,28
MyD88 inhibitor+M. leutus PGN+PGlyRP3 plasmid	0,35 $\pm$ 0,00	0,31 $\pm$ 0,00	0,24 $\pm$ 0,00	9,93 $\pm$ 3,32
MyD88 inhibitor+S. aureu PGN+PGlyRP3 plasmid	0,23 $\pm$ 0,00	0,24 $\pm$ 0,00	0,26 $\pm$ 0,005	6,80 $\pm$ 2,43
MyD88 inhibitor+PGlyRP3 siRNA	1,03 $\pm$ 0,02	1,00 $\pm$ 0,00	1,058 $\pm$ 0,02	78,96 $\pm$ 4,75
MyD88 inhibitor+LGG PGN+PGlyRP3 siRNA	1,00 $\pm$ 0,01	1,00 $\pm$ 0,05	1,01 $\pm$ 0,02	95,73 $\pm$ 5,19
MyD88 inhibitor+B. Subtilis PGN+PGlyRP3 siRNA	0,97 $\pm$ 0,08	0,99 $\pm$ 0,00	1,00 $\pm$ 0,08	109,52 $\pm$ 6,44
MyD88 inhibitor+M. leutus+ PGN + PGlyRP3 siRNA	1,07 $\pm$ 0,07	1,02 $\pm$ 0,03	1,01 $\pm$ 0,02	75,25 $\pm$ 3,92
MyD88 inhibitor+S. aureu+ PGN+PGlyRP3 siRNA	0,99 $\pm$ 0,01	0,98 $\pm$ 0,00	0,96 $\pm$ 0,02	118,03 $\pm$ 8,45

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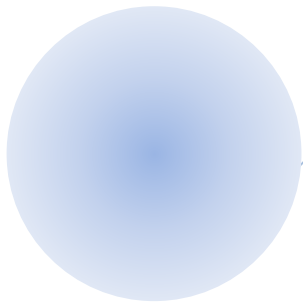
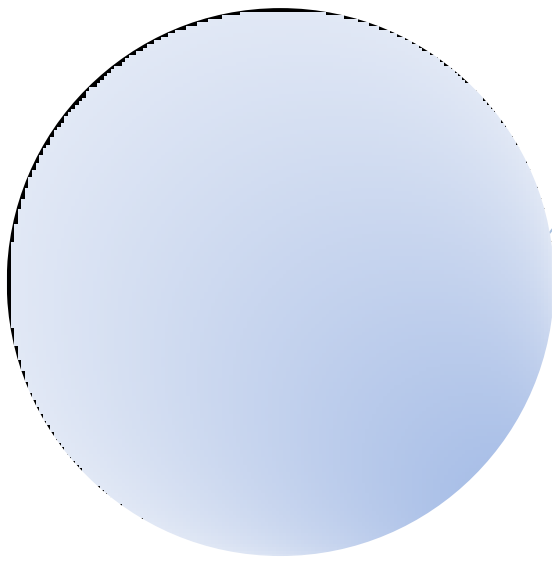
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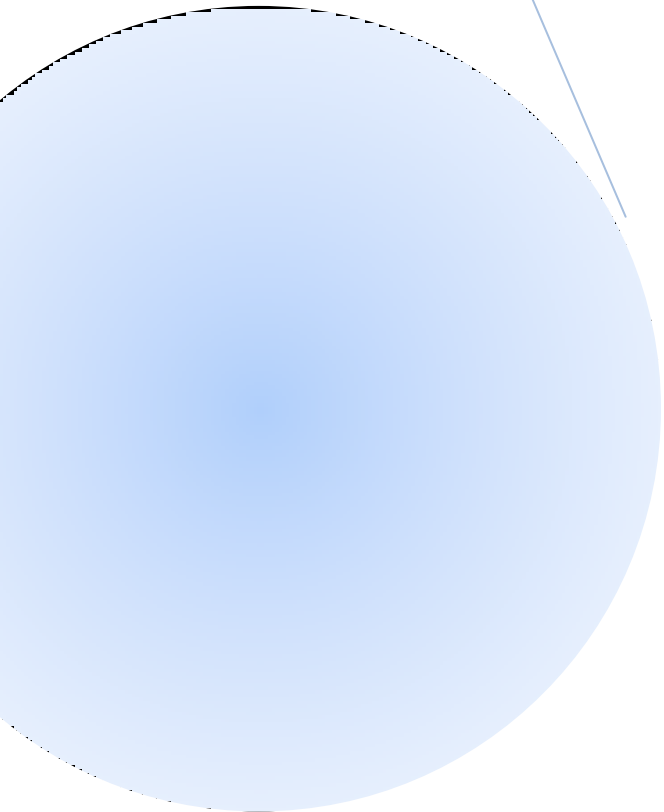
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## **CHAPTER V**

[اكتب العنوان الفرعي للمستند]

### **General Discussion**



## General Discussion

This present study was designed to investigate the potential of nutrients and products of the gastrointestinal microbes to modulate immunity through the interaction with the pattern recognition proteins (PGlyRPs).

It is not surprising that immunity is depending on our dietary habits. The gastrointestinal tract is the organ with the largest defence area and accordingly, it contains the largest part of the immune system, the gastrointestinal immune system (GI immune system). The gastrointestinal tract is a lymphoid organ, which consists of a lymphoid tissue known as gut-associated lymphoid tissue (GALT). The GALT lymphocytes are approximately equivalent in number to those in the spleen. The lymphoid cells in gastrointestinal tract are located in three basic populations according to their location, Peyer's Patches, lamina propria lymphocytes, and intra-epithelial lymphocytes. GI immune system has also other important cell types including for example the M or microfold cell. M cells in Peyer's Patches allow the transport of luminal microorganisms and macromolecules into the underlying lymphoid follicle-associated epithelium (FAE) where they are taken up by local dendritic cells (DC) and macrophages for initiation of the appropriate immune responses. On the other side, DCs themselves detect the presence of microbes by recruiting of pattern recognition receptors (PRRs) that recognize conserved pathogen-associated PAMPS, and are expressed on processes of the DC protruding into the gut lumen (1, 2) . The GI immune system is pivotal in mediating the interactions between luminal microbiota and host (3). In our gut, microbiota have an important role in nutritional and health status by improving a number of protective, immune and metabolic functions. The development and regulation of immune and non-immune host defence are stimulated by gut microbiota via the interaction with the epithelium and the GALT (4). The balance between host protective immunity and regulatory mechanisms is achieved by intestinal cells, which, in

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turn, are providing the intestinal homeostasis and acting through different regulatory pathways (3). This regulation depends on an input arising from the environment surrounding the intestinal cells comprising the bacterial and dietary components of the lumen, which themselves affect the microbial pattern, activity and metabolism.

Our *in vitro* model was the Caco-2 cell line because of several reasons. First, the Caco-2 cell line is a suitable *in vitro* model of the human intestinal epithelium that mimics many important properties of the mucosal epithelium after reaching confluence. Caco-2 cells differentiate in culture and acquire characteristics that are both structurally and functionally similar to that of either crypt cells or villus cells of the small intestine (5-9). Second, the pattern of inflammatory responsiveness in Caco-2 cells is more similar to that of isolated colonocytes than most other colon cell lines. Third, stimulated cytokine secretion in Caco-2 cells is of a similar order of scale to that of normal isolated colonic epithelial cells (10-12). Finally and fortunately, we found that a gene of interest, a member of PGlyRP recognition pattern protein family, namely PGlyRP3, is expressed in this cell line and there were significant differences in its expression during the post-confluence days. It was also reported that treatment of Caco-2 cells with some bioactive food constituents like (butyrate, resveratrol, quercetin and curcumin) lead to changes in some genes and proteins expressions, which resemble *in vivo* observed changes. Moreover, the Caco-2 permeability assay is considered to be the industry gold standard for *in vitro* prediction of *in vivo* human intestinal permeability and bioavailability of orally administered food and drugs (13, 14).

Despite all of the above mentioned information about the similarity of this cell model to the intestinal cells, and the existence of many studies in which Caco-2 cells were used as an *in vitro* model to evaluate the gastrointestinal immune responses of gastrointestinal microbes (15, 16), Caco-2 cells should not

be considered a complete representative of the interaction between the luminal bacteria and the enterocytes *in vivo*. The gut epithelium is impermeable to macromolecules and microorganisms, except in Peyer's patches, where M cells exist, which can transport antigens and microorganisms (17). Rescigno's group (18) suggested the existence of a further road for bacterial invasion. One of them is independent of M cells. In Caco-2–DC co-cultures, only in the presence of DCs bacteria were detected at the basolateral side of the Caco-2 cells, this suggested that bacteria cannot be transported by Caco-2 cells. Interactions between intestinal bacteria and the intestinal epithelium, which are also affected by bacterial metabolism, cannot be investigated *in vitro* by Caco-2 cells (19). Thus, Caco-2 cells do not represent the entire complexity of the *in vivo* system. Other cells are also involved such as M-cells, DCs. This point would be taken in consideration in our future studies. Hence, it is planned to use a mouse model with induced colitis to study the role of PGlyRP3 in regulation of inflammatory responses in intestine to the intestinal bacteria. This study may shed light on the importance of intestinal microbiota and provide a molecular link between microbiota metabolism and immune response.

Inflammation is a special aspect of the immune response to erase local infections efficiently. The inflammation is affected by the immune system from one side and seems to be affected by nutrition from another side. There is abundant evidence that the intestinal microbiota have impact on the pathogenesis of the inflammatory diseases. Many studies have focused on the interference between nutrition and recognition pattern proteins, which are essential for the induction of inflammation. For example, it was found that the balance between saturated and unsaturated fatty acids influence the Toll-mediated immune responses, which in turn can be altered by the dietary fat consumed. TLRs and Nods, the most studied recognition pattern proteins, could be activated by non-microbial molecules and they might also be involved in

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inducing inflammation and immune responses (20-24). This might explain how the different types of fatty acids differentially modulate immune responses. Intestinal microbiota positively affect the immune response and save from the development of inflammatory diseases of the gut (25, 26).

In the same context, we focused on the interference between dietary and microbial factors from one side, and the recognition pattern proteins PGlyRPs from the other side, since less is known about PGlyRPs than TLRs and Nods in the intestinal model.

The metabolic products of the microbiota such as short and branched-chain fatty acids (SCFA, BCFA) have not found much attention, although they have been shown to modulate the expression of various genes and reduce inflammation (27-30). Therefore, our research theory was based on reports about the ability of gastrointestinal microflora such as lactic acid bacteria to produce SCFA or BSFA (lipophilic products) by utilization of the prebiotic oligosaccharides in the intestine (31, 32). These bacterial lipophilic products may regulate genes of immune functions through some transcription factors (TFs). Although this cannot completely be confirmed only by using the *in vitro* model, we thought to test the effects of some oligosaccharides and some fatty acids separately on the PGlyRP3 expression *in vitro* and to investigate whether this effect takes place through a transcription factor. In parallel, we tried also to detect the regulatory role of PGlyRP3 in inflammation and the effects of these different products mentioned above.

In contrast to the insect PGRPs, mammalian PGlyRPs have no known role in stimulating intracellular signalling pathways, but rather appear to act as antibacterial or scavenger molecules. PGlyRP3 is a novel pattern recognition protein, recognizing bacterial peptidoglycan and is up-regulated by bacteria and their products. PGlyRP3 is a bactericidal protein, which functions in antibacterial immunity (33, 34). Large numbers of publications have focused on

TLRs, Nods and PGlyRP2 as inflammatory genes and their role in inflammation and innate immunity. However, there is no available information about PGlyRP3's role in inflammation responses. The phylogenetic analysis of all mammalian and insect PGRPs indicated that mammalian PGlyRP3 was derived from a common ancestor of PGlyRP1 (35). Because PGlyRP1 has an anti-inflammatory role (36, 37), we assumed that PGlyRP3 may also has the same function. The results of the present study confirmed this assumption.

Fatty acids or their metabolites regulate gene transcription by binding directly to specific transcription factors including peroxisome proliferator-activated receptors (PPAR $\alpha$ ,  $\beta$ ,  $\gamma$ 1, and  $\gamma$ 2) (38, 39). Recently, many studies reported that some transcription factors regulate, either directly or indirectly, both metabolic and inflammatory reactions at the same time (40-43). In order to determine common transcription factor binding sites on the PGlyRP3 promoter, an *in silico* analysis using TRANSFAC was applied. TRANSFAC is an internationally knowledgebase with information about experimentally-proven binding sites of transcription factors and regulated genes. TRANSFAC functions to detect genes, which are regulated by transcription factors, determine the binding site for any transcription factors found on these genes' promoter regions and choose the specific DNA sequence(s) that bind these transcription factors (44). In fact, the results of the *in silico* analysis revealed the presence of many transcription factors of lipophilic ligands on the promoter region of PGlyRP3 gene, especially many PPAR $\gamma$  binding sites. PPAR $\gamma$  is a nuclear receptor involved in fatty acid intake, transport and metabolism. PPAR $\gamma$ 's role in chronic diseases was heavily investigated. It is of special interest that PPAR $\gamma$  and its ligands have been reported to exert anti-inflammatory activities in different cell types by strongly down-regulating the transcription factors for many proinflammatory cytokines (45- 50). Therefore, a PPAR $\gamma$  putative binding sequence, as observed from the *in silico* analysis, was

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chosen to perform EMSA. EMSA is a common technique used to study protein-DNA or protein-RNA interactions. This technique can determine whether a protein is able to binding to a given DNA or RNA sequence, and it can also indicate if more than one protein molecule is involved in the binding complex. To demonstrate purposes, the DNA fragment has to be labelled with a biotin, radioactive or fluorescent label. This method is sensitive, but, it is time consuming and it cannot be applied to high-throughput screening. EMSA results revealed the binding of PGlyRP3 gene promoter to the complex PPAR $\gamma$ -RXR. Hence, the recognition pattern PGlyRP3 protein may consequently be responsive to not only bacteria, but also some nutritional elements (FFAs) via its gene activation by the complex PPAR $\gamma$ -RXR.

In our studies, we used two different artificial substances GW1929 and GW9662 that affect PPAR $\gamma$  activity. GW1929 is a PPAR $\gamma$  full agonist, which can activate PPAR $\gamma$ , leading to a wide-ranging gene regulation. On the contrary GW9662 is a potent, selective PPAR $\gamma$  antagonist. GW9662 covalently modifies and inhibit PPAR $\gamma$  in cell culture systems, even in the presence of high concentrations of reducing agent or exogenous protein (51). Different fatty acids were also applied in our research depending on different aspects; carbon chain structure (saturated, unsaturated short and branched chain FFAs), role in inflammation (inflammatory and anti-inflammatory FFAs), relation to PPAR $\gamma$  and FFAs sources (bacterial and dietary source). The results confirmed the assumed responsiveness of PGlyRP3 to the lipophilic ligands and the alteration in PPAR $\gamma$  activity. They also confirmed the link between nutritional factors and their transcription factors from one side, and a bacterial recognition pattern that leads to an immune response from the other side.

In this study we also induced and knocked down the PGlyRP3 gene. Two different transfection methods have been applied. The first method was based on inducing PGlyRP3 plasmid DNA into Caco-2 cells. To get effective gene



transfer, plasmid DNA can be complexed by physico-chemical compounds (transfection reagents) to mediate effective transfer into the cells' nuclei and then mediate subsequent protein expression of the genes of interest. We used lipid-based transfection reagents, which forms strong, stable, positively charged complexes with DNA. This prevents DNA degradation and facilitates transfer of the gene into the cell. This polymer provides the transfection efficiency despite the presence of antibiotics. The second transfection was performed by introducing a double-stranded RNA molecules targeting PGlyRP3 gene into Caco-2 cells, which in turn causes degradation of PGlyRP3 complementary mRNA. Small interfering RNA (siRNA) is a nucleic acid molecule used to silence gene expression. siRNA mediated gene silencing is a quick and an easy method to knockdown a specific gene. It has some disadvantages, especially in those cells that are difficult to transfect, expensive for larger experiments and not useful for long term studies (52, 53).

As proinflammatory markers, the proinflammatory cytokines IL-12 and TNF- $\alpha$  as well as the proinflammatory chemokine IL-8 were chosen. There are small proteins, biologically, highly active levels of them secreted in many different cell lines including intestinal epithelial cells. They regulate the growth, function, and differentiation of different cells and modulate the immune and inflammatory responses (54-56). It was also reported that TNF- $\alpha$ , IL-12 and 8 induce intestinal inflammation, and their up-regulation is associated with inflammatory bowel diseases such as Crohn's disease and ulcerative colitis (57-62). This suggests that these proinflammatory cytokines and chemokines play a key part in the regulation of the pathogenesis of inflammatory bowel disorders. It is also well known that various cytokines such as IL-8, IL-12p35 and TNF- $\alpha$  are expressed and secreted from intestinal epithelial cells as Caco-2 and HT29 (63-65).

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Gene and protein expressions of some cytokines and PGlyRP3 were measured after PGlyRP3 overexpression or silencing to indicate the function of PGlyRP3. The results showed that PGlyRP3 is not only a bactericidal gene as reported before in the literature (33, 34), but also has a role in the regulation of inflammation. PGlyRP3 has an anti-inflammatory effect *per se* without any external induction, since knocking down of the PGlyRP3 gene was sufficient to induce the expression of pro-inflammatory cytokines. Gene expression was measured by quantitative real time PCR, which is a popular technique with broad applications. There are three major advantages of the PCR method: quick and simple since DNA cloning by PCR can be ready in a few hours using relatively simple tools; sensitivity since only very little amounts of target DNA are needed to amplify DNA sequences; and robustness since PCR allowing the amplification of a specific sequences from degraded or embedded DNA (66). The protein level was measured by ELISA or Western blotting. Western blot (protein immunoblot) is the technique in which particular protein could be detected by binding to specific antibodies. This analysis informs us about the size of the protein or how much protein accumulated in cells. It does not mean that it can do for any protein since in the case of quickly protein degradation, it will be hard to detect it well. The antibody is the most important constituent in the Western blot technique since in Western blotting it is used as a probe to detect specific protein. Western blotting can detect proteins in small amounts. But nonspecific result could be getting after reacting of an unintentional protein with the secondary antibody, multiple bands may also appear, bubbles may formed during transfer step or during the incubation with the antibodies, which lead to formation of a skewed band. In Western blot the transfer time should be sufficient, because the higher molecular weight proteins will not transfer properly if the transfer time was not enough, this lead to an incorrect or no band reading at all (67).

ELISA (enzyme-linked immunosorbent assay) is another technique that utilizes antibodies to detect the presence of a specific protein in a sample. ELISA showed sensitivity of 92% and specificity of 98%, quick and convenient, antigens of very low or unknown concentration can be detected, used in wide variety of tests and generally safe. ELISA has also disadvantages. For example, only monoclonal antibodies can be used, which is not easy to find and more expensive than polyclonal antibodies, enzyme-substrate reaction cannot stand for long time so micro-wells must be read immediately. Moreover, when the blocking solution is ineffective, negative controls may give positive results (68).

The transcription factor NF- $\kappa$ B is important for the inducible expression of a wide variety of cellular and viral genes. NF- $\kappa$ B exists in an inactive form in the cytoplasm of most of the body cells, where it bound to the inhibitory I $\kappa$ B proteins. Treatment of cells with various inducers activates a signalling cascade that leads to phosphorylation of I $\kappa$ Bs, resulting in the degradation of I $\kappa$ B proteins. This make the NF- $\kappa$ B dimers free to translocate into the nucleus where they activate the transcription of different target genes. The 5-GGGACTTCC-3 nucleotide sequence is the target of both homo- and heterodimers of the Rel/NF- $\kappa$ B family members (69). The activated NF- $\kappa$ B in the nucleus induces the transcription of proinflammatory genes and has been shown to be a key regulator of immune responses (70). The recognition of PAMPs by different PRRs including TLR and Nods initiates signalling cascade that activates NF- $\kappa$ B. Saturated FFAs activated, while polyunsaturated FFAs inhibited NF- $\kappa$ B and thus cytokine production through TLR and Nods signalling (7-9). As PGlyRP3 is a PRR as TLRs and Nods are, we suggested initially that PGlyRP3 modulates the cytokine production in the same way as both TLRs and Nods through activation/deactivation of the NF- $\kappa$ B pathway. Our results confirmed this assumption; PGlyR3 inhibited the NF- $\kappa$ B translocation through inducing I $\kappa$ B

expression. Traditionally, three different methods were used to study the transcription factors; EMSA, immunoblotting and reporter gene assays. These methods were found to be time consuming and give only semi-quantitative results, and they tend to lack sensitivity. So, in this study we used a new method called TransAM to measure the translocation of NF- $\kappa$ B. TransAM Kits contain a 96-stripwell plate which covered with an oligonucleotide containing the NF- $\kappa$ B consensus-binding site (5-GGGACTTCC-3). The active NF- $\kappa$ B contained in nuclear or whole-cell extract is added to each well, which binds specifically to this oligonucleotide. When NF- $\kappa$ B is activated a primary antibody detect NF- $\kappa$ B recognizes an epitope on p65, p50 or p52. Adding a HRP- conjugated secondary antibody provides a colorimetric readout, which could be quantified by spectrophotometry. TransAM could be defined as an enzyme-linked immunosorbent assay (ELISA) that facilitates the study of transcription factor activation with a high sensitivity. TransAM assay is more sensitive than EMSA and is complete in a few hours. The TransAM method provides a safe measuring method without using radioactivity and gives quantitative results (69).

Recently, it was found that the functional foods or functional food ingredients exert a beneficial effect on host health and reduce some chronic diseases (71, 72). The first generation of functional foods included thoughtful supplementation of food reach with minerals (mainly calcium) and vitamins (72). But, recently the concept has moved to food which has a positive effect on the gut microbiota, introducing pro- and prebiotics (73). Prebiotics are mostly carbohydrate fibres called oligosaccharides that feed and modulate the beneficial bacteria in our gut. The undigestible oligosaccharides remain in the digestive tract and stimulate the growth of beneficial bacteria (73). Although over twenty different types of oligosaccharides are on the world market today (74), only few studies so far have investigated the immunomodulatory effects of

oligosaccharides. Most of the recent oligosaccharides studies concerned about the role of milk oligosaccharides in the protection of the new born intestine against pathogenic microflora, and the ability of fructo-oligosaccharides to be fermented by intestinal bacteria producing SCFA, which in turn reduce the intestinal inflammation. However, based on the reported, not explained anti-inflammatory role of different prebiotic oligosaccharide, we thought to investigate the ability of oligosaccharides to induce the expression of an immune gene (PGlyRP3). Two different oligosaccharides were utilized in the current study, which are from different origins, plant and milk, and widely used as prebiotics in the industry.  $\alpha$ 3-sialyllactose is the most abundant acidic oligosaccharide, which is present in human milk at concentrations of 0.4 to 0.8 mg/ml; levels are much lower in bovine colostrum (0.03-0.06 mg/ml) (75). Recently, sialyllactose is evolving into different commercial products. It is used in foods especially intended for infants, children or adult as well as for medical foods. Raftilose p95 is a substance that is derived from chicory roots, and composed of 95% Fructo-oligosaccharide, mainly oligofructose (76). Raftilose p95 is widely used in industry as a commercial product to improve digestive system and bone health. Our study gives another explanation of the course of the oligosaccharides in the inflammation. Oligosaccharides have anti-inflammatory roles, independently on its source (plant or milk), by enhancing a PPAR $\gamma$ -induced anti-inflammatory PGlyRP3 protein expression, which in turn, inhibits the pro-inflammatory cytokines expression.

The effect of peptidoglycan (PGN), the main ligand of PGlyRPs, on the PGlyRP3 expression was also investigated. Many studies showed the high binding affinity of PGlyRP3 to PGN. Except for what was reported about the up-regulation of the four PGlyRPs mRNA expression and cell surface expression of PGlyRP3 and 4 by synthetic PAMPs (77), nothing was reported about PGN ability to induce PGlyRP3 expression. Administration of probiotic

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strains led to an anti-inflammatory effect and modulated immunity (78). The mechanisms of this immunomodulation are still not fully understood. So, in our study we used different PGNs (probiotic and non-probiotic bacterial PGNs) in order to investigate if the inflammatory role of PGN differs according to its bacterial source, and whether the PGNs from probiotic bacteria have anti-inflammatory effects mediated through PGlyRP3. Unexpectedly, our study showed that all PGNs induce pro-inflammatory cytokines independently on its bacterial source, while in the same time it induces the anti-inflammatory gene (PGlyRP3). PGN induces these pro-inflammatory cytokines through activation of the TLR-pathway. These results also suggested the existence of a competition between the inflammatory effect of PGN through the TLR-pathway and anti-inflammatory effect through PGlyRP3-pathway.

The disclosure of the PGlyRP3 function in immune response is the most interesting conclusion of this study. We found that PGlyRP3 has anti-inflammatory effects through the suppression of NF- $\kappa$ B pathway and inhibition of cytokine expression. The PGlyRP3 – NF- $\kappa$ B pathway is involved in the PPAR $\gamma$  intestinal anti-inflammatory role. PPAR $\gamma$  induces PGlyRP3, which inhibits the cytokine expression on one side and NF- $\kappa$ B translocation on the other side. In the same way, prebiotics inhibit the pro-inflammatory cytokine expression through the stimulation of PGlyRP3 expression. On the other hand, PGNs have inflammatory effects by inducing the pro-inflammatory cytokine expression. The inflammatory role of PGN occurs independently of PGlyRP3. However, the induction of PGlyRP3 reduces the inflammatory effect of PGNs. These results supported our hypothesis that nutrition is capable of modulating immunity through modulation of the recognition pattern protein (PGlyRP3). However, in the current study the role of epithelial PGlyPR3 in inflammation is exclusively investigated in an *in vitro* context. Thus, it is preliminary to conclude for the role of PGlyPR3 in real life intestinal inflammations. So, in

future studies we should provide evidences that epithelial PGlyPR3 has an *in vivo* role in intestinal inflammation by using appropriate *in vivo* model. We would also investigate the role of PGlyRP3 in ulcerative colitis as a step to indicate the role of PGlyRP3 in different inflammatory diseases in human.

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