Immune modulating effects of B-glucan, fish oil and conjugated linoleic acid

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Immune modulating effects of β -glucan, fish oil and conjugated linoleic acid

The logo for NUTRIM, featuring the word "nutrim" in a lowercase, bold, sans-serif font. The letters are black, and the "i" has a dot.

The studies in this thesis were performed within the Nutrition and Toxicology Research Institute Maastricht (NUTRIM) which participates in the Graduate School VLAG (Food Technology, Agrobiotechnology, Nutrition and Health Sciences), accredited by the Royal Netherlands Academy of Arts and Sciences.

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Chapter 1

General introduction

Introduction

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Introduction

The human body is protected against invasion with pathogens such as bacteria, viruses and parasites by the immune system, which is divided in two mutually interactive systems: the innate and adaptive immune system. Inflammation is part of the innate immune response, which occurs at the site where tissues are injured by bacteria (infection), toxins, trauma, heat or any other cause. (1) Inflammation is overall a protective response, although a sustained inflammatory state (chronic inflammation) may be harmful. Chronic inflammation is associated with several common diseases in Western populations, for example cardiovascular diseases (CVD), type II diabetes, rheumatoid arthritis and inflammatory bowel disease (IBD). (2-5) In contrast to a sustained inflammatory response, the response can also be impaired, which may lead to decreased resistance to infections. Especially elderly people may suffer from an impaired immune function. (6) Nutrition can modulate immune responses. Specific nutritional compounds can enhance or dampen the immune response. The role of nutrition in modulating immune responses and consequently influencing inflammation related diseases will be discussed briefly in this chapter. In this respect, the focus will particularly be on CVD and IBD.

Immune system

The immune system protects the human body against invasion with pathogens (a.o. bacteria, viruses and parasites) and is the result of two mutually interactive systems: the innate and adaptive immune system (**Figure 1.1**).

Figure 1.1: Schematic view of the immune system.
Abbreviations: Tc-cells, cytotoxic T-lymphocytes; Th-cells, helper T-lymphocytes.

Epithelial barriers like the skin and linings of the gastrointestinal tract, lungs and urinary tract are the first line of defense of the innate immune system. The innate immune response depends largely on the recognition of conserved microbial structures (pathogen associated molecular patterns, PAMPs) by so-called pattern recognition receptors (PRR). The toll-like receptors (TLRs) are well known examples of PRRs. Examples of PAMPs are cell wall components, such as lipopolysaccharide (LPS) from Gram-negative bacteria, lipoteichoic acid and peptidoglycan from Gram-positive bacteria, and β -glucan from fungi and yeast. Leukocytes that are involved in this early innate immune response are granulocytes (a.o. neutrophils), monocytes/macrophages and natural killer (NK) cells. These cells play an important role in phagocytosis of pathogens, free radical production (oxidative burst), cytokine production, and antigen presentation to lymphocytes. The innate immune response is rapid and functional, but nonspecific. In contrast, the adaptive immune response is somewhat slower, but highly specific. This response depends on 1) the production of antibodies (immunoglobulines) by B-lymphocytes directed against specific antigens present on pathogens (called humoral immune response) and on 2) the attack of infected body cells by cytotoxic and helper T-lymphocytes (called cell-mediated immune response). In addition, due to the presence of memory cells, the adaptive immune response is characterized by enhanced and fast responses after repetitive contacts with the same antigen. Ultimately, an immune response is the result of these two interacting systems. When leukocytes of the innate immune system become activated, they produce cytokines and present antigens to T- and B-lymphocytes, which activate the adaptive immune system. (1, 7)

Cytokines, chemokines and cell adhesion molecules

Cytokines are signaling proteins secreted by a wide variety of cell types and used for inter-cell communication. The types of cytokines produced determine whether a naive helper T-lymphocyte (Th0) develops into a type 1 helper T-lymphocyte (Th1) or a type 2 helper T-lymphocyte (Th2). For example, interleukin (IL)-12, produced by e.g. activated macrophages, stimulates Th1 cell development. This leads to the production of typical Th1 cytokines like IL-1 β , interferon (IFN) γ , IL-2, and tumor necrosis factor (TNF) α , which play an important role in cell-mediated immunity. In contrast, IL-4, produced by e.g. NK-cells, results in the development of Th2 cells, producing IL-5, IL-6, IL-10, and IL-13, which are involved in the humoral immune response. Recently, also various regulatory T-lymphocytes have been described. Their role is most likely to influence the Th1/Th2 balance by inhibiting Th1 activity by producing anti-inflammatory cytokines like TGF β and IL-10. (8)

Besides the above-mentioned cytokines, the immune system also uses specialized cytokines, called chemokines. Chemokines are a specific class of cytokines that are involved in recruiting immune cells to the side of

inflammation. This process is called chemotaxis. IL-8 and monocyte chemotactic proteins (MCPs) are well-known examples of these chemokines, which recruit and subsequently activate respectively neutrophils and monocytes. Increased levels of proinflammatory cytokines and/or chemokines therefore indicate activation of the immune response. (1)

In addition to cytokines and chemokines, a distinct set of cell adhesion molecules (CAMs) - expressed on endothelial and epithelial cells - is required for the binding of leukocytes and their movement towards inflamed areas. For example, vascular cell adhesion molecule (VCAM)-1 and intercellular adhesion molecule (ICAM)-1 are expressed on endothelial cells and function in the transmigration of leukocytes from the blood into the underlying tissue. (1) In addition, ICAM-1 is expressed on the luminal (apical) side of intestinal epithelial cells. Here ICAM-1 binds neutrophils that have been transmigrated from the blood through the epithelial layer into the intestinal lumen. This keeps the neutrophils in close contact with the epithelial layer (prevents flushing away with the bowel content), which may protect against invasion of the mucosa by pathogens. (9) An increased expression of ICAM-1 on intestinal epithelial cells thus indicates activation of the immune response. Indeed, ICAM-1 expression is up-regulated during inflammatory conditions, like inflammatory bowel disease (IBD). (10) Besides specific mediators of inflammation, the activation of leukocytes is eventually assisted by the production of a wide variety of nonspecific mediators, like acute phase proteins (such as C-reactive protein (CRP)), growth factors, metabolites of fatty acids (such as prostaglandins and leukotrienes) and reactive oxygen species (such as nitric oxide).

Regulation of immune responses: NF- κ B and PPARs

As already mentioned, immune cells can be activated directly by binding of PAMPs from pathogens to PRRs or indirectly by binding of cytokines produced by other cells to their respective receptors. After ligand receptor binding, a cascade of intracellular signaling events occurs, ultimately leading to activation of the transcription factor nuclear factor κ B (NF- κ B). NF- κ B activation induces transcription of genes encoding inflammatory proteins like cytokines, chemokines and adhesion molecules (**Figure 1.2**). (11) In resting cells, NF- κ B is present in the cytoplasm, bound to inhibitor κ B (I κ B), a complex that inactivates NF- κ B. After cellular activation by e.g. LPS or cytokines like IL-1 β and TNF α , I κ B kinase (IKK) is activated, which phosphorylates I κ B. This phosphorylation is followed by ubiquitination and degradation of I κ B. NF- κ B then migrates from the cytoplasm into the nucleus, binds to response elements and induces gene transcription.

Figure 1.2: Schematic view of NF-κB pathway and role of PPARs.

Binding of lipopolysaccharide (LPS), tumor necrosis factor (TNF) α or interleukin (IL)-1 β to their respective receptors activates IKK, which phosphorylates I κ B. This leads to ubiquitination (ub) and degradation of I κ B and release of NF- κ B from the inhibiting complex. NF- κ B then migrates from the cytoplasm into the nucleus, where it binds to response elements and induces gene transcription of e.g. cytokines, chemokines and adhesion molecules (e.g. ICAM-1 and VCAM-1). Polyunsaturated fatty acids (PUFAs) and rosiglitazone can bind to PPAR α and/or PPAR γ , which inhibits NF- κ B activation.

One of the genes transcribed encodes for I κ B, which means that after NF- κ B activation the newly synthesized I κ B again forms complexes with the NF- κ B, thereby terminating gene transcription (negative feedback). (11) Dysregulation of this signaling pathway can lead to the development of chronic inflammation. For example, in IBD patients NF- κ B activation in the colon is increased compared to control subjects. (12, 13) The NF- κ B pathway is therefore an attractive target for interventions, both for immune enhancing and dampening strategies.

Activation of NF- κ B is regulated by a complex network of transcription factors, among others peroxisome proliferator-activated receptor (PPAR) α (14) and PPAR γ (15). PPARs are highly expressed in adipose tissue and in the gastrointestinal tract indicating an important role for PPARs in these organs. (16-18) Binding of ligands to PPAR α or PPAR γ activates these transcription factors and thereby inhibit NF- κ B activation (Figure 1.2). Consequently, PPAR ligands might have antiinflammatory effects. A number of natural compounds, like (poly)unsaturated fatty acids and their derivatives like 15-deoxy- $\Delta^{12,14}$ prostaglandin J₂ (15d-PGJ₂), are ligands for PPAR α and PPAR γ . (19) Also thiazolidinediones (TZDs), e.g. rosiglitazone, known for their anti-diabetic effects, are synthetic PPAR γ ligands and thus also may have antiinflammatory effects. (20, 21)

Immune system in the intestine

The intestinal mucosa contains a highly specialized local immune system, which differs in some aspects from the systemic immune system. The intestinal immune system has to distinguish between pathogenic invaders, and harmless commensal gut microflora or components from the food stream. The latter process is called oral tolerance. The gut is therefore normally in a state of restrained immune reactivity, termed 'controlled' or 'physiological' inflammation. (22)

The defensive task of the intestine against infective pathogens is based on three essential constituents: 1) the microflora, 2) the physical barrier, and 3) the local immune system, which contains the gut-associated lymphoid tissue (GALT) (**Figure 1.3**) (23). 1) The gut microflora, mainly present in the colon, plays a major role against exogenous bacteria through colonization resistance. The gut microflora inhibits colonization of the gut with exogenous bacteria by occupying potential binding sites or by secreting inhibitory compounds. (23) 2) The mucosal barrier consists of a single layer of intestinal epithelial cells (enterocytes) that separates the gut lumen from the sterile interior. Pathogens must first pass these epithelial cells to be able to invade the host via the gastrointestinal tract. The epithelial cells are firmly connected to each other by tight-junctions. Tight-junctions consist of a cluster of proteins, including transmembraneous proteins such as claudin and occludin, and zonula occludens (ZO) proteins. (24, 25) These tight-junctions control the paracellular route of entry, which determines the gut permeability. High concentrations of proinflammatory cytokines, such as $\text{TNF}\alpha$ and $\text{IFN}\gamma$, can vigorously disrupt tight-junctions, leading to a loss of intestinal barrier integrity and an increase in gut permeability. (26, 27) The physical barrier is further enhanced by a mucus layer, produced by Goblet cells, and by antimicrobial proteins (defensins), produced by intestinal epithelial cells and Paneth cells. (23, 28) Next to the physical barrier, the intestinal epithelial cells also form an immunological barrier. Binding of microbial pathogens to intestinal epithelial cells triggers a cascade of alarm signals, resulting in the secretion of a wide array of proinflammatory cytokines, chemokines and the expression of adhesion molecules. These inflammatory proteins will initiate an acute inflammatory reaction. (29) 3) The third important player of the intestinal defense system is the specialized GALT system, which is present in the lamina propria and forms clusters of lymphoid follicles, in the small intestine known as Peyer's patches (PP).

Figure 1.3: Schematic view of the intestinal immune system.

The defensive task of the intestine against pathogens is based on three essential constituents: 1) the microbiota (mainly present in the colon), 2) the physical barrier formed by enterocytes (that also produce inflammatory proteins), a mucus layer produced by Goblet cells and antimicrobial proteins like defensins produced by Paneth cells and 3) the gut-associated lymphoid tissue (GALT) system consisting of various immune cells (T-cells, B-cells, dendritic cells and M-cells) in the small intestine clustered in follicles known as Peyer's Patches (PP).

The GALT system consists of various immune cells, such as M cells, T- and B-lymphocytes and dendritic cells. M-cells and dendritic cells continuously monitor the mucosa for invading bacteria, by sampling the luminal content and by transporting antigens to the subepithelial region. (30) In the lymphoid follicles, antigen presentation and activation of T- and B-lymphocytes will take place, initiating an inflammatory response. The intestinal epithelial cells together with the cells of the GALT system will induce an immune response against invading pathogens from the intestinal lumen and are involved in maintaining the intestinal immune response balanced in a normal situation.

Inflammatory bowel disease (IBD)

When the delicate equilibrium of the immune system within the gastrointestinal tract is disrupted, an uncontrolled host defense response against luminal antigens can lead to chronic intestinal inflammation. This uncontrolled immune response will cause intestinal damage and impairs intestinal functioning, leading to abdominal pain and diarrhea. Inflammatory bowel disease (IBD) is such chronic inflammatory condition of the intestine. Two primary distinct forms of IBD exist: ulcerative colitis (UC) and Crohn's disease (CD). Although the

distinction between UC and CD is not always very clear, the main differences are that UC mostly affects the colon, that inflamed areas are continuous (uninterrupted by healthy areas) and that inflammation is limited to the mucosa. CD can affect each part of the gastrointestinal tract, is segmental (in patches) and transmural (deeper into intestinal layers). Both CD and UC are characterized by periods of remission (without inflammation) and exacerbations (active inflammation), called relapses or flare-ups. (5) IBD is rather common in Western Europe, affecting 0.5-1.0% of the population during their lifetime. The incidence has risen over the last decades, but now seems to have reached a plateau. The incidences of UC and CD are highest in early adult life. (31) Although the etiologies of UC and CD are essentially unknown, compelling evidence suggests a role for genetic components triggered by environmental factors. (22) Among environmental factors, smoking and the gut microflora are the ones for which the most solid evidence is currently available. (32) Surprisingly, a role of the diet in inducing or modifying IBD has not drawn much attention so far. In this respect, especially the types of fatty acids in the diet seem to play an important role. (33) Epidemiological studies have shown a low incidence of IBD in Eskimo's as compared to West-European populations. These findings may be related to their high intakes of n-3 fish oil polyunsaturated fatty acids (PUFAs), which may have antiinflammatory effects as will be explained later. (34)

Atherosclerosis

Atherosclerosis is involved in the pathogenesis of cardiovascular diseases (CVD). Morbidity and mortality from CVD, including coronary heart disease (CHD), cerebrovascular diseases and peripheral arterial disease, can largely be explained by the development of an atherosclerotic plaque. Fat, cholesterol, and other substances accumulate in the walls of arteries and form plaques. Besides fats, the atherosclerotic plaque contains large numbers of immune cells, particularly macrophages and T-lymphocytes. Furthermore, the disease is associated with a systemic immune response and signs of inflammation. Therefore, evidence emerges that atherosclerosis is characterized by a chronic condition of low-grade inflammation. (2) Besides the well-known risk factors such as high plasma concentrations of low-density lipoprotein (LDL) cholesterol and smoking, also high plasma concentrations of inflammation markers such as CRP, IL-6 and monocyte chemotactic protein (MCP)-1 are strong predictors of future CVD risk (2, 35-37). Numerous pathophysiologic observations led to the formulation of the response-to-injury hypothesis of atherosclerosis. (2) This hypothesis proposes that endothelial dysfunction is the first step of atherosclerosis and that this leads to a chronic inflammatory process. Possible causes of endothelial injury are elevated and oxidized LDL, free radicals (e.g.

caused by smoking cigarettes), hypertension, diabetes mellitus and infectious microorganisms. Endothelial dysfunction leads to compensatory responses that alter the normal homeostatic properties of the endothelium. The second step is increased accumulation of lipid and (oxidized) LDL beneath the endothelium, presumably from increased transport and/or increased permeability of the endothelial cells. This is rapidly followed by recruitment and attachment (first rolling and later firm adherence) of monocytes and T-lymphocytes to endothelial cells by expression of chemokines (like IL-8 and MCP-1) and adhesion molecules (like E-selectin, VCAM-1 and ICAM-1). Subsequently, transmigration of leukocytes through the endothelium layer into the intima follows, where they start to accumulate. Oxidized LDL in the subendothelium is also a highly chemotactic factor, which recruits monocytes from the blood to the intima where they become activated macrophages. The macrophages subsequently take up oxidized LDL and form foam cells. The accumulation of foam cells in the intima leads to the first stage of atherosclerosis, the fatty streak. Continuation of this process may lead to an expanded, intermediate lesion that contains multiple layers of smooth muscle cells, connective tissue, macrophages and T-lymphocytes. Later, an advanced lesion containing a fibrous cap will be formed. Changes in the fibrous cap, such as ulceration or rupture, can result in the formation of an (occlusive) thrombus that can cause myocardial and cerebral infarctions, two major causes of mortality in Western countries. (2, 38)

Immune modulation

Diet is one of the major exogenous factors that can modulate the activity of the immune system. At the single nutrient level, it has been shown that an adequate nutrient intake is essential for an optimal functioning of the immune system. (39) However, even in well-nourished individuals, human intervention trials have provided evidence that changes in single nutrient intakes affect specific immune functions. (40) The potential to modulate the activity of the immune system by interventions with specific nutrients is called immunonutrition. Although this concept has been applied most frequently in attempts to improve the clinical course of critically ill or surgical patients, it may be applied to any situation in which an altered supply of nutrients is used to modify inflammatory or immune responses. (41) Immunonutrition can have as potential target both systemic inflammation as well as local inflammation. Effects of nutritional compounds on the intestinal immune function are obvious, due to the direct interaction and consequently possible high concentrations of specific nutrients. However, hardly anything is known about these effects. One of the major problems is that it is not easy to examine the intestinal immune system, due to the difficulty in accessing material from the intestine. Also, no generally accepted intestine specific immune biomarkers exist. (40) Therefore,

most of our knowledge is derived from animal (*in vivo*) and cell culture (*in vitro*) studies. In the experiments described in this thesis we used various models to examine effects of different food component on immune function. First, we examined the effects of β -glucan on processes related to intestinal inflammation in different *in vitro* intestinal cell culture models (**Chapter 2**). Next, we examined the effects fish oil fatty acids in an *in vitro* intestinal cell culture model (**Chapter 3**). Furthermore, we examined these effects *in vivo* in a mouse colitis model (**Chapter 4**). In this colitis model we also tested the role of specific PPAR γ activation on intestinal inflammation by using the synthetic ligand rosiglitazone (**Chapter 5**). Finally, we examined the immune modulating effects of conjugated linoleic acid (CLA) on systemic immune parameters in humans (**Chapter 6**).

Diet can make the immune response stronger (immune enhancement), but can also dampen the immune response. Immune enhancement on one hand will increase resistance to infections, which can be especially advantageous for subjects with an impaired functioning of the immune system, such as elderly and diabetes mellitus patients. (6) For example, β -glucan, a fiber present in oat and barley, has possible immune-enhancing effects. (42, 43) Dampening of the immune response on the other hand, will decrease chronic inflammation in for example IBD patients. Consumption of for example fish oil can dampen the systemic immune response. (44) Effects of fish oil on intestinal inflammation in IBD patients and their underlying mechanism are however less known. (34, 45) Conjugated linoleic acid, a fatty acid present in dairy products and also available in capsules as food supplements, is under investigation for its immune modulating effects, but results are inconsistent. (46) In the next paragraphs, these dietary components and their potential immune modulating effects are discussed in more detail.

β -Glucan

β -Glucans are carbohydrates (fibers) consisting of linked glucose molecules, which are major structural components of the cell walls of cereals such as barley and oat, but also of yeast and fungi. In the past decade, β -glucans from oat and barley received a lot of interest because of their cholesterol-lowering activities, whereas studies evaluating the immune modulating effects of oat and barley β -glucans are relatively new. Although immune modulating effects of β -glucans from oat and barley did so far not received much interest, the effects of β -glucans from yeast and fungi have been broadly examined. Because of similarities in structures of these β -glucans, similar responses of the immune system might be expected. There are however also some differences in structure between both groups of β -glucans. The cell wall β -glucans of yeast and fungi consist of β -(1 $\rightarrow$ 3)-linked glycopyranosyl residues with small numbers of (1 $\rightarrow$ 6)-linked branches, whereas the oat and barley cell walls contain unbranched β -glucans with β -(1 $\rightarrow$ 3) and β -(1 $\rightarrow$ 4) linked glycopyranosyl residues (**Figure 1.4**). (42, 43, 47)

Figure 1.4: Structure of fungal, yeast and cereal β -glucan.

In vitro as well as animal studies have found that fungal β -glucans activate leukocytes, as shown by enhanced phagocytic activity, production of reactive oxygen species, and increased production of proinflammatory cytokines and chemokines, like $\text{TNF}\alpha$, IL-1 β , IL-6 and IL-8 (43). Also oat-derived β -glucans enhanced the cytokine production by macrophages and spleen cells *in vitro*. (42) More importantly, in mice resistance against bacterial and parasitic infections was increased after intra-peritoneal or oral injection of oat-derived β -glucan (42, 48-50). Moreover, human intervention studies showed that yeast-derived β -glucan injections reduced serious postoperative infections and mortality after high-risk surgery. (51-53) The mechanisms underlying these effects are unknown. However, the recent discovery of the PRR dectin-1 as the β -glucan receptor (54), has improved our knowledge about the possible molecular routes involved. Dectin-1 recognizes branched and unbranched β -glucans (54) and is mainly expressed on leukocytes, but also on endothelial and epithelial cells (55-57). Binding of β -glucan to dectin-1 activates the transcription factor NF- κ B (58, 59). Although the effects of β -glucans on leukocytes have been examined in detail, possible immune enhancing effects of β -glucans on epithelial cells have not been studied so far. As explained earlier, also enterocytes play an important role in the intestinal defence against pathogens. It is however very difficult to study effects of β -glucans in cell cultures directly, because of problems with solubility. Therefore, to study effects of oat β -glucan in a physiological matrix, ileostomic patients consumed a control

diet or an oat β -glucan enriched diet in a crossover design and we prepared fecal water from the ileostomic content. With this, we conducted an *ex vivo* study in which we examined immune modulating effects of fecal water from ileostomy patients that consumed a diet enriched with oat β -glucan or a control diet on intestinal epithelial cell lines (**Chapter 2**).

Fish oil

Fish oil is rich in the n-3 PUFAs eicosapentaenoic acid (EPA, C20:5 n-3) (**Figure 1.5**) and docosahexaenoic acids (DHA, C22:6 n-3).

Figure 1.5: Structure of the n-6 PUFA arachidonic acid (AA) and the n-3 PUFA eicosapentaenoic acid (EPA).

Because of their antiinflammatory effects, using n-3 PUFAs, and EPA and DHA in particular, have been suggested as treatment for various inflammatory diseases. Except for protective effects on coronary heart diseases (44), fish oil may also have positive effects for IBD patients (34, 45). Epidemiological studies showed low incidences of IBD in Eskimo's as compared to West-European populations, which may be related to their high fish intakes (60). A more recent study from Japan showed a strong correlation of the incidence of Crohn's disease with the n-6/n-3 PUFA ratio in the diet (61). Interestingly, the colon mucosa of IBD patients contains higher proportions of the n-6 PUFA arachidonic acid (AA, C20:4 n-6) as compared to control subjects (62-64). By changing the type of fatty acids in the diet, AA in the colon mucosa can be replaced by for example the fish oil fatty acids EPA and DHA (65). Therefore, it is tempting to suggest that effects of fish oil could be caused by a reduction of the AA content. Alternatively, fish oils may have their own intrinsic anti-inflammatory effects. A direct side-by-side comparison between effects of AA and fish oil on intestinal inflammation has not been performed. Several dietary intervention studies have indeed shown that supplementation of n-3 fish oils may have beneficial effects in IBD patients. (65-75) Although some studies (65, 66) showed rather impressive effects, it should be noted however that not all

intervention studies were that positive (67-75). Based on these intervention studies, the overall conclusion is that fish oil supplementation shows at least minor protective effects. (34, 45) In different mouse and rat models for IBD fish oil has also positive effects (76-81). Unfortunately, in these animal studies control diets were not always adequate to evaluate effects of fish oils versus AA. In other words, the studies carried out so far are not able to conclude whether the observed effects are due to a lowering of n-6 PUFAs during fish oil consumption or due to intrinsic antiinflammatory effects of fish oil. Furthermore, getting more insight in the mechanism underlying the effects of fish oil versus AA may help to explain possible differences in effects on inflammation. The most common mechanism to explain the antiinflammatory effects of fish oil relates to the incorporation of the fatty acids into cell membrane phospholipids. Fatty acids in membrane phospholipids can be mobilized by phospholipase enzymes and free fatty acids can subsequently act as substrates for cyclooxygenase (COX) and 5-lipoxygenase (5-LOX) enzymes, which synthesize eicosanoids. Eicosanoids can modulate the intensity and duration of the inflammatory response. The COX enzymes synthesize prostaglandins and tromboxanes, whereas the 5-LOX enzymes synthesize leukotrienes. The most common fatty acid in the phospholipids of cell membranes is the n-6 PUFA AA. Increased fish oil consumption results in a shift from n-6 to n-3 fatty acids incorporated into phospholipids from cell membranes, which will decrease the capacity to synthesize eicosanoids from AA. EPA itself is also a substrate for COX and 5-LOX, giving rise to derivatives that differ in structure from those produced from AA. These are the serie-3 prostaglandins and tromboxanes instead of serie-2 from AA converted by COX and serie-5 leukotrienes instead of the serie-4 from AA converted by 5-LOX. The eicosanoids produced from EPA are less potent proinflammatory mediators than those synthesized from AA. This may result in a reduced immune stimulating effect and may explain the antiinflammatory effects of n-3 PUFAs compared with n-6 PUFAs. (33) A second mechanism that may explain the antiinflammatory effects of fish oils versus AA, are differences on signaling pathways. One of these pathways is the NF- κ B pathway, which could be inhibited by ligand binding to PPARs. As PUFAs are natural ligands for PPARs (19), this may underlay the anti-inflammatory effects of fish oil. We conducted two studies to compare the effects of fish oils versus AA; an *in vitro* intestinal epithelial cell line model (**Chapter 3**) and an *in vivo* mouse colitis model (**Chapter 4**).

Conjugated linoleic acid (CLA)

Conjugated linoleic acid (CLA) is a mixture of positional (e.g. 9,11 or 10,12) and geometrical (*cis* or *trans*) conjugated isomers of the n-6 PUFA linoleic acid (C18:2 n-6) (**Figure 1.6**).

Figure 1.6: Structure of linoleic acid and conjugated linoleic acid (CLA) isomers.

It is a natural food component, predominantly found in the lipid fraction of meat, milk and other dairy products. *Cis*-9, *trans*-11 (c9, t11) CLA is the most common CLA isomer in nature, whereas commercially available capsules enriched with CLA also contain other isomers like *trans*-10, *cis*-12 (t10, c12). (82, 83) Many health effects have been ascribed to CLA, such as anti-carcinogenic, anti-diabetic, anti-obesity and anti-atherosclerotic effects (82-84). Most of these effects were, however, found in laboratory animals. CLA has also been reported to have immune modulating properties in animals, but results are inconsistent (46, 84). Mechanism that have been suggested underlying these immune modulating effects are - comparable with mechanism of n-3 PUFAs - 1) modification of cell membrane composition which has implications for eicosanoid production, and 2) interactions with PPARs. (85) Regarding the first mechanism, in line with linoleic acid, which is desaturated and elongated into arachidonic acid, CLA is converted by the same enzymes into conjugated arachidonic acid. CLA as well as these metabolites are present in cell membranes of numerous tissues, thereby lowering the AA content. Consequently, this will influence eicosanoid production and inflammatory processes. (85) Regarding the second mechanism, several isomers of CLA are high affinity ligands for PPAR α and to a lesser extend for PPAR γ . (86) Binding to PPARs will activate various cell signaling pathways, which among others leads to inhibition of NF- κ B and thus suggests antiinflammatory effects of CLA.

Unfortunately, data from human studies examining these immune modulating effects of CLA are limited and - if anything - only minor, both immune enhancing and immune dampening effects of mixtures of CLA isomers

on various immune functions have been shown (46, 84). These results were however obtained by evaluating effects of CLA mixtures, and effects may be isomer specific (46). Tricon *et al.* (87) therefore compared side-by-side the effects of *cis*-9, *trans*-11 (c9, t11) and *trans*-10, *cis*-12 (t10, c12) CLA on immune cell function. It was found that both isomers decreased mitogen-induced lymphocyte activation, but no other immune modulating effects were found. The absence of clear effects may be related to the healthy population in that study. We therefore conducted a placebo-controlled study in which we evaluated the effects of consumption of the individual c9, t11 CLA or t10, c12 CLA isomers on inflammation parameters in subjects at increased risk for coronary heart diseases (**Chapter 6**).

Outline of the thesis

The research presented in this thesis describes the potential immune modulating effects of different nutrient components. In the first study (**Chapter 2**) we examined the *ex vivo* immune modulating effects of the dietary fiber β -glucan on enterocytes. In that study we used a new approach to examine effects on intestinal inflammation. We collected ileostomic contents of patients that consumed an oat β -glucan enriched diet or a control diet in a crossover design. Of this ileostomic content we prepared fecal water, which we subsequently supplied to four different cytokine-stimulated enterocyte cell lines.

The immune-suppressive effects of n-3 fish oil PUFAs versus the n-6 PUFA arachidonic acid were examined *in vitro* in an enterocyte cell line (**Chapter 3**) and *in vivo* in a mouse model of colitis (**Chapter 4**). To elucidate the role of the transcription factor PPAR γ - which is supposed to play a role in explaining the antiinflammatory effects of fish oil - on intestinal inflammation, the effects of supplementing the diet of mice with the synthetic PPAR γ ligand rosiglitazone on colitis development was examined (**Chapter 5**).

In the study presented in **chapter 6** we investigated the immune modulating effects of conjugated linoleic acid (CLA) on inflammation parameters in subjects at increased risk for coronary heart diseases.

Chapter 7, the general discussion, combines the results of the different studies and puts the results in perspective.

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Chapter 2

Fecal water from ileostomic patients consuming oat β -glucan enhances immune responses in enterocytes

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Abstract

Yeast, fungal and dietary β -glucans have immune modulating effects *in vitro* and *in vivo*, as thought, mainly by affecting leukocytes. However, effects of oat β -glucan on enterocytes have never been studied. As recognized, supplying oat β -glucans as such to cells in culture directly is difficult because of solubility problems. Therefore, six ileostomic patients consumed, in random order, a control diet or an oat β -glucan enriched diet (5 g) and from the collected ileostomic content, fecal water was prepared and added to two small intestinal cell lines (INT407, Caco-2) and two colon cell lines (HT29, T84) together with a cytokine cocktail ($\text{IL-1}\beta + \text{IFN}\gamma + \text{TNF}\alpha$). Several parameters reflecting immune modulation were measured. As compared to placebo fecal water, β -glucan enriched fecal water significantly increased interleukin (IL)-8 production in HT29 (5.0%; $P = 0.046$) and INT407 cells (22.0%; $P = 0.028$). Intercellular adhesion molecule (ICAM)-1 expression increased in T84 (11.0%; $P = 0.028$) and Caco-2 cells (20.4%; $P = 0.075$). These immune stimulating effects were confirmed by enhancement of inflammatory expression profiles, as determined with an antibody array. Our findings show immune enhancement by fecal water from ileostomic patients consuming oat β -glucan both in small intestinal and colon cell lines after stimulation, which is in agreement with documented effects in leukocytes. Whether these immune stimulating effects on enterocytes contribute to the enhanced protection of the host against invading pathogens as observed both in animals and in humans, as well as the underlying mechanism, needs further evaluation.

Introduction

β -Glucans are carbohydrates consisting of linked glucose molecules with a molecular mass between 50 and 2,300 kDa. They are major structural components of not only the cell walls of yeast and fungi, but also of some cereals such as barley and oat. In nature, there are many different forms of β -glucans varying in length, molecular mass, tertiary structure, and degree of branching. In this respect, the cell wall glucans of yeast and fungi consist of β -(1 $\rightarrow$ 3)-linked glucopyranosyl residues with small numbers of (1 $\rightarrow$ 6)-linked branches, whereas the oat endosperm cell walls contain unbranched β -glucans with β -(1 $\rightarrow$ 3) and β -(1 $\rightarrow$ 4) linkages (1-3). These characteristics influence their effects on physiological functions, such as lipid and glucose metabolism, and inflammatory processes.

It is now well established that fungal β -glucans initiate - by a so far not completely understood mechanism - a very potent immune response in leukocytes (2). Less is known about the immune modulating effects of β -glucans from oats and barley. Estrada *et al.*, however, have demonstrated that culturing macrophages in the presence of oat β -glucans enhanced production of IL-1 α in a dose- and time-dependent manner (1). Also the production of IL-2, interferon (IFN) γ and IL-4 in cultured spleen cells was increased by oat β -glucan (1). *In vivo* studies demonstrated that oat β -glucan enhanced resistance towards bacterial challenges and intestinal parasitic infections (1, 4-6). All together, these findings indicate that β -glucans, not only from yeast and fungi, but also from oat, have immune stimulating effects *in vitro* and *in vivo*.

Moreover, Brown and Gordon (7), have recently suggested that high molecular weight (MW) and/or particulate β -glucans from fungi directly activate leukocytes, while low MW β -glucans from fungi only modulate the response of cells when they are stimulated with e.g., cytokines. Whether the effects of a low MW oat β -glucans are also indirect, i.e. comparable to those of low MW fungi β -glucans, is unknown. In our hands, however, the fecal water with and without low MW oat β -glucans did not induce an immune-stimulation without the presence of an additional inflammatory trigger. Therefore, in this study we have chosen to evaluate immune modulating effects of a low MW oat (1 $\rightarrow$ 3), (1 $\rightarrow$ 4) β -glucan (60 kDa) not as an inducing agent, but particularly as modifier of a cytokine stimulated condition.

Most *in vitro* studies evaluated the effects of β -glucans in different leukocyte populations. However, effects on enterocytes have never been examined. Since it is inevitable that enterocytes play an important role in the intestinal defense against pathogens, the present study evaluated the immune modulating effects of oat β -glucan on human enterocytes. It is however very difficult to study effects of β -glucans in cell cultures directly, because of problems with solubility (8). Therefore, to study effects of oat β -glucan in a physiological matrix, ileostomic patients consumed, in random order, a control diet or an oat β -glucan

enriched diet. From the collected ileostomic contents fecal water was prepared and added together with a cytokine cocktail (IL-1 β , IFN γ and TNF α) to enterocyte cell lines. We have deliberately chosen to study effects in four different enterocyte cell lines (two small intestinal cell lines and two colon cell lines) to exclude the possibility that the presence or absence of the effects found were cell line specific. Next, the cells as well as the supernatants were analyzed for different inflammatory parameters. By this approach we were able to evaluate the effects of dietary oat β -glucan consumption under physiological conditions without making a distinction between direct effects (i.e., β -glucan-enterocyte interactions) or indirect effects (i.e., factors present in ileostomic content derived from effects of β -glucan in the intestinal tract of the patients). As far as we know, this is the first study showing immune enhancing effects of oat β -glucans in enterocytes by such a physiological approach.

Materials and methods

Study design

Six patients (three males and three females) with an ileostomy participated in a double blind, placebo-controlled intervention trial with a crossover design (Figure 2.1).

Figure 2.1: Study design.

To study immune modulating effects of oat β -glucan on enterocytes in a physiological matrix, (A) ileostomic patients consumed a diet enriched with β -glucan or placebo and ileostomic contents were collected. These ileostomic contents were used to prepare fecal water, which (B) was used in an *in vitro* study of stimulated enterocytes. For the first *in vivo* part (A), all ileostomic patients ($n = 6$) received a standardized diet (diet composition see Table 2.1) on the first day of the first period. On the second day, three subjects received a standardized diet and at breakfast and at lunch a beverage (250 mL) enriched with β -glucan (2.5 g, i.e. 5 g in total), while the other three subjects received a placebo beverage enriched with rice starch. One week later, regimes were crossed over. During this second day until the following morning (24 h in total) ileostomic contents were collected at fixed intervals. Fecal water was prepared by dissolving 24-h pooled freeze-dried ileostomic contents in PBS as described in the methods section and subsequently used for *in vitro* cell stimulation experiments. For this second *in vitro* part (B), placebo and β -glucan fecal water was added to four enterocyte cell lines (HT29, T84, INT407 and differentiated Caco-2), which were subsequently stimulated with a proinflammatory cytokine cocktail (IL-1 β , IFN γ and TNF α) for 16 h. After 16 h the culture medium was collected for measuring interleukin (IL)-8 production and inflammatory protein expression profiles and living cells were used for cell membrane intercellular adhesion molecule (ICAM)-1 protein expression measurements.

All subjects were proctocolectomized for ulcerative colitis. The median time since surgery was 6 years (range: 1-17 year). The mean age of the subjects was 51 years (range: 38-74 year). On the first day of the first period, all subjects received a standardized diet (three main meals and three snacks). On the second day, three subjects received again a standardized diet, but in addition, at breakfast and at lunch, a beverage (250 mL) enriched with β -glucan (2.5 g, i.e. 5 g in total). The other three subjects received a placebo beverage enriched with rice starch. One week later, regimes were crossed over. The nutrient compositions of the two diets are presented for men and women in **Table 2.1**.

Table 2.1: Nutrient composition of the diets.

	Men (n = 3)		Women (n = 3)	
	placebo diet	β -glucan diet	placebo diet	β -glucan diet
Energy (kcal)	2170	2170	1813	1813
Protein (g)	98	98	89	89
Protein (En%)	18	18	20	20
Fat (g)	65	65	53	53
Fat (En%)	27	27	26	26
Carbohydrates (g)	300	300	241	241
Carbohydrates (En%)	55	55	53	53
Fibers (g)	11	16	8	13
β -Glucan (g)	0	5	0	5

During the second day of each period ileostomic contents were collected 15 min before the standard meals at 7.45, 11.45, 15.45, 19.45, 21.45 and at 7.45 h the following morning. The ileostomic contents was sealed in a plastic jar immediately after each collection and put on dry ice. The next morning the ileostomic contents were weighed, freeze-dried, weighed again, homogenized and stored at -20 °C. Ethical approval of the study protocol was obtained from the local Research Ethics Committee of Lund University. All subjects gave their written informed consent before participating.

β -Glucan was isolated from Swedish oats (oats var. Sang from Cerealia (Sweden)) at Cereal Base Ceba AB (Lund, Sweden), as described (9). Briefly, oat bran was crushed and milled with water and treated with amylases and proteases, while at the end insoluble fibers were removed by ultrafiltration. The remaining β -glucan fraction was freeze-dried, mixed with water, and used for the production of beverages with a β -glucan concentration of 1% (wt/wt). The β -(1 $\rightarrow$ 3)-(1 $\rightarrow$ 4)-linked unbranched soluble glucan had a mean MW of 60 kDa, determined by VTT Biotechnology, Technical Research Centre of Finland (Espoo, Finland), by SEC-HPLC using μ Hydrogel columns, NaOH-eluent and post-column calcofluor staining with fluorescence detection, as described in ref (10). Control beverages were prepared using rice starch, while sucrose, glucose

syrup and rapeseed oil were added to balance the nutrient composition of the two experimental products.

Ileostomic contents and fecal water

The ileostomic contents from each subject obtained at each period were freeze-dried, after which the MWs and the concentrations of β -glucan in the ileostomic contents were determined, as mentioned above.

The ileostomic contents from each subject used for the cell stimulations, were pooled over the 24-h periods, hereby correcting for differences in volumes from each interval. Fecal water was prepared by dissolving the pooled freeze-dried ileostomic contents in PBS, which was subsequently rotated for 5 h. Next, the dissolved ileostomic contents were centrifuged for 11.5 min at 13,500 rpm, 17 °C. The supernatant (further called fecal water) was carefully collected and used for the *in vitro* cell stimulation experiments. The pH of each fecal water sample was determined.

Intestinal cell cultures

Four different human derived intestinal cell lines - two small intestinal and two colon cell lines - were used. The human small intestinal cell line INT407 was obtained from the European Tissue Type Collection (ETTC) and the human cell line Caco-2 from the American Tissue Type Collection (ATTC). INT407 cells were cultured in minimum essential medium (MEM) (Invitrogen Corporation, Paisley, UK) supplemented with 10% heat inactivated fetal calf serum (FCS) (Greiner Bio-one, Frickenhausen, Germany) and 1% penicillin streptomycin (PS) (Invitrogen Corporation). Caco-2 cells were cultured in Dulbecco's Modified Eagle medium (DMEM) (Invitrogen Corporation) supplemented with 10% FCS, 1% PS, 1% sodium pyruvate (SP) (Invitrogen Corporation) and 1% non-essential amino acids (NEAA) (Invitrogen Corporation). After 18 days culturing of Caco-2 cells in normal DMEM, they were differentiated into small intestinal enterocytes (11). The human colon cell lines HT29 and T84 were kindly supplied by Prof. Dr. W. A. Buurman (Department of Surgery, Maastricht University, The Netherlands). HT29 cells were cultured in RPMI 1640 medium (Invitrogen Corporation) supplemented with 10% FCS and 1% PS. T84 cells were cultured in DMEM nut mix F-12 (DMEM/F12) (Invitrogen Corporation) supplemented with 10% FCS and 1% PS. All cells were cultured at 37 °C in a 5% CO₂ humidified atmosphere. Cells were refreshed every second day and were separated by trypsin-0.03% EDTA (Greiner Bio-one) when they had reached 70-90% confluency.

Cell cytotoxicity

Cell cytotoxicity was measured to determine the maximum concentration of fecal water that could be added to the cell lines without causing cytotoxic effects. For this, different concentrations of fecal water were prepared and tested in two assays, i.e., (i) erythrocytes lytic activity and (ii) phenol red leakage across confluent Caco-2 cell monolayers.

Erythrocytes lytic activity assay

Cytotoxic activity of fecal water was determined by evaluating hemolysis of erythrocytes. For this, human blood with heparin as anticoagulant - from the same volunteer for all experiments - was centrifuged for 10 min at 3,500 rpm to obtain erythrocytes. Plasma was removed and the remaining erythrocytes were washed three times with PBS and centrifuged again for 10 min at 3,500 rpm. The cytotoxicity of fecal water was determined by adding different concentrations of fecal water, prepared by dissolving different amounts of the ileostomic content, to the washed human erythrocytes suspension. After incubation for 45 min at 37 °C, the tubes were centrifuged for 10 min at 3,500 rpm. The supernatant was transferred to a 96-wells plate and diluted 20 times. The hemoglobin concentration in the supernatant, released after lysis of the erythrocytes, was measured spectrophotometrically at 540 nm. Percentage hemolysis was calculated compared to a calibration curve, made from erythrocytes suspension incubated with ascending concentrations distilled water in PBS (0-100% hemolysis). The concentration at which 2% lysis occurred was defined as the critical lytic concentration (CLC) (12). Concentrations below the CLC were determined as noncytotoxic and were used for further experiments.

Phenol red leakage across confluent Caco-2 monolayers

Cytotoxicity of fecal water was also measured by phenol red leakage across confluent Caco-2 monolayers, which can be used as a good marker for cell confluence and tight-junction formation and is an easy-to-use alternative for the electrical measurement of the transepithelial electrical conductance (13). For this, Caco-2 cells were cultured in a polarized transwell system (Costar, Badhoevedorp, the Netherlands). The upper apical chamber contained DMEM (Invitrogen Corporation) with phenol red and the lower basolateral compartment DMEM without phenol red. Disturbance of the confluent Caco-2 cell monolayer due to cytotoxicity of the fecal water resulted in a flux of the small phenol red molecule (MW = 354) across the epithelium, which could be measured in the basolateral compartment by spectrophotometry at 479 nm (13). When the cells were confluent as indicated by the absence of phenol red leakage, cells were refreshed with medium containing fecal water at different concentrations. DMSO (10%) and PBS were used as positive and negative controls, respectively. Cells

were incubated for 6 h with fecal water, 10% DMSO or PBS added apically. Next, the basolateral medium was collected, and refreshed after which the cells were incubated for another 18 h with the same compounds. The phenol red concentration in the basolateral medium (at 6 and 18 h) was measured. Values were corrected for phenol red free medium and compared to the 10% DMSO and PBS controls. Concentrations without phenol red leakage were determined as noncytotoxic and were used for further experiments.

Cell stimulations

To evaluate the immune modulating effects of fecal water with β -glucan *in vitro*, cell stimulation experiments were conducted as represented schematically in **Figure 2.1B**. For this HT29 and T84 cells were plated in six-well plates at an initial density of 1.0×10^6 cells/mL, INT407 at 0.6×10^6 cells/mL, and Caco-2 at 0.5×10^6 cells/mL in a total volume of 1.5 mL. When the HT29, T84, and INT407 cells had reached 70-90% confluency and the Caco-2 cells had differentiated for 18 days, the culture medium was replaced by medium containing fecal water (with or without β -glucan) and a cocktail consisting of the proinflammatory cytokines IFN γ (100 U/mL), IL-1 β (50 U/mL) and TNF α (10 ng/mL). After 16 h of incubation, culture media were collected for analysis of IL-8 concentrations and inflammatory protein expression profiles, while living cells were used to determine cell surface intercellular adhesion molecule (ICAM)-1 protein expression.

IL-8 ELISA and inflammatory protein expression profiles

IL-8 concentrations in culture supernatants were determined using an ELISA as described in ref (14). Briefly, plates (Greiner Bio-one) were coated with monoclonal murine anti-human IL-8 antibodies. Recombinant human IL-8 was used for standard curves. Immobilized IL-8 was detected using a specific biotinylated rabbit-anti-human IL-8 polyclonal antibody, followed by the addition of peroxidase-conjugated streptavidin (Zymed Laboratories, San Francisco, CA) and tetramethylbenzidine (TMB) substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD). The detection limit of the ELISA was 5 pg/mL.

Cell culture supernatants were also used to evaluate expression profiles of multiple inflammatory proteins using the human cytokine antibody array III (Ray Biotech Inc., Norcross, GA). First, cell culture media of all six subjects after 16 h incubation with fecal water containing β -glucan or placebo were pooled. Thus, eight arrays (HT29, T84, INT407, Caco-2 with β -glucan or placebo) were analyzed. One mL of the pooled samples was added to the array membranes. After incubating and washing, the cytokine-bound membranes were incubated with a cocktail of biotin-labeled antibodies, followed by the addition of horseradish peroxidase-conjugated streptavidin. Array spot intensity was detected by using a LAS-3000 Lite Image reader (Raytest GmbH, Straubenhardt,

Germany) based on chemiluminescence imaging. Intensity of the spots was quantified in arbitrary units by densitometry using Aida software version 3.50 (Raytest GmbH), thereby correcting for background staining of the gel. Comparison of protein expression profiles was possible after normalization of each spot on an array using the positive controls, provided by the manufacturer. The cytokines used for stimulation (IFN γ , IL-1 β and TNF α) were excluded from the analysis.

Using the same protocol, this array was also used to determine the amounts of inflammatory proteins possibly present in fecal water. For this, fecal water containing β -glucan or placebo was prepared for each subject and pooled per dietary period. For analysis, the fecal water was not diluted.

Flow cytometry analysis of ICAM-1

ICAM-1 protein expression on the cell surface of living cells was measured by flow cytometry. After 16 h incubation, cells were washed three times with PBS and detached with trypsin-0.03% EDTA. When the cells were detached, the medium was added and cell suspensions were centrifuged for 5 min at 1,200 rpm at room temperature, followed by resuspending the pellets in 500 μ L PBS-1% BSA. Cells were counted and diluted to 1×10^6 cells/mL in PBS-1% BSA. Recombinant-phycoerythrin (R-PE)-conjugated mouse-anti-human CD54 monoclonal antibody (anti-ICAM-1) or isotype-matched control antibody (Becton Dickinson Biosciences, San Diego, CA) 20 μ L/ 10^6 cells was added and incubated for 30 min on ice in the dark. Next, cell suspensions were centrifuged for 5 min at 1,500 rpm and pellets were resuspended in 500 μ L PBS-1% BSA. Because almost all cells were ICAM-1 positive, although they greatly differed in amount of ICAM-1 expression, the mean fluorescence of 10,000 living cells was measured and analyzed with the FACSsort (Becton Dickinson, Franklin Lakes, NJ) and CellQuest analysis software. Percentages of living cells were not different from the different samples and the isotype control antibody showed fluorescence below the threshold.

Statistical analysis

Effects on IL-8 production and ICAM-1 expression were examined with the non-parametric, Wilcoxon signed ranks test. For data presentation, effects from the β -glucan enriched fecal water were also expressed as percentages relative to those of the placebo fecal water. All statistical analyses were performed using SPSS 11.0 (SPSS, Chicago, IL). Values of $P < 0.05$ were considered statistically significant.

Results

Characteristics of ileostomic content and fecal water

The mean MW of the β -glucan in the beverages and in the ileostomic content of four subjects was 60 kDa. For two subjects, only a very slight decrease in MW was observed, indicating that, overall, the β -glucan was not degraded while passing the small intestine.

Fecal water concentrations that were not cytotoxic, as determined by erythrocyte lysis, were in agreement with phenol red leakage assays (data not shown). Experiments in all cell lines were, therefore, carried out with a concentration of 1.44 mg/mL redissolved freeze-dried ileostomic content in PBS. The calculated concentrations of β -glucan added to the cells varied from subject to subject between 0.12 and 0.18 mg/mL.

The mean pH of fecal water containing β -glucan was not significantly different as compared to placebo fecal water (7.4 ± 0.2 and 7.3 ± 0.2 (mean $\pm$ SD), respectively).

To exclude the possibility that the β -glucan enriched fecal water already contained proinflammatory proteins, which may have confounded the results, we measured inflammatory proteins with the antibody array. However, no detectable amounts of inflammatory proteins were present in the fecal water from the β -glucan or placebo period (data not shown).

IL-8 production

Addition of β -glucan enriched fecal water, without stimulation with cytokines, did not induce IL-8 production in any of the four intestinal cell lines (data not shown). However, when β -glucan enriched fecal water was added in combination with a cytokine mixture, we found enhancing effects as compared to placebo fecal water, illustrating that our low MW oat (1 $\rightarrow$ 3), (1 $\rightarrow$ 4) β -glucan (60 kDa), indeed, is not an inducing agent but a modifier of a cytokine stimulated condition. **Figure 2.2A** shows the median IL-8 concentrations (and ranges) after placebo and β -glucan fecal water for all subjects together in the four cell lines, while in **Figure 2.2B** percent changes for the β -glucan fecal water for each subject individually corrected for placebo fecal water are shown. β -Glucan containing fecal water from five subjects increased IL-8 production by HT29 cells. For one subject, no change was found. Overall, this resulted in a significant median increase in IL-8 production of 5.4% (range: -0.3%, 16.6%; $P = 0.046$). Comparable to HT29 cells, IL-8 production by INT407 cells was increased for all six subjects after addition of fecal water containing β -glucan, resulting in a significant increase of 22.0% (range: 6.5%, 29.4%; $P = 0.028$). In contrast, IL-8 production by T84 cells and Caco-2 cells was not significantly changed (median: 5.9%; range: -31.1%, 23.1%; $P = 0.917$ and median: 4.2%; range: -34.8%, 218.3%; $P = 0.463$, respectively).

Figure 2.2: β -Glucan containing fecal water increased IL-8 production by enterocytes.

IL-8 production by HT29, T84, INT407 and Caco-2 cells after 16 h incubation with fecal water containing β -glucan or placebo and stimulation with cytokines (IL-1 β +IFN γ +TNF α) (A, B). (A) The median (and ranges) of IL-8 concentrations (ng/mL) after β -glucan and placebo fecal water and (B) percent changes in IL-8 concentration of each individual after fecal water containing β -glucan corrected for placebo are shown for the four enterocyte cell lines. In panel B, each subject is represented by a dot and the median is represented by a line.

Cell surface ICAM-1 protein expression

In line with effects on IL-8 production, addition of β -glucan enriched fecal water without stimulation with cytokines did not induce ICAM-1 expression in any of the four intestinal cell lines (data not shown), while stimulating effects on ICAM-1 expression were visible in the presence of cytokine stimulation. **Figure 2.3A** shows the ICAM-1 expression after placebo and β -glucan fecal water for all subject together in each of the four cell lines and in **Figure 2.3B** percent changes for the β -glucan fecal water for each subject individually corrected for placebo fecal water are shown.

Figure 2.3: β -Glucan containing fecal water increased ICAM-1 expression in enterocytes. ICAM-1 expression in HT29, T84, INT407 and Caco-2 cells after 16 h incubation with fecal water containing β -glucan or placebo and stimulation with cytokines (IL-1 β +IFN γ +TNF α) (A, B). (A) The median (and ranges) of ICAM-1 expression (arbitrary units) after β -glucan and placebo fecal water and (B) percent changes in ICAM-1 expression of each individual after fecal water containing β -glucan corrected for placebo. In panel B, each subject is represented by a dot and the median is represented by a line.

These figures show that β -glucan containing fecal water from all subjects increased ICAM-1 expression in T84 cells. This resulted in a significant increase of ICAM-1 expression for T84 (median: 11.0%; range: 3.8%, 31.1%; $P = 0.028$). ICAM-1 expression in Caco-2 cells and INT407 cells was increased for five out of the six subjects, with a median ICAM-1 increase of 20.4% (range: -8.8%, 40.5%; $P = 0.075$) and 16.7% (range: -19.0%, 30.0%; $P = 0.249$), respectively. ICAM-1 expression in HT29 cells, however, was not significantly changed (median: -0.4%; range: -8.4%, 10.8%; $P = 0.917$).

Inflammatory protein expression profiles

In general, β -glucan enriched fecal water increased the expression of almost all detectable cytokines, chemokines, colony stimulating factors (CSF) and growth factors as compared to placebo fecal water (**Figure 2.4**). No bar means that the protein was not detectable in the culture medium of that particular cell type or that there was no change in protein expression after β -glucan as compared to placebo fecal water.

Figure 2.4: β -Glucan containing fecal water mostly increased expression of various inflammatory proteins of enterocytes.

Percent changes in expression of chemokines, colony stimulating factors (CSF), growth factors and cytokines after β -glucan as compared to placebo fecal water in HT29, T84, INT407 and Caco-2 cells as measured by an antibody array. For experimental details see materials and methods. GRO, growth regulated protein; IL, interleukin; MCP, monocyte chemotactic protein; MDC, macrophage derived chemokine; MIG, monokine induced by $\text{IFN}\gamma$; MIP, macrophage inflammatory protein; Rantes, regulated upon activation normal T-cell expressed and secreted; TARC, thymus and activation regulated chemokine; GM-CSF, granulocyte-macrophage colony stimulating factor; MCSF, macrophage colony stimulating factor; Tpo, thrombopoietin; EGF, epidermal growth factor; OSM, oncostatin M; PDGF, platelet-derived growth factor; VEGF, vascular endothelial growth factor; TNF, tumor necrosis factor; Ang, angiogenin.

When examining the expression profiles into more detail, it can be seen that especially the changes in chemokines expressions were quite consistent. For example, in all cell lines expressions of all detectable monocyte chemotactic proteins (MCPs) were increased (MCP-2 and 3 expression not measurable in HT29 cells and no MCPs measurable in Caco-2 cells), while that of MIG (monokine induced by $\text{IFN}\gamma$), a chemokine that attracts T-lymphocytes, was decreased (not measurable in T84 and INT407 cells). The increases in IL-8 expressions agreed very well with those observed with the ELISA technique, confirming the validity of the antibody array. It should be noted, however, that the relative change measured with the antibody array was higher in Caco-2. This can be explained by the fact that for the antibody array pooled samples were used and the results therefore represent a mean value. For the ELISA, median values were presented. Changes in CSF and growth factor expressions were less consistent. The T84 cells showed an increased expression for all CSF and growth factors, whereas the HT29 cells showed a decreased expression for all these proteins except for GM-CSF. Also for INT407 and Caco-2 cells increases and decreases for these proteins were observed.

Discussion

Not only β -glucans from the cell wall of fungi and yeast, but also β -glucans from oats have been shown potent immune-stimulators, at least in macrophages and spleen cells (1). However, effects on enterocytes have never been examined. Our results now show that fecal water prepared from ileostomic contents of patients consuming diets enriched with oat β -glucans, induced an immune-stimulating response in enterocytes both from small intestinal as well as from colonic origin. The finding that not all four cell lines responded significantly regarding all parameters, but the fact that nearly all responses observed in the different cell lines were a stimulation, strengthens our believe that oat β -glucans indeed enhanced immune responses in enterocytes. Since our primary goal was to see whether enterocytes responded to β -glucan enriched fecal water at all, we did not attempt to define the mechanism underlying this response in this phase. Therefore, as far as we know now, these findings could be ascribed to direct as well as indirect effects of oat β -glucan.

We measured immune modulating effects of β -glucan by examining IL-8 production and ICAM-1 expression of enterocytes. IL-8 is a chemokine and ICAM-1 is an adhesion molecule whose expression is both increased in case of an infection by pathogens. IL-8 production by enterocytes will induce recruitment of inflammatory cells such as neutrophils, basophils and lymphocytes to the inflamed tissue (15). ICAM-1 expression on enterocytes may help to keep leukocytes that have transmigrated through the epithelial layer towards the intestinal lumen in close contact with the intestinal epithelium (16). This will prevent the further invasion of the mucosa by pathogens. Fecal water with β -glucan increased the production of IL-8 and the cell surface expression of ICAM-1, which thus indicates an enhanced immune response. This agrees with the results of an *in vivo* study with C57BL/6 mice showing that oral administration of (1 $\rightarrow$ 3), (1 $\rightarrow$ 6)- β -glucan isolated from baker's yeast increased the number of intraepithelial lymphocytes in the intestine (17). Although effects of yeast glucans and oat glucans may, because of structural differences, not as such be extrapolated to each other, the similarity in responses is suggestive.

Inflammatory signatures obtained from the antibody arrays suggest a potential role of other inflammatory players, because β -glucan elevated the expression of numerous inflammatory proteins. An important group of proteins that showed highly consistent elevations in all four enterocyte cell lines, were the MCPs. The elevation of these chemokines, which preferably attract monocytes, suggests a role of these cells (besides the granulocytes and lymphocytes attracted by IL-8) in the β -glucan induced improvement in immune function as well. Our results therefore suggest that the elevated IL-8 production and increased ICAM-1 expression and probably also the increased production of MCPs by enterocytes may contribute to the increased resistance to

pathogens after β -glucan consumption, as shown in mice (1, 4) and humans (18, 19). No correlation between the β -glucan concentration in the fecal water and the overall inflammatory response (in all cell lines) of each subject was present, probably due to the small β -glucan concentration interval (0.12-0.18 mg/mL) and the small number of subject. We showed that the four cell lines did not always respond in the same manner. For example, in T84 cells the IL-8 production was not significantly different, whereas ICAM-1 expression significantly increased. This suggests that individual inflammation markers are differently regulated in the various intestinal cell lines. Therefore, we decided to measure a whole range of inflammation related markers in all four cell lines. Although there are differences between the cell lines and not all effects were significant, the overall conclusion is that they are nearly all in the same direction, indicating immune stimulating effects of β -glucan enriched fecal water in enterocytes.

To what extent our findings can be extrapolated to other types of β -glucans is not known. β -Glucans are a complex group of molecules with different degrees of branching, polymer lengths, tertiary structures and solubilities (3, 20). We have used a soluble unbranched β -glucan with β -(1 $\rightarrow$ 3) and β -(1 $\rightarrow$ 4) linkages, that was extracted from oat, with a mean MW of 60 kDa. Brown and Gordon have recently suggested that high MW and/or particulate β -glucans of fungi directly activate cells. In contrast, low MW β -glucans might not directly activate cells, but may modulate the response to another challenge. Results of these low MW β -glucans, however, are conflicting, as both primed and suppressed secondary responses have been reported (7). There is no clear explanation for these different results, but it is known that small differences between β -glucan molecules besides MW also have an impact on their immune modulating effects (7, 20). Unfortunately, characteristics of the β -glucans used in other studies have not always been reported (7). In the present study, we have now shown that a low MW oat (1 $\rightarrow$ 3), (1 $\rightarrow$ 4) β -glucan (60 kDa) in the physiological matrix of fecal water induced an enhanced immune response in enterocytes after cytokine stimulation. Moreover, the finding that addition of the β -glucan enriched fecal water without stimulation with cytokines did not induce an inflammatory response (data not shown) supports the hypothesis that low MW β -glucans can modulate, but not evoke, an inflammatory response. However, despite numerous barriers and mechanisms counteracting bacterial translocation and too strong inflammatory responses, it is very likely that there is always some translocation also in a normal situation. (21) Therefore, it is very likely that a local inflammatory microenvironment is always present in the intestine (21), suggesting that adding cytokines to stimulate the enterocytes might be considered rather physiological. With respect to the physiological relevance of the β -glucan induced immune enhancement, the finding that numerous immune mediators all change in the same direction, suggests that this can have a strong physiological effect. However, in contrast to the *in vivo* situation in the intestine,

the *in vitro* cultured enterocytes do not produce mucus, however mucus is not expected to prevent direct contact between enterocytes and β -glucan since β -glucan is water soluble and is expected to easily pass the mucus layer.

Dectin-1 plays a central role to explain the immune modulating effects of β -glucans (3). Of the four epithelial intestinal cell lines we have used, dectin-1 mRNA expression has been shown in Caco-2 cells (22). As far as we know, the expression of dectin-1 in the other cell lines is not examined yet. The expression of dectin-1 on intestinal epithelial cells *in vivo* is contradictory. Presence and absence on mRNA and protein level and on murine and human intestinal cells has been reported (23-25). Because it is not evident if dectin-1 is expressed on enterocytes, we cannot exclude the possibility that the observed effects of β -glucan are not mediated by dectin-1. The present study was not initiated to mechanistically examine the effects of β -glucan. Whether our results are dectin-1 mediated or due to indirect changes in the fecal water composition induced by oat β -glucan will hopefully be addressed in future studies.

As suggested above, the observed effects in our *in vitro* model may also be caused by an increased or decreased production of another, yet unknown, factor. If true, this implies that effects of β -glucan fecal water on enterocytes are indirect and would in that case never have been found when - if possible - the oat β -glucans were applied to the cells directly. As an example of indirect effects, β -glucan could have induced changes in inflammatory proteins in the fecal water. However we could not identify any inflammatory protein in the fecal water with the highly sensitive antibody array. Although the amount of LPS in the fecal water is not determined, effects of LPS as explanation for the observed changes in the intestinal cell lines used can be neglected since all cell lines were found nonresponsive to even high concentrations of LPS (data not shown). However, measuring LPS is not the solution since potential effects of other microbial products in fecal water cannot be ruled out. However, since each volunteer was his own control, potential disturbing effects of these factors are not very likely. Another possible explanation for the observed effects is an increased production of antimicrobial peptides like defensins, after consumption of β -glucan. These peptides, which will then be present in elevated amounts in the fecal water, may also influence intestinal inflammation by stimulating chemokine secretion and attracting leukocytes (26). Another possible mechanism explaining the observed effects is degradation of β -glucan into short chain fatty acids (SCFA). SCFA, for example butyrate, are known to modulate the intestinal flora and intestinal inflammation (27). However, in our study we worked with ileostomic patients and β -glucan did therefore not pass the colon, the only place where fibers can be degraded into SCFA by bacteria. Also the molecular weight of β -glucan in the ileostomic contents was not substantially changed, which excludes the formation of degradation products. It is therefore not likely that the reported effects are caused by differences in SCFA composition of the fecal water. Finally, it is known that consumption of β -glucan

enriched diets increases fecal bile acid excretion (28). If anything, however, bile acids will have immune suppressive effects (29).

In conclusion, we found that β -glucan enriched fecal water enhanced the immune response in stimulated intestinal cell lines, which should allow the host to defend itself better against invading pathogens. This is to our opinion the first time that responsiveness of enterocytes towards β -glucans has been shown. Whether the reported effects of β -glucan enriched fecal water are direct effects of β -glucan (potentially via dectin-1) or indirect (via so far unknown mechanism) need further investigation. Finally, extrapolation of our findings to the *in vivo* situation remains to be established.

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Chapter 3

Arachidonic acid (n-6 PUFA) activates NF- κ B and elevates ICAM-1 expression as compared to EPA (n-3 PUFA) and oleic acid (n-9 PUFA) in enterocytes

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Submitted

Abstract

In patients with inflammatory bowel disease (IBD), intestinal activation of the transcription factor NF- κ B as well as ICAM-1 expression - which is involved in recruiting leukocytes to the side of inflammation - are increased. Moreover, colonic arachidonic acid (AA) proportions are increased and oleic acid (OA) proportions are decreased. Fish oils are protective in IBD patients, however a side-by-side comparison between effects of fish oils, AA and OA has not been made. We therefore compared effects of eicosapentaenoic acid (EPA; n-3 polyunsaturated fatty acid (PUFA)) versus AA (n-6 PUFA) and OA (n-9 monounsaturated fatty acid (MUFA)) on ICAM-1 expression in Caco-2 enterocytes. To validate our model we showed that dexamethasone, sulfasalazine and PPAR α (GW7647) and PPAR γ (troglitazone) agonists significantly lowered ICAM-1 expression. ICAM-1 expression of non-stimulated and cytokine stimulated Caco-2 cells cultured for 22 days with AA was significant higher as compared to EPA and OA. Furthermore, AA increased NF- κ B activation in a reporter cell-line as compared to EPA. Finally, we showed that cyclooxygenase (COX)-inhibition by indomethacin could not explain these results. Antibody array analysis of multiple inflammatory proteins particularly showed an increased MCP-1 and angiogenin production and a decreased IL-6 and IL-10 production by AA as compared to EPA. Our results might give an explanation for the elevated NF- κ B activation in intestinal mucosa of IBD patients since elevated AA proportions have consistently been reported. It also suggests that replacement of AA by EPA or OA might have beneficial effects. Finally, we suggest that the proinflammatory effects of AA versus EPA and OA are not related to PPAR γ activation or eicosanoid formation.

Introduction

Although the etiologies of ulcerative colitis (UC) and Crohn's disease (CD) - the two major manifestations of inflammatory bowel disease (IBD) - are essentially unknown, compelling evidence suggests a role for genetic components triggered by environmental factors. This will ultimately result in an uncontrolled host defense response against luminal antigens. Among the environmental factors, nutrition and more specifically the type of fatty acids in the diet might play a role (1). Intakes of certain fatty acids are reflected by the fatty acid composition of plasma phospholipids, which correlates in healthy volunteers with that of the mucosa. In IBD patients, however, relationships between fatty acid patterns of plasma and intestinal mucosa are less pronounced (2). Therefore, focusing on the mucosal fatty acid composition seems warranted. These studies consistently showed elevated proportions of the n-6 polyunsaturated fatty acid (PUFA) arachidonic acid (AA) in colon mucosa of both UC and CD patients as compared to those of control subjects (2, 3). Although the proportion of the n-3 PUFA eicosapentaenoic acid (EPA) in the mucosa was in most studies not statistically different between IBD patients and healthy controls, one study reported a tendency towards lower EPA proportions in IBD patients (3). Since n-6 PUFAs are considered to be proinflammatory as compared to n-3 PUFAs, this characteristic mucosal fatty acid pattern in IBD patients seems relevant. Also epidemiological studies have suggested that fish oils protect against IBD (4). Moreover, a higher intake of fish oil resulted in the replacement of arachidonic acid by the less inflammatory fish oil fatty acids in the colon mucosa of IBD patients, which was associated with significantly reduced corticosteroid requirements (5) and lower relapse rates (6).

During acute exacerbations of chronic colitis, changes in histology are dominated by a massive influx of neutrophils from the circulation across the epithelial lining. Besides cytokines and chemokines, a distinct set of cell adhesion molecules (CAMs) is required for the binding and movement of leukocytes towards inflamed areas. Regarding IBD, intercellular adhesion molecule-1 (ICAM-1) has received a lot of interest. Indeed, in IBD patients intestinal ICAM-1 expression (7) and plasma levels of soluble ICAM-1 are increased (8). Also, associations between IBD and polymorphisms in the gene encoding for ICAM-1 suggest that ICAM-1 plays a role in the pathogenesis of IBD (9). Moreover, animal models have shown that blocking with ICAM-1 antibodies (10) and with ICAM-1 antisense oligonucleotides (11) inhibited intestinal inflammation. In humans, results of both intravenous (12) and subcutaneous (13) administration of the antisense oligonucleotide Alicaforsen® were disappointing, but direct administration of Alicaforsen® into the intestine significantly improved short- and long-term clinical disease activity of IBD (14). These studies therefore point towards an important role for ICAM-1 in intestinal inflammation. A side-by-side comparison of the n-3 PUFA EPA, and the n-6

PUFA AA on ICAM-1 expression of intestinal cells has never been performed. We also compared the effects of AA with the n-9 MUFA oleic acid (OA) since OA is the most abundant fatty acid present both in our diet (15) as well as in colon mucosa (3). Interestingly, also the proportion of OA is decreased in colon mucosa of IBD patients (2, 3). However it should be realized that decreasing mucosal AA levels may be easier achievable by increasing fish oil intake than by increasing OA intake.

The transcription factor NF- κ B is a key regulator of the inflammatory response. Activation of NF- κ B also seems to play a critical role in the initiation and perpetuation of intestinal inflammation in IBD (16). NF- κ B activity in the colon is increased during active episodes in IBD patients and inhibition of NF- κ B activity by glucocorticoid treatment is associated with reduced disease activity (17, 18). In animal models, NF- κ B blockade by antisense oligonucleotides abolished established colitis (19). As for ICAM-1, effects of EPA versus AA on NF- κ B activation of intestinal cells have never been compared. Therefore the aim of the present study was to evaluate and compare the effects of the n-3 PUFA EPA and the n-6 PUFA AA on ICAM-1 expression and NF- κ B activation in an enterocyte cell line.

Materials and methods

Reagents

Bovine serum albumin (BSA; endotoxin and fatty acid-free), sulfasalazine, dexamethasone, GW7647, oleic acid (OA), arachidonic acid (AA), eicosapentaenoic acid (EPA) and indomethacin were obtained from Sigma Chemical Company (St Louis, MO). Troglitazone was purchased from Biomol (Plymouth Meeting, PA). Recombinant human IL-1 β and interferon (IFN) γ were purchased from Roche Molecular Biochemicals (Mannheim, Germany). DMEM, trypsin, penicillin streptomycin (PS), sodium pyruvate (SP) and non-essential amino acids (NEAA) were obtained from Invitrogen Corporation (Paisley, UK). Fetal calf serum (FCS; South-American) was obtained from Greiner Bio-one (Frickenhausen, Germany).

Intestinal cell cultures

The human cell line Caco-2 was purchased from the American Tissue Type Collection (ATTC). Caco-2 cells were cultured in DMEM supplemented with 10% heat-inactivated FCS and 1% PS, 1% SP and 1% NEAA. Cells were cultured at 37 °C in a 5% CO₂ humidified atmosphere, refreshed every second day and separated by trypsin-0.03% EDTA, when they had reached 70-90% confluence. To evaluate the immune modulating effects of various interventions, Caco-2 cells were plated in 6-well tissue culture plates at an initial density of 0.5x10⁶ cells/mL in a total volume of 1.5 mL. Medium was replaced every other day for 24 days. After 24 days Caco-2 wells were fully differentiated into small intestinal enterocytes (20). First, effects of immune-suppressive pharmacological compounds (sulfasalazine, dexamethasone and troglitazone) were tested to validate the model. Although these pharmacologic compounds have known immune-suppressive effects, effects on ICAM-1 expression in Caco-2 cells have - as far as we know - not been reported before. Therefore, after Caco-2 cells were fully differentiated, medium was replaced by medium containing the compound of interest in combination with an inflammation-inducing cocktail consisting of the cytokines IFN γ (100 U/mL) and IL-1 β (50 U/mL). The compounds of interest were pre-incubated 30 minutes (sulfasalazine) or 2 hours (dexamethasone, troglitazone or GW7647) before stimulation with the cytokine cocktail. After 16 hours of cytokine stimulation, cells were used to determine cell surface ICAM-1 protein expression.

Fatty acid experiments

The effects of various fatty acids were evaluated using the same Caco-2 cell model. For this, various fatty acids were added at indicated concentrations 2 days after plating the cells and again for the following 22 days each time when the medium was refreshed. The fatty acids were dissolved in ethanol up to a

final ethanol concentration in the medium of maximal 0.5% (v/v). Before addition to the cell cultures, the fatty acids dissolved in ethanol were pre-incubated for 30 min at 37 °C in full culture medium which also contained 0.1% BSA. Caco-2 cells were cultured with respectively 160 μ M OA (C18:1 n-9) versus 130 μ M AA (C20:4 n-6) plus 30 μ M OA (in total 160 μ M fatty acids) or 6 μ M EPA (C20:5 n-3) plus 154 μ M OA (in total also 160 μ M fatty acids). By this approach, the total molarity of fatty acids supplied was similar in all experiments, while supplying different amounts of the fatty acid of interest (i.e. 130 μ M AA or 6 μ M EPA). These relatively low concentrations of OA (160 μ M), AA (130 μ M) and EPA (6 μ M) were chosen because they are 4 times higher than normally present in culture medium of Caco-2 cells containing 10% FCS. We cultured the cells for 22 days with these relatively low concentrations of fatty acids to simulate a realistic long-term *in vivo* change in dietary fatty acid intake. After 22 days culturing the cells with fatty acids, medium was replaced by medium enriched with the different fatty acids plus the cytokine cocktail (IFN γ (100 U/mL) and IL-1 β (50 U/mL)). After 16 hours stimulation, ICAM-1 expression on living cells was measured and culture media were collected to determine inflammatory protein expression profiles. To evaluate the effects of the fatty acids on NF- κ B activity and the role of cyclooxygenase (COX)-enzymes, the experiments with the fatty acids AA and EPA were repeated but now in our NF- κ B reporter Caco-2 cell line with and without indomethacin (20 μ M) added 2 hours before and during cytokine stimulation. Prostaglandin (PG)E $_2$ levels in the supernatant were quantitated using a PGE $_2$ Biotrak enzyme-immunoassay (EIA) system (Amersham Biosciences Ltd, Buckinghamshire, UK) according to the high sensitivity enzyme immunoassay protocol 2.

Flow cytometry analysis of ICAM-1

In order to quantify cell surface ICAM-1 protein expression on living Caco-2 cells we developed a flow cytometry assay. After 16 hours of stimulation, the cells were washed three times with PBS and detached with trypsin-0.03% EDTA. Next, medium was added and cell suspensions were centrifuged for 5 min at 1,200 rpm at room temperature, followed by resuspending the pellets in 500 μ L PBS-1% BSA. Cells were counted and diluted to 10 6 cells/mL in PBS-1% BSA. Recombinant-phycoerythrin (R-PE)-conjugated mouse-anti-human CD54 monoclonal antibody (anti-ICAM-1) or isotype-matched control antibody (Becton Dickinson Biosciences, San Diego, CA; 20 μ L/10 6) cells was added and incubated for 30 minutes on ice in the dark. Next, cell suspensions were centrifuged for 5 min at 1,500 rpm and pellets were resuspended in 500 μ L PBS-1% BSA. The amount of fluorescence of 10,000 living cells was counted and analyzed with the FACSORT and CellQuest analysis software (Becton Dickinson, Franklin Lakes, NJ).

Stable transfection of NF- κ B in Caco-2 cells

For evaluating the effects of the various interventions on transcriptional activity of NF- κ B, a stable NF- κ B reporter Caco-2 cell line was created. The 6 κ B-TK-luciferase (NF- κ B reporter) plasmid and neomycin resistance plasmid were both kindly provided by Dr. R.C. Langen (Department of Pulmonology, Maastricht University, The Netherlands). Cells were transfected using Lipofectamine 2000 (Invitrogen Corporation, Paisley, UK) according to the manufacturers' instructions. Positive clones were selected by culturing with geneticin (1 mg/mL). To determine luciferase activity, non-stimulated and 3 hours cytokine (100 U/mL IFN γ and 50 U/mL IL-1 β) stimulated cells were lysed in luciferase lysis buffer (Promega, Madison, WI) and stored at -80 °C. Luciferase (Promega) activity was measured according to the manufacturers' instructions and expressed relative to total protein (Bio-rad assay; Bio-rad, Hercules, CA).

Peroxisome proliferator-activated receptor (PPAR) γ and PPAR α mRNA expression of differentiated Caco-2 cells

Total RNA was extracted from differentiated Caco-2 cells with Trizol according to the manufacturer's instructions (Gibco BRL, Gaithersburg, MD). Next, cDNA was made as described (21), and mRNA expression of PPAR γ and PPAR α was determined using commercially available Taqman gene expression assays (Applied Biosystems, Foster city, CA). Data was normalized against β -actin as housekeeping gene.

Fatty acid composition of Caco-2 cells

Fatty acid incorporation into the Caco-2 cells was evaluated using extraction and analysis procedures as previously described (22). Briefly, total lipids were extracted from 500 μ L cell suspension in PBS-1% BSA according to the method of Bligh and Dyer (23). Aminopropyl-bonded silica columns (Varian, Harbor City, CA) were used to separate phospholipids from the total lipid extract (24). The phospholipids were then saponified, and the resultant fatty acids were methylated into their corresponding fatty acid methyl esters (FAMES) (25). Fatty acids were separated on an Autosystem (Perkin-Elmer, Norwalk, CT) gas chromatograph that was fitted with a silica-gel column (Cp-sil 88 for FAME, 50 m x 0.25 mm, 0.2- μ m film thickness; Chrompack, Middelburg, The Netherlands) with helium gas (130 kPa) as the carrier gas. Both the injection and detection temperatures were set at 300 °C. The starting temperature of the column was 160 °C. Ten minutes after injection, the temperature was increased up to 190 °C at a rate of 2.5 °C/min. After 20 min at 190 °C, the temperature was increased up to 230 °C at a rate of 4 °C/min. The final temperature of 230 °C was maintained for 10 min. Data were analyzed by using CHROMCARD software (version 1.21; CE Instruments, Milan, Italy). The

fatty acid compositions of the Caco-2 cells are expressed in relative amounts (% of total fatty acids identified; wt/wt).

Inflammatory protein expression profiles using an antibody array

Protein expression patterns of multiple cytokines, chemokines and growth factors, were detected simultaneously in Caco-2 cell culture media with the human cytokine antibody array III (Ray Biotech Inc., Norcross, GA) according to the manufacturer's instructions. First, duplicates of cell culture media of Caco-2 cells cultured with AA and EPA after cytokine stimulation were pooled. One mL of the pooled samples was added to the array membranes. After incubating and washing, the protein-bound membrane was incubated with a cocktail of biotin-labeled antibodies, followed by the addition of horseradish peroxidase-conjugated streptavidin. Array spot intensity was detected by using a LAS-3000 Lite Image reader (Raytest GmbH, Straubenhart, Germany) based on chemiluminescence imaging. Intensity of the spots was quantified in arbitrary units (a.u.) by densitometry using Aida software version 3.50 (Raytest GmbH), thereby correcting for background staining of the gel. Comparison of protein expression profiles was possible after normalization of each spot on an array using the positive controls, provided by the manufacturer. The sensitivity of the array is not the same for the various proteins. Differences in heights of bars from different proteins do therefore not necessarily represent differences in concentrations. The cytokines used for stimulation (IFN γ and IL-1 β) were excluded from analysis.

Detection of ICAM-1 on Caco-2 frozen sections

To determine the localization of ICAM-1 in our *in vitro* Caco-2 cell model, Caco-2 cells were cultured and differentiated into small intestinal enterocyte on collagen-coated polyfluoroethylene transwell membrane inserts with a 0.4 μ m membrane pore size (Corning Costar, Cambridge, MA). Differentiated Caco-2 cells were stimulated with IFN γ (100 U/mL) and IL-1 β (50 U/mL) for 16 hours, embedded in Tissue-Tek (Sakura Finetek, Zoeterwoude, The Netherlands) and rapidly frozen in 2-propanol (Fluka, Zwijndrecht, The Netherlands), dry-ice-cooled and stored at -80 °C. Serial cryosections (10 μ m) were obtained using a Leica CM3050 cryostat (Leica Microsystems GmbH, Wetzlar, Germany) and thaw mounted on uncoated glass slides. Before processing or storage at -80 °C, the samples were air dried overnight. To detect ICAM-1 the sections were incubated 30 minutes in the dark at room temperature with recombinant-phycoerythrin (R-PE)-conjugated mouse-anti-human CD54 monoclonal antibody or isotype-matched control antibody (Becton Dickinson Biosciences, San Diego, CA) 1:50 diluted in PBS-1% BSA. To detect cytokeratin (CK)-19 the sections were simultaneously incubated with a monoclonal antibody directed to

CK-19, kindly obtained from Dr. E.B. Lane (University Dundee, Dundee, UK) 1:10 diluted. Then the sections were washed three times for 5 minutes in PBS. After that the secondary antibody goat anti-mouse IgG1 (ALEXA555) (Molecular Probes Europe, Leiden, The Netherlands) (1:500) against anti-CD54 (to evade fast quenching of the PE-label) and goat anti-mouse IgG2b (FITC) (Southern Biotech, Sanbio BV, Uden, The Netherlands) (1:50) against CK19 diluted in PBS-1% BSA was added to the sections and incubated for 30 minutes. Again the sections were washed three times for 5 minutes with PBS. Finally, sections were mounted in Mowiol-TRIS pH 8.5 (Calbiochem, Omnilabo International, Etten-Leur, The Netherlands) containing 0.5 g/mL 4-6-diamino-2-phenylindole (DAPI; Molecular Probes Europe) to stain the nuclei. All sections were examined using a Nikon E800 fluorescence microscope (Uvikon, Bunnik, The Netherlands) coupled to a Basler A101C progressive scan color CCD camera. By just a simple shift in filters, images were grabbed in fluorescence using the ALEXA excitation filter (540–580 nm), the FITC excitation filter (465–495 nm) and DAPI UV excitation filter (340–380 nm) in the red, green and blue channel, respectively. The images acquired were merged to examine the cellular localization and level of expression of ICAM-1.

Statistical analysis

Data were expressed as means and standard deviations (SD) for the non-stimulated condition, the cytokine stimulated condition and cytokine stimulated – non-stimulated (called net stimulated) condition. To determine statistical significance unpaired t-tests (comparison between two interventions) or ANOVA (comparison between 3 interventions) with a Bonferonni post-hoc test when differences between interventions were significantly different, were performed. First non-stimulated values between interventions were tested to examine differences in basal values. Then, net stimulated values between interventions were tested to examine differences in cytokine-induced changes. Furthermore cytokines stimulated values were tested to examine differences in 'end' values. All statistical analyses were performed using SPSS 11.0 for Mac Os X (SPSS, Chicago, IL). *P*-values of less than 0.05 were considered statistically significant.

Results

Model validation

Stimulation with IL-1 β and IFN γ increased cell-surface ICAM-1 protein expression on Caco-2 cells, which resulted in a net stimulated ICAM-1 expression of about 50 a.u. (**Figure 3.1**, panels A and B).

Figure 3.1: Model validation.

(A) ICAM-1 expression [in arbitrary units a.u.] on living control Caco-2 cells and after 2 hours pre-treatment with 1 μ M dexamethasone or 30 minutes 5 mM sulfasalazine with and without cytokine stimulation for 16 hours (100 U/mL IFN γ and 50 U/mL IL-1 β) and (B) net stimulated (stimulated – non-stimulated) ICAM-1 expression. Results represent means $\pm$ SD; n=2. ^a $P < 0.05$ versus control non-stimulated, ^b $P < 0.05$ versus control cytokine stimulated, ^c $P < 0.05$ versus control net stimulated. (C-F) Immunohistochemistry of ICAM-1 (red) on frozen sections of Caco-2 cells on a transwell showed increased apical expression after cytokine stimulation (100 U/mL IFN γ and 50 U/mL IL-1 β). (C) Hematoxylin staining (D) Non-stimulated Caco-2 cells (E) Apical cytokine stimulated Caco-2 cells (F) Isotype control staining of ICAM-1. Red staining, ICAM-1; green staining, cytoskeleton (cytokeratin-19); blue staining, nucleus. Magnification 40x.

After pre-treatment of the cells for 2 hours with dexamethasone, a corticosteroid with known therapeutic effects in IBD patients, the cytokine stimulated ICAM-1 expression was significantly decreased as compared to control ($P = 0.044$). Also the net stimulated ICAM-1 expression (25 a.u.) was significantly reduced as compared to control ($P = 0.019$). Next, we examined the effect of another frequently used therapeutic drug for IBD patients, sulfasalazine. Effects of

sulfasalazine were comparable to those observed for dexamethasone. However, also ICAM-1 expression without cytokine stimulation ($P = 0.004$) (Fig. 3.1, panel A) was lowered. Altogether, these results show that in our cell model ICAM-1 expression is related to the clinical outcomes of drugs proven to treat IBD and could therefore be used as the main outcome parameter in the following experiments. To further validate characteristics of our *in vitro* model, we also localized the site of ICAM-1 expression on the Caco-2 cells by means of immunohistochemistry on frozen sections. As shown in Figure 3.1 panel C to F, ICAM-1 was expressed on the apical (lumen) side of the polarized Caco-2 cells. Besides a low constitutive expression (panel D), there was a clear increase after stimulation with the cytokine cocktail (panel E). This localisation is in line with the apical ICAM-1 expression, as found in intestinal biopsies from IBD patients (122).

Effects of PPAR agonists on ICAM-1 expression and NF- κ B activation

Since PPARs are known modulators of inflammation and fatty acids are natural ligands for PPARs, we first examined PPAR expression in our differentiated Caco-2 cells. We found that PPAR α and PPAR γ mRNA are expressed in equal amounts (data not shown). Next we examined the effects of the PPAR γ agonist troglitazone (a thiazolidinedione (TZD)) and the PPAR α agonist GW7647 on ICAM-1 expression. Although both troglitazone and GW7647 significantly lowered the cytokine stimulated ICAM-1 expression as compared to control ($P < 0.001$ and $P = 0.009$ respectively), only troglitazone significantly ($P = 0.008$) reduced the net stimulated ICAM-1 expression (**Figure 3.2**, panels A and B). Troglitazone and GW7647 were used at a concentration where they are selective for their receptor subtypes (27, 28). It is known that PPARs suppress inflammation by inhibiting the transcription factor NF- κ B, which is a key regulator of inflammation (29). Therefore, we also examined effects of troglitazone on NF- κ B transactivation in our Caco-2 NF- κ B reporter cell line. These results showed that the cytokine cocktail induced after already 3 hours a more than 200 percent increase in NF- κ B transactivation. This increase of NF- κ B transactivation was significantly ($P = 0.008$) lower after 2 hours pre-treatment with the PPAR γ agonist troglitazone (Fig. 3.2, panels C and D). Moreover, the inhibitory effect of pre-treatment with the PPAR γ agonist troglitazone on cytokine induced NF- κ B transactivation was significantly ($P = 0.001$) larger as compared to pre-treatment with the PPAR α agonist GW7647 (data not shown).

Figure 3.2: Effects of PPAR agonists on ICAM-1 expression and NF-κB transactivation in Caco-2 cells.

(A-B) ICAM-1 expression [in arbitrary units a.u.] on living control Caco-2 cells and after pre-treatment with PPAR agonists (2 hours; PPAR γ troglitazone 100 μ M and PPAR α GW7647 1 μ M) and (C-D) NF-κB transactivation measured by luciferase activity [in RLU/mg] in a NF-κB reporter Caco-2 cell-line of control cells and after troglitazone pre-treatment with and without cytokine stimulation for 3 hours (100 U/mL IFN γ and 50 U/mL IL-1 β) and net stimulated (stimulated – non-stimulated). Results represent means $\pm$ SD; n=2. ^a $P < 0.05$ versus control cytokine stimulated, ^c $P < 0.05$ versus control net stimulated.

Fatty acid incorporation in phospholipids

Table 3.1 shows that the fatty acids supplied for 22 days in culture medium were incorporated into the phospholipid fraction (% of total fatty acids) of the Caco-2 cells. Fatty acid incorporation into phospholipids showed the same pattern as the fatty acid composition of total lipids (data not shown), although changes in the total lipids were more pronounced. Moreover, the fatty acid profiles of phospholipids (table 3.1) and total lipids (data not shown) were similar in non-stimulated and stimulated Caco-2 cells. Cells cultured with AA showed in particular an increase in the proportion of long chain n-6 PUFAs (mainly AA) and a decrease in n-9 MUFAs (mainly OA) as compared with cells cultured with only OA. The cells cultured with EPA showed an increase in the proportion of n-3 fatty acids (mainly EPA) whereas the proportions of n-9 MUFAs and n-6 PUFAs did not change much as compared with cells cultured with only OA.

Table 3.1: Fatty acid composition in phospholipids of Caco-2 cells without (-) and with (+) cytokine stimulation (IL-1 β and IFN γ) supplemented with different fatty acids (% of total fatty acids).

Fatty acids	OA (160 μ M)		AA + OA (130 + 30 μ M)		EPA + OA (6 + 154 μ M)	
	-	+	-	+	-	+
16:0	16.0	16.1	18.7	19.5	16.3	15.8
18:0	5.9	6.1	9.6	10.5	6.7	6.5
18:1 trans	3.0	3.1	6.1	5.7	2.7	2.7
18:1 n-7	3.4	3.4	2.6	2.5	3.3	3.3
18:1 n-9	47.1	46.3	14.4	13.8	45.2	45.0
18:2 n-6	1.7	1.7	1.1	1.1	1.7	1.6
20:1 n-9	2.1	2.1	0.3	0.3	1.7	1.7
20:4 n-6	9.3	9.4	29.3	28.9	8.9	8.9
20:5 n-3	0.4	0.4	0.0	0.0	2.1	2.2
22:1 n-9	0.8	0.8	1.5	1.3	0.8	0.8
24:1 n-9	2.0	2.1	0.9	1.0	2.0	2.1
22:4 n-6	0.4	0.4	7.7	7.8	0.3	0.3
22:5 n-3	0.5	0.5	0.4	0.4	1.3	1.3
Σ	92.6	92.4	92.6	92.8	93.0	92.2
Σ SAFA	21.9	22.2	28.3	30.0	23.0	22.3
Σ MUFA	58.4	57.8	25.8	24.6	55.7	55.6
Σ PUFA	12.3	12.4	38.5	38.2	14.3	14.3
Σ n-3	0.9	0.9	0.4	0.4	3.4	3.5
Σ n-6	11.4	11.5	38.1	37.8	10.9	10.8
Σ n-9	52.0	51.3	17.1	16.4	49.7	49.6

Σ is SUM of all fatty acids belonging that particular class. Abbreviations: AA, arachidonic acid; EPA, eicosapentaenoic acid; MUFA, monounsaturated fatty acids; OA, oleic acid; PUFA, polyunsaturated fatty acids; SAFA, saturated fatty acids; - non-stimulated; + cytokine stimulated.

Effects of fatty acids on ICAM-1 expression and NF- κ B activation

As shown in **Figure 3.3** (panel A), AA significantly elevated ICAM-1 expression as compared to OA or EPA (non-stimulated $P < 0.001$ AA versus OA and AA versus EPA, and stimulated $P = 0.010$ AA versus OA, and $P = 0.013$ AA versus EPA).

Figure 3.3: Effects of oleic acid (OA), arachidonic acid (AA) and eicosapentaenoic acid (EPA) on ICAM-1 expression on Caco-2 cells.

(A-B) ICAM-1 expression [in arbitrary units a.u.] on living Caco-2 cells cultured for 22 days with 160 μ M oleic acid (OA) or 130 μ M arachidonic acid (AA) plus 30 μ M OA or 6 μ M eicosapentaenoic acid (EPA) plus 154 μ M OA with and without cytokine stimulation for 16 hours (100 U/mL IFN γ and 50 U/mL IL-1 β) and net stimulated (stimulated – non-stimulated). Results represent means $\pm$ SD; $n=2$. ANOVA between groups, non-stimulated $P < 0.001$, stimulated $P = 0.006$ and net stimulated $P = 0.134$. ^a Bonferroni $P < 0.05$ versus OA and EPA unstimulated, ^b Bonferroni $P < 0.05$ versus OA and EPA cytokine stimulated.

Interestingly, stimulated and non-stimulated ICAM-1 expressions on cells cultured with EPA did not significantly differ from those cultured with OA (Figure 3.3, panel A). Despite these clear differences in non-stimulated and stimulated ICAM-1 expression between AA and EPA or OA, the net stimulated ICAM-1 expression did not differ between the groups (ANOVA $P = 0.134$). Since dietary interventions aiming at lowering mucosal AA content are most likely more successful by dietary EPA enrichments than by OA enrichment - because OA is the most abundant fatty acid present in our diet (113), making higher intakes not realistic - in further experiments we have focused on effects of AA versus EPA. First, we examined the effects on NF- κ B activation in the stable NF- κ B reporter Caco-2 cell line (**Figure 3.4**). NF- κ B transactivation in stimulated Caco-2 cells cultured with AA was significantly increased as compared to that in stimulated cells cultured with EPA (Fig. 3.4, panels A and B) (stimulated $P = 0.017$ and net stimulated $P = 0.001$). To evaluate whether the effects were related to the production of different families of eicosanoids we measured PGE $_2$ production and examined the effects of the different fatty acids in the presence of the cyclooxygenase (COX)-inhibitor indomethacin. As expected, PGE $_2$ production by Caco-2 cells cultured with AA was significantly higher than by cells cultured with EPA (data not shown).

Figure 3.4: Effects of arachidonic acid (AA) and eicosapentaenoic acid (EPA) on NF- κ B transactivation in Caco-2 cells and role of cyclooxygenase (COX)-inhibition.

NF- κ B transactivation with and without cytokine stimulation for 3 hours (100 U/mL IFN γ and 50 U/mL IL-1 β) (A, C, E) and net stimulated (stimulated – non-stimulated) (B, D, F) in a NF- κ B reporter Caco-2 cell line measured by luciferase activity [in RLU/mg]. (A-B) Reporter Caco-2 cells cultured for 22 days with 130 μ M arachidonic acid (AA) plus 30 μ M OA or 6 μ M eicosapentaenoic acid (EPA) plus 154 μ M OA. (C-D) Control reporter Caco-2 cells and after 2 hours pre-treatment with the cyclooxygenase (COX)-inhibitor indomethacin (20 μ M). (E-F) Reporter Caco-2 cells cultured for 22 days with 130 μ M arachidonic acid (AA) plus 30 μ M OA or 6 μ M eicosapentaenoic acid (EPA) plus 154 μ M OA and 2 hours pre-treatment with the COX-inhibitor indomethacin (20 μ M). Results represent means $\pm$ SD; $n=2$. ^b $P < 0.05$ versus EPA cytokine stimulated, ^c $P < 0.05$ versus EPA or EPA + indomethacin net stimulated.

Stimulation of the cells with the cytokine cocktail did however not influence PGE₂ production. Since cytokine stimulation did enhance NF- κ B activation, this does suggest that PGE₂ is not directly involved in NF- κ B activation. Indomethacin decreased PGE₂ production of Caco-2 cells by approximately 70% (data not shown), but had no effect on NF- κ B transactivation (Figure 3.4, panels C and D) as compared to untreated Caco-2 cells. Also ICAM-1 expression was not affected (data not shown). However, as shown in Figure 3.4, the effects of AA versus EPA in the presence of indomethacin treatment

(panel E) showed an identical pattern as observed without indomethacin (panel A). Also in the presence of indomethacin the net cytokine stimulated NF- κ B transactivation was significantly higher after AA as compared to EPA ($P = 0.047$) (Figure 3.4, panel F). This indicates that the increased basal PGE₂ production by AA cannot explain the different effects of AA and EPA on NF- κ B activation.

Effects of fatty acids on inflammatory proteins expression profiles

Finally, to explore the effects of AA and EPA on a broader scale, we evaluated protein expression profiles consisting of various inflammatory mediators by using antibody arrays. **Figure 3.5** shows that AA treatment particularly increased expression of monocyte chemotactic protein (MCP)-1 and angiogenin, while EPA treatment increased IL-10, IL-6, macrophage inflammatory protein (MIP)-1 δ , and growth regulated protein (GRO) expression.

Figure 3.5: Effects of arachidonic acid (AA) and eicosapentaenoic acid (EPA) on protein expression profiles in culture medium of Caco-2 cells.

Protein expression profile in the culture medium of Caco-2 cells cultured for 22 days with 130 μ M arachidonic acid (AA) plus 30 μ M OA or 6 μ M eicosapentaenoic acid (EPA) plus 154 μ M OA after 16 hours cytokine stimulation (100 U/mL IFN γ and 50 U/mL IL-1 β) measured with an antibody array. Results are represented as means $\pm$ SD; $n=2$. Abbreviations: CSF, colony stimulating factor; ENA, epithelial-derived neutrophil activating protein; MCP, monocyte chemotactic protein; MDC, macrophage derived chemokine; MIG, monokine induced by gamma interferon; MIP, macrophage inflammatory protein; SDF, stromal cell-derived factor; GRO, growth regulated protein; MCSF, Macrophage colony stimulating factor; Tpo, thrombopoietin; EGF, epidermal growth factor; OSM, oncostatin M; VEGF, Vascular endothelial growth factor; SCF, stem cell factor; IL, interleukin; TNF, tumor necrosis factor; Ang, angiogenin.

Discussion

ICAM-1 may be important in the pathology of IBD (7). Whether effects of fish oils - which protect against relapse rates in IBD patient on remission (6) - are mediated by ICAM-1, is however unknown. In addition, a direct side-by-side comparison of fish oils with AA - which may have proinflammatory effects (1) and is elevated in the intestinal mucosa of IBD patients (2, 3) - has never been made. The transcription factor NF- κ B is important in regulating intestinal inflammation and is elevated in IBD patients (17, 18). We have now shown that the n-3 fatty acid EPA, as compared to the n-6 fatty acid AA, clearly reduced cytokine stimulated NF- κ B activation and ICAM-1 expression in enterocytes. Remarkably, effects of OA on ICAM-1 expression were comparable to those of EPA. Because EPA and OA resulted in comparable proportions of AA in the phospholipids, the reported effects may be ascribed to the increased AA proportion in the AA cultured cells. Thus, decreasing cellular AA levels seems to be a crucial step, which could be achieved by increasing EPA or by increasing OA. However since OA is already the most abundant fatty acid present in our diet (15) and in the colon mucosa (3), decreasing mucosal AA levels may be easier by increasing fish oil intake than by increasing OA intake. It is tempting to suggest that high levels of NF- κ B activation in IBD patients (17) are caused by the high AA colonic contents in IBD patients and might be reversed by replacing AA for EPA. In line with our observations, n-6 PUFAs also increased NF- κ B activation as compared to n-3 PUFAs in monocytes (30) and macrophages (31).

Earlier *in vitro* studies have demonstrated that fish oils reduced cytokine stimulated ICAM-1 expression in endothelial cells (32) and monocytes (33) as compared to conditions without addition of fatty acids. Also *in vivo* ICAM-1 expression (surface and mRNA) on peritoneal macrophages was reduced in mice fed fish oils compared to that in mice fed coconut oil (34). In humans, dietary fish oil supplementation lowered expression of ICAM-1 on *ex vivo* stimulated monocytes as compared to no supplementation (35). However, our study is the first that examined effects of EPA versus AA on ICAM-1 expression in enterocytes. We used an approach of supplying different amounts of the fatty acid of interest (i.e. 130 μ M AA or 6 μ M EPA), while the total molarity of fatty acids supplied was similar in all experiments by adding OA. These different concentrations of AA and EPA were deliberately chosen because they were both four times the amount in which the cells grow normally (i.e. the fatty acid composition of normal complete culture medium). Using iso-molaric total concentrations of fatty acids is essential, because an increase in total fat can be immune suppressive (36). Therefore we used OA as a reference fatty acid to make total fatty acid concentrations between experimental fatty acid conditions iso-molaric, i.e. OA was exchanged for AA or EPA. In addition, we evaluated the condition of OA only. The results of this latter condition showed us that

decreasing AA levels in the mucosa seems to be more important than increasing EPA levels. The findings of our *in vitro* model that not only pharmacological agents (dexamethasone and sulfasalazine) known to be effective in IBD treatment lowered ICAM-1 expression, but also the fish oil EPA compared to AA suggest that a decreased ICAM-1 expression may explain the protective effects of fish oil and stresses the importance of lowering intestinal AA content.

Regarding the pathways underlying the anti-inflammatory effect of fish oils, several suggestions have been made. As compared to n-6 fatty acids, the n-3 PUFAs may have different effects on (1) signal transduction pathways, and (2) the types and levels of eicosanoids synthesized (1). Regarding the first mechanism, our finding that EPA lowered NF- κ B transactivation and protein ICAM-1 expression as compared to AA indeed showed that EPA and AA differently affected the NF- κ B signal transduction pathway. In this respect, two peroxisome proliferators-activated receptors (PPARs), PPAR α and PPAR γ , seem relevant. Fatty acids are natural ligands for these PPARs, which have anti-inflammatory effects by inhibiting NF- κ B activation. The expression of PPAR α and PPAR γ and possibly also their physiological role is different in various tissues. In human intestines, expression of PPAR γ seems to be higher than expression of PPAR α (37). However, in a direct comparison, Huin and colleagues showed that PPAR α expression in Caco-2 cells was higher than PPAR γ expression (38), while we found no differences between PPAR α and PPAR γ expression in differentiated Caco-2 cells. More importantly, PPAR γ (39-41) and PPAR α ligands (42) have been shown to be protective against colonic inflammation in different animal models. In humans, the PPAR γ ligand rosiglitazone may also have positive effects as suggested in an open-label single arm trial for UC patients (43). In our *in vitro* model the PPAR γ agonist troglitazone inhibited NF- κ B transactivation, and cytokine and net stimulated ICAM-1 expression, while the PPAR α agonist GW7647 only inhibited cytokine stimulated ICAM-1 expression. This suggests that both PPAR γ and PPAR α have anti-inflammatory effects within enterocytes, in which effects of PPAR γ seem more pronounced. This assumption is also in agreement with results from the reported effects that PPAR γ agonists but not a PPAR α agonist inhibited IL-8 expression by Caco-2 cells and HT29 cells (39). As mentioned before, fatty acids are natural PPAR ligands. Differences between fatty acids in activation of PPAR γ might explain the inhibition of cytokine stimulated NF- κ B transactivation by EPA as compared to AA in our reporter Caco-2 cells. This is, however, not supported by our findings as PPAR γ binding affinities of EPA and AA are about the same (44), while we used much lower EPA concentrations than AA concentrations. Secondly, not only EPA but also OA lowered intestinal ICAM-1 expression while OA is a poor PPAR γ ligand (44). Therefore, although PPAR γ activation certainly protects against inflammation, our results do not suggest that the protective effects of EPA and OA as compared to AA on intestinal

inflammation are PPAR γ -mediated. Therefore, there should be another explanation to explain why effects of OA and EPA were comparable but different from those of AA.

The mechanism underlying a possible second explanation relates to the incorporation of fatty acids into cell membrane phospholipids. Fatty acids in membrane phospholipids can be mobilized by phospholipase enzymes, and the obtained free FA can subsequently act as a substrate for cyclooxygenase (COX) and 5-lipoxygenase (5-LOX) enzymes for eicosanoids synthesis. Eicosanoids can modulate the intensity and duration of the inflammatory response. Replacement of AA by EPA in the culture medium of the cells resulted in a decrease in the proportion of AA in cell membranes and an increase in the EPA proportion, which will subsequently give rise to less serie-2 and serie-4 eicosanoids and more serie-3 and serie-5 eicosanoids. In general eicosanoids produced from EPA are less potent than those synthesized from AA, which could explain the anti-inflammatory effects of EPA compared to AA seen in *in vivo* experiments. Indeed the PGE₂ production of Caco-2 cells cultured with EPA was lower than PGE₂ production of cells cultured with AA. However PGE₂ production was not different between non-stimulated and stimulated cells, while stimulation resulted in an abundant elevation of NF- κ B transactivation. To our opinion this rules out the role of PGE₂ in the NF- κ B transactivation of AA and EPA. Moreover, although in our experiments indomethacin - a blocker of COX - indeed inhibited PGE₂ production, it did not lower NF- κ B transactivation. This is in line with findings of De Catherina *et al.* showing that in endothelial cells the effects of DHA on VCAM-1 expression could not be inhibited by indomethacin, although prostacyclin production was completely suppressed (32). Therefore, we fully agree that in an *in vivo* situation proinflammatory effects of AA are probably mediated by eicosanoids synthesized from AA, however these effects are merely derived from effects on attracting leucocytes, temperature set point regulation etc, and not by a direct eicosanoid mediated activation of NF- κ B as shown in our model.

Finally, we evaluated inflammatory protein signatures of Caco-2 cell culture medium after 3 weeks treatment with EPA versus AA. Since these signatures were analyzed in pooled material we cannot draw any conclusions based on statistical analysis. However, this array was intended to generate hypotheses about differences in immune modulating effect of AA versus EPA based on the protein expression profiles. These profiles indicate, that both fatty acids induced specific changes in various inflammation mediators. Interestingly most - but not all - of these proteins are regulated by NF- κ B. For example, IL-6 protein expression was higher after EPA as compared to AA, whereas NF- κ B activation was higher after AA. It should however be considered that although highly important, NF- κ B is not the only transcription factor involved in the regulation of IL-6 and other inflammatory proteins. Thus, expression of individual proteins cannot be predicted by NF- κ B activation solely. Besides this, we found clear

differences between EPA and AA, i.e. that some proteins were expressed higher after EPA (IL-6, IL-10, MIP-1 δ and GRO), while others were higher expressed after AA (MCP-1, and angiogenin). The precise role of these individual proteins in the process of intestinal inflammation is not clear. However, the increased MCP-1 expression after AA treatment is in line with the finding that MCP-1 expression is upregulated in the mucosa of IBD patients and correlates with disease activity (45). The increased expression of the Th2 cytokine IL-10 after EPA treatment fits with the anti-inflammatory role for IL-10 in IBD pathology. In this respect, IL-10 knockout mice develop chronic intestinal inflammation and delivery of recombinant IL-10 to the intestinal mucosa by the bacterium *L. Lactis* attenuated mucosal inflammation in two mouse models (46). In humans, a pilot study using these bacteria showed also promising effects (47).

In conclusion, we showed proinflammatory effects of AA as compared to EPA or OA on ICAM-1 cell surface protein expression in enterocytes, which can be explained by an elevated NF- κ B transactivation. Our results might give an explanation for the elevated inflammatory activity and elevated NF- κ B activation in intestinal mucosa of IBD patients, who are characterized by increased mucosal AA proportions. Finally, we hypothesize that the anti-inflammatory effects of lowering AA proportions in membranes by EPA or OA is not related to PPAR γ activation or eicosanoid formation. This illustrates that there is an open question regarding the mechanism underlying the protective effects of fish oils in NF- κ B activation and consequent inflammatory bowel disease.

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Chapter 4

An arachidonic acid-enriched diet does not result in more colonic inflammation as compared to fish oil- or oleic acid-enriched diets in mice with experimental colitis

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Abstract

Fish oils (FO) - rich in EPA and DHA - may protect against colitis development. Moreover, inflammatory bowel disease (IBD) patients have elevated colonic arachidonic acid (AA) proportions. So far, effects of dietary AA vs FO on colitis have never been examined. We therefore designed 3 iso-energetic diets, which were fed to female wild-type C57BL/6 mice for 6 weeks preceding and during 7 days dextran sodium sulphate (DSS) colitis induction. The control diet was rich in oleic acid (OA). For the other two diets, 1.0% (wt/wt) OA was exchanged for EPA + DHA (FO group) or AA. After 7 days DSS (1.5% wt/v) colitis-induction, the AA group had gained weight ($0.46 \pm 0.54\text{g}$), whereas the FO and OA groups had lost weight ($-0.98 \pm 0.81\text{g}$ and $-0.79 \pm 1.05\text{g}$, respectively; $P < 0.01$ vs AA). The AA group had less diarrhea than the FO and OA groups ($P < 0.05$). Weight and length of the colon, histological scores and cytokine concentrations in colon homogenates showed no differences. Myeloperoxidase (MPO) concentrations in plasma and polymorphonuclear cells infiltration in colon were decreased in the FO group as compared to the OA group. We conclude that in mice an AA-enriched diet consumed 6 weeks preceding DSS-colitis induction increased colonic AA content, but did not result in more colonic inflammation as compared to FO- and OA-enriched diets. These results contrast other animal studies showing protective effects of FO as compared to linoleic acid (LA)-rich diets, which however did not increase colonic AA contents. Future studies should elucidate if dietary LA and AA have differential effects on colitis development.

Introduction

Epidemiological studies have shown a low incidence of inflammatory bowel disease (IBD) in Eskimo's as compared to West-European populations. These findings may be related to their high intakes of n-3 fish oil PUFA, which may have antiinflammatory effects as compared to in particular n-6 PUFA (1). Interestingly, other studies have found that the fatty acid composition of phospholipids from the colonic mucosa of IBD patients contained more of the n-6 PUFA arachidonic acid (AA) as compared to colonic mucosa of control subjects (2-4). By increasing the intake of fish oil, the n-6 PUFA arachidonic acid in the colon mucosa is replaced by the n-3 fish oil PUFA eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (5). Furthermore, in an *in vitro* study we have recently shown that culturing enterocytes with AA increased inflammatory parameters as compared to enterocytes cultured with EPA or OA (6). Therefore, it is tempting to suggest that the antiinflammatory effects of fish oils are caused by a reduction of the AA content. Alternatively, fish oils may have their own intrinsic antiinflammatory effects. Several dietary intervention studies have indeed shown that supplementation of n-3 fish oils have beneficial effects in IBD patients (5, 7-16). Although some studies showed rather impressive effects (5, 7), it should be noted however that not all clinical studies were that positive (8-16). Based on these intervention studies, the overall conclusion was that fish oil supplementation may have at least minor protective effects (1). Also in various mouse and rat IBD intervention studies fish oil has positive effects (17-23), whereas some studies did not find protective effects (24, 25). Unfortunately, due to the composition of the diets these studies could not evaluate specifically effects of fish oils versus those of AA.

Therefore, the aim of the present study was to examine the *in vivo* effects of fish oil (FO) versus AA on intestinal inflammation. A diet enriched with the n-9 MUFA oleic acid (OA) was used as control. The OA of the control diet was iso-energetic exchanged for the n-3 PUFA of FO or the n-6 PUFA AA. These diets were fed for 6 weeks to female C57BL/6 mice. At the end of this period, dextran sodium sulphate (DSS) colitis was induced for 7 days and the severity of colonic inflammation and related parameters were evaluated.

Materials and methods

Animals, diets and experimental design

Experiments were approved by the Animal Studies Ethics Committee of the University of Amsterdam, The Netherlands. Thirty four-weeks old female wild-type C57BL/6 mice were obtained from Charles River (Horst, The Netherlands). The mice were housed under standard conditions (AM-III 15 mm, Hope Farms, Woerden, The Netherlands) and had free access to water and food. The mice were randomly assigned to 3 groups of ten mice, which received one of the 3 diets for 6 weeks before colitis induction. The diets were also fed during the week of DSS colitis induction. All three diets contained the same amounts of protein, carbohydrates and fat providing respectively 21 En%, 66 En% and 14 En%. Of this 14 En% or 6.0% (wt/wt), 8 En% (or 3.4% wt/wt) was part of the standard (CRM(E)) chow, while 6 En% (or 2.6% wt/wt) was specifically added as enrichment. Thus, the only difference between the diets was the source of fat added to the chow. The fish oil (FO) group received normal chow enriched with fish oils (2.6% wt/wt) (Loders Crocklaan, Wormerveer, The Netherlands). Of this 2.6% (wt/wt), 1.0% (wt/wt) was EPA plus DHA with an EPA to DHA ratio of 1.4) (**Table 4.1**). The arachidonic acid (AA) group received the same chow but now enriched with arachidonic ethyl ester oil (Nu-Chek-Prep, Elysian, MN) (1.1% wt/wt). Of this 1.1% (wt/wt), 1.0% (wt/wt) was the arachidonic ethyl ester. In addition, cacao butter (1.5% wt/wt) (SDS, Essex, UK) was added to the AA diet. In this way, in total 2.6% (wt/wt) fat was added to the chow, which made total fat intake equal to that of the FO diet. The control (OA) group also consumed the normal chow, but now enriched with the same iso-energetic amount (2.6% wt/wt) of fatty acids from cacao butter (1.2% wt/wt) plus high oleic sunflower oil (1.4% wt/wt) (Loders Crocklaan, The Netherlands). All diets were enriched with a tocopherol blend (3 mg tocopherols per g fat; Loders Crocklaan). All diets were made by Special Diet Services (SDS, Essex, UK) and irradiated at 25 kGy. The fatty acid composition of the diets is shown in table 4.1. As can be seen, the main difference between the diets is the proportion of OA which was replaced by EPA + DHA or AA.

Table 4.1: Fatty acid composition of the fish oil (FO), arachidonic acid (AA) and oleic acid (OA) diets (g/100g diet).

Fatty acid	Diet		
	FO	AA	OA
C14:0	0.15	0.02	0.02
C16:0	0.91	0.97	0.93
C18:0	0.19	0.60	0.62
C16:1 n-7	0.13	0.02	0.02
C18:1 n-7	0.10	0.06	0.07
C18:1 n-9	0.85	1.15	2.04
C20:1 n-9	0.07	0.04	0.05
C18:2 n-6	1.94	1.96	1.91
C20:4 n-6	0.04	0.78	<0.01
C18:3 n-3	0.22	0.20	0.19
C20:5 n-3	0.58	0.02	0.02
C22:6 n-3	0.41	0.05	0.04
Others	0.43	0.14	0.09
Σ SAFA	1.32	1.64	1.64
Σ MUFA	1.26	1.28	2.17
Σ PUFA	3.42	3.08	2.18
Σ n-9	0.96	1.20	2.09
Σ n-6	2.02	2.78	1.92
Σ n-3	1.39	0.29	0.26
Ratio n-6/n-3	1.5	9.6	7.4

Σ is SUM of all fatty acids belonging that particular class.

Abbreviations: MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SAFA, saturated fatty acids.

Body weights of the mice were recorded every third day during the first 6 weeks. One mouse of the FO group died in week 4 because of an elephant tooth. After 6 weeks on the experimental diets, colitis was induced in the 10 weeks old mice by administration of 1.5% (wt/v) dextran sulphate sodium (DSS; MW 40 kDa; TdB Consultancy, Uppsala, Sweden) to the drinking water for one week. During the week of colitis induction, body weights of the mice were recorded daily. After 7 days DSS treatment, all mice were anaesthetized with fentanyl-fluanisone-midazolam (FFM) and sacrificed via a cardiac puncture and exsanguinations. Blood was sampled in EDTA tubes (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ) and plasma was obtained by centrifugation at 2,000 *g* for 20 minutes at 4 °C and stored at -80 °C until analysis for myeloperoxidase (MPO) and serum amyloid P component (SAP) concentrations. Through a midline incision, the colons were removed. First, the total length was measured as an indicator of disease-related colon shortening. In addition, fecal material was removed and collected to score diarrhea severity; i.e. 0: normal feces, 1: loose stool, 2: watery diarrhea, 3: slimy diarrhea, little blood and 4: very severe diarrhea. Next, the colons were opened longitudinally

and the wet weight of the distal 6 cm was recorded and used as an index of disease-related intestinal wall thickening. Subsequently, the colons were divided longitudinally into two parts and both parts were rolled up. These samples were frozen in liquid nitrogen and stored at -80 °C until analysis. For all 29 mice, one role was used for histological analysis. In 5 randomly selected mice per group, one half of the other role was used for cytokine detection, while the other half of this role was used for analysis of intestinal fatty acid composition.

Fatty acid composition of the diets and colon homogenates

Fatty acid compositions of the diets and colon samples were analyzed by gas chromatography. Extraction and analysis procedures have been described previously. (26) Briefly, total lipids were extracted from about 50 mg diet and 7 mg dried-frozen colon samples according to the method of Bligh and Dyer. (27) Aminopropyl-bonded silica columns (Varian, Harbor City, CA) were used to separate phospholipids from the total lipid extract. (28) The phospholipids were then saponified, and the resultant fatty acids were methylated into their corresponding fatty acid methyl esters (FAMES). (29) Fatty acids were separated on an Autosystem (Perkin-Elmer, Norwalk, CT) gas chromatograph that was fitted with a silica-gel column (Cp-sil 88 for FAME, 50 m x 0.25 mm, 0.2- μ m film thickness; Chrompack, Middelburg, The Netherlands) with helium gas (130 kPa) as the carrier gas. Both the injection and detection temperatures were set at 300 °C. The starting temperature of the column was 160 °C. Ten minutes after injection, the temperature was increased up to 190 °C at a rate of 2.5 °C/min. After 20 min at 190 °C, the temperature was increased up to 230 °C at a rate of 4 °C/min. The final temperature of 230 °C was maintained for 10 min. Data were analyzed by using CHROMCARD software (version 1.21; CE Instruments, Milan, Italy). The fatty acid compositions are expressed in relative amounts (% of total fatty acids identified; wt/wt).

Histological analysis

The longitudinally divided rolled-up parts of the colon, which were used for routine histology, were directly fixed in 4% formaldehyde and embedded in paraffin. Three transverse slices (5 μ m), taken from each colonic sample, were stained with hematoxylin-eosin and examined by light microscopy. Colonic inflammation was evaluated in a blind manner by estimating the 1) percentage of area involved, 2) the amount of follicles, 3) edema, 4) erosion/ulceration, 5) crypt loss and infiltration of 6) polymorphonuclear cells and 7) mononuclear cells. The percentage of area involved, erosion/ulceration and the crypt loss was scored on a scale ranging from 0 to 4 as follows: 0: normal, 1: less than 10%, 2: 10-25%, 3: 25-50%, 4: more than 50%.

Follicle aggregates were counted and scored as follows: 0: 0-1 follicles, 1: 2-3 follicles, 2: 4-5 follicles, 3: 6 follicles or more. The severity of the other parameters was scored on a scale 0 to 3 as follows: 0: absent, 1: weak, 2: moderate, 3: severe. All scores on the individual parameters together could result in a total score ranging from 0 to 24.

Homogenisation and cytometric bead array (CBA)

For analysis of cytokine concentrations, homogenates were made from colon samples with a tissue homogenizer in 9 volumes (wt/v) Greenberger lysis buffer (300 mmol/L NaCl, 15 mmol/L Tris, 2 mmol/L $MgCl_2$, 2 mmol/L Triton X-100 (Sigma, St. Louis, MO), pepstatin A, leupeptin, aprotinin (Roche, Mannheim, Germany), all 20 ng/mL; pH 7.4). The colon homogenates were lysed in the Greenberger lysis buffer for one hour on ice and centrifuged for 7 minutes at 3,000 rpm and for 10 min at 14,000 rpm. The supernatant was collected and stored at -80 °C until cytokine analysis using a cytometric bead array. The mouse inflammation and Th1/Th2 cytometric bead arrays (CBA) (Becton Dickinson Biosciences, San Diego, CA) were used in colon homogenates to determine simultaneously the concentrations of IL-2, IL-4, IL-6, IL-10, IL-12p70, MCP-1, $IFN\gamma$ and $TNF\alpha$ according to the instructions of the manufacturer. Briefly, 5 μ L of sample or the cytokine standard mixture was mixed with 5 μ L of the mixed capture beads and 5 μ L of the detection antibody-phycoerythrin (PE) reagent and incubated at room temperature for 2 hours in the dark. Two-colour flow cytometric analysis was performed using a FACScan® flow cytometer (Becton Dickinson, Immunocytometry Systems (BDIS), San Jose, CA). Data were acquired and analyzed using Becton Dickinson Cytometric Bead Array (CBA) software.

Myeloperoxidase (MPO) in colon homogenates and plasma

To quantify the extent of colonic neutrophil accumulation and systemic neutrophil amounts, MPO concentrations in colon homogenates and EDTA plasma were analyzed by ELISA (HBT, Uden, The Netherlands). All samples of all groups were analyzed on one plate. The detection limit of the MPO ELISA was 1.02 ng/mL. The intra-assay variation was less than 7.5%.

Serum amyloid P component (SAP) in plasma

To evaluate the acute phase reaction activity, SAP concentrations were analyzed in EDTA plasma by ELISA, as described (30). Briefly, a 96 well plate (Greiner Bio-one, Frickenhausen, Germany) was coated with 3 $\mu\text{g/mL}$ sheep-anti-mouse SAP (Calbiochem, San Diego, CA) antibodies. Immobilized SAP was detected using a specific biotinylated rabbit-anti-mouse SAP antibody (Calbiochem), followed by the addition of peroxidase-conjugated streptavidin (Zymed Laboratories, San Francisco, CA) and tetramethylbenzidine (TMB) substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD). All samples of all groups were analyzed on one plate. The detection limit of the SAP ELISA was 5 ng/mL. The intra-assay variation was less than 5%.

Statistics

All data, except body weights (means $\pm$ SEM), are expressed as dot plots in which the median is indicated. Differences between the three groups (AA vs FO vs OA) were analyzed using the Kruskal Wallis test. If a significant difference was found ($P < 0.05$) the Mann-Whitney U test was used as a post-hoc test. If the Mann-Whitney post-hoc test was performed, a $P < 0.017$ was considered as statistically significant. Weight changes in time were tested by one-way ANOVA ($P < 0.05$) with a Bonferonni post-hoc test when differences between interventions were significantly different. Correlation analyses were performed using the Spearman correlation test. Values of $P < 0.05$ were considered statistically significant. SPSS 10 for Macintosh (SPSS, Chicago, IL) was used for the analysis.

Results

Fatty acid composition of the diets and colon homogenates

Table 4.1 (pag. 79) showed that the fatty acid compositions of the three diets were as anticipated. The OA diet contained more MUFA and less PUFA than the other two diets. Further, the total amounts of MUFA and PUFA were not different between the AA and FO diets. However, the AA diet provided more of the n-6 PUFA AA, while the FO diet contained more of the n-3 PUFA EPA and DHA. This resulted in a higher n-6/n-3 ratio in the AA diet than in the FO diet. The only other difference was a slightly lower SAFA content in the FO diet as compared to the OA and AA diets. **Table 4.2** shows that differences in fatty acid composition of the colons between groups paralleled those of the diets.

Table 4.2: Fatty acid composition of phospholipids in the colon from DSS colitis mice fed a fish oil (FO) diet, arachidonic acid (AA) diet and oleic acid (OA) diet (% of total fatty acids).

Fatty acid	Diet		
	FO	AA	OA
C16:0	22.3 ± 0.2 ^b	21.7 ± 0.6 ^{ab}	20.5 ± 1.5 ^a
C18:0	15.2 ± 0.1 ^a	15.8 ± 0.8 ^a	15.7 ± 1.0 ^a
C22:0	1.1 ± 0.1 ^a	1.1 ± 0.1 ^a	1.0 ± 0.1 ^a
C24:0	1.2 ± 0.2 ^b	1.0 ± 0.1 ^{ab}	0.9 ± 0.0 ^a
C16:1 n-7	1.3 ± 0.2 ^a	1.1 ± 0.4 ^a	1.0 ± 0.4 ^a
C18:1 n-7	2.3 ± 0.1 ^a	2.4 ± 0.5 ^a	2.4 ± 0.4 ^a
C18:1 n-9	10.7 ± 0.7 ^c	9.1 ± 0.6 ^b	12.8 ± 0.7 ^a
C24:1 n-9	1.6 ± 0.3 ^a	1.4 ± 0.2 ^a	1.8 ± 0.2 ^a
C18:2 n-6	10.5 ± 0.8 ^c	4.3 ± 1.0 ^b	8.8 ± 0.6 ^a
C20:3 n-6	2.3 ± 0.3 ^{ab}	1.9 ± 0.3 ^a	2.8 ± 0.4 ^b
C20:4 n-6	8.9 ± 0.2 ^c	23.6 ± 0.6 ^b	15.8 ± 1.4 ^a
C22:4 n-6	0.7 ± 0.1 ^c	4.1 ± 0.3 ^b	2.4 ± 0.3 ^a
C22:5 n-6	0.2 ± 0.0 ^c	1.0 ± 0.1 ^b	0.4 ± 0.0 ^a
C20:5 n-3	4.4 ± 0.6 ^b	0.3 ± 0.1 ^a	0.6 ± 0.2 ^a
C22:5 n-3	2.8 ± 0.4 ^c	0.7 ± 0.1 ^b	0.9 ± 0.1 ^a
C22:6 n-3	9.8 ± 0.6 ^c	5.4 ± 0.3 ^b	7.1 ± 0.6 ^a
Others	5.0 ± 0.2 ^a	5.3 ± 0.4 ^a	5.1 ± 0.6 ^a
Σ SAFA	41.9 ± 0.4 ^a	41.7 ± 0.6 ^a	40.0 ± 1.0 ^b
Σ MUFA	16.9 ± 0.5 ^a	15.4 ± 1.1 ^a	19.3 ± 1.3 ^b
Σ PUFA	41.1 ± 0.6 ^a	42.9 ± 0.7 ^a	40.7 ± 1.6 ^a
Σ n-9	12.8 ± 0.6 ^c	11.3 ± 0.5 ^b	15.5 ± 0.6 ^a
Σ n-6	23.8 ± 0.8 ^c	35.9 ± 0.9 ^b	31.5 ± 1.7 ^a
Σ n-3	17.1 ± 0.4 ^c	6.6 ± 0.3 ^b	9.0 ± 0.5 ^a
Ratio n-6/n-3	1.4 ± 0.1 ^c	5.4 ± 0.4 ^b	3.5 ± 0.3 ^a

Values are means ± SD of 5 mice per diet group.

Σ is SUM of all fatty acids belonging that particular class.

Means for a variable in a row with superscripts without a common letter differ; $P < 0.017$ (Post hoc Mann Whitney test with Bonferroni correction). Abbreviations see table 4.1.

In the AA group, the proportions of n-6 PUFA (C20:4 and longer) were increased, whereas the proportion of C18:2 n-6 was decreased compared to the OA and FO groups. Moreover, the n-3 PUFA in the AA group were decreased, which resulted in an increased n-6/n-3 ratio compared to the OA and FO group. In the FO group, the proportions of n-3 PUFA were increased, while those of n-6 PUFA were decreased compared to the OA and AA groups. This resulted in a decreased n-6/n-3 ratio as compared to the OA and AA groups.

Body weight before and after DSS colitis induction.

During the 6 weeks preceding colitis induction weight changes were not significantly different between the three groups ($P = 0.290$) (**Figure 4.1A**).

After 7 days DSS treatment, both the FO and the OA fed mice had lost weight ($-0.98 \pm 0.81\text{g}$, or -4% of their initial weight at DSS induction for the FO group and $-0.79 \pm 1.05\text{g}$, or -4% for the OA group) (**Figure 4.1B**). In contrast, weight of the AA fed mice first decreased until day 5, but was slightly increased after 7 days ($0.46 \pm 0.54\text{g}$, or 2% of their initial weight at DSS induction). At day 5 the AA fed mice had lost significantly more weight than the FO and OA fed mice ($P < 0.001$), but at day 7 the AA group had gained significantly more weight than the FO ($P = 0.002$) and control groups ($P = 0.007$).

Diarrhea

Seven days after the induction of DSS colitis, diarrhea of the mice was scored (**Figure 4.1C**). In agreement with the increase in body weight of the AA fed mice, these mice had less diarrhea (median score 1.5) than the FO (score 3.0, $P = 0.002$) and OA (score 2.5, $P = 0.029$) fed mice. Body weight changes correlated negatively with diarrhea score ($r = -0.574$, $P = 0.001$), indicating that a higher diarrhea score was associated with more body weight loss.

Length and weight of the colon

After sacrificing the mice, the total length and the weight of the last six centimeters of the colon were determined and used as indicators of disease-related colon shortening and intestinal wall-thickening respectively. Although body weight changes correlated with colon length ($r = 0.461$, $P = 0.012$), colonic lengths of the mice were however not significantly different between the groups ($P = 0.497$) (**Figure 4.1D**). Colonic weights were also comparable ($P = 0.138$) (**Figure 4.1E**). As a reference, the approximate colonic weight and length of non-diseased C57BL/6 mice on a standard chow diet is around 140 mg and 7.5 cm, respectively.

Figure 4.1: Effects of a fish oil (FO), arachidonic acid (AA) and oleic acid (OA) diet on dextran sodium sulphate (DSS) colitis disease severity.

Ten female mice consumed a FO enriched diet, an AA enriched diet or an OA enriched diet for 43 days prior to DSS colitis induction. Colitis was induced by 1.5% (wt/v) DSS in the drinking water for 7 days. Body weight was recorded prior to colitis induction (A) and after colitis induction as a measure of disease activity (B). Furthermore disease severity was measured at 7 days after DSS colitis induction by a diarrhea score (C), colon length (D), and colon weight of last 6 cm (E). * $P < 0.05$

Histology

Colonic inflammation was also evaluated by histological analysis. Irrespective of the diet consumed, all mice showed common pathologic features as crypt loss, ulceration of the mucosa, edema, and granulocyte and mononuclear cell infiltration in the mucosa. There were no significant differences in total colitis scores between the three diet groups (FO score 11.5, AA score 13.0, OA score 12.3; $P = 0.854$) (**Table 4.3**). Also when the scores of the individual parameters were examined, no significant differences between the groups could be found (Table 4.3). The most interesting difference was the polymorphonuclear cell infiltration (score 1.0) in the FO group, which tended to be lower ($P = 0.047$) as compared to the OA group (score 1.75), whereas the differences between the other groups were not significantly different (Table 4.3).

Table 4.3: Histology scores of DSS colitis mice fed a fish oil (FO) diet, arachidonic acid (AA) diet and oleic acid (OA) diet.

Histologic parameter	Diet		
	FO (n = 9)	AA (n = 10)	OA (n = 10)
Total score	11.5 (8.0-16.0)	13.0 (3.5-14.5)	12.3 (8.0-16.0)
Area involved ^a	4.0 (3.0-4.0)	3.0 (1.0-4.0)	4.0 (3.0-4.0)
Follicle aggregates ^b	0.0 (0.0-2.0)	1.0 (0.0-2.0)	0.0 (0.0-2.0)
Edema ^b	2.0 (1.0-2.0)	1.8 (0.5-2.0)	1.8 (1.0-2.0)
Ulceration ^a	1.0 (0.0-2.0)	1.0 (0.0-2.0)	1.0 (1.0-3.0)
Crypt loss ^a	2.0 (1.0-3.0)	1.0 (1.0-2.0)	2.0 (1.0-3.0)
Polymorphonuclear cells ^b	1.0* (0.0-2.0)	1.5 (0.5-2.0)	1.8 (0.5-2.5)
Mononuclear cells ^b	1.5 (1.0-2.0)	1.8 (0.5-2.0)	1.5 (1.0-2.0)

^a Median scores (min-max) on a scale of 0-4; 0 = normal, 1 = less than 10%, 2 = 10-25%, 3 = 25-50%, 4 = more than 50%.

^b Median scores (min-max) on a scale of 0-3; 0 = absent, 1 = weak, 2 = moderate, 3 = severe.

* $P = 0.047$ versus OA group.

Cytokines, MPO and SAP concentrations in colon homogenates and/or plasma

The concentrations of eight different cytokines in colon homogenates were analyzed by a cytometric bead array to evaluate the extent and characteristics of the local colonic inflammation. However, the colonic concentrations of IL-12p70, TNF α , IFN γ , MCP-1, IL-10, IL-6, IL-4 and IL-2 were not significantly different between the three groups (Table 4.4).

Table 4.4: Cytokine concentrations in colon homogenates of mice fed a fish oil (FO) diet, arachidonic acid (AA) diet and oleic acid (OA) diet.

Cytokines	Diet		
	FO (n = 5)	AA (n = 5)	OA (n = 5)
IL-12p70	8.4 (6.5-14.5)	9.4 (5.6-11.8)	10.0 (9.1-17.2)
TNF α	53 (31-94)	54 (25-161)	84 (41-229)
IFN γ	11.2 (3.3-11.3)	7.0 (1.7-12.8)	8.0 (3.4-37.2)
MCP-1	693 (220-2086)	510 (185-603)	477 (391-950)
IL-10	8.3 (0.0-20.4)	7.3 (0.0-11.5)	9.6 (7.5-17.8)
IL-6	750 (145-2378)	374 (48-585)	390 (96-1327)
IL-4	2.5 (0.0-3.4)	2.2 (0.0-5.3)	0.0 (0.0-5.0)
IL-2	2.7 (2.0-6.8)	2.0 (1.5-3.4)	2.8 (2.1-6.3)

Concentrations in pg/mL.

Values are medians (min-max) for 5 mice per diet group.

Abbreviations: IFN, interferon; IL, interleukin; MCP, monocyte chemotactic protein; TNF, tumor necrosis factor.

To determine colonic neutrophil accumulation and systemic neutrophil levels, MPO concentrations were analyzed in colon homogenates and in plasma. Although differences in plasma MPO concentration were small, the MPO concentration was significantly lower in the FO group compared to the OA group ($P = 0.010$) (**Figure 4.2A**). However, in colon homogenates no significant differences in MPO concentrations between the three groups were found (**Figure 4.2B**). MPO concentrations in colon homogenates correlated with IL-12p70 ($r = 0.546$, $P = 0.035$), IFN γ ($r = 0.712$, $P = 0.003$) and MCP-1 ($r = 0.743$, $P = 0.002$) concentrations in these homogenates.

To determine the extent of the acute phase response, we analyzed plasma SAP concentrations. SAP concentrations did not differ between the three groups ($P = 0.121$) (**Figure 4.2C**). SAP concentrations correlated with $\text{TNF}\alpha$ ($r = 0.611$; $P = 0.016$) and $\text{IFN}\gamma$ ($r = 0.522$; $P = 0.046$) concentrations in colon homogenates.

Figure 4.2: Effects of a fish oil (FO), arachidonic acid (AA) and oleic acid (OA) diet on myeloperoxidase (MPO) concentrations in colon homogenates and plasma and serum amyloid P component (SAP) concentrations in plasma.

Ten female mice consumed a FO enriched diet, an AA enriched diet or an OA enriched diet for 43 days prior to DSS colitis induction. Colitis was induced by 1.5% (wt/v) DSS in the drinking water for 7 days. MPO concentrations were determined in colon homogenates (A) and in plasma (B) and SAP concentrations were determined in plasma (C) 7 days after colitis induction.

Discussion

In the present study we have shown that 6 weeks consumption of an AA-enriched diet versus a FO- or OA-enriched diet preceding DSS colitis induction in mice did not result in more pronounced colonic inflammation. In fact, after 7 days, weight loss and diarrhea score were less in the AA group as compared to the FO and OA groups. Thus, increasing AA intake was not harmful, but may even have been protective in this DSS-induced colitis model. This finding is at least surprising since AA is considered as a fatty acid with proinflammatory characteristics, at least when compared to the n-3 fatty acid from fish oils (1). However, there are also indications that prostaglandin E₂ (PGE₂), which is produced from AA, has antiinflammatory properties (31, 32). In line with this, cyclooxygenase (COX)-2 inhibitors - although having much more effects than only lowering PGE₂ production - indeed worsen colitis disease activity (33). Therefore, both AA as well as fish oils may have protective effects, but achieved by different mechanisms.

Although weight loss at 7 days after colitis induction suggested protective effects of AA, at 5 days after colitis induction weight loss in the AA group was significant larger than in the FO and OA groups. Thus the time point of evaluating the effects of the diets seems essential and in future studies different time points should be evaluated. Moreover, although in our study some parameters showed protective effects of AA supplementation, all other inflammatory parameters, i.e. weight and length of the colon, histological scores and cytokine concentrations in colon homogenates, showed no differences between the groups at 7 days after DSS colitis induction. Also no protective effects of fish oil compared to OA were shown. Only MPO concentrations in plasma and polymorphonuclear cells infiltration in the colon were slightly decreased in the FO group as compared to the OA group. This could suggest that the protective effects of FO had just started and FO might have more protective effects on later time points (i.e. > 7 days), e.g. in the resolution phase of colitis. This has also been suggested by the production of resolvins and protectins, generated from fish oil fatty acids during the resolution phase of inflammation (34). In contrast, the effects of AA seems most pronounced in the early stage (day 7), e.g. in the induction phase. Thus comparing effects of AA versus FO both on earlier and later time points after colitis induction should be evaluated in future studies.

In the next paragraph, we will compare our results to findings from other intervention studies in animals. Several aspects will be highlighted, i.e. effects of fish oil vs n-6 PUFA (AA and/or linoleic acid (LA)), effects of fish oil vs OA and whether effects might depend on the time of supplementation or might be specific for the type of model used. In various mouse and rat IBD intervention studies fish oil has positive effects (17-23), whereas some studies did not find protective effects (24, 25). In several of the animal studies that showed

protective effects, effects of fish oil were compared versus those of the n-6 fatty acid linoleic acid (LA; C18:2 n-6), while we made a comparison between fish oil and AA on colitis development. Although dietary LA can be converted into AA, the only two colitis dietary intervention studies that measured colonic fatty acid composition showed no differences in colonic contents of AA between the LA rich and FO rich diets (17, 18). Also studies that did not have colitis as end point, but examined tissue fatty acid incorporation showed only small effects of LA supplementation on AA incorporation into various tissues. (35-37) In line with our findings, however, other studies showed that increasing dietary AA resulted in pronounced increases in tissue AA contents (37-39) and that n-3 PUFA supplementation reduced tissue AA contents (37, 40, 41). These findings support our approach of using dietary AA itself instead of n-6 precursor PUFA to compare effects of AA and fish oils on colitis development. Furthermore, we showed an increase in colonic LA content in the FO group versus the AA and OA group, whereas the two other studies found no change or a decrease in colonic LA content after fish-oil supplementation. (17, 18) To summarize, the two studies that used a LA rich control diet and showed no differences in colonic contents of AA, showed protective effects of fish oil (17, 18). However, in line with our results, Camuesco *et al.* (18) did not show a protective effect of fish oil versus an OA rich olive oil diet. Whether the differences in effect on colic fatty acid composition can explain the possible differential effects between fish oil studies remains to be determined. However, also several other differences in the methodology of these two studies compared to our study could explain the differences in outcome. In our study colitis was induced in mice by DSS, whereas Nieto *et al.* (17) induced experimental colitis in rats by TNBS. Furthermore, they supplied the different diets after colitis induction (therapeutic approach), whereas we supplied the different diets for a period of 6 wk preceding colitis induction (preventive approach). Camuesco *et al.* (18) started - in line with our approach - experimental diets before DSS colitis induction in rats, but only 2 weeks instead of 6 weeks. Thus, it is also possible that results depend on the time of supplementation or the type of model used. That the model may be important can also be concluded from inconsistent effects of FO versus control diets in various mice studies. Protective effects in a CD45RB^{hi} T-cell transfer model (22), no effects in a DSS-colitis model (24), or even increased colitis in an IL-10 knockout chronic colitis model (25) have been reported.

Despite the fact that AA seemed protective against colitis as compared to FO and OA, no differences were seen on colonic cytokine concentrations, while MPO concentrations in plasma were lowered in FO fed animals versus OA. Although we showed no effects on colonic MPO, three other studies showed a decrease in MPO activity in the colon after FO supplementation, but in these studies FO was also protective on major (pathological) parameters (17, 18, 20). Although suppression of proinflammatory cytokine production has been

suggested to contribute to the protective effects of FO (42), only two other studies measured cytokine concentrations after FO supplementation in colitis models and showed a decrease, whereas we showed no effects. Thus, MPO seems a sensitive marker for FO effects, whereas effects on cytokines are less consistent and need more attention.

In our study, AA was supplied as ethyl ester while OA, and EPA and DHA were supplied as triglycerides. Although ethyl esters may be less efficiently absorbed in the intestine as compared to triglycerides (43-45), the fatty acid composition of the colon clearly represented the fatty acid composition of the diets, which suggests appropriate intestinal AA absorption. Moreover, many fish oil capsules that have been successfully used in human studies (e.g. in the GISSI-trial) contained EPA and DHA as ethyl esters (46). Therefore, AA being supplied as ethyl esters is not an explanation for the results observed.

In conclusion, we have shown that an AA-enriched diet consumed 6 weeks preceding colitis induction increased colonic AA content, but did not result in more colonic inflammation. The AA-enriched diet may even have been protective in a DSS induced colitis model as shown by a decreased weight-loss and lower diarrhea scores as compared to FO- or OA-enriched diets 7 days after colitis induction. This result seems in contrast to earlier studies that compared effects of LA-rich diets versus FO. However, in contrast to our study, these studies showed no differences in colonic AA contents. Future studies should elucidate if dietary LA and AA have differential effects on colitis development.

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Chapter 5

The PPAR γ agonist rosiglitazone impairs colonic inflammation in mice with experimental colitis

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Abstract

Various animal models showed that peroxisome proliferator-activated receptor (PPAR) γ agonists, when given as a gavage shortly preceding colitis induction, protect against IBD. We have now examined the effects of 16 days rosiglitazone treatment via the diet prior to dextran sodium sulphate (DSS)-induced colitis in mice. After 7 days DSS in the drinking water rosiglitazone fed mice had lost significantly more weight than control mice. Rosiglitazone treated mice had more diarrhea, weight of colon and spleen were increased, and length of colon was decreased. Histology showed that rosiglitazone treated mice had more severe colitis, mainly caused by more ulceration, crypt loss and edema. Immunofluorescence showed a loss of tight junction structure Zonula Occludens protein 1 (ZO-1) in colons of rosiglitazone treated mice as compared to control mice. Also, serum amyloid P component (SAP) concentrations in plasma were increased. However, concentrations of tumor necrosis factor (TNF) α and interferon (IFN) γ in colon homogenates, and TNF α in spleen homogenates were significantly decreased, whereas interleukin (IL)-10 in spleen homogenates was increased. Other cytokines (IL-2, IL-4, IL-6, IL-12p70 and monocyte chemotactic protein (MCP)-1) and myeloperoxidase (MPO) concentrations showed no differences. In conclusion, 16 days pretreatment with rosiglitazone impaired DSS-induced colitis in mice.

Introduction

Peroxisome proliferator-activated receptor (PPAR) γ is a nuclear hormone receptor controlling the expression of numerous genes, among others those involved in inflammation (1). PPAR γ activation dampens the inflammatory response by decreasing NF- κ B activation. PPAR γ is highly expressed in the intestine, suggesting an important role in the regulation of the intestinal inflammatory response (2, 3). In line with this assumption, heterozygous PPAR γ deficient (+/-) mice are more susceptible to experimental induced colitis than wild-type mice (4, 5). In addition, treatment with PPAR γ agonists protected against colonic inflammation in different animal models of inflammatory bowel disease (IBD) (4-12). Also in humans, the PPAR γ ligand rosiglitazone may have positive effects as suggested by the number of patients that achieved clinical and endoscopic remission in an open-label single-arm trial for ulcerative colitis (UC) patients (13). Unfortunately, this study did not include a control group, which makes it difficult to draw any firm conclusions. Yet, these studies together do suggest that PPAR γ activation dampens intestinal inflammation. However, in studies concerning animals, only the effects of treatment with PPAR γ agonists at the first day of colitis induction (4, 11, 12), one or two days before colitis induction (4, 5, 7, 8, 10) or after colitis induction (5, 6, 12) were examined. However, in reality, IBD patients should be treated with PPAR γ agonists such as rosiglitazone for longer periods, either when applied preventive to avoid the occurrence of relapses (as a maintenance therapy), or curative during acute exacerbations. Looking at the current literature, the latter approach, i.e., 14 days chronic rosiglitazone treatment during chronic trinitrobenzensulfonic acid (TNBS) colitis induction (6) has been evaluated, whereas the first longer-term preventive approach has not been done. Thus, studies are lacking which evaluates effects of longer term PPAR γ agonist treatment before colitis induction. The aim of the present study was, therefore, to examine the *in vivo* effects of longer-term preventive treatment with the PPAR γ ligand rosiglitazone on dextran sodium sulphate (DSS)-induced colitis.

Materials and methods

Experimental design

All experiments were approved by the Animal Studies Ethics Committee of the University of Amsterdam, The Netherlands. Twenty 8-weeks-old female wild-type C57BL/6 mice were obtained from Charles River (Horst, The Netherlands). The mice were housed under standard conditions (AM-III 15 mm, Hope Farms, Woerden, The Netherlands) and had free access to water and food. The mice were randomly assigned to 2 groups, which received either control chow (control group) or control chow enriched with 12 mg/100g rosiglitazone (Avandia, GlaxoSmithKline, Brentford, UK) (rosiglitazone group) for 16 days before colitis induction. Treatments were continued during the week of colitis induction. Both diets consisted of 21 En% protein, 66 En% carbohydrates, and 14 En% fats. Both diets were made by Special Diet Services (SDS, Essex, UK) and irradiated at 25 kGy. Colitis was induced by administration of 1.5% (wt/v) DSS (MW 40 kDa; TdB Consultancy, Uppsala, Sweden) to the drinking water of the mice for one week. Body weights of the mice were recorded every third day, but were recorded daily in the week of colitis induction. After 7 days DSS treatment, all mice were anesthetized with fentanyl-fluanisone-midazolam (FFM) and sacrificed via a cardiac puncture and exsanguinations. Blood was sampled in EDTA tubes (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ) and plasma was obtained by centrifugation at 2,000 *g* for 20 minutes at 4 °C and stored at -80 °C until analysis for myeloperoxidase (MPO) and serum amyloid P component (SAP) concentrations. Through a midline incision, the colons were removed. First, the total length was measured as an indicator of disease-related colon shortening. In addition, fecal material was removed and collected to score diarrhea severity: 0; normal feces, 1; loose stool, 2; watery diarrhea, 3; slimy diarrhea, little blood, and 4; very severe diarrhea. Next, the colons were opened longitudinally and the wet weight of the distal 6 cm was recorded and used as an index of disease-related intestinal wall thickening. When the colons were shorter than 6 cm, the entire colon was removed and weight was recalculated for 6 cm. Subsequently, the colons were divided longitudinally into two parts and both were rolled up. For all animals, one role was used for histological analysis. In 5 randomly selected animals from each group, the other role was used for cytokine detection by means of a cytometric bead array (CBA) and for immunofluorescence of Zonula Occludens protein 1 (ZO-1) in the other 5 mice from each group. Besides the colon, for all animals, spleens were also sampled, weighed, and used for cytokine detection. All sampled tissues were frozen in liquid nitrogen and stored at -80 °C until analysis.

Histological analysis

The longitudinally divided rolled-up parts of the colon, which were used for routine histology, were directly fixed in 4% formaldehyde and embedded in paraffin. Three transverse slices (5 μ m), taken from each colonic sample, were stained with hematoxylin-eosin and examined by light microscopy. Colonic inflammation was evaluated in a blind manner by estimating the 1) percentage of involved area, 2) the amount of follicles, 3) edema, 4) erosion/ulceration, 5) crypt loss, 6) infiltration of polymorphonuclear cells, and 7) infiltration of mononuclear cells. The percentage of area involved, erosion/ulceration and the crypt loss was scored on a scale ranging from 0 to 4 as follows: 0; normal, 1; less than 10%, 2; 10-25%, 3; 25-50%, 4; more than 50%. Follicle aggregates were counted and scored as follows: 0; 0-1 follicles, 1; 2-3 follicles, 2; 4-5 follicles, 3; 6 follicles or more. The severity of the other parameters was scored on a scale from 0 to 3 as follows: 0; absent, 1; weak, 2; moderate, 3; severe. All scores on the individual parameters together could result in a total score ranging from 0 to 24.

Immunofluorescence of Zonula Occludens protein-1 (ZO-1)

To examine the distribution of tight junctions, we stained 3 colon sections of 5 mice per group for ZO-1. Frozen sections of rolled-up colons were cut (6 μ m), allowed to air dry, fixed with 4% paraformaldehyde, and then washed three times with cold PBS. The sections were blocked with 1:10 normal goat serum and then incubated for 1 hour at room temperature with rabbit anti-ZO-1 polyclonal antibody (Zymed, San Francisco, CA). As a negative control, sections were incubated with PBS-0.1% BSA. Thereafter, sections were washed 3 times with cold PBS, incubated with Texas Red conjugated goat antirabbit antibody (Jackson, West Grove, PA) for 50 minutes at room temperature and washed 3 times with PBS. Next, sections were incubated with 4',6-diamino-2-phenyl indole (DAPI) (0.5 mg/mL, Sigma, St. Louis, MO) for 2 minutes, washed 3 times with PBS, dehydrated in ascending ethanol series, and mounted in mounting solution (Dako Cytomation, Ely, UK). Distribution of tight junctions was recorded with Metasystems Image Pro System (Metasystems, Sandhausen, Germany) mounted on a Leica DM-RE fluorescence microscope (Leica, Wetzlar, Germany). Photomicrographs were recorded at a 200x magnification.

Homogenisation and cytometric bead array (CBA)

For analysis of cytokine concentrations, homogenates were made from colon and spleen samples with a tissue homogenizer in 9 volumes (wt/v) Greenberger lysis buffer (300 mmol/L NaCl, 15 mmol/L Tris, 2 mmol/L MgCl_2 , 2 mmol/L Triton X-100 (Sigma, St. Louis, MO), Pepstatin A, Leupeptin, Aprotinine (Roche, Mannheim, Germany), all 20 ng/mL; pH 7.4). The tissues were lysed in the Greenberger lysis buffer for 1 hour on ice and centrifuged for 7 minutes at 3,000 rpm and for 10 minutes at 14,000 rpm. The supernatant was collected and stored at -80 °C until cytokine analysis using a cytometric bead array. The mouse inflammation and Th1/Th2 CBA (Becton Dickinson Biosciences, San Diego, CA) were used for colon homogenates and the mouse inflammation CBA in spleen homogenates to determine simultaneously the concentrations of various cytokines in colon (interleukin (IL)-2, IL-4, IL-6, IL-10, IL-12p70, monocyte chemotactic protein (MCP)-1, interferon (IFN) γ and tumor necrosis factor (TNF) α and spleen (IL-6, IL-10, IL-12p70, MCP-1 and TNF α) homogenates according to the instructions of the manufacturer. Briefly, 5 μL of sample or the cytokine standard mixture was mixed with 5 μL of the mixed capture beads and 5 μL of the detection antibody-phycoerythrin (PE) reagent and incubated at room temperature for 2 hours in the dark. Two-colour flow cytometric analysis was performed using a FACScan® flow cytometer (Becton Dickinson Immunocytometry Systems (BDIS), San Jose, CA). Data were acquired and analyzed using Becton Dickinson CBA software.

Myeloperoxidase (MPO) in colon homogenates and plasma

To quantify the extent of colonic neutrophil accumulation and systemic neutrophil amounts, MPO concentrations in colon homogenates and EDTA plasma were analyzed by ELISA (HBT, Uden, The Netherlands). All samples of both groups were analyzed on one plate. The detection limit of the MPO ELISA was 1.02 ng/mL. The intraassay variation was less than 7.5%.

Serum amyloid P component (SAP) in plasma

To evaluate the acute phase reaction activity, SAP concentrations were analyzed in EDTA plasma by ELISA, as described in (14). Briefly, 96 well plates (Greiner Bio-one, Frickenhausen, Germany) were coated with 3 $\mu\text{g/mL}$ sheep-antimouse SAP (Calbiochem, San Diego, CA) antibodies. Immobilized SAP was detected using a specific biotinylated rabbit-antimouse SAP antibody (Calbiochem), followed by the addition of peroxidase-conjugated streptavidin (Zymed Laboratories, San Francisco, CA) and TMB substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD). All samples of both groups were analyzed on one plate. The detection limit of the ELISA was 5 ng/mL. The intraassay variation was less than 5%.

Statistical analysis

All data, except body weights (means $\pm$ SEM), are expressed as dot plots in which the median is indicated. Differences between the groups were analyzed using the Mann-Whitney U test. Two ANCOVA models were used to analyze changes in body weight. The first one evaluated the effects of rosiglitazone on body weight at the end of 16 days rosiglitazone treatment using weight at the start of rosiglitazone treatment (day 0) as covariate and the second model evaluated the effects of rosiglitazone on body weight at the end of 7 days DSS treatment (day 23) using weight at the start of DSS treatment (day 16) as covariate. Correlation analyses were performed using the Spearman correlation test. Values of $P < 0.05$ were considered statistically significant. SPSS 10 for Macintosh (SPSS, Chicago, IL) was used for the analysis.

Results

Effects of rosiglitazone on colitis severity

During the 16 days preceding colitis induction, mice treated with rosiglitazone gained more weight ($15.3 \pm 2.7\%$) than control mice ($5.6 \pm 2.1\%$) ($P < 0.001$) (**Figure 5.1A**). After 7 days DSS the rosiglitazone treated mice had lost significantly more weight ($-13.5 \pm 4.0\%$ of their initial weight at DSS induction) than the control mice ($-3.8 \pm 5.0\%$) ($P < 0.001$) (**Figure 5.1B**).

Figure 5.1: Treatment with rosiglitazone increased dextran sodium sulphate (DSS) colitis disease severity.

Ten female mice consumed a control diet or the same diet with 12 mg/100g rosiglitazone for 16 days prior to DSS colitis induction. Colitis was induced by 1.5% (wt/v) DSS in the drinking water for 7 days. Body weight was recorded prior to colitis induction (A) and after colitis induction as a measure of disease activity (B). Disease severity was measured 7 days after DSS colitis induction by a diarrhea score (C), colon length (D), colon weight of last 6 cm (E) and spleen weight (F).

Seven days after DSS-colitis induction, diarrhea of the mice was scored (**Figure 5.1C**). In agreement with the more pronounced loss of body weight, the rosiglitazone treated mice had more diarrhea (median score 4.0) than control mice (score 2.5) ($P = 0.003$).

After sacrificing the mice, the total length of the colon and the weight of the last 6 cm of the colon were determined and used as indicators of disease-related colon shortening and intestinal wall-thickening. The weight of the colon (217 mg) was significantly increased and the length (5.5 cm) of the colon was significantly decreased in the rosiglitazone group ($P = 0.005$ and $P = 0.009$ respectively) as compared to the control group (197 mg and 6.5 cm, respectively) (**Figures 5.1D-E**). Also, the weight of the spleen was significantly increased in the rosiglitazone treated mice (145 v 93 mg) ($P = 0.002$) (**Figure 5.1F**). Colon weight correlated positively with spleen weight ($r = 0.466$, $P = 0.038$) and diarrhea score ($r = 0.509$, $P = 0.022$).

Colonic inflammation was also evaluated by histological analysis. **Figures 5.2A-B** show representative pictures of the colons from control and rosiglitazone treated mice. Colonic inflammation in the control mice was mostly continuous (most severe at the anal side), whereas the colonic inflammation of the rosiglitazone treated mice was characterized by skipped-lesions (isolated affected areas with intervening quite normal mucosa). The total colitis score was significantly increased in the rosiglitazone group (16.5 points) as compared to the control group (12.3 points; $P = 0.006$) (**Figure 5.2C**), which was mainly caused by an increase in ulceration ($P = 0.011$), crypt loss ($P = 0.007$) and edema ($P = 0.043$) (**Table 5.1**). Total histological score correlated positively with colon weight ($r = 0.514$, $P = 0.020$).

Immunofluorescence showed that ZO-1 appearance in colons of rosiglitazone treated mice was less distinct, diffusely localized, and even a loss of ZO-1 protein was observed in several parts of the tissue sections as compared to control mice (**Figures 5.2D-F**).

Figure 5.2: Treatment with rosiglitazone increased histological score and induced a loss of tight junction protein ZO-1.

Ten female mice consumed a control diet or the same diet with 12 mg/100g rosiglitazone for 16 days prior to DSS colitis induction. Colitis was induced by 1.5% (wt/v) DSS in the drinking water for 7 days. (A-C) Histological examination was scored in haematoxylin-eosin (HE) stained sections of colons at 7 days after DSS colitis induction (magnification 25x). (A) HE staining of representative section of a control mouse and (B) rosiglitazone treated mouse. (C) Total histological score of control and rosiglitazone treated mice. (D-F) Immunolocalization of ZO-1 (red) showed a relative regular distribution in the colon of control colitis mice (D). ZO-1 is localized in the upper part of the enterocytes, showing a normal distribution in association with the cellular surface. Although parts of the colon of control colitis mice showed ZO-1 loss, rosiglitazone treatment (E) led to significant more loss of ZO-1. Moreover, the colon tissue was more disrupted and disorganized, illustrated by an irregular distribution of nuclei (blue). Although tissue sections of rosiglitazone treated mice showed parts with a normal distribution of ZO-1 the difference with the control mice was striking. (F) Negative control staining of a colon section of a control mouse. The histology shown is representative for all tissue sections studied. (magnification 200x)

Table 5.1: Histology scores of the colon of rosiglitazone treated and control DSS colitis mice.

	Rosiglitazone	Control
	(n = 10)	(n = 10)
Total score	16.5* (12.5-21.0)	12.3 (8.0-16.0)
Area involved ^a	4.0 (4.0-4.0)	4.0 (3.0-4.0)
Follicle aggregates ^b	1.0 (0.0-3.0)	0.0 (0.0-2.0)
Edema ^b	2.5* (1.0-3.0)	1.8 (1.0-2.0)
Ulceration ^a	2.0* (2.0-3.0)	1.0 (1.0-3.0)
Crypt loss ^a	3.0* (2.0-3.0)	2.0 (1.0-3.0)
Polymorphonuclear cells ^b	2.0 (1.5-3.0)	1.8 (0.5-2.5)
Mononuclear cells ^b	1.5 (1.0-2.0)	1.5 (1.0-2.0)

^aMedian scores (min-max) on a scale of 0-4; 0 = normal, 1 = less than 10%, 2 = 10-25%, 3 = 25-50%, 4 = more than 50%.

^bMedian scores (min-max) on a scale of 0-3; 0 = absent, 1 = weak, 2 = moderate, 3 = severe.

* $P < 0.05$ versus control group.

Effects of rosiglitazone on colon and spleen cytokine concentrations

The concentration of different cytokines in colon and spleen homogenates were analyzed by a CBA to evaluate the extent of the local immune responses. The colonic concentrations of $\text{TNF}\alpha$ ($P = 0.028$) and $\text{IFN}\gamma$ ($P = 0.009$) were decreased in the rosiglitazone group (**Table 5.2**). IL-2, IL-4, IL-6, IL-10, IL-12p70, and MCP-1 concentrations in the colon homogenates were not significantly different between the two groups. IL-12p70 concentrations in colon homogenates correlated with $\text{TNF}\alpha$ and $\text{IFN}\gamma$ (both $r = 0.664$, $P = 0.044$) and $\text{TNF}\alpha$ also correlated with $\text{IFN}\gamma$ ($r = 0.891$, $P = 0.001$). Furthermore, MCP-1 correlated with IL-6 ($r = 0.685$, $P = 0.029$). Not only in the colon, but also in the spleen, $\text{TNF}\alpha$ concentrations were significantly ($P = 0.028$) decreased. However, spleen IL-10 concentrations were significantly ($P = 0.013$) increased in the rosiglitazone treated mice (Table 5.2). IL-6, IL-12p70, and MCP-1 concentrations in spleen were not different between the two groups.

Table 5.2: Cytokine concentrations in colon and spleen homogenates.

Cytokines ^a	Colon		Spleen	
	Rosiglitazone (n = 5)	Control (n = 5)	Rosiglitazone (n = 10)	Control (n = 10)
IL-12p70	8.6 ^b (6.6-17.9)	10.0 (9.1-17.2)	4.1 (0.0-5.7)	1.4 (0.0-6.7)
TNF α	48* (38-83)	84 (41-229)	57* (30-106)	82 (54-106)
IFN γ	1.9* (1.6-3.0)	8.0 (3.4-37.2)	ND	ND
MCP-1	867 (455-1131)	477 (391-950)	135 (54-191)	152 (101-342)
IL-10	8.4 (0.0-22.4)	9.6 (7.5-17.8)	3.3* (0.0-36.3)	0.0 (0.0-0.0)
IL-6	390 (96-1327)	272 (151-1249)	25 (16-33)	36 (13-290)
IL-4	0.0 (0.0-3.9)	0.0 (0.0-5.0)	ND	ND
IL-2	2.6 (2.0-3.4)	2.8 (2.1-6.3)	ND	ND

^a Concentrations in pg/mL.^b Median (min-max).* $P < 0.05$ versus control group.

Abbreviations: IFN, interferon; IL, interleukin; MCP, monocyte chemotactic protein; ND, not determined; TNF, tumor necrosis factor.

Effects of rosiglitazone on MPO and SAP concentrations in colon homogenates and/or plasma

To determine colonic neutrophil accumulation and systemic neutrophil levels, MPO concentrations were analyzed in colon homogenates and plasma. No significant differences between the groups were found, neither in colon homogenates nor in plasma ($P = 0.754$ and $P = 0.174$, respectively) (**Figures 5.3A-B**). MPO concentrations in colon homogenates correlated positively with MCP-1 concentrations in colon homogenates ($r = 0.673$, $P = 0.033$).

To determine the extent of the acute phase response, we analyzed plasma SAP concentrations. The SAP concentration of the plasma of rosiglitazone treated mice was significantly increased compared to control mice (885 ng/mL versus 196 ng/mL) ($P = 0.041$) (**Figure 5.3C**). SAP concentration in plasma correlated positively with total histological score ($r = 0.540$, $P = 0.014$) and diarrhea score ($r = 0.619$, $P = 0.004$).

Figure 5.3: Treatment with rosiglitazone did not change myeloperoxidase (MPO) concentrations in colon homogenates and plasma, but increased serum amyloid P component (SAP) concentrations in plasma.

Ten female mice consumed a control diet or the same diet with 12 mg/100g rosiglitazone for 16 days prior to DSS colitis induction. Colitis was induced by 1.5% (wt/v) DSS in the drinking water for 7 days. MPO concentrations were determined in colon homogenates (A) and in plasma (B) and SAP concentrations were determined in plasma (C) 7 days after colitis induction.

Discussion

The present study showed that in mice, 16 days pre-treatment with rosiglitazone in the diet increased colonic inflammation after experimentally DSS-induced colitis. In contrast with all earlier studies, this is the first study that showed adverse effects of a PPAR γ agonist in a colitis model. Earlier studies administrated various PPAR agonists (troglitazone, pioglitazone, rosiglitazone, netoglitazone) by oral gavage on the first day of colitis induction (4, 12), 1 or 2 days before colitis induction (4, 5, 8, 10) or after colitis induction (5, 6, 12) until sacrificing the mice. Each of these studies showed decreased disease activity, less mortality, and less colonic (Th1) cytokine production (4-6, 8, 10, 12). Also, intraperitoneal injection of pioglitazone - starting at the day of DSS colitis induction until day 7 - showed preventive effects (11). Moreover, rosiglitazone in the diet, 2 days before colitis induction protected against acute DSS-colitis (7). The most striking difference in the design of all these studies as compared to ours is the time at which PPAR γ agonist treatment was initiated. In our experimental model, we have deliberately chosen to test the effects of a PPAR γ agonist in a longer term preventive manner to lower the risk of relapses. We therefore applied rosiglitazone for 16 days prior to DSS-induced colitis induction. Only in one other study, rosiglitazone was added to the diet for a longer period (9). In that study, Lytle *et al.* examined the effects of rosiglitazone treatment in the diet for a period of 12 weeks on the spontaneous development of colitis in IL-10 knockout mice. In contrast to our study, this study showed that longer term treatment with rosiglitazone slowed down the onset of spontaneous severe colitis compared to a control diet. However, rosiglitazone did not prevent colitis development and in the end disease severity was comparable between rosiglitazone treated and control animals. However, in contrast to our study, rosiglitazone did not worsen the disease state. It should be realized, however, that the IL-10 knockout model is a different model as compared to the DSS model. Whereas the IL-10 knockout model depends on a dysregulated immunological response, which results in spontaneous colitis development, the DSS model is related to a disruption of epithelial cell barrier function, which results in acute colitis.

In all studies in which rosiglitazone was administrated as a gavage, daily intakes varied between 1-50 mg/kg. Optimal (preventive and therapeutic) effects were found at 20 mg/kg, while higher doses had no additional beneficial effects (4-6, 12). In the two studies in which rosiglitazone was mixed with powdered chow and used in a preventive mode, animals consumed daily about 20 mg/kg (7, 9). It can be calculated that in our study daily intake was comparable. Therefore, differences in effects on colitis cannot be explained by differences in rosiglitazone dosage. However, in future studies, a dose response relationship for the longer term preventive (dietary) mode should be evaluated. Another possibility could be differences in susceptibility between

mice of various genetic backgrounds. We used C57BL/6 mice whereas other DSS studies used not only C57BL/6 (5, 8), but also balb/c (10, 11) and Swiss-Webster mice (12). However, these DSS studies using gavages did not show any differences in effects between strains. A possible explanation therefore is that - in contrast to the earlier studies - longer term rosiglitazone treatment before colitis induction induced changes in the intestinal barrier, which made the intestine more susceptible to intestinal damage. In the DSS model, which depends on intestinal barrier disruption, this increased susceptibility may induce more severe intestinal damage. This hypothesis however needs to be tested in future studies, for example, by comparing acute curative effects of rosiglitazone versus long-term preventive effects side-by-side in one experiment and by confirming this data in another model of IBD.

Changes in weight loss, diarrhea, colon weight and length, spleen weight, histological scores, and plasma SAP concentration indicated that colitis was more severe in rosiglitazone treated mice. Although the mice gained more weight before colitis induction, they also lost more weight after 7 days DSS colitis. Weight gain is a known adverse effect of rosiglitazone treatment in humans (15, 16) and in mice (17), which is also mentioned in the instructions of the Avandia tablets. Moreover, also other parameters - diarrhea, colon weight and length, spleen weight, histological scores, and plasma SAP concentration - indicate that colitis was more severe in rosiglitazone treated mice. However, $\text{TNF}\alpha$ levels in colon and spleen and $\text{IFN}\gamma$ levels in colon were decreased, and those of IL-10 (an antiinflammatory cytokine) in the spleen were increased in the rosiglitazone treated mice. Moreover, other cytokines (IL-2, IL-4, IL-6, IL-12p70 and MCP-1) and MPO concentrations in colon and/or spleen homogenates and/or plasma showed no differences. Why these inflammatory proteins did not show an increase as expected from disease severity is not known. At first, the combination of results seems contradictory, i.e., lower expression of proinflammatory cytokines in the colon, no change in colonic MPO concentrations and a more severe DSS-induced colitis phenotype as evaluated by - among others - weight loss and histological scores. There are, however, different possibilities explaining the simultaneous appearance of these characteristics. One possible explanation is that mice were too sick to produce cytokines at high concentrations, as has also been found in another study (18). This would imply that the acute innate inflammatory response during the first days of DSS treatment in the rosiglitazone animals was so strong that at day 7, when the mice were sacrificed, the local inflammatory cells were exhausted. However, the increased IL-10 concentration does not support this explanation and suggests an enhanced antiinflammatory immune response. Therefore, a more likely explanation is that the absence of elevated cytokine concentrations is caused by the down-regulation of the proinflammatory response of intestinal and immune cells by rosiglitazone. Other studies with $\text{PPAR}\gamma$ agonists also showed decreased colonic Th1 cytokines (4, 5, 10, 12) and an increase in

colonic IL-10 mRNA (5). However, in contrast to our study, in these earlier studies, colitis was attenuated. We now suggest that the down-regulated immune response due to long-term rosiglitazone pretreatment will ultimately result in a situation that there is no adequate immune response toward the injured epithelium by DSS. This will enhance the possibility of bacterial translocation, consequently leading to more pronounced disease severity. Also, differences in the distribution of the colonic damage (continuous from anal towards the small intestine in control animals and skipped-lesions in rosiglitazone animals) suggest a different type of colitis/colonic injury due to rosiglitazone interference. In relation to this latter explanation, it is more likely that rosiglitazone pretreatment makes the intestinal wall more vulnerable for DSS-induced tissue injury. Although in a future study we should measure tight-junction distribution in rosiglitazone treated mice before colitis induction, the striking loss of ZO-1 protein in rosiglitazone treated colitis mice as compared to control colitis mice seems suggestive. We do realize that this does not provide any evidence for the assumption that 16 days rosiglitazone pretreatment makes the intestinal wall more susceptible for DSS-induced tissue injury; however, with respect to the severe outcome we did not want to postpone this knowledge for too long. Moreover, the increase in spleen weight in the rosiglitazone treated animals is of potential concern. It could simply be a result of the more severe colitis in the rosiglitazone treated animals. At this moment, however, we cannot exclude that it is the result of rosiglitazone treatment per se.

In conclusion, our results indicate that rosiglitazone when added to the diet in a preventive approach for a longer period (16 days) results in a higher susceptibility for DSS-induced colitis. This finding is in sharp contrast to promising results of earlier studies in which PPAR γ agonists were given shortly (i.e., 1 or 2 days) before experimental colitis induction and longer term dietary treatment in a spontaneous IL-10 knockout colitis model. We hypothesize that rosiglitazone pretreatment makes the intestinal wall more susceptible for DSS-induced tissue injury ultimately resulting in a more pronounced disease severity. The phenotypic characteristics suggest that the PPAR γ activation keeps the inflammatory response dampened despite the higher tissue injury by DSS. Future research is warranted to unravel the mechanism underlying these unexpected effects and confirm this data in other IBD models.

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Chapter 6

Effects of the individual isomers *cis*-9, *trans*-11 versus *trans*-10, *cis*-12 conjugated linoleic acid (CLA) on inflammation parameters in moderately overweight subjects with LDL-phenotype B

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Abstract

Immune modulating effects of conjugated linoleic acid (CLA) have been reported in animals, but results are inconsistent. In humans, CLA has shown none or only minor effects on immune function. The objective of this study was to evaluate the immune modulating effects of 3 g *cis*-9, *trans*-11 (c9, t11) versus *trans*-10, *cis*-12 (t10, c12) CLA isomers in a population with a high risk of coronary heart disease (CHD) characterized by moderately overweight (BMI 25-32.5 kg/m²) in combination with LDL-phenotype B ($\geq 35\%$ small LDL cholesterol, density ≥ 1.040 g/mL). After a run-in period of one week, forty-two men and women were randomly allocated to the c9, t11 CLA group, the t10, c12 CLA group or the placebo group. Effects of 13 weeks of consumption of 3 g of CLA isomers on cytokine production by *ex vivo* lipopolysaccharide (LPS) stimulated peripheral blood mononuclear cells (PBMC) and whole blood and on plasma C-reactive protein (CRP) concentrations were evaluated. To generate hypotheses for future studies, protein expression patterns of 42 cytokines, chemokines and growth factors were evaluated with an antibody array in pooled, nonstimulated, fasting plasma samples. LPS induced interleukin (IL)-6, IL-8 and tumor necrosis factor (TNF) α productions by PBMC and whole blood as well as plasma CRP concentrations were not significantly changed by the c9, t11 and the t10, c12 CLA isomers. The cytokine expression profile in nonstimulated plasma suggested that both CLA isomers induced a specific inflammatory signature, in which the c9, t11 CLA group showed more activity in terms of numbers of proteins regulated. We conclude that a daily consumption of 3 g of c9, t11 or t10, c12 CLA isomer did not affect LPS-stimulated cytokine production by PBMC or whole blood and plasma CRP levels. Inflammatory signatures in fasting, non-stimulated plasma as determined by an antibody array may indicate enhanced immune function by both CLA isomers. These specific effects however need to be addressed in future studies.

Introduction

Conjugated linoleic acid (CLA) is a mixture of positional (e.g. 9,11; 10,12) and geometrical (*cis* or *trans*) conjugated isomers of linoleic acid (C18:2 n-6). It is a natural food component, predominantly found in the lipid fraction of meat, milk and other dairy products. Many health effects have been described to CLA. The earlier studies mainly focused on its anticarcinogenic properties (1, 2), but later studies also examined additional health benefits such as anti-diabetic, anti-obesity and anti-atherosclerotic effects (3). Most of these effects were, however, found in laboratory animals. CLA has also been reported to have immunomodulatory properties in animals, but results are inconsistent (3, 4).

Data from human studies are limited and - if anything - only minor effects of mixtures of CLA isomers on immune functions have been shown (3, 4). Effects may, however, be isomer specific (4). Tricon *et al.* (5) therefore compared side-by-side *cis*-9, *trans*-11 (c9, t11) and *trans*-10, *cis*-12 (t10, c12) CLA. Both isomers decreased mitogen-induced lymphocyte activation but had no effects on lymphocyte subpopulations, *ex vivo* cytokine production, and serum C-reactive protein (CRP) concentration. The absence of clear effects may be related to the healthy population in that study. It is very well possible that effects are more apparent if the immune system is already triggered. In the present placebo-controlled study we therefore evaluated the effects on inflammation parameters of consumption of 3 g of the individual c9, t11 CLA or t10, c12 CLA isomers in moderately overweight subjects. It is known that overweight and obese persons are at increased risk for coronary heart diseases (CHD), which may be related to their proinflammatory serum profiles (6). In addition, subjects were characterized by LDL-phenotype B, which is typified by the presence of increased proportions of the highly atherogenic small dense LDL particles (7, 8). Subjects with LDL phenotype B have an increased risk for CHD that might be explained by the increased oxidative susceptibility of the small dense LDL particles (9). Since leukocytes are easily triggered by oxidized LDL particles (10, 11), potential positive effects on leukocyte immunoreactivity may be more evident in this population. Leukocyte function was determined *ex vivo* by measurement of interleukin (IL)-6, IL-8 and tumor necrosis factor (TNF) α production after lipopolysaccharide (LPS) stimulation of isolated peripheral blood mononuclear cells (PBMC) and of whole blood. However, plasma concentrations of inflammation markers such as CRP, IL-6 and monocyte chemoattractant protein-1 (MCP-1) are also strong predictors of future CHD risk (12-14). Therefore, we also measured plasma concentrations of CRP and of 42 different cytokines, chemokines and growth factors in pooled plasma samples using an antibody array.

Subjects and methods

Subjects

Forty-two apparently healthy, moderately overweight (BMI 25-32.5 kg/m²), middle-aged (35-65 years), men (n=22) and women (n=20), classified as having LDL-phenotype B ($\geq 35\%$ small LDL cholesterol, density ≥ 1.040 g/mL), were included in the study. Volunteers were recruited through announcements in local newspapers. Eligible subjects completed a standard blood test and filled in a medical questionnaire. All subjects were non-hypercholesterolemic (mean serum total cholesterol < 7.0 mmol/L and mean serum triacylglycerol < 3.0 mmol/L), as measured on two separate occasions with at least 3 days interval after an overnight fast. Exclusion criteria were diastolic blood pressure > 85 mmHg or systolic blood pressure > 150 mmHg; unstable body weight or attempts to lose weight during the previous three months; presence of proteinuria or glucosuria; use of medication, a diet or a clinical condition known to affect lipid or glucose metabolism; drug or alcohol abuse; history of CHD, decompensatio cordis (Class III or IV), cardiomyopathy or kidney, liver and pancreatic disease or malignancies < 5 years ago; pregnancy or breast-feeding; and participation in another biochemical trial < 30 days ago. The subjects were requested not to change their usual diets, levels of physical exercise, smoking habits, or use of alcohol during the study. The Ethical Committee had approved the study protocol, and all subjects signed informed consent forms before entering the study.

Study design

This study was part of a larger project on the health effects of CLA in overweight middle-aged Europeans (Fifth European Commission framework program QLK1-1999-00076). Of this larger multi-center study, forty-two participants from Maastricht were used to measure the immune modulating effects of CLA. The study was designed as a placebo-controlled, double-blind parallel design. During the first week of the study (run-in period), subjects consumed 100 mL of a drinkable dairy product enriched with 3 g of a high oleic acid sunflower oil (placebo) each day. Thereafter, the volunteers were randomly allocated to one of three treatment groups. Randomization was balanced for males and females, BMI and LDL-phenotype. For the next 13 weeks of the study (intervention period), the first group continued to consume the placebo drinkable yogurt-like dairy product enriched with 3 g of a high oleic acid sunflower oil (100 mL/day). The second group consumed the same product enriched with 3 g of c9, t11 CLA instead of the oleic acid, while the third group consumed this product enriched with 3 g of t10, c12 CLA. No extra antioxidants were added to the experimental products.

The two CLA isomers were given as a triacylglycerol (TAG) and were produced by Natural Lipids LTD (Hovdebygda, Norway), as described (15). The CLA was incorporated into a yogurt-like dairy product (by Danone; Palaiseau, France), which contained (wt/wt) 67% water, 20% milk (3.2% proteins, 5% lactose, 0.7% minerals (1250 ppm Ca) and 0.05% fat), 4.1% oils, 8% saccharose, 0.4% pectin, 0.35% citric acid, and 0.12% flavorings. The c9, t11 CLA concentrate contained > 80% c9, t11 CLA, < 5% t10, c12 CLA, and other isomers in minor amounts. The t10, c12 CLA concentrate contained > 80% t10, c12 CLA, < 5% c9, t11 CLA, and other isomers in minor amounts.

During the study, subjects recorded in diaries any signs of illness or any side effects experienced. Food intake was measured at the end of the run-in and the intervention period, using three-day dietary records. Energy and nutrient intakes were calculated using the Dutch Food Composition Table (NEVO-tabel) (16).

Both at the end of the run-in period (week 1) and the end of the intervention period (week 14) blood was sampled after an overnight fast of at least 10 hours. Subjects were not allowed to consume alcohol 24 hours before blood sampling and were not allowed to smoke the morning before blood sampling. Blood was sampled in EDTA tubes and serum tubes (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ). Plasma was obtained by centrifugation at 2,000xg for 30 minutes and used for CRP analysis and antibody arrays. Serum was obtained by centrifugation at 2,000xg for 30 minutes and was used for lipid and lipoprotein analysis. Plasma and serum samples were stored at -80 °C until analysis. In addition, blood was sampled in endotoxin-free heparinized tubes (Becton Dickinson Vacutainer Systems; final heparin concentration 10 U/ml) for PBMC and whole blood stimulation.

PBMC and whole blood stimulation

To examine *ex vivo* cytokine production by PBMC, cells were isolated from whole blood using Lymphoprep (Nycomed Pharma, Oslo, Norway) under sterile conditions. After isolation, PBMC were immediately plated in 24-well flat-bottom culture plates (2.5×10^6 cells/mL per well; 200-600 μ L per well), and mixed with LPS (E Coli 055:B5, Sigma, St Louis, MO; final concentrations 1 and 10 ng/mL in endotoxin-free buffered saline) or 20 μ L polymixin B (Sigma; final concentration 1 mg/mL in endotoxin-free buffered saline). RPMI-1640 was used as the culture medium, containing 1% penicillin/streptomycin, 1% sodium pyruvate and 1% of a heat-inactivated human serum pool. The cells were incubated for 6 hours at 37°C. After incubation, the culture media were aspirated. The aspirated media were centrifuged at 1,000xg for 30 minutes to obtain cell-free media, which were stored at -80 °C until analysis.

To examine *ex vivo* cytokine production by PBMC in whole blood, 2 mL of whole blood was immediately mixed with 20 μ L LPS (final concentrations 1 and 10 ng/mL) or polymixin B (final concentration 1 mg/mL). The blood samples were incubated for 6 hours at 37 °C. After incubation, samples were centrifuged

at 1,000xg for 30 minutes and platelet-poor plasma was stored at -80 °C until analysis.

Cytokine and C-reactive protein analysis

IL-6, IL-8 and TNF α concentrations in platelet poor plasma of stimulated whole blood and cell-free media of stimulated PBMC were assessed by sandwich ELISA as previously described (17-20). Briefly, plates (Greiner Bio-one, Frickenhausen, Germany) were coated with monoclonal murine anti-human IL-6, IL-8 or TNF α antibodies. Recombinant human IL-6, IL-8 or TNF α were used for their respective standard titration curves. Immobilized IL-6 or IL-8 was detected using a specific biotinylated rabbit-anti-human IL-6 or IL-8 polyclonal antibody, followed by the addition of peroxidase-conjugated streptavidin (Zymed Laboratories, San Francisco, CA) and tetramethyl-benzidine (TMB) substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD). Immobilized TNF α was detected using a specific rabbit-anti-human TNF α antibody, followed by the addition of goat-anti-rabbit peroxidase (Jackson ImmunoResearch, West Grove, PA) and TMB substrate.

Plasma CRP concentrations were measured with a highly sensitive immuno-turbidimetric assay (Kamiya Biomedical Company, Seattle, WA) (21).

Inflammatory protein expression profiles

To generate hypotheses for future studies, expression patterns of multiple cytokines, chemokines and growth factors, were detected simultaneously with the human cytokine antibody array III (Ray Biotech Inc., Norcross, GA) according to the manufacturer's instructions. We therefore evaluated changes in protein expression profiles, or patterns of protein clusters to be able to define possible differential immune modulating effects of the two CLA isomers. For this, fasting EDTA plasma samples from all participants of each group were pooled at the end of the run-in period and at the end of the intervention period. This means that six arrays (placebo group, c9, t11 CLA group and t10, c12 CLA group at the end of the run-in period and at the end of the intervention period) were analyzed. In brief, 1 mL of the pooled plasma samples was added to the array membranes. After incubating and washing, the cytokine-bound membrane was incubated with a cocktail of biotin-labeled antibodies, followed by adding horseradish peroxidase-conjugated streptavidin. Array spot intensity was detected by using a LAS-3000 Lite Image reader (Raytest GmbH, Straubenhardt, Germany) based on chemiluminescence imaging. Finally, intensity of the spots was quantified by densitometry using Aida software version 3.50 (Raytest GmbH), thereby correcting for background staining of the gel. Comparison of protein expression profiles was possible after normalization of each spot on an array using the positive controls provided by the manufacturer. For each group, responses to the dietary supplements were calculated as the percentage

change between values at the end of the run-in period (week 1) and those at the end of the intervention period (week 14). Next, for each cytokine the change in the control group was subtracted from the change in the intervention groups.

Lipids, apolipoproteins and LDL-phenotype

Total, LDL, HDL cholesterol and TAG concentrations were determined as described (22). LDL-phenotype was determined by Y. A. Carpentier from L. Deloyers Laboratory for Experimental Surgery of the Université Libre de Bruxelles (Brussels, Belgium) as described in ref (23).

Fatty acid composition of plasma phospholipids

Fatty acid composition of plasma phospholipids was measured in EDTA plasma as described by Sebedio *et al.* (24).

Statistics

For each subject, responses to the dietary supplements were calculated as the change between values at the end of the run-in period (week 1) and those at the end of the intervention period (week 14). Since the concentrations of IL-6, IL-8 and TNF α as well as fatty acid compositions were not normally distributed, the differences in changes were evaluated with the non-parametric Kruskal-Wallis test, followed by the Mann-Whitney test if the Kruskal-Wallis test showed a significant difference between the groups. The differences in changes of CRP were evaluated by analysis of variance (ANOVA). CRP concentrations at the end of the run-in period (week 1) were different between the 3 groups. Therefore, also an ANCOVA with week 1 concentrations as co-variant was carried out. Correlation analyses were performed using the Spearman correlation test.

All statistical analyses were performed using SPSS for Macintosh 10.0 (SPSS, Chicago, IL). Normally distributed values are presented as means $\pm$ SD, non-normally distributed values as medians (ranges). A *P*-value for the diet-effect of < 0.05 was considered as statistically significant. If a Tukey or Mann-Whitney test was performed, a *P*-value < 0.017 was considered as statistically significant.

Results

Baseline characteristics of the 38 subjects that completed the study are presented in **Table 6.1**. Three subjects dropped out during the intervention period, because of appearance of rashes ($n=1$, control group), because of participation in another clinical trial ($n=1$, t10, c12 CLA group), or because of a lung infection ($n=1$, t10, c12 CLA group). After the study, one man from the control group was excluded from the analysis, because of a non-fasting blood sample at the end of the intervention period. Seven subjects were smokers (1 subject from the control group and 3 subjects from each CLA group). One woman from the c9, t11 CLA group and 2 women from the t10, c12 CLA group used oral contraception. Five women from the control group, 5 women from the c9, t11 CLA group, and 4 women from the t10, c12 CLA group were postmenopausal. At baseline, there were no significant differences between the three groups, except for serum TAG concentrations (Table 6.1).

Table 6.1: Baseline characteristics of subjects before start of the study.^a

	Control ($n = 12$)	c9, t11 CLA ($n = 14$)	t10, c12 CLA ($n = 12$)
Age (y)	58 ± 5	53 ± 7	56 ± 6
Sex, M:F	6:6	7:7	6:6
BMI (kg/m^2)	29 ± 2	29 ± 2	29 ± 3
Visceral adiposity (waist/hip ratio)	0.95 ± 0.08	0.94 ± 0.08	0.94 ± 0.07
Systolic blood pressure (mmHg)	138 ± 14	140 ± 20	136 ± 13
Diastolic blood pressure (mmHg)	86 ± 9	88 ± 9	88 ± 5
Total cholesterol (mmol/L) ^b	6.22 ± 1.15	6.17 ± 0.79	5.80 ± 0.92
LDL cholesterol (mmol/L) ^b	4.19 ± 1.12	4.02 ± 0.92	3.95 ± 0.92
HDL cholesterol (mmol/L) ^b	1.54 ± 0.79	1.31 ± 0.74	1.18 ± 0.35
Triacylglycerol (mmol/L) ^{b,c}	1.08 ± 0.39	1.82 ± 0.90	1.45 ± 0.65
LDL-phenotype (%) ^d	40.9 (34.5–60.7)	41.2 (33.2–58.1)	41.3 (33.9–55.1)

^a Values are means $\pm$ SD except for LDL-phenotype medians (ranges).

^b Fasting serum concentrations of total cholesterol, LDL cholesterol, HDL cholesterol and triacylglycerol were measured during the first and second screening visit, with an interval of at least three days.

^c Significant difference between the groups ($P = 0.043$).

^d LDL-phenotype is expressed as the proportion of LDL cholesterol in LDL particles with a density $\geq 1.040 \text{ g/mL}$.

Compliance to the drinks was confirmed by the incorporation of the CLA isomers into plasma phospholipids (**Table 6.2**). For the c9, t11 CLA group, the change in the proportion of c9, t11 CLA was significantly higher as compared with the changes in the control ($P < 0.01$) and the t10, c12 CLA groups ($P < 0.01$). The t10, c12 CLA group had significantly higher increases in the proportions of the t10, c12 CLA as compared with those in the control ($P < 0.001$) and the c9, t11 CLA groups ($P < 0.01$).

Table 6.2: Proportions of fatty acids in phospholipids (% of total fatty acids (wt/wt)).^a

	Control	c9, t11 CLA	t10, c12 CLA	P-value
	(n = 12)	(n = 14)	(n = 14)	
c9, t11 CLA				
Run-in period	0.126 (0.000–0.224)	0.145 (0.094–0.731)	0.108 (0.091–0.185)	
Intervention period	0.136 (0.081–0.290)	0.694 (0.078–1.280)	0.183 (0.145–0.196)	
Change	–0.013 (–0.042–0.196)	0.579 ^{b,c} (–0.126–1.135)	0.077 (–0.010–0.139)	0.001
t10, c12 CLA				
Run-in period	0.000 (0.000–0.000)	0.000 (0.000–0.098)	0.000 (0.000–0.557)	
Intervention period	0.000 (0.000–0.208)	0.088 (0.000–0.183)	0.505 (0.000–0.755)	
Change	0.000 (0.000–0.208)	0.088 ^{c,d} (–0.053–0.183)	0.489 (0.000–0.755)	< 0.001

^a During the run-in period of 1 week subjects consumed a drinkable dairy product enriched with 3 g of a high oleic acid sunflower oil (placebo) per day. During the intervention period of 13 weeks the control group continued to consume the placebo product, the c9, t11 CLA group consumed 3 g of c9, t11 CLA, while the t10, c12 CLA group consumed 3 g of t10, c12 CLA. Values are medians (ranges).

^b c9, t11 CLA group vs control group $P < 0.01$.

^c c9, t11 CLA group vs t10, c12 CLA group $P < 0.01$.

^d t10, c12 CLA group vs control group $P < 0.001$.

Daily intakes of energy, and the percentages of energy from fat, saturated fatty acids, monounsaturated fatty acids, PUFA, protein and carbohydrates as well as daily intake of cholesterol, fiber and alcohol, did not differ during the run-in and intervention period (data not shown).

PBMC and whole blood stimulation at the end of the run-in period

Stimulation of PBMC and whole blood with 1 and 10 ng/mL LPS resulted in dose-dependent increases in IL-6, IL-8 and TNF α productions. Conclusions however did not depend on the dose of LPS used and therefore only the 10 ng/mL LPS results are presented. All polymixin B controls were negative (data not shown).

IL-6, IL-8 and TNF α productions by PBMC at the end of the run-in period varied between the groups, but differences did not reach statistical significance (**Table 6.3**).

Table 6.3: Effects of c9, t11 CLA or t10, c12 CLA on *ex vivo* cytokine production by isolated PBMC in response to stimulation with LPS.^a

	Control	c9, t11 CLA	t10, c12 CLA	<i>P</i> -value
IL-6 (ng/mL)				
n	11	13	9	
Run-in period	4.71 (0.89–41.65)	15.01 (0.53–65.21)	3.74 (0.63–32.21)	
Intervention period	6.96 (1.12–52.23)	9.42 (2.19–67.15)	11.76 (1.21–50.86)	
Change	0.59 (–9.05–46.17)	1.66 (–40.34 –38.26)	4.40 (–9.74–48.26)	0.439
IL-8 (ng/mL)				
n	10	12	9	
Run-in period	8.29 (1.89–46.83)	18.62 (1.10–42.91)	7.97 (3.12–42.00)	
Intervention period	17.37 (3.53–53.80)	16.08 (4.15–51.97)	21.38 (7.07–35.80)	
Change	2.61 (–7.48–46.23)	1.84 (–14.27 –30.20)	4.90 (–6.20–22.03)	0.427
TNFα (ng/mL)				
n	11	13	9	
Run-in period	1.06 (0.16–2.74)	1.31 (0.10–4.77)	0.61 (0.28–2.44)	
Intervention period	1.60 (0.24–4.75)	1.56 (0.47–6.65)	2.44 (0.34–5.39)	
Change	0.12 (–0.39–2.01)	0.48 (–3.33–4.10)	1.30 (–0.08–4.78)	0.175

^a For experimental details: see table 6.2. Peripheral blood mononuclear cells (PBMC) were isolated from whole blood and stimulated for 6 hours with 10 ng/mL lipopolysaccharide (LPS) from *E Coli* 055:B5. Values are medians (ranges). The results of five subjects (one of the control, one of the c9, t11 and three of the t10, c12 CLA group) were missing, because PBMC isolation from the blood was not successful. The results of the interleukin (IL)-8 production by PBMC of one subject of the control group and one subject of the c9, t11 CLA group were missing, because the amount of the samples was limited.

Abbreviations: IL, interleukin; TNF, tumor necrosis factor.

At the end of the run-in period, the IL-6 and IL-8 production in whole blood was about 20 ng/mL, whereas the TNF α production was much lower (about 4 ng/mL). There were no statistically significant differences between the three groups at the end of the run-in period (**Table 6.4**).

Table 6.4: Effects of c9, t11 CLA or t10, c12 CLA on *ex vivo* cytokine production by PBMC in whole blood in response to stimulation with LPS.^a

	Control	c9, t11 CLA	t10, c12 CLA	P-value
IL-6 (ng/mL)				
Run-in period	25.28 (5.63–63.61)	20.14 (10.14–43.40)	20.65 (4.54–32.89)	0.121
Intervention period	16.42 (3.19–41.24)	17.60 (3.58–33.64)	16.84 (3.16–27.33)	
Change	–8.82 (–22.37–5.84)	–6.06 (–20.08–17.35)	–2.10 (–14.57–6.07)	
IL-8 (ng/mL)				
Run-in period	21.28 (14.80–43.30)	20.09 (9.72–49.71)	19.28 (6.74–40.12)	0.506
Intervention period	8.86 (3.16–17.51)	8.68 (3.32–39.50)	9.40 (2.65–20.56)	
Change	–14.60 (–25.79–0.85)	–10.21 (–23.89–4.87)	–8.83 (–32.82–4.77)	
TNFα (ng/mL)				
Run-in period	4.12 (1.57–14.89)	4.51 (0.70–7.03)	4.00 (1.60–8.88)	0.659
Intervention period	2.53 (0.77–7.56)	3.42 (0.99–4.50)	2.38 (1.50–7.32)	
Change	–1.61 (–9.53–2.95)	–1.37 (–5.13–3.07)	–0.86 (–4.84–2.11)	

^a For experimental details: see table 6.2. Values are medians (ranges). Whole blood was stimulated for 6 hours with 10 ng/mL LPS from *E Coli* 055:B5. Values are medians (ranges). The results of one subject of the c9, t11 CLA group were missing, because not enough blood was drawn.

For abbreviations see Table 6.3.

As shown in **Table 6.5**, Spearman correlations between the different cytokine concentrations produced by stimulated PBMC at the end of the run-in period were high ($P < 0.0001$). Spearman correlations between the cytokines in whole blood were weaker than those of PBMC, but still reached statistical significance ($P < 0.05$).

Table 6.5: Spearman Rank correlations at the end of the run-in period between different cytokine concentrations after stimulation of PBMC or whole blood and between cytokine concentrations after stimulation of PBMC vs whole blood.^a

	r	P-value
PBMC		
IL-6 vs IL-8	0.91	<0.0001
IL-6 vs TNF α	0.81	<0.0001
IL-8 vs TNF α	0.74	<0.0001
Whole blood		
IL-6 vs IL-8	0.51	0.002
IL-6 vs TNF α	0.47	0.005
IL-8 vs TNF α	0.33	0.048
PBMC vs whole blood		
IL-6	0.29	0.113
IL-8	0.31	0.094
TNF α	0.17	0.340

^a PBMC and whole blood were stimulated for 6 hours with 10 ng/mL LPS from *E Coli* 055:B5. For abbreviations see Table 6.3.

Effects of c9, t11 or t10, c12 CLA on PBMC and whole blood stimulation

Effects of the dietary interventions on IL-6, IL-8 and TNF α production in PBMC and whole blood after LPS stimulation are shown in Tables 6.3 and 6.4. In PBMC, IL-6, IL-8 and TNF α production increased in both intervention groups and in the control group. Although the increase in the three cytokines was the highest in the t10, c12 CLA group, the differences between the groups did not reach significance (*P*-values for diet effects: IL-6, *P* = 0.439; IL-8, *P* = 0.427; TNF α , *P* = 0.175; Table 6.3). In whole blood, IL-6, IL-8 and TNF α production decreased in both intervention groups and in the control group. The decrease in the three cytokines was less in the t10, c12 CLA group, but again the differences between the groups did not reach significance (*P*-values for diet effects: IL-6, *P* = 0.121; IL-8, *P* = 0.506; TNF α , *P* = 0.659; Table 6.4).

C-reactive protein

Two subjects of the control group and two subjects of the c10, t12 CLA group with CRP values higher than 8 mg/mL were excluded from the analysis. At the end of the run-in period, CRP concentrations were significantly different between the groups (*P* = 0.042; **Table 6.6**). Changes between groups, however, were not significantly different (*P* = 0.557; Table 6.6). Also ANCOVA analysis with week 1 concentrations as co-variant resulted in the same conclusion.

Table 6.6: Effects of c9, t11 CLA or t10, c12 CLA on plasma C-reactive protein (CRP) concentrations.^a

	Control	c9, t11 CLA	t10, c12 CLA	<i>P</i> -value
	(n = 9)	(n = 14)	(n = 10)	
CRP (mg/L)				
Run-in period ^b	0.90 ± 0.50	2.77 ± 2.21	1.68 ± 1.50	
Intervention period	0.75 ± 0.55	2.97 ± 2.40	2.01 ± 1.39	
Change	-0.15 ± 0.36	0.19 ± 0.99	0.33 ± 1.32	0.557

^a For experimental details: see table 6.2. Values are means ± SD. Two subjects of the control group and two subjects of the t10, c12 group with values higher than 8 mg/L were excluded for analysis. Of one subject of the control group no CRP analysis was performed, because of lack of the samples.

^b Significant difference between the groups at the end of the run-in period (*P* = 0.042).

Inflammatory protein expression profiles

In the pooled plasma samples, twenty-seven cytokines, chemokines or growth factors of the 42 different spots on the antibody arrays were detectable and could be semi-quantified. **Figure 6.1** shows the absolute values (in Arbitrary Units) of the control group at the end of the run in period. **Figure 6.2** shows that the number as well as the extent of the changes were more pronounced in the c9, t11 CLA group as compared to those in the t10, c12 CLA group. The observed changes were mostly increases, but decreases were also found. Consumption of the c9, t11 and t10, c12 CLA isomers caused changes into the same directions (e.g. TARC and IL-1 α), as well as into opposite directions (e.g. EGF and TNF β). Further, some cytokines showed only a change in the c9, t11 CLA group (e.g. SDF-1, IL-1 β and IL-2) or in the t10, c12 CLA group (e.g. GRO). The increases of the three MCP subclasses in the c9, t11 CLA group were very consistent, while some interleukins showed changes into different directions (e.g. IL-1 α and IL-1 β). When the cytokines were divided into different classes (chemokines, colony stimulating factors (CSF), growth factors, and stimulating and suppressive cytokines), the profile remained complex. However, in both CLA groups the concentrations of chemokines that attract granulocytes (neutrophils, eosinophils and basophils) (except GRO in the t10, c12 CLA group) increased, while concentrations of chemokines that mainly attract monocytes, macrophages and T-lymphocytes decreased (except MIP-1 δ and RANTES in the c9, t11 CLA group). Furthermore, concentrations of MCSF and thrombopoietin (Tpo), two CSF, were clearly elevated in both CLA groups, but more pronounced in the c9, t11 CLA group. Also concentrations of IL-10, an example of a suppressive cytokine, were increased, an effect that was stronger in the t10, c12 CLA group. Effects on growth factors and stimulating cytokines were less uniform. Leptin plasma concentrations were decreased in both CLA groups.

Figure 6.1: Absolute protein expression in control group.

The absolute protein expression (in arbitrary units) of different cytokines, chemokines and growth factors of the control group at the end of the run-in period as measured by an antibody array. The sensitivity of the array is not the same for the different proteins, therefore the heights of the bars do not represent concentrations. For experimental details see Subjects and Methods. The proteins are divided into different classes i.e. chemokines, colony stimulating factors (CSF), growth factors, and stimulating (+) and suppressive (-) cytokines. ENA, epithelial-derived neutrophil activating protein; GRO, growth regulated protein; IL, interleukin; MCP, monocyte chemotactic protein; MDC, macrophage derived chemokine; MIP, macrophage inflammatory protein; Rantes, regulated upon activation normal T-cell expressed and secreted; SDF, stromal cell-derived factor; TARC, thymus and activation regulated chemokine; MCSF, macrophage colony stimulating factor; Tpo, thrombopoietin; EGF, epidermal growth factor; OSM, oncostatin M; PDGF, platelet-derived growth factor; VEGF, vascular endothelial growth factor; IFN, interferon; TNF, tumor necrosis factor; Ang, angiogenin.

Figure 6.2: Inflammatory protein expression profile. Effects of 13 weeks c9, t11 CLA or t10, c12 CLA consumption on plasma concentrations of different cytokines, chemokines and growth factors as measured by an antibody array. The percentage changes for each protein in the intervention groups were calculated compared to the control group. For experimental details see subjects and methods section. The proteins are divided into different classes i.e. chemokines, colony stimulating factors (CSF), growth factors, and stimulating (+) and suppressive (-) cytokines. The chemokines are furthermore subdivided into chemokines that attract granulocytes (gran) (neutrophils (neu), basophils (baso) and eosinophils (eos)), monocytes/macrophages (mono/macro), and T-lymphocytes (T lymph). For abbreviations see Figure 6.1.

Correlations

At the end of the run-in period, no significant correlations were found between the proportions of c9, t11 and t10, c12 CLA in plasma phospholipids with cytokine production. Body weight, however, correlated positively with TNF α production in whole blood ($r = 0.359$, $P = 0.029$), as well as with IL-6 or IL-8 production by PBMC ($r = 0.423$, $P = 0.014$ and $r = 0.406$, $P = 0.023$). In line with this, TNF α production by PBMC correlated with BMI ($r = 0.395$, $P = 0.023$), and IL-6 production by whole blood and PBMC with visceral adiposity ($r = 0.438$, $P = 0.007$ and $r = 0.436$, $P = 0.011$, respectively). Also BMI correlated with plasma CRP concentrations ($r = 0.568$, $P = 0.001$). At the end of the intervention period, we found a significant correlation of 0.698 ($P = 0.008$) only between the changes in the proportions of c9, t11 CLA in plasma phospholipids and the changes in TNF α production in whole blood in the c9, t11 CLA group.

Discussion

In animals, inconsistent effects of CLA on immune parameters have been reported, whereas in most humans no effects of CLA mixtures could be shown at all (3, 4). It has been suggested that these inconsistencies might be due to opposite effects of the two most common CLA isomers (4). Recently, however, Tricon *et al.* (5) showed no effects of the c9, t11 or t10, c12 CLA isomers on *ex vivo* cytokine production by LPS and concanavalin A stimulated PBMC. It is possible that this lack of effect is related to the fact that the study population consisted of young, healthy subjects. Another explanation might be the absence of effects on cytokine production by stimulated PBMC, whereas CLA may affect plasma cytokine concentrations in non-stimulated conditions. In our study we have therefore evaluated the effects of the two individual CLA isomers side-by-side in moderately overweight subjects at increased risk for CHD, which are expected to have a proinflammatory profile based on their BMI, both after LPS stimulation as well as in non-stimulated plasma. We found positive correlations between different indices of obesity (weight, BMI, visceral adiposity) and LPS stimulated production of different cytokines (IL-6, IL-8 and TNF α) by PBMC and in whole blood. It is thus conceivable that our study population is more responsive to interventions with potential effects on inflammation parameters. However, in the present study we also found no effects of a daily consumption of 3 g of c9, t11 or t10, c12 CLA for 13 weeks on *ex vivo* cytokine production by isolated PBMC or by PBMC present in whole blood after stimulation with LPS. Our results not only agree with those of Tricon *et al.* (5), but also with earlier studies in which mixtures of the various CLA isomers did not alter the *ex vivo* cytokine production by LPS stimulated PBMC in healthy men and women (25, 26). Taken together, however, studies in various population groups do not suggest that consumption of the purified CLA isomers or mixtures of CLA isomers affects *ex vivo* cytokine production by stimulated PBMC or whole blood.

Effects of CLA on immune cell functions have also been studied in various animal models. Results were equivocal and varied from stimulation to inhibition, depending on the parameter examined. In fact, conflicting results for the same parameter have even been found within the same animal species, for which there is no clear explanation (3). It is possible, however, that effects may depend on the composition of the background diet. When rats were fed a soybean oil-based diet supplemented with a CLA mixture, *ex vivo* basal and LPS-stimulated secretions of IL-6 by resident peritoneal macrophages were reduced (27). These effects were not observed when the CLA mixture was added to a menhaden and safflower oil-based diet (27). In the same study, CLA feeding also decreased basal, but not LPS-stimulated, secretion of TNF α , but in this case effects did not depend on the background diet (27). When mice were fed a diet supplemented with purified c9, t11 or t10, c12 CLA, increases in

TNF α and IL-6 secretion were found by *ex vivo* cultured splenocytes after LPS stimulation. Effects were comparable for both CLA groups (28). These two animal studies examined immune function in a comparable manner as we did and found both immune-stimulating and -suppressing effects. Many other animal studies have examined other aspects of the immune system, like lymphocyte proliferation and antibody responses, and also reported both immune stimulation and suppression (3, 4).

The reason that we observed no significant effects of each isomer cannot be explained by non-compliance, as shown by the significant changes in fatty acid composition of plasma phospholipids. Further, it has been demonstrated that both c9, t11 and t10, c12 CLA are incorporated into PBMC lipids, although less efficiently than into plasma phosphatidylcholine (PC) and cholesteryl esters (29). In our study CLA was given to the volunteers with a dairy product and not by capsules, as in many other studies. This, however, did not affect the bioavailability of the CLA, as we have recently reported that the proportion of CLA incorporated into plasma phospholipids was in good agreement with studies that have used capsules (30). In our study, plasma CRP concentrations increased slightly, in particular in the t10, c12 CLA group. Differences between the groups did not, however, reach statistical significance. This agrees with the findings of Tricon *et al.* (5) who also showed no effects of a daily intake of 2.5 g of the individual CLA isomers on serum CRP concentrations in healthy subjects. Risérus *et al.* (31), however, observed that 12 weeks supplementation with 3.4 g of the purified t10, c12 CLA isomer increased plasma CRP concentrations of obese men with metabolic syndrome. It is possible that these inconsistencies can be explained by differences in intakes, which was the highest in the study of Risérus *et al.* As BMI is a major determinant of CRP concentrations, another explanation is that t10, c12 CLA may affect CRP concentrations in subjects with increased CRP concentrations. Again, the mean BMI of the subjects in the study of Risérus *et al.* was the highest (30 kg/m²) and in the study of the Tricon *et al.* the lowest (25 kg/m²).

Besides cytokine production of LPS stimulated PBMC, effects of CLA consumption on plasma concentrations of cytokines were also evaluated. To our opinion, in this way two different processes are measured. When stimulating PBMC with LPS, the potency of leukocytes to respond to a bacterial infection is simulated. Plasma cytokines do not originate only from leukocytes and they may also be secreted from other cells, such as adipocytes (32),(33). In particular, fasting levels of various cytokines, acute phase proteins and/or chemokines are valuable predictors of future coronary risk (12-14). Risérus *et al.* (31) measured fasting plasma concentrations of TNF α and IL-6 but showed no changes after intake of the CLA mixture (containing 35.4% c9, t11 and 35.9% t10, c12 CLA) and the purified t10, c12 CLA isomer. We extended these observations by measuring an inflammatory signature consisting of twenty-seven different cytokines, chemokines or growth factors in pooled plasma with

an antibody array. Our array data, however, suggested slight changes in fasting plasma TNF α concentrations, which increased in the t10, c12 CLA group and decreased in the c9, t11 CLA group. Since these signatures were analyzed in pooled material, we cannot draw any conclusions based on statistical analysis. However, as already indicated, this array was intended to generate hypotheses on possible differential immune modulating effects of the two CLA isomers, which will be discussed below.

When expression profiles of different cytokines, chemokines and growth factors in pooled plasma samples were evaluated, we found that the c9, t11 and t10, c12 CLA isomers induced different protein expression profiles, whereas changes in the c9, t11 CLA group were more pronounced. It has indeed been suggested that the two CLA isomers have different effects on immune functions (4). Furthermore, in general, both isomers induced more increases in protein concentrations than decreases, which indicates an enhanced immune function. This observation was particularly apparent for the c9, t11 CLA isomer. When looking in more detail at the protein expression profiles, we observed that plasma concentrations of chemokines that attract granulocytes, which are important in the first phase of an acute inflammation caused by microorganisms (neutrophils) or parasites (eosinophils and basophils), were increased by mainly the c9, t11 CLA isomer. On the other hand, concentrations of chemokines more related to monocyte and lymphocyte migration were hardly affected and even tended to be lowered. Therefore, the expression profiles of both CLA isomers, and particularly that of c9, t11 CLA, suggested improved resistance against pathogens. Animal studies have indeed reported that CLA increased resistance to infections (4). Also in humans, 12 weeks consumption of 1.7 g/d of a 50:50 mixture of c9, t11:t10, c12 CLA isomers beneficially affected the initiation of a specific antibody response towards hepatitis B vaccination, indicating enhanced immune function (25). However, a similar approach to test the effects of consumption of 3.9 g/d of a CLA mixture for nine weeks on antibody response towards an influenza vaccine was not successful (34). The lack of effect in this study could be due to the wide range of CLA isomers in the mixture (t10, c12, 22.6%; c11, t13, 23.6%; c9, t11, 17.6%; t8, c10, 16.6%; other isomers 19.6%). The effects on growth factors and cytokines cannot easily be interpreted. Again, effects of the c9, t11 CLA on each of the detectable proteins were more pronounced. The concentration of the colony stimulating factor MCSF (also called CSF-1) and of interferon γ (IFN γ), which both stimulate monocyte/macrophage cells, were increased. The cytokine IL-1 α , which is produced by macrophages, was, however, decreased. The increase in IL-2 expression in the c9, t11 CLA group has also been reported in mice (4). Whether our findings are favorable or not is difficult to conclude. On one hand, the changes in the inflammation parameters could result in increased resistance against pathogens. On the other hand, increased plasma concentrations of inflammatory

markers (e.g. MCP-1) may be related to increased cardiovascular risk, especially in a high CHD risk population (12-14).

The antibody array did suggest modest decreases in leptin concentrations in both CLA groups. Decreases in leptin levels were also shown in mice models and a similar tendency was seen in rats (35). In humans effects on leptin are contradictory. Medina *et al.* (36) only showed a significant decrease after 7 weeks, which returned to baseline after 9 weeks of intervention with a CLA mixture. Two other studies showed no effects on leptin levels after 12 weeks and after 1 year (37, 38).

Thus, there are no effects of a daily consumption of 3 g of the c9, t11 or t10, c12 CLA isomer on *ex vivo* LPS-stimulated cytokine production by PBMC and by whole blood in moderately overweight subjects with a high risk for CHD. Also, plasma CRP concentrations did not change. However, inflammatory signatures in non-stimulated fasting plasma raised the hypothesis that both CLA isomers enhanced immune function - in particular an increased resistance against pathogens - with more pronounced effects of the c9, t11 CLA isomer. These effects, however, should be confirmed in future studies for the individual cytokines, chemokines and growth factors.

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Chapter 7

General discussion

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Introduction

The major objective of the studies presented in this thesis was to examine the immune modulating potential of specific nutritional components. For this, we have considered both immune enhancing and immune suppressive food components. Although the emphasis was on intestinal immune function, also systemic immune function was evaluated in a human intervention study. In the first part of this discussion the immune modulating effects of the various nutritional components - β -glucan, fish oil and CLA - will be discussed and in the second part these results will be placed into a broader perspective.

β -Glucan

Dietary oat β -glucans are known for their cholesterol lowering effects, but immune modulating effects can also be expected due to similarities in structure with fungi and yeast β -glucans. These fungi and yeast β -glucans have known immune modulating effects especially on lymphocytes. (1-3) Because dietary β -glucans first reach the intestine, in the first study (**chapter 2**) we examined the immune modulating effects of dietary oat β -glucan on enterocytes. For this, we enriched the diets of ileostomic patients (all patients were proctocolectomized) with oat β -glucan or a control fiber (rice starch) and collected ileostomic contents for 24 hours. From these ileostomic contents fecal water was prepared and supplied to four different enterocyte cell lines (two small intestinal cell lines and two colon cell lines) in an *in vitro* cell culture system. In this way, β -glucan present in the fecal water had passed the small intestine and was in a physiological matrix when added to the enterocyte cell lines. Because we did not had the possibility to directly measure intestinal immune function in the patients by taking for example biopsies, this was the most physiological approach and a standardized way to examine effects of β -glucan on enterocytes. Another approach that we could have followed, i.e. supplying β -glucan directly to enterocytes *in vitro*, would have been less physiological and has additional problems. The most important problem herein is the poor solubility of β -glucan in water. We found that fecal water with β -glucan enhanced the ICAM-1 and IL-8 response of small intestinal cells as well as colon epithelial cells as compared to placebo fecal water, when supplied together with cytokines to stimulate the enterocytes. Also various inflammatory proteins (chemokines - especially monocyte chemotactic proteins (MCPs) -, growth factors, colony stimulation factors and cytokines) showed an increased expression when measured simultaneously by using an antibody array. Altogether these results indicate that fecal water enriched with β -glucan stimulated the cytokine-induced immune response of enterocytes. However, with this approach we cannot distinguish between direct and indirect effects of

β -glucan. With direct effects we mean effects of β -glucan present in the fecal water on the intestinal cell of the *in vitro* model. Indirect effects on the enterocytes of the *in vitro* system can be due to changes in the composition of the fecal water, as a result of effects of β -glucan on various cells in the patients *in vivo*.

Concerning effects of β -glucan, the pattern recognition receptor (PRR) dectin-1 plays a central role, but also other receptors could mediate β -glucan effects. (4) So far, it is not convincing whether intestinal epithelial cells, and especially the various cell lines we have used (Caco-2, INT407, HT29 and T84), express dectin-1. Dectin-1 mRNA has been shown in Caco-2 cells (5), but the expression of dectin-1 protein on intestinal epithelial cells *in vivo* is under debate. To examine whether the *in vitro* effects we reported were dectin-1 mediated, our experiments should be repeated while blocking the dectin-1 receptor. Concerning indirect effects, these can be ascribed to many different – maybe unknown – factors. As an example of indirect effects, β -glucan can affect the secretion of antimicrobial peptides like defensins by several cell types in the gastrointestinal tract of the ileostomy patients and thereby changing their presence in the fecal water. Because of the many possibilities when considering indirect effects, the only way to elucidate whether these effects are important, is to add purified β -glucans directly to the intestinal cells *in vitro*. To circumvent problems related to the low water-solubility of oat β -glucan, alternative approaches should therefore be developed.

β -Glucans from different sources (yeast, fungi, oat, and barley) have both stimulating and inhibiting effects on specific immune functions. However, effects may not only be source-dependent. Also effects of β -glucans from the same source can differ, because of differences in characteristics such as length, molecular mass, tertiary structure and degree of branching. (6) For example, large MW and/or particulated fungal β -glucans can directly stimulate leukocytes, while fungal β -glucans with a low MW may suppress monocyte function. (6, 7) These differences in characteristics of β -glucans from the same source can depend on the isolation and processing of the β -glucans. A direct comparison of β -glucans from different sources and the same source, but with different characteristics, have not been performed so far and should be addressed in future studies.

Not only exploring the mechanisms behind the effects of β -glucans, but also evaluating the physiological implications of enhancement of the immune response by oat β -glucans should be addressed in future studies. Our finding that oat β -glucan does not induce an immune response in a non-cytokine stimulated condition, but enhances the cytokine-stimulated immune response is in agreement with known effects of low MW fungal β -glucans. (6) This characteristic is important when considering physiological possibilities. The fact that β -glucans enhance the immune response after triggering the immune system, could explain the increased resistance to infections as has been found

in other studies. (3, 8-10) For example, Estrada and colleagues showed in mice that intraperitoneal, subcutaneous and intragastric injection of oat β -glucan could increase resistance to systemic bacterial and intestinal parasitic infections. (3, 8-10) Moreover, human intervention studies showed that intravenous injections of yeast β -glucan reduced postoperative infections and mortality after high-risk surgery. (11-13) These findings showed that there is a possible role for β -glucans to increase resistance to infection, which can be especially interesting for subjects with a reduced immune function, such as elderly and diabetes mellitus subjects.

Fish oil

Fish oils have well-known health benefits on cardiovascular diseases, for which many mechanisms have been described, like reducing serum triglycerides and antithrombotic, antiarrhythmic and antiinflammatory effects. (14) These potential antiinflammatory benefits of fish oils have also been studied in inflammatory bowel disease (IBD) patients. (15) These patients suffer from chronic intestinal inflammation accompanied by increased colonic arachidonic acid (AA) proportions and decreased oleic acid (OA) proportions. (16-18) We therefore hypothesized that the antiinflammatory effects of fish oil might be caused by a reduction of the colonic AA content. To systematically examine the effects of fish oil versus AA and OA on intestinal inflammation we have performed an *in vitro* (**chapter 3**) and an *in vivo* (**chapter 4**) study. In the *in vitro* study we showed that enterocytes cultured with AA had an increased ICAM-1 expression and NF- κ B activation as compared to those cultured with EPA. Interestingly, effects of OA on ICAM-1 expression were comparable to those of EPA. Because EPA and OA resulted in comparable proportions of AA in the phospholipids, we concluded that the reported effects may be ascribed to the increased AA proportions in the AA cultured cells. Thus, decreasing cellular AA levels seems to be a crucial step. Since OA is already the most abundant fatty acid present in our diet (19) and in the colon mucosa (18), we suggested that decreasing mucosal AA levels may be easier by increasing fish oil intake than by increasing OA intake. Therefore we performed an *in vivo* study to examine the effects of fish oil (FO) or OA as compared to AA on experimentally induced colitis. In this study we found that mice fed an AA-enriched diet for 6 weeks preceding DSS-colitis induction did - as expected - have increased colonic AA contents, but did not have more severe colitis as compared to mice fed an OA- or FO-rich diet. On the contrary, after 7 days DSS-colitis induction, weight loss and diarrhea scores were less in the AA group as compared to the FO and OA groups. Thus, in the experimental model used, increasing AA intake seems not harmful, but may be even protective. Furthermore, we could only show some small protective effects of fish oil treatment as compared to OA; only MPO

concentrations in plasma and polymorphonuclear cells infiltration in the colon were slightly decreased in the FO group as compared to the OA group, but not as compared to the AA group. However, all other inflammatory parameters (weight and length of the colon, histological scores and cytokine concentrations in colon homogenates) showed no differences between the groups. Thus, although the proinflammatory effects of the n-6 PUFA AA versus the n-3 PUFA EPA *in vitro* were convincing, this could not be confirmed in our *in vivo* study. Although other animal studies have shown some protective effects of fish oil (20-26), well-controlled studies with clear effects are limited. Some studies also showed no protective effects or even showed increased colitis development (23, 27, 28). This indicates that effects of fish oil on inflammation *in vivo* may be small and highly specific. A concern when using experimental colitis models is that the type of inflammation depends on the model. Because fish oils may have effects on specific parts of an immune response, this might be apparent in one model but not in another model. The various animal models are for example Th1 (e.g. DSS colitis) or Th2 (e.g. TNBS colitis in balb/c mice) dependant (29). We used the dextran sodium sulphate (DSS)-induced colitis model in mice. In this model the physical agent DSS induces intestinal barrier disruption, which promotes cellular exposure to normal mucosal microflora. This will activate macrophages, which will induce a Th1 immune response. This model was in our hands not sensitive to the preventive effects of fish oils. Our study results are in contrast with those of Camuesco *et al.* (23) who showed protective effects of 2 weeks pretreatment of fish oil in the DSS model in rats. Genetic factors influence the susceptibility of different mice and rats strains for DSS colitis (29) and thus may influence effectiveness of treatments. Nieto *et al.* (26) showed positive effects of fish oil in a TNBS model in rats by using a therapeutic approach. After TNBS colitis induction the diets were supplemented with different fatty acids for 1 and 2 weeks. Thus the effect may also depend on the time point of supplementing the diets. Another possibility explaining differences in effects between studies is that conclusions on the effects of fish oil could depend on the compositions of the control diets, which were quite different between the various studies. Therefore, the complete fatty acid composition of the fish oil and control diets should be considered. In our study, the AA and FO diets contained equal amounts of PUFAs, which were either AA or n-3 PUFAs (EPA+DHA), whereas the OA diet contained less PUFAs and more MUFAs, which was mainly OA. More specifically, we exchanged 1% (wt/wt) of OA for AA or EPA+DHA. No other study compared the effects of fish oil versus AA. In stead, control diets with high contents of the n-6 PUFA linoleic acid (LA) were used. For example, in the study of Nieto *et al.* (26) protective effects of fish oil were shown as compared to a LA rich olive oil+soybean oil+coconut oil diet, whereas Camuesco *et al.* (23) showed protective effects of fish oil as compared to a LA-rich soybean oil diet, but not as compared to an OA-rich olive oil diet. Furthermore, the fatty acid composition of the colon

depends on the diets and will influence the local immune response. Therefore, the fatty acid composition of the colon should be examined in relation to the effects of dietary interventions. Although LA is a precursor for AA and FO supplementation should decrease tissue AA contents, in the studies of Nieto *et al.* (26) and Camuesco *et al.* (23) (the only two studies that determined colonic fatty acid composition) the colonic AA contents were not different between the diets. Moreover, we showed an increase in colonic LA content in the FO group versus the AA and OA group, whereas the two other studies found no change or a decrease in colonic LA content after fish-oil supplementation. (23, 26) Whether these differences in effect on colic fatty acid composition can explain the possible differential effects between fish oil studies remains to be determined. Also, when the outcomes of clinical trials with IBD patients are carefully considered, only some have shown major protective effects of fish oil (30, 31), while most studies have found only minor effects (32-40). This indicates that - although supported by epidemiological studies (41, 42) - effects of fish oil in intervention studies are not that clear. (15) Thus, although fish oil could have some positive effects for IBD patients, it should be realized that effects may be small and highly specific.

Conjugated linoleic acid (CLA)

Many health effects have been ascribed to CLA, such as anticarcinogenic, antidiabetic, antiobesity and antiatherosclerotic effects (43, 44). Moreover, CLA may have immune modulating properties (43, 45). Although animal studies have shown impressive effects of CLA on immune function, studies in humans so far did not show any effects (46-50) or only minor effects (51, 52). Furthermore, differences in effects may be due to differences in the composition of the CLA isomer mixture used or the use of purified isomers, because the c9, t11 and t10, c12 isomer may have distinct physiologic effects. (45) In our human study (**chapter 6**) we showed no significant effects of 13 weeks consumption of each of the two CLA isomers, c9, t11 and t10, c12, on *ex vivo* LPS-stimulated cytokine production by PBMCs or whole blood, and on plasma hs-CRP concentrations. If anything, we showed rather proinflammatory than suppressive effects. In non-stimulated plasma we showed for both isomers specific inflammatory signatures, in general indicating an enhanced immune function. For subjects with an increased risk of coronary heart diseases this could mean that CLA has undesirable effects. CLA supplementation is intended for this population because of the claims that CLA may favorably affect the lipid and lipoprotein profile and induces weight loss. (43) However, again most evidence for these effects is derived from animal studies, whereas the existence of these effects in humans is under debate. (53) Moreover, our finding that especially the t10, c12 isomer may have adverse effects on immune

parameters is supported by others. Riserus *et al.* (54) have reported earlier that the t10, c12 CLA isomer increased CRP concentrations in obese men with metabolic syndrome. Also a recent study of Taylor *et al.* (55) showed that endothelial function - as measured by flow mediated dilatation (FMD) - was impaired when healthy overweight subjects were supplemented with a commercially available isomeric mixture of CLA (35% c9, t11 + 36% t10, c12 + minor CLA isomers and other fatty acids) for 12 weeks. Altogether, this suggests that CLA is not suitable as a dietary supplement to reduce the risk for cardiovascular disease (CVD).

In contrast to the huge interest concerning the potential antiinflammatory effects of CLA in relation to CVD risk, the possibility of using CLA for the treatment of inflammatory bowel diseases (IBD) has been scarcely investigated. Again, clear protective effects of CLA mixtures were shown in various IBD animal models by the group of Hontecillas and Bassaganya-Riera (56-58). They demonstrated that mucosal damage was decreased by dietary supplementation with a CLA mixture in two porcine and two mouse colitis models. Moreover, they showed that this protection was not seen in mice devoid of PPAR γ expression in the intestine (57) and that CLA supplementation upregulated colonic PPAR γ expression (56), suggesting that this nuclear receptor has an important role in the protective effects of CLA. Especially the c9, t11 CLA isomer is a very potent natural ligand for PPARs with a binding affinity in the nM range. (59)

To examine these potential antiinflammatory effects of the c9, t11 and/or t10, c12 CLA isomers on intestinal inflammation we also performed a pilot *in vitro* study (unpublished data and not described in this thesis). We cultured intestinal epithelial Caco-2 cells, according to the methods described in chapter 3, for 20 days (ICAM-1 and IL-8) or 5 days (NF- κ B) with c9, t11 or t10, c12 CLA or control fatty acids in an iso-molaric approach. The control cells were cultured with 160 μ M oleic acid (OA) or 70 μ M linoleic acid (LA) + 90 μ M OA, whereas the CLA cells were cultured with 4 μ M CLA (either c9, t11 or t10, c12) + 66 μ M LA and 90 μ M OA. After this, the cells were stimulated with a cytokine mixture (IL-1 β and IFN γ). In **Figures 7.1 A-C** it is shown that both CLA isomers significantly increased cytokine stimulated IL-8 production and NF- κ B activation compared to LA+OA cultured cells, whereas we could not show significant differences on cytokine stimulated ICAM-1 expression. Although differences in effects between the CLA isomers have been suggested (45), in this model both isomers showed the same proinflammatory effects. These *in vitro* proinflammatory effects of the two CLA isomers are in sharp contrast to the anti-inflammatory effects of CLA mixtures in animal colitis models and needs further investigation. These results are also in contradiction with the PPAR binding affinities of CLA (59), because we have also shown that PPAR γ stimulation decreased NF- κ B activation in our model (chapter 3).

Figure 7.1: Immune modulating effects of conjugated linoleic acid (CLA) isomers, oleic acid (OA) and linoleic acid (LA) on enterocytes *in vitro*.

IL-8 production [in ng/mL] (A), ICAM-1 protein expression [in arbitrary units] (B), and NF- κ B transactivation measured by luciferase activity [in RLU/mg] (C) of enterocytes cultured for 20 (ICAM-1 and IL-8) or 5 days (NF- κ B) with 160 μ M OA, or 70 μ M LA + 90 μ M OA or 4 μ M c9, t11 CLA + 66 μ M LA + 90 μ M OA, or 4 μ M t10, c12 CLA + 66 μ M LA + 90 μ M OA with and without cytokine (50 U/mL IL-1 β and 100 U/mL IFN γ) stimulation for 16 (ICAM-1 and IL-8) or 3 hours (NF- κ B). Data are expressed as means and standard deviations. Statistical significance is determined with ANOVA and a post-hoc Bonferonni test, when differences between interventions were statistical different. ^a $P < 0.05$ vs OA, ^b $P < 0.05$ vs LA, ^c $P = 0.073$ vs OA and ^d $P = 0.074$ vs LA.

In conclusion, both CLA isomers and CLA mixtures may have minor effects on systemic immune parameters (and if anything the t10, c12 isomer seems proinflammatory), whereas effects on intestinal immune parameters are - although not extensively examined - inconsistent. Together with inconsistent or only minor effects on other health related parameters like body weight, serum lipids and lipoproteins and insulin resistance, there are no indications that either of the two CLA isomers or CLA mixtures are beneficial for health.

PPAR γ

PPAR γ is a transcription factor controlling the expression of numerous genes involved in many functions, like lipid and lipoprotein metabolism, glucose homeostasis, cell proliferation and differentiation, and apoptosis. (60) More recently it was demonstrated that PPAR γ also plays a role in the control of inflammatory responses. (61) Therefore, the role of PPAR γ in IBD is currently also an area of interest. (62) Protein levels of PPAR γ in colonic epithelial cells from ulcerative colitis patients are decreased as compared to those of healthy controls, while expression of PPAR γ in inflammatory cells of these patients is similar as compared to controls. (63) Since PPAR γ has antiinflammatory effects by inhibiting NF- κ B activation (64), which will down-regulate proinflammatory protein synthesis, decreased PPAR γ levels in epithelial cells can contribute to the elevated NF- κ B expression (65) and thus to the onset or maintenance of intestinal inflammation in IBD patients. Also, PPAR γ +/- heterozygous mice exhibited an increased susceptibility to experimental colitis (66, 67), indicating that expression of PPAR γ may have a regulatory role in the control of colitis. Moreover, overexpression of PPAR γ by an adenoviral construct in mucosal epithelial cells in mice with experimental colitis was associated with amelioration of inflammation. (68) Thiazolidinediones (TZDs) - currently used as insulin sensitizing agents in the treatment of type II diabetes mellitus - are high affinity synthetic ligands of PPAR γ . (69) Due to the antiinflammatory properties of PPAR γ , therapeutic efficacy of TZDs in various animal models of experimental colitis (e.g. DSS, TNBS and IL-10 knockout mice) has been evaluated. These animal studies indeed showed protective effects of PPAR γ ligands. (63, 64, 67-73) In contrast, in our mice study (**chapter 5**) rosiglitazone treatment for 16 days before DSS-colitis induction enhanced colonic inflammation. Changes in weight loss, diarrhea, colon weight and length, spleen weight, histological scores and plasma SAP concentrations indicated that colitis was more severe in rosiglitazone treated animals as compared to controls. These unexpected results might be explained by longer-term pre-treatment in comparison with earlier studies in which TZDs were given at the first day of colitis induction (66, 70, 71), one or two days before (66, 67, 72-74) or after colitis induction (67, 70, 75). Based on our data we speculated that this longer-term pre-treatment - which we applied to mimic a preventive approach - may increase susceptibility to intestinal injury by e.g. inducing intestinal barrier changes. One reported adverse effect of rosiglitazone in diabetic patients is edema, which was suggested by Lebovitz (76) to be caused by increased vascular permeability. Also in an *in vitro* study rosiglitazone increased permeability across a layer of pulmonary endothelial cells. (77) We have indeed shown a striking loss of expression of the tight-junction protein ZO-1 in rosiglitazone treated colitis mice. These results support our hypothesis that (longer-term) rosiglitazone treatment may increase intestinal barrier

permeability. Unfortunately, we had no control and rosiglitazone group in which we did not induced colitis, so we cannot conclude if rosiglitazone without colitis induction may induce intestinal barrier disruption. Thus in future (long-term) rosiglitazone studies, evaluation of intestinal barrier function before colitis induction should be performed to obtain more insight into the functional consequences of this TZD effect. Also, potential side effects in human interventions studies should be evaluated carefully.

In contrast to the measurements that indicated increased colitis severity, in the rosiglitazone treated mice $\text{TNF}\alpha$ protein levels in colon and spleen and $\text{IFN}\gamma$ colon levels were decreased, and those of IL-10 in the spleen were increased. Thus effects on these cytokines indicate antiinflammatory effects of rosiglitazone. Other studies showed the same effects on cytokines, but in these studies disease severity was also diminished. (66, 67, 70, 74) We hypothesize that our contradicting results of cytokines levels versus disease severity may be due to a non-adequate immune response to intestinal damage. This could be the result of too strong antiinflammatory effects caused by the longer-term pre-treatment with rosiglitazone. This could ultimately enhance bacterial translocation and consequently lead to more severe intestinal damage. Although other studies used the same rosiglitazone concentration in (shorter) therapeutic and preventive approaches, in future studies a dose response relationship for the longer-term preventive (dietary) mode should be evaluated. Although rosiglitazone binding to $\text{PPAR}\gamma$ is quite specific, also $\text{PPAR}\gamma$ independent effects of rosiglitazone are known (72) and could be responsible for the adverse effects of especially long-term treatment. Therefore differences in effects of various specific $\text{PPAR}\gamma$ agonists should be evaluated.

In conclusion, our results, indicating that rosiglitazone, when added to the diet in a preventive approach for a longer time period, results in a higher susceptibility for DSS-induced colitis, are in sharp contrast to promising results of earlier studies. Future research is warranted to unravel the mechanism underlying these unexpected effects and to confirm our results in other colitis models.

Considerations about modulating immune function

As explained into detail in the previous paragraphs, we conducted various *in vitro* and *in vivo* (animal and human) studies in which we used different methods to examine effects of nutritional components on intestinal or systemic immune function. We have tried to interpret these results in terms of health effects for humans. In the following paragraphs some general considerations will be discussed.

Great variation exists in parameters of immune function of normal healthy subjects. Thus it is not easy to interpret changes in this broad normal range and

translate them into benefits for individuals. Furthermore, there is not one single test that can define either the status or functional capacity of the total immune system, although it is possible to measure many parameters related to immune function. One opportunity is measuring effects on many inflammatory proteins simultaneously in one sample using for example an antibody array. This gives a lot of information about effects on various inflammatory proteins, which sometimes can be in a clear direction. E.g. in the *in vitro* study with β -glucan (chapter 2) we showed that in four different intestinal cell lines the expression of almost all inflammatory proteins was increased. However, effects are not always this consistent, which complicates interpretation. For example, *in vitro* enterocytes cultured with AA as compared to cells cultured with EPA (chapter 3) increased MCP-1 and angiotensin, whereas EPA increased IL-10, IL-6, MIP-1 α and GRO. The AA-induced increase in MCP-1 expression - which is also upregulated in IBD patients (78) - is in line with the observed proinflammatory effects of AA. Also the EPA-induced increase in IL-10 expression is in line with known antiinflammatory effects of bacterial delivery of recombinant IL-10 in IBD patients (79, 80) and fits with the assumed antiinflammatory effects of EPA. In contrast, the increased IL-6 expression by EPA as shown by the protein expression profile of the array is less easy to explain. IL-6 is an NF- κ B regulated protein, but although EPA decreased NF- κ B expression it increased IL-6 levels. However, not only NF- κ B is involved in IL-6 expression regulation. Thus, measuring inflammatory protein expression profiles by using antibody arrays will generate a lot of new questions to examine.

Another approach to get better insight into the various aspects of immune function is to examine the activation of transcription factors, which are involved in regulation of many inflammatory processes. NF- κ B is known as one of the most important key regulators of inflammation. (81) Activation of NF- κ B may therefore indicate stimulation of many immune parameters, whereas reduced NF- κ B activity may indicate inhibition of immune parameters. We therefore developed a NF- κ B reporter Caco-2 cell line to measure immune modulating effects of different food components.

Another problem when trying to measure immune function in humans is that only blood can easily be sampled. It should be remembered that only 2% of lymphocytes circulate at any given time (82). The activity of blood lymphocytes might not be a good indicator for that of tissue lymphocytes, which limits interpretation of data using blood cells. It is further not only important what happens in lymphocytes of targeted tissues, but also in tissue cells itself. The immune function of targeted tissues e.g. the intestine is a combination of tissue lymphocytes (as part of the GALT) and other tissue cells like enterocytes. In humans, the digestive tract is often the target tissue for health claims related to functional foods. A large market already exists for gut-functional foods, which e.g. claim to help maintaining a beneficial balance of intestinal bacteria, thereby contributing to a healthy digestive system and thus supporting the body's

natural defenses. However, one of the main problems, which hampers the development of gut health related functional foods is the lack of validated markers that relate to intestinal (barrier) function and especially intestinal immune function. Thus, in this active new area of research, new methodologies have to be developed. One promising marker of intestinal inflammation is calprotectin, a protein present in neutrophils, which appears in feces caused by the presence of neutrophils in the intestinal lumen during inflammation. (83) Indeed, calprotectin was elevated in IBD patients. (84) Using calprotectin in healthy subjects as a measure of subclinical intestinal inflammation has only recently been initiated as will be discussed in the next paragraphs.

Relation gut and cardiovascular diseases

Although the gut is a gate-keeper organ, hardly anything is known about the influence of gut functioning on the rest of the body. However, several lines of evidence suggest that there are relations between gut-barrier function and CVD. This makes evaluation of intestinal inflammation - even in a milder form as present in IBD patients - relevant from a different perspective. Nowadays it is generally accepted that chronic low-grade inflammation is an important aspect of CVD. (85) Three main risk factors - high LDL cholesterol concentration in the blood, irritating substances of cigarette smoke, and a high blood pressure - can cause endothelial (blood vessel wall) activation, which results in a chronic inflammation reaction and consequent foam cell formation, the first steps in CVD development. (85, 86) Recently, it has been suggested that each (chronic) inflammation process somewhere in the human body can increase CVD risk. For instance, people with chronic inflammation disorders like rheumatoid arthritis (87) or Crohn's disease (88) have an increased CVD risk. The role of intestinal inflammation in this respect was also illustrated by Krack *et al.* (89). They hypothesized that a leaky bowel (i.e. increased gut permeability), leads to bacterial and endotoxin translocation from the intestine into the circulation, which increases (systemic) immune activation. Immune activation will result in cytokine production, which can activate the endothelium, which - as explained - is the first step in CVD. Thus, it can be concluded that enhancing gut barrier functioning could result in a lower CVD risk. More research is necessary to address this new possible role of the gut in CVD.

Gut inflammation and lifestyle factors

A leaky bowel is suggested to be relative common in the general population. Poullis *et al.* (84) recently showed a positive association between the body mass index (BMI) and subclinical intestinal inflammation (as measured by fecal calprotectin). Thus, subjects with a higher BMI are in general characterized by higher levels of calprotectin. Fecal calprotectin levels do not vary much from day-to-day (84), suggesting that calprotectin is a marker for a chronic inflammation process. It is nowadays generally accepted that obesity is a risk factor for CVD, which is associated with systemic inflammation. There is ample evidence that this proinflammatory profile is due to cytokine (e.g. $\text{TNF}\alpha$, IL-6, CRP, MCP-1) production by adipocytes. A relatively new paradigm is the potential association between obesity and increased gut permeability or even local inflammation in the intestine. (90) Also in mice it was shown that obesity leads to an increased intestinal permeability. (91) Whether local gut inflammation causes or otherwise enhances systemic inflammation, or the other way around, needs further investigation. Besides a correlation with BMI, there is also a positive relationship between fecal calprotectin and increased age, physical inactivity, and an inverse relationship with fiber intake and vegetable consumption. (84) This increase in fecal calprotectin with age is possibly a result of failing (intestinal) immune function when getting older. Physical inactivity and increasing age are also risk factors for CVD and are associated with low-grade systemic inflammation as indicated by increased serum CRP and IL-6 levels. (84, 92)

The presence of a leaky gut in 'healthy' subjects has not been systematically investigated so far. However, many factors are known to play a role in intestinal functioning and a leaky gut could thus be quite common. Besides the already mentioned factors, other factors that could be thought of causing a leaky gut are (http://drkaslow.com/html/leaky_gut.html):

- food allergy (e.g. gluten intolerance, Celiac disease)
- imbalance of gut microflora (e.g. low amounts of bifidobacteria)
- malnutrition or malabsorption of nutrients
- genetic predisposition (e.g. subjects with Crohn's disease and their relatives)
- chronic stress
- use of medication (e.g. antibiotics, hormones (prednisone), NSAIDs)
- excess alcohol intake

More research is therefore necessary to examine the role of various lifestyle factors on gut functioning and the possible role of nutrition to influence this function.

Conclusions and recommendations

Nutritional components can both enhance and dampen the immune function. Depending on the target population this will have beneficial or adverse effects. For example, by stimulating intestinal barrier function via enhancing immune responses, the resistance to infections may be increased. In this respect, our findings showing immune enhancing effects of oat β -glucan in enterocytes are promising. The mechanisms underlying these effects are currently under investigation. By inhibiting exaggerated intestinal immune responses, chronic intestinal inflammation can be prevented. In this respect, antiinflammatory effects of fish oil on IBD development are widely accepted, but results are not consistent. Furthermore, only minor (stimulating) effects of CLA on systemic inflammation were shown and effects on intestinal inflammation have not been examined extensively so far. In contrast to results from others, our pilot data on enterocytes *in vitro* are not very promising for IBD patients.

In general, nutritional components are a promising tool to modulate intestinal and systemic immune function, but more properly designed studies are needed. Furthermore, it should be realized that gut barrier function and intestinal inflammation is influenced by many lifestyle and nutritional factors. Moreover, well-defined and validated tools and biomarkers to examine effects on immune function and especially intestinal function have to be developed. Finally, it is a challenge for future research to elucidate the possible role of subclinical intestinal inflammation in CVD risk development.

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Summary

Summary

The immune system protects the human body against invasions with pathogens such as bacteria, viruses and parasites. If the function of the immune system is suppressed or impaired, this may lead to an increased infection risk. However, when reactions of the immune system are unbalanced and sustained, this may lead to chronic inflammation. Chronic inflammation is associated with several common diseases in Western countries, e.g. cardiovascular diseases (CVD), rheumatoid arthritis and inflammatory bowel disease (IBD). In this thesis the focus was on immune responses related to IBD and CVD. IBD is a chronic inflammatory condition of the intestine, of which two distinct forms exist, namely ulcerative colitis (UC) and Crohn's disease (CD). CVD pathogenesis involves the accumulation of fat, cholesterol and immune cells in the walls of arteries, which form atherosclerotic plaques. Thus, for both diseases the immune system is clearly involved. Nutritional compounds can enhance or dampen immune responses. In this thesis we have examined the potential immune modulating effects of β -glucan, fish oil and conjugated linoleic acid (CLA).

β -Glucans are carbohydrates (fibers), which are major structural components of the cell walls of cereals, such as oat and barley, but also of yeast and fungi. In the past decade, cereal β -glucans received a lot of interest because of their cholesterol-lowering activities. Although the immune modulating effects of β -glucans from yeast and fungi have been broadly examined, studies evaluating the immune modulating effects of oat and barley β -glucans are relatively new. In **chapter 2** we examined the effects of oat β -glucan on intestinal inflammation related processes. Because direct examination of effects of β -glucans in cell cultures is difficult, we supplied ileostomic patients with a β -glucan enriched diet or a control diet in a crossover design and prepared fecal water from the ileostomic content. We found that fecal water enriched with β -glucan as compared to placebo fecal water enhanced the interleukin (IL)-8 and inter-cellular adhesion molecule (ICAM)-1 response of small as well as colon intestinal epithelial cell lines *in vitro*, when supplied together with cytokines to stimulate the enterocytes. IL-8 and ICAM-1 are both involved in the recruiting, binding and activation of immune cells, thus an increased expression indicates an enhanced immune response. Also various inflammatory proteins showed an increased expression, when measured with an antibody array. Altogether these results indicate that fecal water containing β -glucan stimulated the cytokine-induced immune response of enterocytes *in vitro*. However, with this approach we were not able to distinguish between direct effects of β -glucan present in the fecal water, and indirect effects due to changes in the composition of the fecal water as a result of effects of β -glucan consumption on various cells in the ileostomic patients *in vivo*. Exploring the mechanisms behind the effects of β -glucans and physiological implications of enhancement of the immune response by oat β -glucans should be addressed in future studies. The fact that β -glucans from oat

enhance the immune response after triggering the immune system, might explain the increased resistance to infections as is found in other animal and human studies with oat and yeast β -glucans. This might be especially relevant for subjects with a reduced immune function, such as elderly and diabetes mellitus patients.

Fish oils are rich in the n-3 polyunsaturated fatty acids (PUFAs) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Because of their anti-inflammatory effects, using n-3 PUFAs, of which EPA and DHA in particular, have been suggested as treatment for various inflammatory diseases, such as IBD. Interestingly, the colon mucosa of IBD patients contains higher proportions of the n-6 PUFA arachidonic acid (AA) and lower proportions of the n-9 monounsaturated fatty acid oleic acid (OA) as compared to control subjects. Therefore, we made a direct side-by-side comparison between effects of OA, AA and fish oil (FO) on intestinal inflammation first *in vitro* (**chapter 3**) and later on *in vivo* in mice (**chapter 4**). With this approach we tried to elucidate if the protective effects of fish oil are due to a lowering of intestinal n-6 PUFA content or due to intrinsic antiinflammatory effects of fish oil. In the *in vitro* study (**chapter 3**) we have shown that enterocytes cultured with AA had an increased ICAM-1 expression and an increased activation of the transcription factor NF- κ B as compared to EPA and OA. NF- κ B activation induces transcription of genes encoding many proinflammatory proteins and NF- κ B is thus an important regulator of inflammation. In IBD patients NF- κ B activation in the colon is increased compared to control subjects. Thus, culturing enterocytes with AA induced proinflammatory effects versus EPA and OA. Because EPA and OA resulted in comparable proportions of AA in phospholipids of the enterocytes, we concluded that the reported effects may be ascribed to increased AA proportions in AA cultured cells. Therefore, decreasing cellular AA levels seems to be a crucial step, which may be easier to achieve by increasing fish oil intake than by increasing OA intake, since OA is already highly present in our diet. To verify our *in vitro* results, we performed an *in vivo* study (**chapter 4**) in which we compared an OA- versus an AA- and an FO-enriched diet on experimentally induced colitis. In this study we found that mice fed an AA-enriched diet for 6 weeks preceding dextran sodium sulphate (DSS)-colitis induction did - as expected - have increased colonic AA contents, but did not have more severe colitis as compared to mice fed an OA- or FO-rich diet. On the contrary, after 7 days DSS-colitis induction, weight loss and diarrhea score were less in the AA group as compared to the FO and OA groups. Thus, in the experimental *in vivo* model, increasing AA intake seems not harmful, but may be even protective. Furthermore, we could only show some small protective effects of FO treatment as compared to OA. Although the proinflammatory effects of the n-6 PUFA AA versus the n-3 PUFA EPA *in vitro* were convincing, this could not be confirmed in our *in vivo* study in mice.

To elucidate the role of the transcription factor PPAR γ - which is supposed to play a role in explaining the antiinflammatory effects of fish oil - on intestinal

inflammation, effects of supplementing the diet of mice with the synthetic PPAR γ ligand rosiglitazone on colitis development was examined in **chapter 5**. In contrast to earlier studies showing protective effects of PPAR γ activation, in our mice study rosiglitazone treatment for 16 days before DSS-colitis induction enhanced colonic inflammation. Changes in weight loss, diarrhea, colon weight and length, spleen weight, histological scores and plasma concentrations of the acute phase protein SAP (serum amyloid P component) indicated that colitis was more severe in rosiglitazone treated animals as compared to controls. These unexpected results might be explained by longer-term pre-treatment in comparison with earlier studies in which PPAR γ agonists were given at the first day of colitis induction, one or two days before or after colitis induction.

The last nutritional component of which immune modulating effects were evaluated was CLA. CLA is a mixture of positional (e.g. 9,11 or 10,12) and geometrical (*cis* or *trans*) conjugated isomers of the n-6 PUFA linoleic acid. *Cis*-9, *trans*-11 (c9, t11) CLA is a natural food component, predominantly found in the lipid fraction of meat, milk and other dairy products. *Trans*-10, *cis*-12 (t10, c12) CLA is particularly present in food supplements. Many health effects have been ascribed to CLA, among which immune modulating properties in animals and humans. Results - which are inconsistent - were however obtained mostly by evaluating effects of mixtures of CLA isomers. Since effects may be isomer specific, we conducted a placebo-controlled study in which we evaluated the effects of consumption of the individual c9, t11 or t10, c12 CLA isomers on inflammation parameters in subjects at increased risk for CVD (**chapter 6**). We were not able to show a significant effect of 13 weeks consumption by any of the two CLA isomers, c9, t11 and t10, c12, on *ex vivo* LPS-stimulated cytokine production by blood mononuclear cells, and on plasma C-reactive protein (CRP) concentrations. If anything, we showed rather proinflammatory than suppressive effects. Also in non-stimulated plasma we showed for both isomers specific inflammatory protein signatures, in general indicating an enhanced immune function. Altogether, this suggests that CLA is not suitable as a dietary supplement to reduce inflammatory responses.

In conclusion, nutritional components might be promising tools to modulate systemic and intestinal immune function, but more properly design studies in humans are needed. In this thesis we have showed that: 1) fecal water of ileostomic patients that consumed a β -glucan enriched diet enhanced the immune response of enterocytes *in vitro*, 2) the suggested proinflammatory effect of the n-6 PUFA AA versus n-3 PUFAs of fish oil in models of intestinal inflammation is not consistent, 3) using fish oil and oleic acid results in comparable effects on inflammation in an *in vitro* intestinal cell culture model and in an *in vivo* mice model of colitis, 4) the CLA isomers c9, t11 and t10, c12 have no effects on *ex vivo* LPS-stimulated cytokine production of blood mononuclear cells. However, additional research is needed to establish the importance of these findings *in vivo* in humans.

Samenvatting

Samenvatting

Het afweersysteem (ook wel het immuunsysteem genoemd) beschermt het menselijk lichaam tegen het binnendringen van ziekteverwekkers zoals bacteriën, virussen en parasieten. Als het functioneren van het immuunsysteem verminderd of verslechterd is, kan dit leiden tot een verhoogd risico op infecties. Aan de andere kant kan een ongecontroleerde of te sterke reactie van het immuunsysteem leiden tot chronische ontstekingsreacties (inflammatie). Chronische ontsteking is geassocieerd met verschillende, in Westerse landen, veel voorkomende ziekten, zoals hart- en vaatziekten, reumatoïde artritis en chronische inflammatoire darmziekten (in het Engels afgekort als IBD). In de studies, die in dit proefschrift zijn beschreven, lag de nadruk op immunoreacties die gerelateerd zijn aan chronische inflammatoire darmziekten en hart- en vaatziekten. Bij chronische inflammatoire darmziekten is (een deel van) de darm chronisch ontstoken. Hierbij wordt onderscheid gemaakt tussen twee verschillende vormen, namelijk colitis ulcerosa en de ziekte van Crohn. Hart- en vaatziekten ontstaan door het ophopen van vet, cholesterol en cellen van het immuunsysteem in de wand van bloedvaten. Dit proces wordt ook wel atherosclerose genoemd. Dus bij beide ziekten is het immuunsysteem duidelijk betrokken. Voedingsmiddelen kunnen het immuunsysteem stimuleren of remmen, hetgeen ook wel immunomodulatie wordt genoemd. In dit proefschrift hebben we de mogelijk immunomodulerende effecten van β -glucan, visolie en geconjugeerd linolzuur (CLA) onderzocht.

β -Glucanen zijn koolhydraten (voedingsvezels) en vormen een belangrijke component van de celwand van granen, zoals haver en gerst, maar ook van de celwand van gisten en schimmels. De laatste decennia is er veel aandacht voor de cholesterolverlagende effecten van β -glucanen uit granen geweest. Hoewel de immunomodulerende effecten van β -glucanen uit gisten en schimmels vaak zijn onderzocht, zijn er nog maar weinig studies uitgevoerd met β -glucanen uit haver en gerst. In **hoofdstuk 2** onderzochten wij de effecten van β -glucan uit haver op processen behorende bij een darmontsteking. Omdat het onderzoeken van effecten van β -glucanen in celstudies moeilijk is, hebben we eerst ileostoma patiënten in willekeurige volgorde een voeding verrijkt met β -glucan en een controle voeding gegeven. Vervolgens is van de ileostoma inhoud fecaal water gemaakt. We vonden dat fecaal water verrijkt met β -glucanen in vergelijking met controle fecaal water de interleukine (IL)-8 productie en expressie van het adhesie molecuul ICAM-1 van dunne en dikke darm cellijnen *in vitro* verhoogt, als het fecaal water tegelijk werd gegeven met cytokines om de darmcellen te stimuleren. IL-8 en ICAM-1 zijn beide betrokken bij het aantrekken, binden en activeren van immuuncellen. Een verhoogde productie en expressie van deze eiwitten duidt dan ook op een versterkte immunoreactie. Ook verschillende inflammatoire eiwitten (gemeten met een antilichaam array) lieten een verhoogde expressie zien. Alles bij elkaar duiden

deze resultaten erop dat fecaal water met β -glucan de cytokine-geïnduceerde immuunreactie van darmcellen *in vitro* stimuleert. Echter, met deze aanpak waren we niet in staat onderscheid te maken tussen de effecten van het β -glucan aanwezig in het fecaal water en de effecten ten gevolge van veranderingen in de samenstelling van het fecaal water door effecten van β -glucan consumptie op verschillende cellen in de ileostoma patiënten *in vivo*. Mechanismen achter de gevonden effecten van β -glucanen en fysiologische implicaties van de versterkte immuunreactie door β -glucanen uit haver moeten worden onderzocht in vervolgstudies. Het feit dat β -glucanen uit haver de immuunreactie versterken na stimulatie van het immuunsysteem zou mogelijk een verklaring kunnen zijn voor de verhoogde weerstand tegen infecties, zoals gevonden in dierstudies en humane studies met β -glucan uit haver en gist. Dit zou met name relevant kunnen zijn voor mensen met een verminderde immuunfunctie, zoals bijvoorbeeld ouderen en mensen met type II diabetes.

Visolie is rijk aan de n-3 meervoudig onverzadigde vetzuren (MOV's) eicosapentaeenzuur (EPA) en docosahexaeenzuur (DHA). Vanwege de anti-inflammatoire effecten van n-3 MOV's en van EPA en DHA in het bijzonder, wordt het gebruik van deze vetzuren gesuggereerd als behandeling voor verschillende inflammatoire ziekten, zoals IBD. Wetenswaardig is dat de mucosa van de dikke darm van IBD patiënten een hoger aandeel van het n-6 MOV arachidonzuur (AA) bevat en een lager aandeel van het n-9 enkelvoudig onverzadigde vetzuur oliezuur (OA) ten opzichte van controle personen. Daarom vergeleken wij de effecten van OA, AA en visolie (FO) op processen behorende bij een darmontsteking, eerst *in vitro* (**hoofdstuk 3**) en vervolgens *in vivo* in muizen (**hoofdstuk 4**). Met behulp van deze aanpak probeerden we te ontrafelen of de beschermende effecten van visolie het gevolg zijn van een verlaging van de hoeveelheid n-6 MOV's in de darm of het gevolg is van intrinsieke anti-inflammatoire effecten van visolie. In de *in vitro* studie hebben we laten zien dat darmcellen gekweekt met AA een verhoogde expressie van ICAM-1 en een verhoogde activatie van de transcriptie factor NF- κ B hebben ten opzichte van darmcellen gekweekt met EPA en OA. NF- κ B activatie induceert de transcriptie van genen die coderen voor pro-inflammatoire eiwitten en NF- κ B is dus een belangrijke regulator van ontsteking. In IBD patiënten is NF- κ B activatie verhoogd ten opzichte van die in controle personen. Dus het kweken van darmcellen met AA heeft pro-inflammatoire effecten ten opzichte van EPA en OA. Omdat het kweken met EPA en OA resulteerde in gelijke hoeveelheden van AA in fosfolipiden van de darmcellen concludeerden we dat de gerapporteerde effecten mogelijk toegeschreven konden worden aan de verhoogde AA hoeveelheden in de met AA gekweekte cellen. Daarom lijkt het verlagen van cellulaire AA niveaus de cruciale stap. Dit kan mogelijk makkelijker worden bereikt door het verhogen van visolie inname dan door het verhogen van OA inname, omdat OA al in grote hoeveelheden in onze voeding voorkomt. Om onze *in vitro* resultaten te verifiëren hebben we een *in vivo* studie

uitgevoerd (**hoofdstuk 4**), waarin we de effecten van een OA verrijkte voeding vergeleken met een AA en een visolie verrijkte voeding op experimenteel geïnduceerde colitis in muizen. In deze studie vonden we dat muizen die een AA rijk voer kregen gedurende 6 weken voorafgaande aan met dextraan natriumsulfaat (DSS) geïnduceerde colitis zoals verwacht een verhoogde hoeveelheid AA in de dikke darm hadden. Echter, de AA gevoede muizen kregen geen verergerde colitis ten opzichte van muizen die OA of FO verrijkt voer kregen. In tegenstelling tot een verslechtering vonden we na 7 dagen DSS-colitis inductie dat het gewichtsverlies en de mate van diarree in de AA groep minder erg was dan in de FO en OA groep. In dit experimentele *in vivo* colitis model lijkt een verhoogde AA inname dus niet schadelijk, maar mogelijk zelfs beschermend. Verder konden we alleen enkele minimale beschermende effecten van FO ten opzichte van OA laten zien. Hoewel de pro-inflammatoire effecten van het n-6 MOV AA ten opzichte van het n-3 MOV EPA *in vitro* duidelijk was, konden we dit niet bevestigen in onze *in vivo* studie in muizen.

Om de rol van de transcriptie factor PPAR γ , die mogelijk een rol speelt bij het verklaren van de anti-inflammatoire effecten van visolie, op darmontsteking te ontrafelen, hebben we in **hoofdstuk 5** de effecten onderzocht van het toevoegen van de synthetische PPAR γ ligand rosiglitazone aan het voer van muizen op colitis ontwikkeling. In tegenstelling tot eerdere studies die beschermende effecten van PPAR γ activatie lieten zien, zorgde in onze muizenstudie rosiglitazone behandeling gedurende 16 dagen voorafgaand aan DSS colitis inductie, voor een verslechtering van de darmontsteking. Veranderingen in gewichtsverlies, diarree, gewicht en lengte van de dikke darm, gewicht van de milt, histologie score en plasma concentraties van het acute fase eiwit SAP (serum amyloïd P component) leiden tot de conclusie dat colitis ernstiger was in rosiglitazone behandelde muizen ten opzichte van controle muizen. Deze onverwachte resultaten kunnen mogelijk verklaard worden door de langere duur van de voorbehandeling ten opzichte van eerdere studies waarin PPAR γ agonisten werden gegeven op de eerste dag van de colitis inductie, of slechts één of twee dagen voorafgaand of na de colitis inductie.

De laatste voedingscomponent waarvan de immuunmodulerende effecten zijn bekeken is CLA. CLA is een mengsel van positioneel (b.v. 9, 11 of 10, 12) en geometrisch (*cis* of *trans*) geconjugeerde isomeren van het n-6 MOV linolzuur. *Cis*-9, *trans*-11 (c9, 11t) CLA is een natuurlijke voedingscomponent die voornamelijk voorkomt in de vetfractie van vlees, melk en andere zuivelproducten. *Trans*-10, *cis*-12 (t10, c12) CLA komt met name voor in voedingssupplementen. Er zijn veel gezondheidseffecten beschreven voor CLA, waaronder immuunmodulerende effecten in dieren en mensen. Deze (veelal inconsistente) resultaten, zijn voornamelijk verkregen middels onderzoek naar effecten van mengsels van CLA isomeren. Aangezien effecten isomeer specifiek kunnen zijn, hebben wij een placebo-gecontroleerde studie uitgevoerd

waarin we de effecten van consumptie van de individuele c9, t11 of t10, c12 CLA isomeren op inflammatie parameters hebben bekeken in proefpersonen met een verhoogd risico op hart- en vaatziekten (**hoofdstuk 6**). We waren niet in staat om een significant effect van 13 weken consumptie van één van de twee CLA isomeren, c9, t11 of t10, c12, te laten zien op *ex vivo* LPS-gestimuleerde cytokine productie door mononucleaire bloedcellen en op plasma C-reactive protein (CRP) concentraties. De geringe effecten die we zagen, waren het eerder pro-inflammatoire effecten dan remmende effecten. Ook in niet gestimuleerd plasma zagen we voor beide isomeren specifieke inflammatoire eiwit profielen, die over het algemeen op een versterkte immuunfunctie wezen. Alles bij elkaar suggereert dit, dat voedingssuppletie met CLA niet bruikbaar is om inflammatoire reacties te verminderen.

Concluderend kunnen we stellen dat voedingscomponenten veelbelovend zouden kunnen zijn om de immuunfunctie in het algemeen, en specifiek van de darm, te moduleren, maar meer goed uitgevoerd onderzoek bij mensen is nodig. In dit proefschrift hebben we laten zien dat: 1) fecaal water van ileostoma patiënten die producten rijk aan β -glucanen uit haver hebben gegeten de immuunreactie van darmcellen *in vitro* versterkt, 2) het veronderstelde pro-inflammatoire karakter van het n-6 MOV arachidonzuur ten opzichte van de n-3 MOV's van visolie in modellen voor darmontsteking niet éénduidig is, 3) het gebruik van visolie en oliezuur vergelijkbare effecten op inflammatie heeft, zowel in *in vitro* studies met darmcellen als in een *in vivo* muizenmodel voor colitis, 4) de CLA isomeren *cis*-9, *trans*-11 en *trans*-10, *cis*-12, geen effect hebben op *ex vivo* LPS-gestimuleerde cytokine productie door mononucleaire bloedcellen. Echter, aanvullend onderzoek is noodzakelijk om het belang van deze bevindingen voor mensen *in vivo* aan te tonen.

Abbreviations

Abbreviations

a.u.	arbitrary units
AA	arachidonic acid
ANCOVA	analysis of covariance
Ang	angiogenin
ANOVA	analysis of variance
BMI	body mass index
BSA	bovine serum albumine
c9, t11	<i>cis</i> -9, <i>trans</i> -11
Caco-2	human colon adenocarcinoma cell line; differentiated small intestinal cell line
CAM	cell adhesion molecule
CBA	cytometric bead array
CD	Crohn's disease
CHD	coronary heart disease
CLA	conjugated linoleic acid
COX	cyclooxygenase
(hs)-CRP	(high sensitive)-C-reactive protein
CSF	colony stimulating factor
CVD	cardiovascular disease
DHA	docosahexaenoic acid
DSS	dextran sodium sulphate
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
En%	energy percentage
ENA	epithelial-derived neutrophil activating protein
EPA	eicosapentaenoic acid
FCS	fetal calf serum
FO	fish oil
GALT	gut-associated lymphoid tissue
GF	growth factor
GM-CSF	granulocyte/macrophage colony stimulating factors
GRO	growth regulated protein
HDL	high-density lipoprotein
HT29	human colon adenocarcinoma cell line
IBD	inflammatory bowel disease
ICAM	intercellular adhesion molecule
IFN	interferon
IL	interleukin
INT407	human small intestinal cell line
LA	linoleic acid
LDL	low-density lipoprotein

LOX	lipoxygenase
LPS	lipopolysaccharide
MCSF	macrophage colony stimulating factor
MCP	monocyte chemotactic protein
MDC	macrophage derived chemokine
MIG	monokine induced by interferon- γ
MIP	macrophage inflammatory protein
MPO	myeloperoxidase
MUFA	monounsaturated fatty acid
MW	molecular weight
ND	not determined
NEAA	non-essential amino acids
NF- κ B	nuclear factor kappa B
NK cell	natural killer cell
NSAID	non-steroidal anti-inflammatory drug
OA	oleic acid
OSM	oncostatin M
PAMP	pathogen associated molecular pattern
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PDGF	platelet-derived growth factor
PG	prostaglandin
PP	Peyer's patches
PPAR	peroxisome proliferator-activated receptor
PRR	pattern recognition receptor
PUFA	polyunsaturated fatty acid
Rantes	regulated upon activation normal T-cell expressed and secreted
RLU	relative light units
SAFA	saturated fatty acid
SAP	serum amyloid P component
SCF	stem cell factor
SCFA	short chain fatty acid
SD	standard deviation
SDF	stromal cell-derived factor
SEM	standard error of mean
t10, c12	<i>trans</i> -10, <i>cis</i> -12
T84	human colon carcinoma cell line
TARC	thymus and activation regulated chemokine
Tc	cytotoxic T-lymphocyte
TGF	transforming growth factor
Th	helper T-lymphocyte
TLR	toll-like receptor
TNBS	2,4,6-trinitrobenzene sulfonic acid

TNF	tumor necrosis factor
Tpo	thrombopoietin
TZD	thiazolidinediones
UC	ulcerative colitis
VCAM	vascular cell adhesion molecule
VEGF	vascular endothelial growth factor
vs	versus
ZO	zonula occludens

Dankwoord

Dankwoord

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Julian

List of publications

List of publications

Full papers

Julian D. Ramakers, Jean-Louis Sébédio, Jogchum Plat, Ronald P. Mensink. Effects of individual isomers *cis*-9, *trans*-11 versus *trans*-10, *cis*-12 conjugated linoleic acid (CLA) on inflammation parameters in moderately overweight subjects with LDL-phenotype B. *Lipids* 2005;40(9):909-918.

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Curriculum vitae

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Julian Desirée Ramakers was born on December 1, 1978, in Sittard. She completed secondary school at Serviam in Sittard in 1997. In the same year she started her academic education at Maastricht University, where she studied Biological Health Sciences and graduated in 2001. During her first internship in 2000 at the department of Health Risk Analysis and Toxicology of the Maastricht University, she examined the hydroxyl radical scavenging capacity of flavonoids. During the second internship in 2001 at the department of General Surgery of the Maastricht University she studied the role of fibroleukin and Toll-Like Receptor (TLR)-9 in experimental colitis. From January 2002 until December 2006 she was appointed as a PhD-student at the department of Human Biology of the Maastricht University. During this period she conducted both *in vitro* studies using intestinal cell lines as well as *in vivo* studies in animals to examine the effects of different (food) components (among others fish oils and β -glucan) on processes related to intestinal inflammation and to unravel the (molecular) mechanisms underlying these effects. She also studied the effects of conjugated linoleic acids (CLA) on inflammation markers in healthy subjects. Currently she is working as a post-doc at the department of Human Biology as member of the Nutrigenomics Consortium.