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DISSERTATION

Effects of omega-3 fatty acids docosahexaenoic acid and eicosapentaenoic acid and their metabolites in acute inflammation

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Abbreviations:

A-MuLV	Abselon murine leukaemia virus
AT-RvD$_{1-4}$	Aspirin-triggered resolvin D$_{1-4}$
AA	Arachidonic acid
ALA	α-Linolenic acid
ALX	Lipoxin A$_4$ receptor
BAD	Bcl-xL/Bcl-2 associated death promoter
BAX	Bcl-2–associated X protein
BCL-2	B cell lymphoma 2
BCL-X$_L$	B cell lymphoma X$_L$
BLT1	Leukotriene B$_4$ receptor 1
CCL3/4/5/8	C-C motif chemokine ligand 3/4/5/8
CCR5	C-C motif chemokine receptor 5
ChemR23	Chemokine receptor 23
CMKLR1	Chemokine-like receptor 1
COX-1/2	Cyclooxygenase 1/2
DHA	Docosahexaenoic acid
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DPA	Docosapentaenoic acid
DTA	Docosatetraenoic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EPA	Eicosapentaenoic acid
FBS	Fetal bovine serum
GC	Gas chromatography
GISSI	Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto Miocardico
GPR32	G protein-coupled receptor 32
HBSS	Hank's buffered saline solution
HDHA	Hydroxydocosahexaenoic acid
HEPE	Hydroxyeicosapentaenoic acid

HPLC	High performance liquid chromatography
IL-1β	Interleukin 1 beta
IL-1 ra	Interleukin 1 receptor antagonist
I.p.	Intraperitoneal
LA	Linoleic acid
LTA4H	Leukotriene A_4 hydrolase
5-LO	5-Lipoxygenase
15-LO	15-Lipoxygenase
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LTB_4	Leukotriene B_4
Ltb4r1	Leukotriene 4 receptor 1
LXA_4	Lipoxin A_4
MCP-2	Monocyte chemotactic protein 2
MIP-1α	Macrophage inflammatory protein 1α
MIP-1β	Macrophage inflammatory protein 1β
M/z ratio	Mass to charge ratio
N-3	Omega 3
N-6	Omega 6
NAD+	Nicotinamide adenine dinucleotide
NF-κB	Nuclear factor kappa b
NPD_1	Neuroprotectin D_1
NSAID	Non-steroidal anti-inflammatory drug
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PD_{1-4}	Protectin D_{1-4}
PMN	Polymorphonuclear leukocyte (granulocyte)
PUFA	Polyunsaturated fatty acid
RANTES	Regulated on activation, normal T expressed and secreted
RvE_1	Resolvin E_1
RvD_{1-4}	Resolvin D_{1-4}
SEM	Standard error of the mean
SPE buffer	Solid phase extraction buffer
TNF-α	Tumor necrosis factor alpha
WT	Wild type

1. Introduction

Fatty acids are known to be involved in a multitude of physiologic as well as pathophysiologic functions in the human organism. While energy storage might be the most prominent and apparent one, fatty acids are also in the focus of research concerning signaling pathways, particularly with regard to their influence on inflammation.

1.1 Properties and structure of poly unsaturated fatty acids

Fatty acids are carboxyl acids with variably long aliphatic carbon chains. They frequently occur esterified as phospholipids with amphiphilic properties. As double layers, they are one of the main components of cell membranes in the human organism.

If the carbon chain does not contain any double bonds, the fatty acid is classified as saturated. If one or more double bonds occur, it is called mono- or polyunsaturated (PUFA = polyunsaturated fatty acid). The number of carbon atoms located between the first double bond and the last carbon atom of the chain, called ω atom according to the last letter of the Greek alphabet, is important for the nomenclature of fatty acids. All ω-3 fatty acids carry the first double bond at the third atom, counted from the ω end, all ω-6 fatty acids at the sixth. Alternatively they are called n-3 or n-6 fatty acids. These double bonds usually occur in cis-position.

In terms of n-3 PUFAs, eminent ones are eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), as for n-6 fatty acids arachidonic acid (AA) (Fig. 1). These compounds modulate a range of physiologic processes, in particular immune defense mechanisms.
Furthermore, for nomenclatural facilitation, fatty acids are often systemized according to their carbon chain length as well as the total number of double bonds. EPA, for example, with a carbon chain length of 20 atoms and 5 double, bonds is correspondingly abbreviated 20:5.

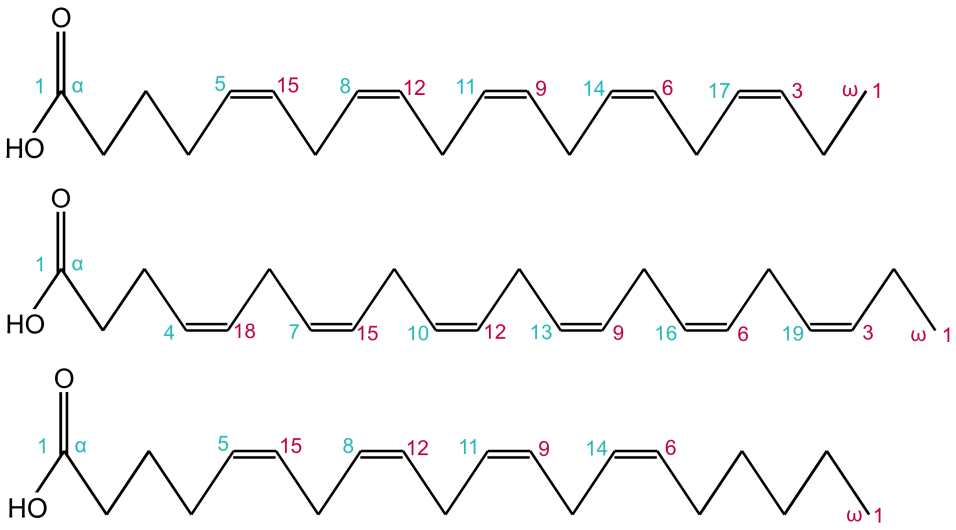

Fig. 1.1: *Examples of n-3 and n-6 PUFAs*

Eicosapentaenoic acid, a 20:5 ω-3 fatty acid (top), docosahexaenoic acid, a 22:6 ω-3 fatty acid (middle) and arachidonic acid, a 20:4 ω-6 fatty acid (bottom).

1.2 Effects of n-3 and n-6 PUFAs

N-6 PUFAs and their metabolites were characterized earlier than n-3 PUFAs. Arachidonic acid is probably the most prominent n-6 PUFA, and its physiological functions have been thoroughly investigated. Already in 1939, von Euler named a substance that he had isolated from certain genital glands “Prostaglandins” [1]. In 1982, Borgeat *et al.* coined the term leukotrienes [2], another group of bioactive compounds that, like prostaglandins, are present in inflammatory processes. Over time, it was elucidated how these substances are integrated into the orchestra of inflammation signals and pathways. Today, arachidonic acid-derived mediators are called eicosanoids and comprise the subgroups of prostaglandins, thromboxanes, prostacyclines and leukotrienes and are widely known for their mainly pro-inflammatory activities [3, 4].

While the molecular mechanisms of n-6-derived eicosanoid signal transduction and their effect on the organism have been well characterized, we know less about n-3 PUFAs. Interest in these compounds arose from the observations made by Danish physicians Jorn Dyerberg and Hans Olaf Bang, who noticed that the mortality from coronary heart

disease among Greenland Eskimos was only about 10% of that registered in epidemiologic studies for Danes and Americans [5, 6]. Even though the amount of total fat in the diet of Eskimos and Americans was roughly the same, fat in the diet of native Eskimos was largely from whale blubber and seal as well as fish fat, all of which are rich in n-3 PUFAs [7]. This led to the assumption that the high n-3 PUFA content could be responsible for these differences.

Subsequent studies confirmed the importance of n-3 PUFAs such as DHA and EPA for the prevention of cardiovascular disease and decrease of cardiac mortality. The GISSI (Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto Miocardico) Prevenzione Trial found a significant reduction of death, cardiac death, nonfatal myocardial infarction and stroke in subjects consuming high amounts of n-3 fatty acids [8].

Besides coronary heart disease, the Greenland Eskimo population showed a lower prevalence of further diseases, such as psoriasis, inflammatory bowel disease, asthma, rheumatoid arthritis and other autoimmune diseases [9-12]. The inflammatory background of these illnesses led to the assumption that n-3 PUFAs may unfold their effects by down-regulation of inflammatory stimuli.

Animal studies demonstrated an anti-inflammatory effect of n-3 PUFAs in models of colitis [13], asthma [14] or hepatitis [15]. Due to the duality of n-3 and n-6 PUFAs, it was assumed that n-3 PUFAs might function as direct antagonists, competing with binding sites of n-6 PUFA-derived mediators and thereby inhibiting their pro-inflammatory effects by blockage of prostaglandin or leukotriene formation [16].

However, recent studies identified n-3 PUFA as precursors of a distinct set of lipid mediators with separate pathways through which they unfold anti-inflammatory effects. These new n-3-derived, anti-inflammatory mediators have been named protectins/neuroprotectins and resolvins. Resolvins have first been identified in inflammatory exudates of murine air pouchitis during the resolution phase, and have been accordingly named “resolution phase interaction products” [17, 18].

Protectins were initially found to attenuate damage of brain ischemia-reperfusion injury and thus named “neuroprotectins” [19]. As they were subsequently also discovered in other tissues, the prefix “neuro-“ was dropped. In reference to their site of synthesis, they are still called neuroprotectins when formed in neural tissue.

To make matters more complex, similar compounds were described that also mediate anti-inflammatory effects but are derived from n-6 PUFAs. These compounds are called lipoxins and were first described in 1984 by Serhan *et al.* [20]. Lipoxins show strong similarity to n-3-derived mediators, both in their formation as well as the way they unfold their anti-inflammatory effects (Fig. 1.2).

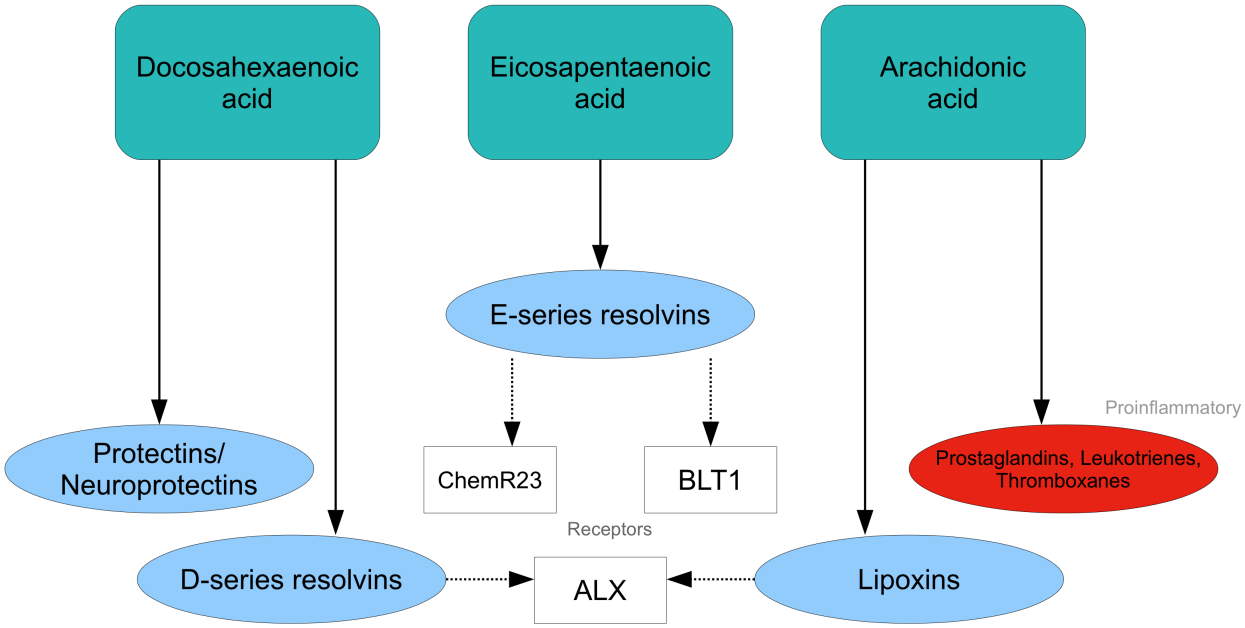

Fig. 1.2: *N-3 and n-6-derived lipid mediators*

Overview of n-3 and n-6-derived mediators and their receptors. DHA and EPA are precursors to anti-inflammatory protectins and resolvins, while both anti- and pro-inflammatory mediators can be synthesized from arachidonic acid, i.e. lipoxins, as well as prostaglandins, leukotrienes and thromboxanes. Receptors ChemR23 (chemokine receptor 23) and BLT1 (leukotriene B_4 receptor 1) have been described as binding site for E-series resolvins, as well as receptor ALX (lipoxin A_4 receptor) for both D-series resolvins and lipoxins.

1.3 Inflammation resolution

Inflammation is an important component in the pathogenesis of numerous diseases. In general, inflammation can be defined as an organism's defense reaction to harmful stimuli. These stimuli may be, amongst other things, microorganisms as well as agents of chemical or physical nature. The elaborately concerted process of local inflammation

is characterized not only by its swift initiation but also by its controlled resolution in order to enable healing and to prevent inflammation from spinning out of control. This active down-regulation of inflammation and return of the tissue to its prior physiological function is called catabasis [21].

Catabasis is nowadays thought to be more than a mere passive process in which inflammation simply “fizzles out” [22]. A key characteristic of inflammation resolution is the understanding that inflammation does not only come to an end by a decline of pro-inflammatory stimuli and the subsequent fading of their effects until homeostasis is reached; the new notion is that at a certain point of inflammation, i.e. the resolution phase, a different pattern of mediators is released. These mediators then have an active impact on resolution, not simply by antagonizing pro-inflammatory mediators but rather actively promoting the return to health [22]. Consequently, return to health can only be achieved if the causative stimulus is effectively eliminated and, subsequently, the endogenous regulatory mechanisms of resolution work effectively. If this is not the case, acute inflammation might perpetuate or even become chronic.

1.4 Lipid mediators in the course of inflammation

Even though n-3-derived lipid mediators and lipoxins were initially isolated in the resolution phase of inflammation [17], numerous studies have shown that they possess both anti-inflammatory as well as pro-resolving characteristics that affect inflammation in different stages.

Inflammation usually starts with the secretion of pro-inflammatory mediators from immune cells as a response to various inflammatory stimuli, e.g. microorganisms, toxins or mechanical irritation, with the ultimate aim of neutralizing these threats to the organism’s integrity. Many of these mediators are n-6 PUFA-derived compounds, such as eicosanoids. Key enzymes in the conversion of arachidonic acid to eicosanoids are cyclooxygenases (COX-1 and COX-2), as well as lipoxygenases (LO). A hallmark of inflammation initiation is the influx of first line leukocytes, i.e. granulocytes (also called polymorphonuclear leukocytes or PMNs) into the inflamed tissue. Upon recognition of

antibody or complement-covered cells, these leukocytes secrete their granules, which contain e.g. myeloperoxidase, neutrophil elastase as well as other antimicrobial enzymes. Beyond that, these cells are capable of phagocytosis. With the secretion of these substances into the tissue, PMNs are responsible for pus formation. After the influx of PMNs and the ongoing formation of proinflammatory mediators, macrophages enter the site and are responsible for phagocytosis of pathogens.

Phagocytosis of apoptotic cells is a central task for macrophages, especially during inflammation resolution. In this process, which is sometimes called efferocytosis [23], macrophages recognize apoptotic PMNs by cell surface receptors such as $\alpha_v\beta_3$ integrin, CD36 and phosphatidylserine receptors [24]. Both DHA as well as EPA-derived lipid mediators have been reported to upregulate phagocytosis of PMNs by macrophages in a non-phlogistic manner, meaning that, in addition to engulfment and ingestion of apoptotic PMNs, an increase of anti-inflammatory (e.g. interleukin 10, IL-10) as well as a decrease of pro-inflammatory (e.g. interleukin 6, IL-6) mediators occurs [25].

When inflammation is effectively combated, the active resolution phase is initiated. Studies have shown that through the influence of prostaglandin E_2 (PGE2) and prostaglandin D_2 (PGD_2), a class switch occurs. This switch shifts the lipid mediator profile from pro-inflammatory, mainly n-6-derived eicosanoids, to pro-resolving mediators, such as lipoxins, resolvins and protectins [26] (Fig. 1.4).

Various mechanisms are involved in this process. For instance, leukotriene B_4 (LTB_4), a strong pro-inflammatory eicosanoid, is a product of 5-LO, an enzyme that has been shown to decrease in activity when exposed to PGE_2. At the same time, PGE_2 as well as PGD_2 strongly increase 15-LO activity, which, using the same substrate (arachidonic acid) does not synthesize LTB_4 but lipoxin A_4 (LXA_4), which has potent anti-inflammatory effects [26]. The finding that animals can be protected from inflammatory diseases through over-expression of 15-LO underlines the importance of this enzyme in limiting inflammation [27].

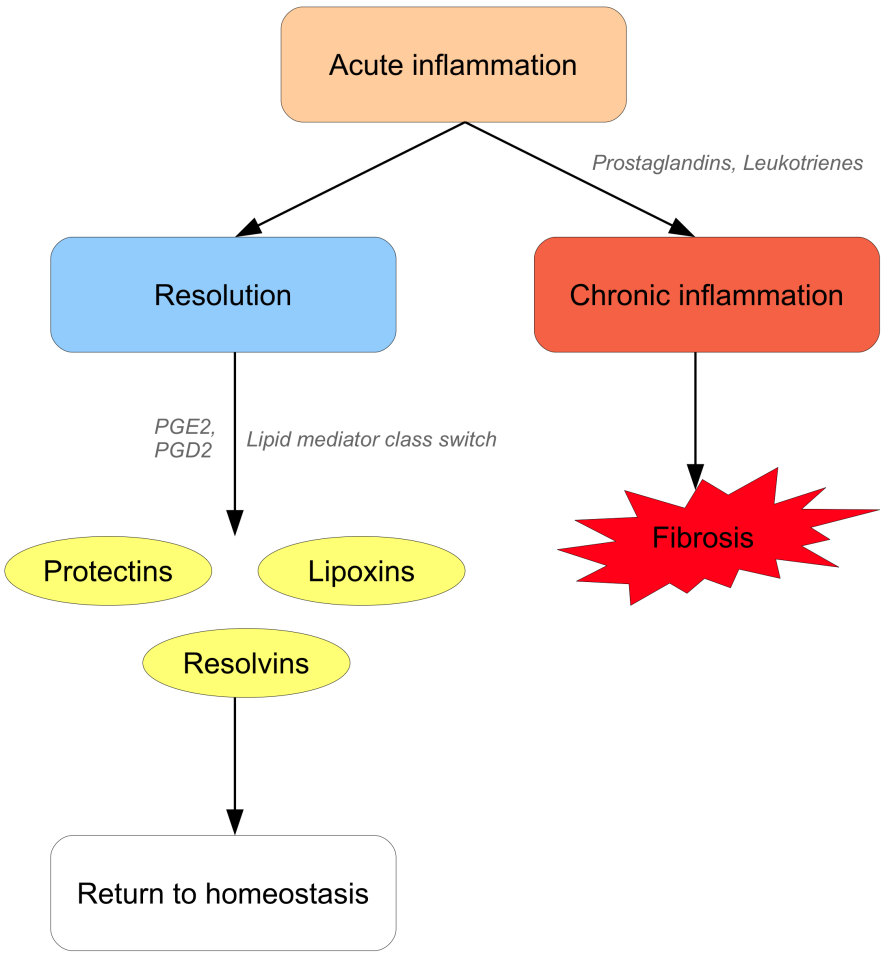

Fig. 1.4: *Illustration of mediators in the course of inflammation and resolution*

Schematic diagram of mediators involved in resolution or chronification of inflammation. If pro-inflammatory mediators persist, inflammation becomes chronic. To initiate resolution, substances like PGE_2 and PGD_2 can induce a lipid mediator class switch towards pro-resolving mediators that promote catabasis. Adapted from [22].

As discussed above, lipoxygenases are not the only enzymes that metabolize n-3 as well as n-6 fatty acids. Cyclooxygenases are important for the formation of prostaglandins. These enzymes are widely known for their pro-inflammatory potential, and inhibiting them with drugs such as aspirin, ibuprofen and other non-steroidal anti-inflammatory drugs (NSAIDs) is one of the most important therapeutic concept in medicine today. In the context of inflammation resolution, it is of interest to note that the iso-enzyme COX-2 is of particular importance in the synthesis of both lipoxins as well as n-3-derived mediators.

The role of aspirin in the formation of pro-resolving mediators was first recognized with regard to lipoxin formation [28]. It was found that COX-2 acetylation through aspirin

blocks prostaglandin formation, but enhances both lipoxin as well as n-3 mediator synthesis [22, 29]. Through this effect, and in contrast to other NSAIDs, aspirin could be of great importance for the initiation of resolution. In contrast, disruption of the enzymatic cascade of lipid mediator formation through administration of selective COX-2 as well as LOX inhibitors has been shown to delay resolution of leukocyte clearance [25, 30]. These findings indicate that these enzymes, previously thought to be mainly involved in the formation of pro-inflammatory mediators, are essential for resolution as well.

1.5 EPA-derived mediators

1.5.1 Effects of EPA-derived mediators

EPA is the precursor for E-series resolvins, of which resolvin E_1 (RvE_1) and resolvin E_2 (RvE_2) have been characterized so far. Several studies have tried to identify specific receptors for these mediators.

ChemR23, initially described as an orphan receptor (i.e. a receptor with a not yet identified ligand), is the binding site for two ligands of entirely different molecular structure, namely chemerin (a polypeptide) and RvE_1 (a lipid mediator). This receptor shares 36.4% homology of its amino acid sequence with the lipoxin receptor ALX [31]. ChemR23, abundantly expressed on antigen presenting cells such as macrophages and dendritic cells [32, 33], is a G-protein coupled receptor (GPCR), which, after binding chemerin, promotes chemotaxis of antigen presenting cells such as immature dendritic cells and macrophages *in vitro* [33]. Through the binding of RvE_1, on the other hand, dendritic cell migration and IL-12 production in response to pathogens were suppressed *in vivo* [31], and phagocytic activity of macrophages was significantly enhanced both *in vivo* and *in vitro* [25].

BLT1, on the other hand, was initially identified as a receptor for the pro-inflammatory cytokine LTB_4. RvE_1 has been shown to attenuate BLT1's LTB_4-dependent pro-inflammatory signals such as mobilization of intracellular calcium and NF-κB activation

[31]. It has been reported that RvE_1 possesses similar antagonistic potency as LTB_4-antagonist U-75302 [34].

Consequently, it appears that RvE_1 unfolds its anti-inflammatory effects by antagonizing pro-inflammatory stimuli of LTB_4 through BLT_1 blockage, and promotes resolution by enhancing phagocytic activity as agonist of the ChemR23 receptor.

A remarkable finding was the upregulation of CCR5 (C-C motif chemokine receptor 5) on late apoptotic PMNs through RvE_1 [35]. This was shown to function as a scavenging mechanism, since CCR5 is a receptor for pro-inflammatory cytokines such as CCL3 (C-C motif chemokine ligand 3, also known as macrophage inflammatory protein 1α or MIP-1α), CCL4 (also known as MIP-1β), CCL5 (also known as RANTES as acronym for "regulated on activation, normal t expressed and secreted") and CCL8 (also known as monocyte chemotactic protein 2 or MCP-2) [36]. Binding of these cytokines to late apoptotic PMNs and subsequent degradation through macrophage ingestion can thus lead to a strong reduction of pro-inflammatory cytokines. In the past, proteolysis was believed to be the main mechanism of chemokine inactivation, and CCR5 scavenging may therefore be a new mechanism for the termination of inflammatory cytokine signal transduction [37]. The observation that RvE_1 selectively upregulates CCR5 on late apoptotic cells only, i.e. on cells that mainly occur in the resolution phase of inflammation, also emphasizes the specific importance of this mediator for inflammation resolution.

Other studies have examined the effect of RvE_1 in various disease models *in vivo*. In a rabbit model of localized aggressive periodontitis, RvE_1 was reported to reduce neutrophil infiltration, prevent connective tissue and bone loss, promote healing of diseased tissues and regenerate lost soft tissue and bone [38, 39]. Furthermore, in experimental settings of murine disease models, RvE_1 has attenuated the severity of peritonitis [21, 25], prevented neovascularisation after oxygen-induced retinopathy [40] and improved survival rates in colitis [13].

It is of particular interest to note that the formation process of RvE_1 leads to both 18R-RvE_1 (generally referred to as RvE_1) as well as 18S-RvE_1 (see below). Both compounds share the same sites of action, i.e. ChemR23 and BLT1. While the 18S epimer was reported to have a significantly higher affinity to these receptors, it is inactivated more rapidly than RvE_1 [41]. These distinct properties explain the differences in anti-

inflammatory and pro-resolving actions of these two compounds. Oh *et al.* report that in a murine zymosan peritonitis model, for instance, RvE_1 has a stronger impact on the reduction of PMN infiltration and formation of pro-inflammatory cytokines such as interleukin 1β (IL-1β), interleukin 6 (IL-6) or tumor necrosis factor alpha (TNF-α) than its 18S epimer at equimolar dosages [41].

1.5.2 Formation of EPA-derived mediators

As with lipoxins, aspirin seems to have a great impact on the formation of E-series resolvins, since aspirin led to an increase of RvE_1 in both human and murine endothelial cells [17].

The initial step of E-series resolvin synthesis consists of formation of 18-hydroxyeicosapentaenoic acid (18-HEPE). Oh *et al.* have reported that stereochemistry is an important factor in the formation of E-series resolvins. Without supplementation of aspirin, EPA is mainly converted to 18R-HEPE, with an 18R/18S-HEPE ratio of 1.5:1. Not only did aspirin increase total 18-HEPE formation around 6 fold, it also shifted the R/S ratio to 1:1 [41].

Subsequently, both 18R- and 18S-HEPE are further metabolized in the presence of 5-lipoxygenase (5-LO) from PMNs. This cell-cell interaction between COX-2 synthesizing cells, e.g. endothelial cells, as well as 5-LO synthesizing cells, e.g. PMNs, is a vital step in this synthesis pathway. 5-LO initially converts 18-HEPE to a 5S-hydroperoxy-18R-HEPE or 5S-hydroperoxy-18S-HEPE intermediate. These compounds are then either reduced to RvE_2 (which refers to 18R-RvE_2) and 18S-RvE_2, or further processed to 5S,6-epoxy-18R-HEPE or 5S,6-epoxy-18S-HEPE by 5-LO. The following step is dependent on yet another enzyme that is widely recognized for its pro-inflammatory properties, i.e. LTA_4 hydroxylase (LTA4H). Otherwise pivotal for the synthesis of leukotrienes, it catalyses the final step from the 15S,6-epoxy-18-HEPE intermediate to both RvE_1 (which refers to 18R-RvE_1) and 18S-RvE_1 [29, 41] (Fig. 1.5.2).

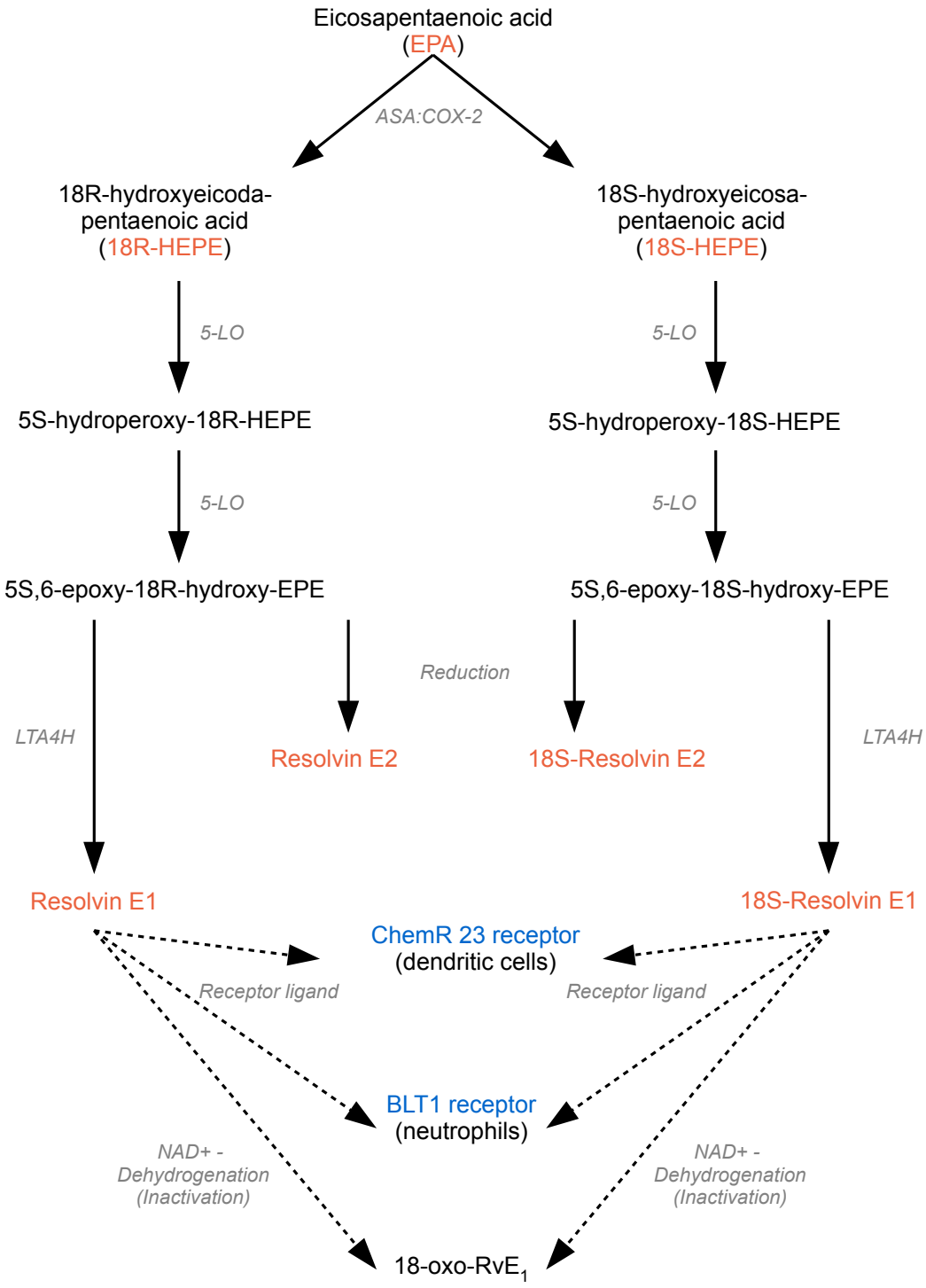

Fig. 1.5.2: *Synthesis of E-series resolvins*

E-series resolvins are synthesized from eicosapentaenoic acid. Initially, EPA is converted to 18-HEPE. Subsequently, 5-lipoxygenase is responsible for further processing into 5S-hydroperoxy-18-HEPE. Finally, either resolvin E_1 is formed by enzymatic epoxidation, or resolvin E_2 is formed through reduction. Each compound exists as R or S epimer, depending on its precursor molecule (with 18-R-resolvin E_1/E_2 usually being referred to as resolvin E_1/E_2). While targets of RvE_2 have yet to be elucidated, two receptors for RvE_1 have been described, i.e. ChemR23 and BLT1. RvE_1 18-oxo-RvE_1 is biologically inactive. Adapted from [29, 41].

1.6 DHA-derived mediators

1.6.1 Effects of DHA-derived mediators

DHA constitutes the origin for all D-series resolvins, as well as for a second family of mediators, i.e. protectins and neuroprotectins. Recently, receptors have been described for RvD_1 [42]. ALX was initially identified as receptor for lipoxins, the anti-inflammatory mediators that derive from n-6 fatty acids [43]. Human G protein-coupled receptor 32 (GPR32) on the other hand is an orphan receptor. It was reported that both receptors act as high affinity binding site for RvD_1, and that RvD_1 enhances the phagocytic and clearance functions of human macrophages through these receptors [42].

RvD_1 and aspirin-triggered (AT-) RvD_1 are diastereomeres with very similar bioactivity. Both showed equipotent reduction of leukocyte infiltration in murine inflammatory exudates [44]. Other properties of D-series resolvins include blockage of TNF-induced transcription of IL-1 in microglial cells [18], reduction of damage after ischemia-reperfusion injury to the kidneys as well as mitigation of subsequent kidney scarring [45].

DHA is also precursor of a second sort of lipid mediators, protectins and neuroprotectins. Structurally the same compound, the only difference between these two mediators is their site of origin. Since neuroprotectins were first discovered in neuronal tissue, they received the prefix *neuro* to specify their site of action [46]. A high affinity binding site has been identified on neutrophils that might constitute a novel receptor for protectins [47, 48]. Various neural tissues have been identified as place a of formation and action of neuroprotectins, amongst others human microglia cells, brain cells [18] as well as retinal epithelium [46]. Beyond that, protectin D_1 (PD1) also has proven anti-inflammatory effects on macrophages [25], T-cells [49] as well as PMNs [35].

Neuroprotectin D1 (NPD_1) was reported to reduce IL-1β gene transcription in murine and human microglial cells [18, 50] and reduce oxidative stress-induced apoptosis in human retinal pigment epithelium cells [46]. NPD_1 as well as resolvins have been shown to reduce pathologic neovascularization through enhanced vessel regrowth after vascular loss and injury in murine oxygen-induced retinopathy. Through production of

NPD_1, retinal pigment epithelium cells undergoing oxidative stress up-regulate expression of anti-apoptotic proteins BCL-2 (B cell lymphoma 2) and BCL-X_L (B cell lymphoma X_L) and down-regulate pro-apoptotic proteins of the same family, i.e. BAX (Bcl-2–associated X protein) and BAD (Bcl-xL/Bcl-2 associated death promoter) [40]. In the context of Alzheimer's disease pathogenesis, NPD_1 has been reported as a protective factor regulating amyliod-β secretion and thereby improving neuronal survival as well as brain cell function [51].

PD_1 significantly reduces PMN transmigration, both *in vivo* and *in vitro* [52]. Similar to RvE_1, PD_1 was able to upregulate CCR5 expression on PMNs [35] and to enhance phagocytic activity of macrophages [25]. Beyond that, PD_1 has several inflammation dampening effects on human T cells [49]. In an experimental asthma model using ovalbumin aerosol challenge in mice, i.v. application of PD_1 significantly reduced the ovalbumin-induced T-cell and eosinophil reaction and thereby attenuated airway hyperresponsiveness [14].

1.6.2 Formation of DHA-derived mediators

For DHA mediator formation, an aspirin-dependent and a microbial oxygenase dependent pathway have been described (Fig. 1.6.2 A). Beyond that, a third independent pathway was identified for these compounds which uses the organism's own enzymatic systems and is thus not dependent on aspirin or microbial enzymes (Fig. 1.6.2 B) This independent formation happens through 15-LO and 5-LO interaction. So far, four different D-series resolvins are known, i.e. resolvin D_1, D_2, D_3 and D_4 (RvD_1-D_4).

Biosynthesis of D series resolvins through aspirin or microbial enzymes leads to so-called "aspirin-triggered resolvins D_1 – D_2" (AT-RvD_1 – D_4). Since aspirin-triggered and non-aspirin-triggered resolvins differ in the stereochemical configuration of their hydroxyl groups at carbon atom 17, aspirin-triggered resolvins are also called 17R- and their aspirin independent counterparts 17S D-series resolvins. In the same manner, their precursor molecules are called 17R-HDHA and 17S-HDHA, respectively.

It is noteworthy that it was possible to synthesize RvD_1 *in vitro* with a single one-pot reaction using lipoxygenase incubation as well [44]. This observation suggests that there may be other, yet unidentified biosynthetic pathways of resolvin D_1 formation *in vivo*.

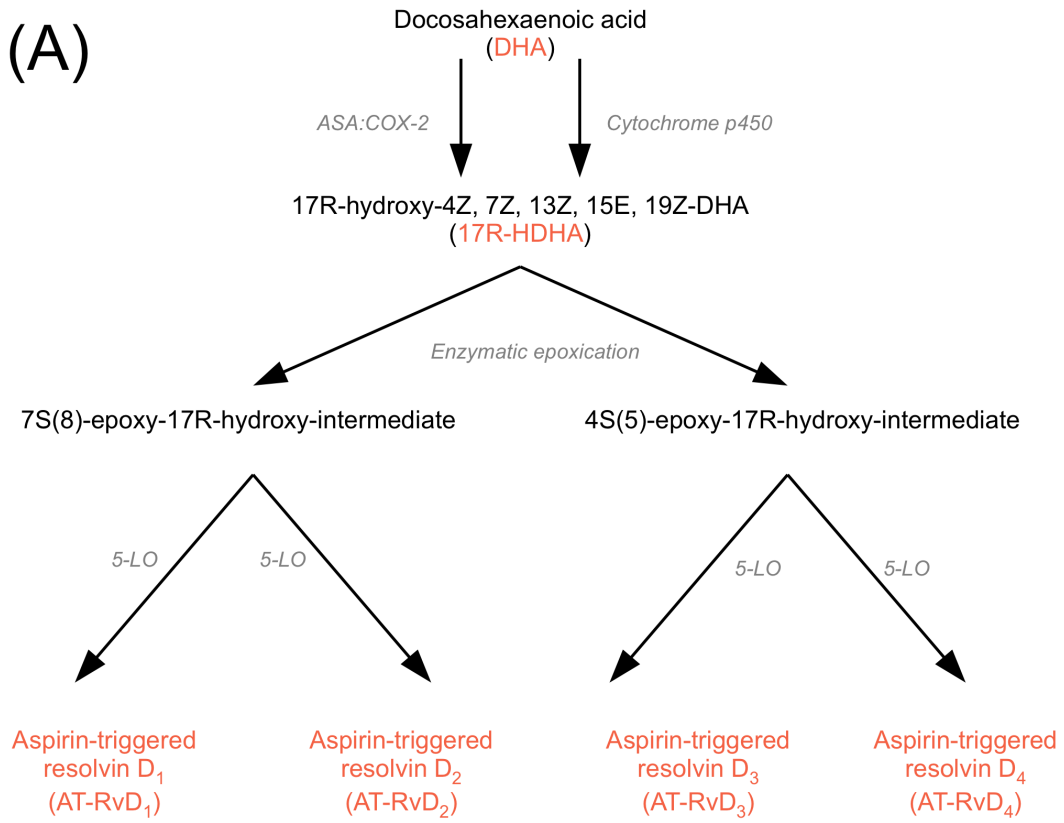

Fig. 1.6.2 A: *Synthesis of aspirin-triggered D series resolvins*

D-series resolvins can be formed through microbial cytochrome p450 or acetylated COX-2, similar to E-series resolvins (see above), and subsequent processing through 5-LO. Final products are aspirin-triggered resolvins D_1-D_4. Adapted from [29].

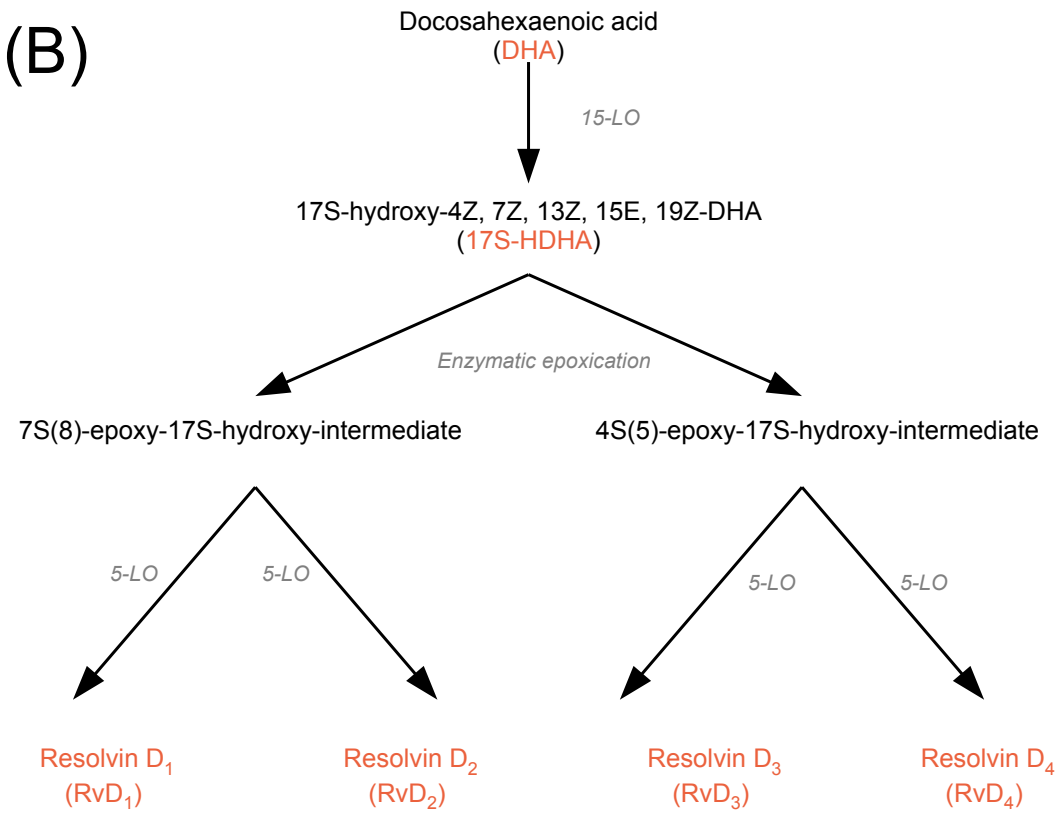

Fig. 1.6.2 B: *Synthesis of D series resolvins*

An alternative pathway is dependent on sequential action of 15-LO and 5-LO, therefore this pathway is aspirin-independent and leads to formation of resolvins D1-D4. Adapted from [29].

For PD_1, a single pathway has been described so far. As in the formation of D-series resolvins, DHA is initially converted to 17S-HDHA, followed by further enzymatic steps that culminate in the synthesis of protectin D1 (Fig. 1.6.2 C).

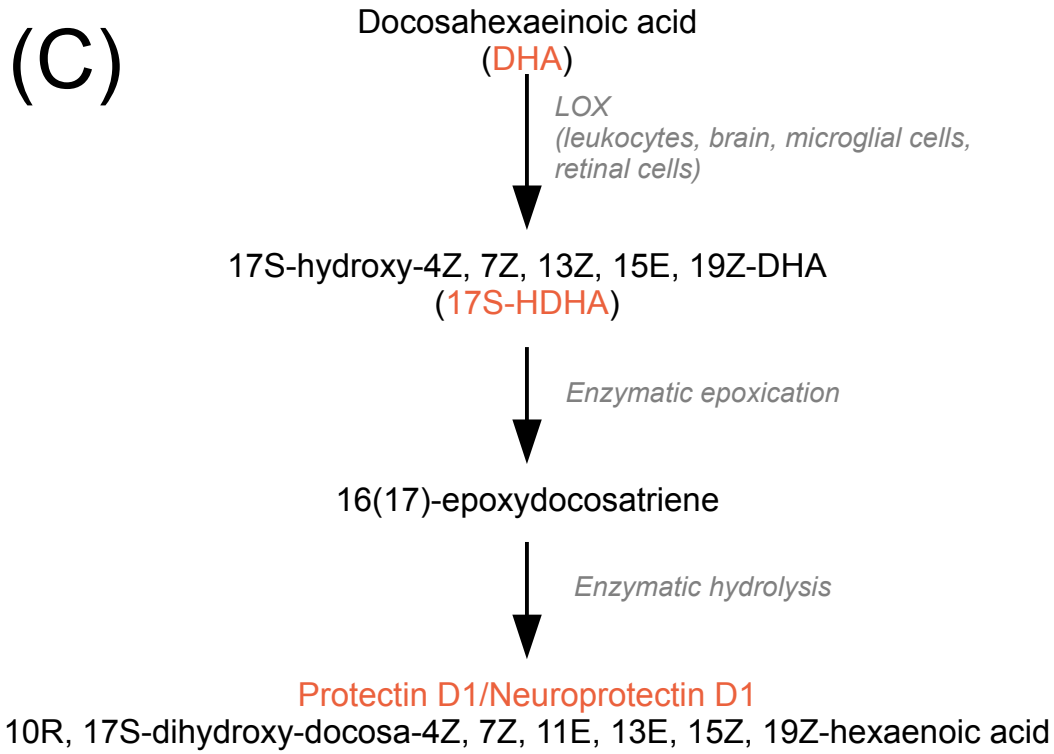

Fig. 1.6.2 C: *Synthesis of protectin/neuroprotectin D1*

Through 15-LO conversion and subsequent epoxidation and hydrolysis, protectin D_1 (PD_1) and neuroprotectin D_1 (NPD_1, if synthesized in neural tissue) are formed from DHA. Adapted from [29].

When comparing the synthesis of DHA-derived resolvins and protectins, it becomes obvious that all pathways and mediators described share 17-HDHA as initial precursor molecule [18, 46, 52]. Thus, similar to the formation of E-series resolvins through 18-HEPE, 17-HDHA is a good indicator of the initial step of D-series resolvin and protectin synthesis.

2. Research goals

A large number of studies indicate that inflammation can be dampened through administration of omega-3 fatty acids [14, 15, 40, 45, 53-56].

An important recent finding is that n-3 PUFAs apparently exert their effects through synthesis of n-3-derived hydroxylated mediators, such as resolvins and protectins. In order to further investigate this concept of n-3 PUFAs mediating anti-inflammatory effects through metabolites and mediators, the aim of this study was to extend observations of n-3 PUFAs EPA and DHA themselves to the monohydroxylated metabolites 17-HDHA and 18-HEPE that were identified as the pivotal intermediate metabolites in the process of resolvin and protectin synthesis.

We therefore investigated the effect of these metabolites as well as their precursor fatty acids EPA and DHA in a murine macrophage model *in vitro*. In order to assess and quantify their ability to dampen inflammation and improve resolution *in vitro*, the effects of fatty acids and their monohydroxylated metabolites were assessed by measuring LPS-induced TNF-α secretion and bioparticle-triggered phagocytosis in the RAW 264.7 cell culture model.

In a next step, the effect of endogenously increased n-3 PUFA tissue content in the transgenic fat-1 mouse model was assessed in a zymosan A peritonitis model, which is a well-established model for the systematic and quantitative assessment of the inflammatory response and allows evaluation of the inflammatory process on a cellular level. Macrophages and PMNs are the main cells that are involved in this model [57-60], and n-3 PUFAs and their derived mediators where previously shown to have effects on these cells.

3. Methods

3.1 Cell culture

3.1.1 RAW 264.7 cells and passaging procedure

For cell culture experiments, we used RAW 264.7 cells. These adherent murine macrophages were initially established from the ascites of a tumor induced in a male mouse by intraperitoneal injection of Abselon murine leukemia virus (A-MuLV) and are thus suitable for use in immunologic cell culture experiments. Cells were cultured in growth medium that consisted of high glucose DMEM (Dulbecco's modified Eagle's medium, GIBCO, Invitrogen, Carlsbad, CA, USA) with 10% fetal calf serum (Atlanta Biologicals, Lawrenceville, GA, USA) 20 mM HEPES, 2 mM l-glutamine, 100 U/ml penicillin and 100 U/ml streptomycin (all from GIBCO, Invitrogen, Carlsbad, CA, USA). Unless stated otherwise, all incubation steps were performed in an incubation chamber at 37^{o} Celsius and 5% CO_2

Cell stocks had been previously frozen in growth medium with 10% DMSO (dimethyl sulfoxide, Sigma Aldrich, St. Louis, MO, USA) at 5 x 10^6 cells/ml and 1 ml per vial. For cryo conservation, cells were placed overnight in Nalgene Mr. Frosty 1^{o}C Freezing Container (Nalgene, Penfield, NY, USA) at 80^{o}C and stored in liquid nitrogen until required for experiments. About 3 weeks prior to experiments, frozen stocks were thawed. Therefore, frozen cells were placed in 37^{o}C water bath. The cell suspension was immediately gently aspirated, transferred into 9 ml of equilibrated growth medium and centrifuged at 400 g for 5 minutes. Supernatant was aspirated and discharged, the cell pellet was resuspended in 10 ml growth medium and the suspension was transferred to a 100 mm tissue culture petri dish (Thermo Fisher Scientific, Waltham, MA, USA). Cells were incubated for 48 hours until the first passage.

For passaging, medium was aspirated and cells were washed with phosphate-buffered saline (PBS) without calcium/magnesium (GIBCO, Invitrogen, Carlsbad, CA, USA), exposed to 4 ml 0.25% trypsin-EDTA (ethylenediaminetetraacetic acid, GIBCO, Invitrogen, Carlsbad, CA, USA) and incubated for 5 minutes. After mechanically

detaching cells by gently tapping the petri dish sideways, 16 ml cell culture medium was added and cells were dislodged by slowly pipetting the suspension up and down. The cell suspension was filled into a 50 ml conical tube and centrifuged at 400 g for 5 minutes, supernatant was aspirated and the pellet was resuspended in 10 ml growth medium. Aliquots were taken to assess cell vitality by means of a trypan blue stain as well as to calculate cell numbers in the suspension. Cells were counted in an improved Neubauer hemocytometer (Sigma Aldrich, St. Louis, MO, USA). An adequate volume of growth medium was added in order to yield a 1 x 10^6 cells in 10 ml growth medium per dish. Cells were seeded in a 100 mm petri dish (Thermo Fisher Scientific, Waltham, MA, USA) at a confluence of about 10% and passaged every two days. For passage over the weekend, 7.5 x 10^5 cells were seeded under otherwise identical conditions. Cells were used for experiments after 10 to 15 passages.

3.1.2 TNF-α ELISA

Murine macrophage RAW 264.7 cells were cultured as described above. For experiments, cells were planted in 24-well plates (Greiner Bio-One, Kremsmünster, Austria) at a density of 5 x 10^5 cells/well and incubated overnight. All experiments were performed in a humidified atmosphere under 5% CO_2 at 37°C.

RAW 264.7 cells were preincubated for 2 hours with either 20 μM EPA or DHA, or 500 nM 17-HDHA or 18-HEPE (Cayman Chemicals, Ann Arbor, MI, USA); controls were treated with equal volumes of vehicle solvent solution (ethanol). After preincubation the medium was aspirated and cells were stimulated with 0.5 μg/ml *E. coli* LPS (Sigma Aldrich, St. Louis, MO, USA) in medium for 4 hours. Supernatants were then taken and frozen at -80°C until TNF-α was assayed. TNF-α was measured in cell culture supernatants using an ELISA (enzyme-linked immunosorbent assay) kit (eBioscience, San Diego, CA, USA) following the manufacturer's protocol.

Results are observations from four individual experiments. For every experiment, TNF-α levels of each group (negative and positive control, DHA, EPA, 17-HDHA and 18-

HEPE) were measured in three different samples, with each sample measured in triplicates.

3.1.3 Phagocytosis assay

RAW 264.7 cells were grown as described above. Cells were plated in 96-well plates at a density of 1 x 10^5 cells/well and incubated overnight. To quantify phagocytosis, cells were incubated with fluorescein-labelled bioparticles (Vybrant Phagocytosis Assay, Invitrogen, Carlsbad, CA, USA), diluted 1:2. Uptake was measured after 4 hours in a fluorescence plate reader (Victor 1420 Multilabel Counter, Wallac 1420 Workstation Software Version 3.00 Revision 2, Perkin Elmer, Wellesley, MA, USA), essentially following the manufacturer's protocol. Cells were pre-treated with 20 μM DHA and EPA as well as 1 μM 17-HDHA and 18-HEPE for 2 hours. Positive controls with equivalent volumes of vehicle (ethanol) and negative controls were performed in parallel.

Observations from two sets of experiments are shown. Each experiment included the same groups (i.e. negative control, positive control, DHA, EPA, 17-HDHA, 18-HEPE), and each group consisted of 4 different samples (n = 4 per group).

3.2 Animal model

3.2.1 Mice

Fat-1 mice offer a well-established model to investigate the effects of n-3. Through transgenic engineering, the genome of fat-1 mice was equipped with a desaturase from the roundworm *Caenorhabditis elegans*. The key function of this enzyme is not a *de-novo* synthesis of n-3 PUFAs, but a conversion of n-6 fatty acids into their n-3 counterparts.

Fat-1 mice were created as described in [61] and backcrossed at least four times onto a C57BL/6 background. All fat-1 animals used in this study were heterozygous. Due to insufficient availability of laboratory animals, wild type (WT) mice were bought from Charles River Laboratories Int. (Wilmington, MA, USA) at the age of one month and received the same diet as fat-1 mice (see below) for two months. Only male animals were used for experiments. Animal groups used in experiments are treated fat-1, treated WT, and untreated WT control animals. Due to lack of availability of fat-1 mice, it was not possible to include an untreated fat-1 control group. At the age of three months, mice were sacrificed using isoflurane anesthesia.

Animals were kept in a specific pathogen-free, air-conditioned environment in filter-topped cages with a controlled light cycle of 12 hours and received autoclaved food and water only, according to National Institute of Health guidelines.

Mice were fed with a diet high in n-6 and low in n-3 fatty acids (TestDiet® AIN-76A Semi-Purified Diet 58B0 with 10% total corn oil, Cat. no. 1812692) and drinking water *ad libitum*. Prior to one month of age, WT mice were fed with Purina 5L79 and were then switched to modified AIN-76A Diet.

All studies were approved by The Massachusetts General Hospital Subcommittee on Research Animal Care.

3.2.2 Gas chromatography

In order to determine the genotypic background of each animal, lipids were extracted from tail samples and analyzed by means of gas chromatography, as described previously [62]. After homogenization, boron trifluoride and hexane were added in equal parts to the samples, vials were sealed with nitrogen and incubated at 100º+ C for 60 minutes. After letting cool to room temperature, double distilled water was added and the mixture was shaken vigorously. The hexane phase containing fatty acids was removed, concentrated under a nitrogen fume hood, and samples were analyzed by an automated HP5890 system (Hewlett-Packard, Palo Alto, CA, USA) equipped with a

flame-ionization detector [63]. Peaks were identified through comparison with fatty acid standards (Nu Chek Prep, Elysian, MN, USA), and each peak's area percentage was analyzed using a PerkinElmer M1 integrator (PerkinElmer, Waltham, MA, USA) [62].

3.2.3 Induction of peritonitis and intraperitoneal injection

Peritonitis was induced by intraperitoneal (i.p.) injection of zymosan A, a glucan isolated from the cell wall of the yeast *Saccharomyces cerevisiae*. It has been previously used and tested for its capability to induce an inflammatory response of the peritoneum in mice [21]. Zymosan A (Sigma Aldrich, St. Louis, MO, USA) was diluted in sterile saline solution at a concentration of 1 mg per ml. The dosage of administered zymosan was set to be weight-dependent at 1 mg per 20 g body weight. Control mice received an i.p. injection of a weight-adjusted dose of vehicle (sterile saline) without zymosan. 24 hours after injection, mice were anesthetized by isoflurane anesthesia. Immediately after commencement of anesthesia, percutaneous cardiocentesis followed and peritoneal lavage was performed. Blood samples were filled into sterile, heparinized tubes and subsequently centrifuged to obtain pure serum. Serum and tissue samples were frozen in liquid nitrogen and stored at -20ºC until used for further analysis.

Different techniques of i.p. injection were tested in order to assure a consistent and uniform application of zymosan. Therefore, we conducted a preliminary trial run in which we injected 1 ml of 0.1% crystal violet solution (Sigma Aldrich, St. Louis, MO, USA) i.p. using different injection techniques. Shortly after injection, mice were sacrificed and a laparotomy was performed in order to examine the abdomen. We tested both a one-man as well as a two-man procedure on 10 animals per procedure.

The one-man procedure was carried out by injection of the desired volume into the lower left quadrant of the animal's abdomen while holding the animal in the left hand. The skin fold on the dorsal side of the animal's neck was held between thumb and index finger, while the tail was fixed between the little finger and the hypothenar eminence (Fig. 3.2.3 A).

The two-men procedure was conducted by one person holding the animal's ears between the left hand's thumb and index finger and the lower left extremity with the right hand. The second person held the animal's lower right extremity between the little finger and ring finger of the left hand while raising a fold of the abdominal wall with the left hand. The peritoneal cavity was subsequently penetrated by inserting the needle into the lower left quadrant perpendicularly to the skin fold (Fig. 3.2.3 B).

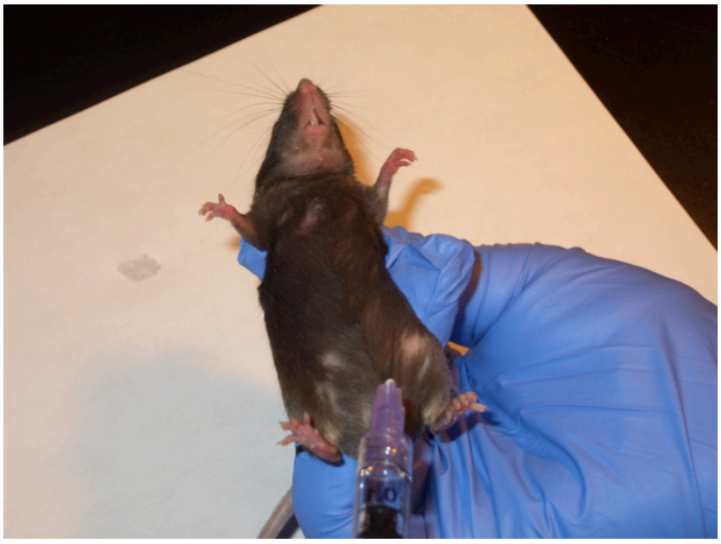

Fig. 3.2.3 A: *I.p. injection, one-man procedure*

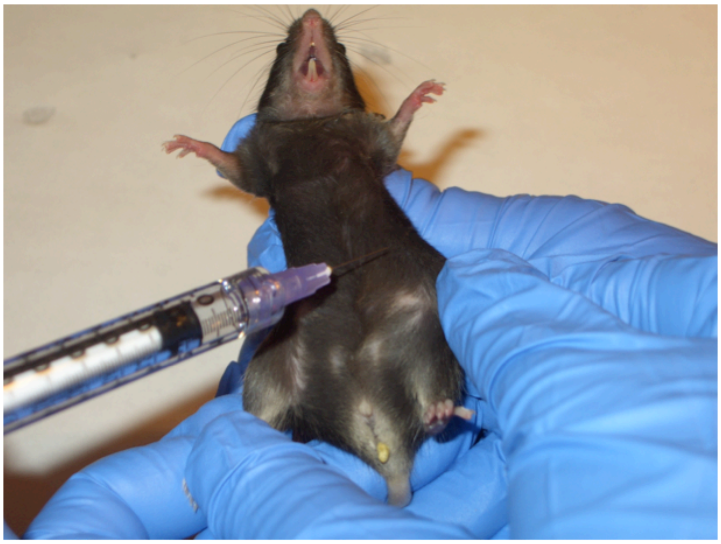

Fig. 3.2.3 B: *I.p. injection, two-man procedure*.

3.2.4 Peritoneal lavage

Prior to commencement of peritoneal lavage, the animal's abdomen was cleaned with 70% alcohol. A small midline incision of only a few millimeters within the skin layer was made with a pair of scissors, and the abdominal skin was retracted with forceps to expose the intact peritoneal wall. The peritoneum was penetrated with a needle, beveled end of needle facing up. Whilst passing the needle through the peritoneum, the syringe plunger was pushed lightly to avoid hitting the intestine by squirting a jet of water. 10 ml of sterile saline solution were injected into the peritoneal cavity. The needle was withdrawn and the mouse was carefully moved to allow cells to evenly distribute within lavage fluid. The needle was re-inserted into the peritoneum, beveled end down. By raising the needle slightly, the peritoneal wall was tented to prevent inner organs from congesting the needle opening. Fluid was withdrawn slowly. The average recovery rate was ~ 8 ml per mouse. Unless used immediately, cell suspensions and aliquots were kept on ice before further processing.

3.2.5 Cell count

Immediately after peritoneal lavage, cells were counted using an improved Neubauer hemocytometer (Sigma Aldrich, St. Louis, MO, USA). The suspension was diluted at a ratio of 1:10 with sterile saline solution (GIBCO, Invitrogen, Carlsbad, CA, USA), and the hemocytometer's two counting chambers were filled and counted twice for calculation of mean values.

3.2.6 Wright-Giemsa Stain of exudates smears

Parts of the cellular suspension were used for preparing a Wright-Giemsa stain (Sigma Aldrich, St. Louis, MO, USA) in order to determine the cellular composition of the

exudates. Therefore, an aliquot containing 2 x 10^6 cells was centrifuged and the supernatant was discharged. The pellet was resuspended in 5 µl FBS (fetal bovine serum, Sigma Aldrich, St. Louis, MO, USA) in order to achieve conditions similar to a blood smear. This approach was chosen since preliminary smears carried out with a suspension of cells in saline solution with subsequent alcohol fixation resulted both in poor cell morphology as well as low overall cellularity on the slides. Given the circumstance that the suspension extracted from the peritoneal cavity offered a very limited number of cells to work with, it was important to handle them efficiently and keep cellular loss to a minimum.

Following the manufacturer's instructions for the preparation of a blood smear, the suspension was applied on a microscopy slide, struck out and air dried. Thereupon, the slide was dipped into the Wright-Giemsa stain for 30 seconds, removed, and placed in a basin of deionized water for 15 minutes. Finally, the slide was air-dried once more before sealing it with mounting medium and a coverslip. For each mouse sample, two slides were made. The cellular composition was determined by light microscopy. 400 cells were counted on each of the two slides per animal, with a total of 800 cells counted per animal. Identified cells were macrophages/monocytes, neutrophil granulocytes, eosinophil granulocytes, basophil granulocytes/mast cells as well as lymphocytes.

3.2.7 TNF-α ELISA

To quantify the systemic inflammatory response to zymosan, we conducted a TNF-α ELISA from mouse serum samples. TNF-α is a central cytokine in inflammatory processes, and since its level correlates with severity of inflammation, it is a practical marker for its assessment.

Shortly after commencement of isoflurane anesthesia, blood was drawn by means of percutaneous cardiocentesis. A sterile needle was inserted in the left midaxillary line. Blood was drawn as slowly as possible to avoid hemolysis. Depth of anesthesia was supervised by a second person. After a sufficient volume of blood was drawn, mice

were killed by an isoflurane overdose and blood samples were centrifuged. The upper serum phase was transferred into another tube, snap-frozen in liquid nitrogen and stored at -20ºC.

A commercially available TNF-α ELISA kit from eBioscience (San Diego, CA, USA) was used as described above.

3.2.8 Liquid chromatography tandem mass spectrometry

A substantial leap forward regarding research in the field of n-3 and n-6 fatty acids has been made through liquid chromatography with electrospray ionization tandem mass spectrometry (LC-MS/MS), since this technology allows direct measurement of compounds like n-3-derived mediators in various tissues. Besides quantification, this method can also be used for structural analysis.

As the name implies, LC-MS/MS uses two distinctive techniques, i.e. high performance liquid chromatography (HPLC) for extraction of compounds from tissue samples as well as tandem mass spectrometry for detection. Following HLPC, the mobile phase is conveyed into a gas phase through molecular nitrogen (N_2) and compounds are ionized by electrospray ionization for analysis. This is essential since ions are filtered according to their mass to charge (m/z) ratio in a triple quadrupole mass analyzer and subsequently captured in a detector.

Thirty milligrams ground and frozen liver tissue was mixed with methanol and internal standard (LTB4-d4) and hydrolyzed with 300 µl of 10 M sodium hydroxide for 30 min at 60°C. The solution was neutralized with 60% acetic acid and pH was adjusted to 6.0 with sodium acetate buffer. A solid phase extraction was performed with an anion exchange column (Bond Elute Certify II, Agilent, Santa Clara, CA). For elution, an n-hexane - ethyl acetate extraction mixture 25:75 with 1% acetic acid was used. The eluate was evaporated on a heating block at 40°C under a stream of nitrogen to obtain a solid residue. Residues were then dissolved in 70 µl acetonitrile. An Agilent 1200 HPLC system and a solvent system consisting of acetonitrile/0.1% formic acid in water

were used. The gradient elution was started with 15% acetonitrile, this was increased within 10 min up to 90% and held for 10 min. The high-performance liquid chromatography was coupled with an Agilent 6410 Triplequad mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) with electrospray ionization source. Analysis of lipid mediators was performed using multiple reaction monitoring in negative mode.

3.3 Statistical analysis

Data analysis was performed with Prism 3.02 software (GraphPad, La Jolla, CA, USA). Comparisons were made using the two-tailed Student's t-test. All values are presented as mean ± standard error of the mean (SEM) or as indicated. Statistical significance was set to $p = 0.05$. Asterisks indicate level of significance, with "*" if $p < 0.05$, "**" if $p < 0.01$ and "***" if $p < 0.001$

4. Results

4.1 Results of cell culture study

4.1.1 *In vitro* TNF-α ELISA

For further elucidation of the effect of n-3 PUFAs and its mediators on macrophages, we carried out an *in vitro* assessment of TNF-α levels in RAW 264.7 murine macrophage cell cultures. Cells were preincubated with n-3 fatty acids or metabolites 17-HDHA or 18-HEPE and subsequently stimulated with LPS. TNF-α levels were measured with a TNF-α ELISA kit (eBioscience). Values are normalized and expressed as increase vs. negative control. Results are observations from four individual experiments. For every experiment, TNF-α levels of each group were measured in three different samples, with each sample measured in triplicates.

In comparison to the positive control group (6.50 ± 0.29 fold increase), both DHA (5.93 ± 0.23 fold increase) and EPA (5.85 ± 0.15 fold increase) showed a reduction of TNF-α levels, however, not significantly. Metabolites 17-HDHA (5.57 ± 0.10 fold increase) as well as 18-HEPE (5.06 ± 0.14 fold increase), on the other hand, led to a significant decrease of TNF-α levels at lower doses than DHA and EPA (20 μM DHA/EPA vs. 1 μM 17-HDHA/18-HEPE). Furthermore, it is of interest to note that EPA led to a stronger decrease of TNF-α than DHA, while EPA-derived metabolite 18-HEPE showed an even stronger decrease than DHA-derived metabolite 17-HDHA (Fig. 4.1.1.).

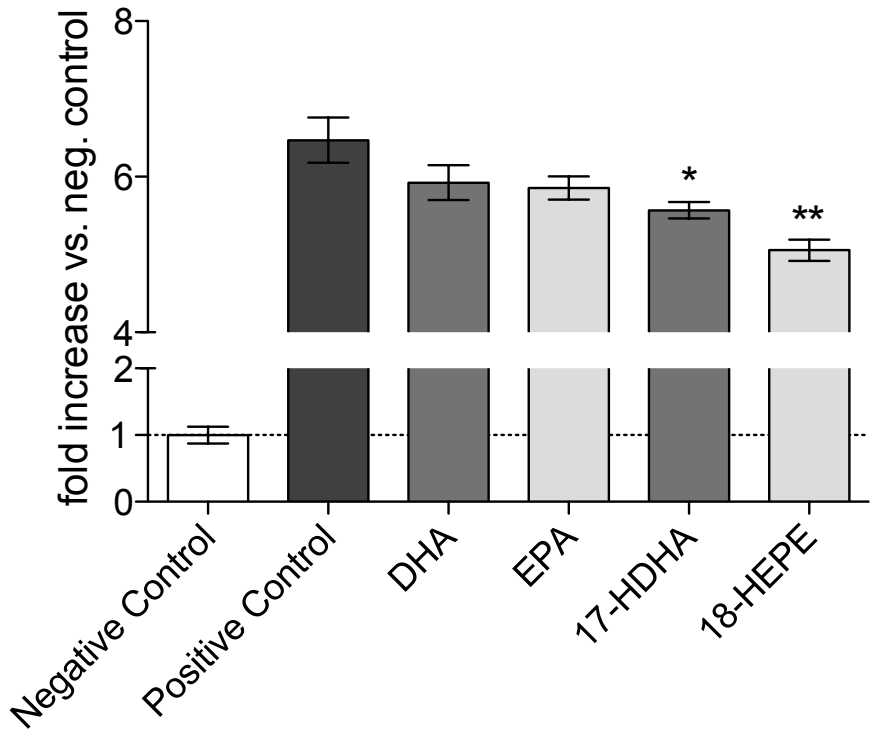

Fig. 4.1.1: *TNF-α levels in cell culture*

Cells were preincubated with fatty acids or metabolites and subsequently stimulated with LPS. TNF-α levels were measured with an ELISA kit, results are expressed as increase vs. negative control. n = 4 for each group. Asterisks indicate level of significance (vs. positive control), with "*" if $p < 0.05$ and "**" if $p < 0.01$.

4.1.2 *In vitro* phagocytosis assay

As key marker of inflammation resolution, phagocytic activity is of particular interest in the evaluation of inflammation. We conducted an *in vitro* phagocytosis assay of fluorescein-labeled *Escherichia coli* particles in order to assess whether DHA, EPA, 17-HDHA and 18-HEPE have an effect thereon. Results shown are from two separate experiments carried out on two days and expressed as fold induction vs. positive control (Fig. 4.1.2. A and B).

Both experiments show a general increase of phagocytosis through n-3 fatty acids and its metabolites.

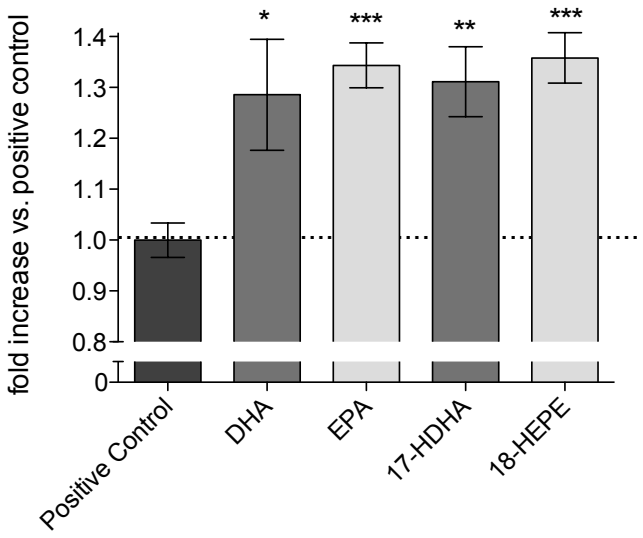

Fig. 4.1.2 A: *Results of phagocytosis assay number 1*

Values show fluorescence of RAW 264.7 cells as fold induction vs. positive control after fatty acid preincubation and subsequent incubation with fluorescein-labeled *Escherichia coli* particles. n = 4 for each group. Asterisks indicate level of significance (vs. positive control), with "*" if $p < 0.05$, "**" if $p < 0.01$ and "***" if $p < 0.001$.

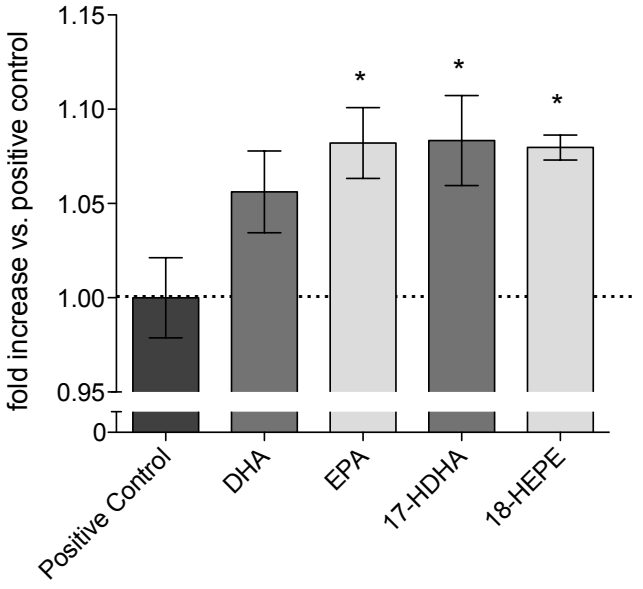

Fig. 4.1.2 B: *Results of phagocytosis assay number 2*

Values show fluorescence of RAW 264.7 cells as fold induction vs. positive control after fatty acid preincubation and subsequent incubation with fluorescein-labeled *Escherichia coli* particles. n = 4 for each group. Asterisks indicate level of significance (vs. positive control), with "*" if $p < 0.05$.

Results of set 1 show a significant increase of phagocytosis in each group (DHA: 1.29 ± 0.11; EPA: 1.34 ± 0.09; 17-HDHA: 1.31 ± 0.68; 18-HEPE: 1.36 ± 0.05 fold increase) vs. positive control, with the lowest level of significance found in the DHA group. All groups but DHA also show significant results vs. positive control on set 2 as well (DHA: 1.06 ± 0.02; EPA: 1.08 ± 0.02; 17-HDHA: 1.08 ± 0.02; 18-HEPE: 1.08 ± 0.01 fold increase), with generally lower levels of significance.

4.2 Results of animal model study

In order to evaluate the effects of n-3 PUFAs and their mediators *in vivo*, we carried out an animal model using transgenic fat-1 mice with endogenously high levels of n-3 PUFAs. Fatty acid profiles of mice were measured using gas chromatography. 24 hours after induction of peritonitis with intra-peritoneal injection of zymosan A, exudates were collected by means of peritoneal lavage. For assessment of inflammatory parameters, cellular composition of exudates was analyzed in Wright-Giemsa smears. Moreover, we performed a TNF-α ELISA from serum samples and measured concentrations of n-3 mediators 17-HDHA and 18-HEPE in liver samples in order to assess utilization of n-3 fatty acids DHA and EPA.

4.2.1 Gas chromatography

Mice were kept on a diet high in n-6 and low in n-3 fatty acids with a n-6/n-3 ratio > 20. Fatty acid levels were determined by lipid extraction and subsequent gas chromatography of tail samples. We found significantly higher levels of n-6 PUFAs arachidonic acid and docosatetraenoic acid in WT mice compared to fat-1 mice. Linoleic acid, which is a chief ingredient of the diet that mice were kept on, was higher in WT animals, however not significantly. In terms of n-3 fatty acids, we found significantly higher levels of α-linolenic acid, eicosapentaenoic acid and docosapentaenoic acid in

fat-1 animals. Eicosapentaenoic acid levels were not detectable in WT animals. Docosahexaenoic acid was insignificantly lower in WT animals than in fat-1 animals.

Overall, WT animals showed higher levels of n-6 PUFAs and lower levels of n-3 PUFAs in comparison with fat-1 animals. All analyzed fatty acids but DHA showed significantly different values in WT vs. fat-1 animals. While WT animals were found to have an n-6 (LA+AA+DTA)/n-3 (ALA+EPA+DPA+DHA) ratio of 9.57, it was 1.57 in fat-1 animals (Tab. 4.2.4.).

PUFA		*Wild Type*	*Fat-1*	*Significance (WT vs. fat-1)*
n-3 PUFAs				
α-Linolenic acid	(ALA) 18:3	0.08 ± 0.06%	2.77 ± 0.15%	$p < 0.0001$ ***
Eicosapentaenoic acid	(EPA) 20:5	0.00	2.22 ± 0.10%	$p < 0.0001$ ***
Docosapentaenoic acid	(DPA) 22:5	0.71 ± 0.20%	2.07 ± 0.08%	$p < 0.0001$ ***
Docosahexaenoic acid	(DHA) 22:6	1.97 ± 0.12%	2.10 ± 0.09%	$p = 0.37$
n-6 PUFAs				
Linoleic acid	(LA) 18:2	16.52 ± 0.71%	12.90 ± 1.33%	$p < 0.05$ *
Arachidonic acid	(AA) 20:4	6.31 ± 0.32%	0.80 ± 0.10%	$p < 0.0001$ ***
Docosatetraenoic acid	(DTA) 22:4	3.98 ± 0.31%	0.15 ± 0.09%	$p < 0.0001$ ***
Total n-3		2.76%	9.16%	
Total n-6		26.43%	13.85%	
Ratio n-6/n-3		9.57	1.51	

Table 4.2.1: *Fatty acid profile of tail tissue*

Tail tissue samples were used for lipid extraction and determination of fatty acid profiles. Numbers given are percentages of each fatty acid of total fatty acid levels ± SEM.

4.2.2 Intraperitoneal injection

In order to ensure an optimized experimental framework, we conducted a trial run in which we tested different methods of intraperitoneal injection that were described above (see chapter 3.2.3). We tested a one-man as well as a two-man procedure.

Accuracy of injection was determined by evaluation of injection of crystal violet solution into the peritoneal cavity of 10 animals per group through subsequent laparotomy. As dye of intense violet color, it was easy to determine visually whether a crystal violet injection made hit the correct anatomic compartment. We encountered residues of misplaced injections in the small bowel of 2 animals in the two-man procedure group and none in the one-man procedure group (Fig. 4.2.1).

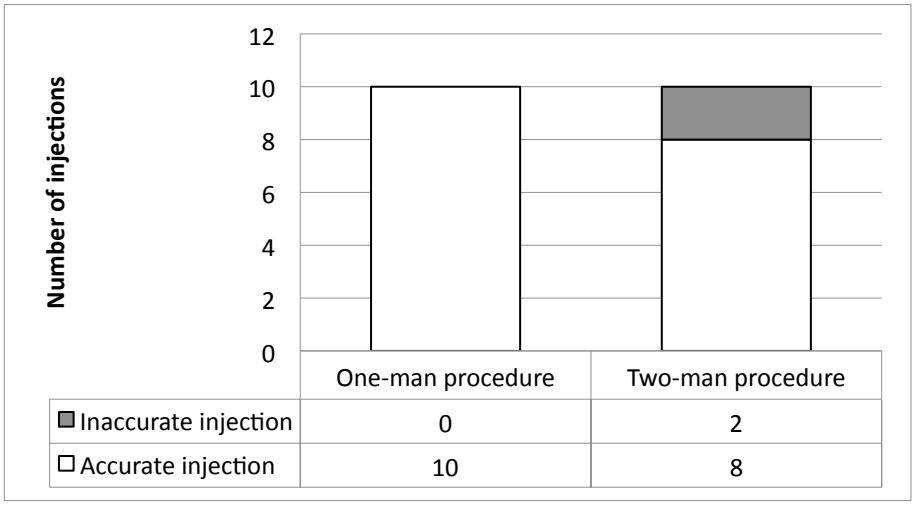

Fig. 4.2.2: *Comparison of i.p. injection methods*

0.1% crystal violet solution was injected intraperitoneally. Success was evaluated by inspection of peritoneal cavity after laparotomy. Accurate injections resulted in dye accumulation within the peritoneal cavity, inaccurate ones in dye accumulation in hollow organs, e.g. small bowel (n = 10 for each group).

These findings spoke in favor of the one-man procedure, which we retained throughout the experiments.

4.2.3 Analysis of peritoneal exudate cellularity

Both treated WT and fat-1 mice showed similar cellularity (2.37 ± 0.29 x 10^7 cells/ml for WT and 2.58 ± 0.18 x 10^7 cells/ml for fat-1) with no statistically significant difference. Control animals (= untreated WT animals) showed a significantly lower number of cells (0.38 ± 0.04 x 10^7 cells/ml) in comparison with treated WT ($p < 0.05$ for WT vs. untreated control) and fat-1 animals ($p < 0.05$ for fat-1 vs. untreated control) (Fig. 4.2.2. A).

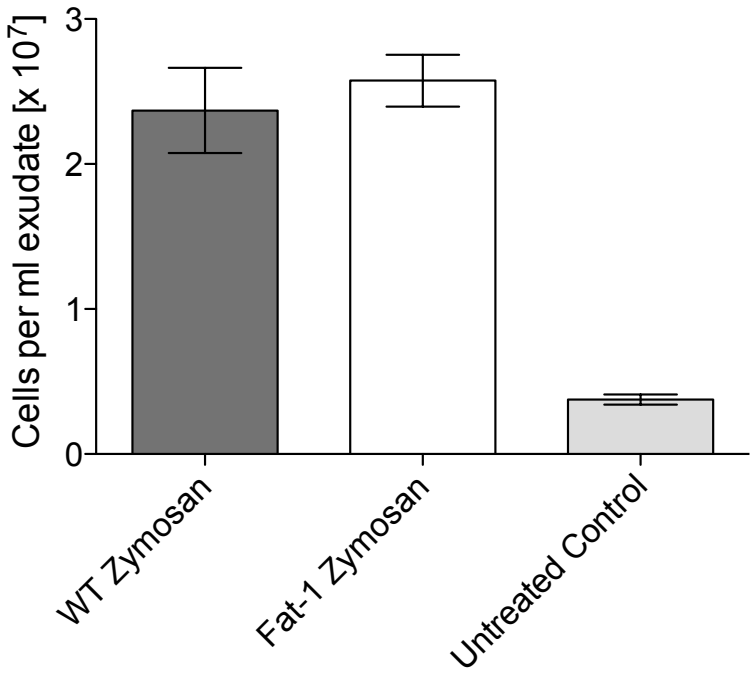

Fig. 4.2.3 A: *Total numbers of exudates cells*

Absolute number of exudate cells per ml, including red blood cells, 24 hours after zymosan A injection. n = 6 for WT, n = 8 for fat-1, n = 3 for control. Untreated control = untreated WT animals.

In the next step, Wright-Giemsa stains of exudates smears were prepared to assess the cellular composition of the exudates. We had to take into consideration that exudate cell counts might have been falsified by accidental contamination with red blood cells through damaging small blood vessels during injection. Therefore, the percentage of red blood cells of each sample, determined by light microscopic evaluation of each smear, was subtracted.

In regard to the proportion of red blood cells relative to the total number of cells, control animals showed a significantly smaller number (0.60 ± 0.21%) than zymosan-treated fat-1 (16.53 ± 4.17%, $p < 0.05$ for fat-1 vs. untreated control) or WT (12.57 ± 2.13%, $p < 0.01$ for WT vs. untreated control) animals (Fig. 4.2.2. B).

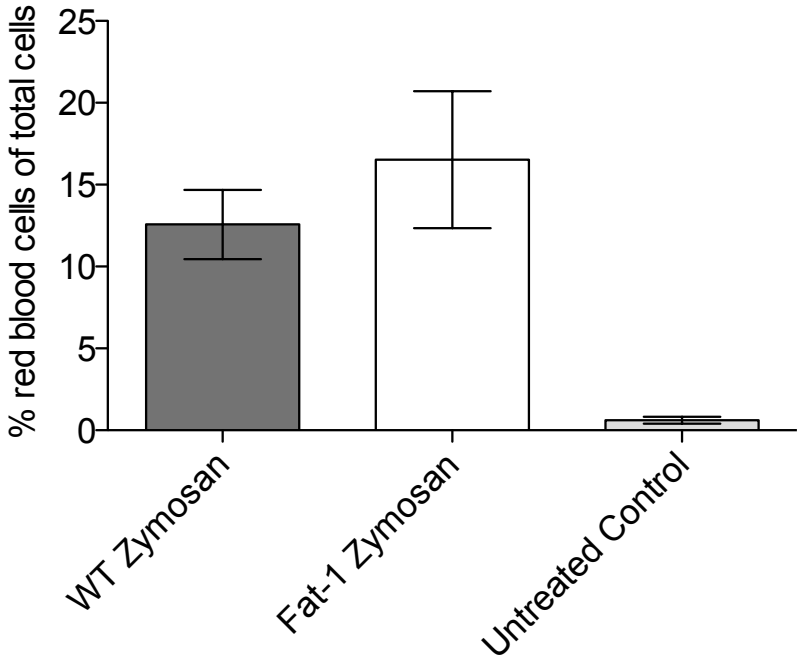

Fig. 4.2.3 B: *Percentages of red blood cells on total exudates cells*

After evaluation of the exudate's cellular composition through light microscopy, percentages of red blood cells relative to the total number of exudate cells were calculated. n = 6 for WT, n = 8 for fat-1, n = 3 for control. Untreated control = untreated WT animals.

Therefore, red blood cell infiltration is most likely a result of inflammatory cell influx into the peritoneal cavity than an artifact, since the control animal group was exposed to the same technique and number of pinpricks as both treated animal groups.

Moreover, we conducted a differential cell count to obtain further data on the exudates' composition. Cell types counted were macrophages/monocytes, neutrophils, eosinophils, lymphocytes and mast cells/basophils (Fig. 4.2.2. C).

The exudates of control animals consisted mainly of macrophages and lymphocytes, with few basophil granulocytes. When looking at total cell numbers in the exudates in

untreated animals, it is apparent that macrophages (1.68 ± 0.15 x 10^6 cells/ml) and lymphocytes (1.81 ± 0.21 10^6 cells/ml) were represented in roughly equal shares, whereas neutrophils (0.01 ± 0.01 x 10^6 cells/ml) only made up a minor part. The number of mast cells/basophils (0.20 ± 0.04 x 10^6 cells/ml) and eosinophils (0.04 ± 0.01 x 10^6 cells/ml) were negligibly small.

When examining exudates of zymosan-treated animals, the most striking difference is a strong increase of neutrophils (WT: 8.15 ± 1.05 x 10^6 cells/ml and fat-1: 8.10 ± 1.12 x 10^6 cells/ml) and monocytes/macrophages (WT: 9.36 x 10^6 ± 1.48 cells/ml & fat-1: 9.38 ± 0.75 x 10^6 cells/ml) in comparison to untreated control animals. Moreover, there is a notable rise in eosinophils in untreated vs. treated animals (WT: 1.32 ± 0.31 x 10^6 cells/ml and fat-1: 0.66 ± 0.09 x 10^6 cells/ml). The change in lymphocyte (WT: 1.81 ± 0.51 x 10^6 cells/ml and fat-1: 3.15 ± 0.45 x 10^6 cells/ml) and mast cell/basophil (WT: 0.06 ± 0.03 x 10^6 cells/ml and fat-1: 0.05 ± 0.03 x 10^6 cells/ml) numbers between control and treated animals is not as obvious as for other cell types. This shows that the result of an i.p. zymosan application is an influx of mainly monocytes/macrophages and neutrophil granulocytes into the peritoneal cavity.

When comparing WT and fat-1 animals, no significant differences can be found in monocyte/macrophage (p = 0.949), neutrophil (p = 0.851), lymphocyte (p = 0.141), eosinophil (p = 0.059) and mast cell/basophil (p = 0.662) populations.

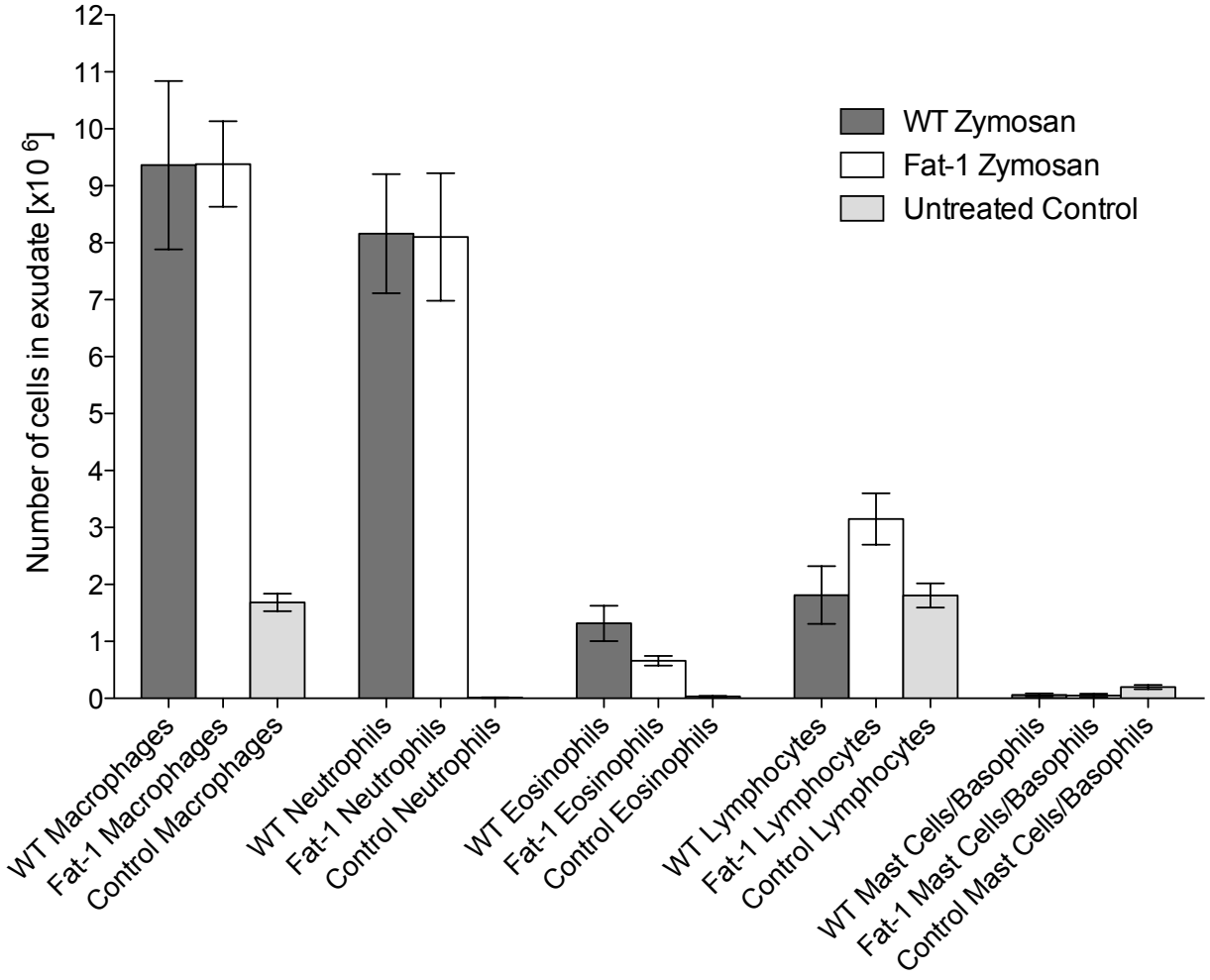

Fig. 4.2.3 C: *Cell counts of total white blood cells in peritoneal exudates*

A differential cell count was carried out from Wright-Giemsa stained exudate smears. n = 6 for WT, n = 8 for fat-1, n = 3 for control. Untreated control = untreated WT animals.

4.2.4 TNF-α ELISA from serum samples

For assessment of TNF-α levels in serum, blood samples previously obtained by cardiocentesis were used. TNF-α levels of untreated control mice (2.27 ± 0.13 pg/ml, n = 4) were significantly lower than those of treated fat-1 (3.85 ± 0.28 pg/ml n = 6, $p < 0.05$ for fat-1 vs. untreated control) and WT mice (3.59 ± 0.41 pg/ml, n = 7, $p < 0.01$ for WT vs. untreated control), there was, however, no significant difference between fat-1 and WT animals.

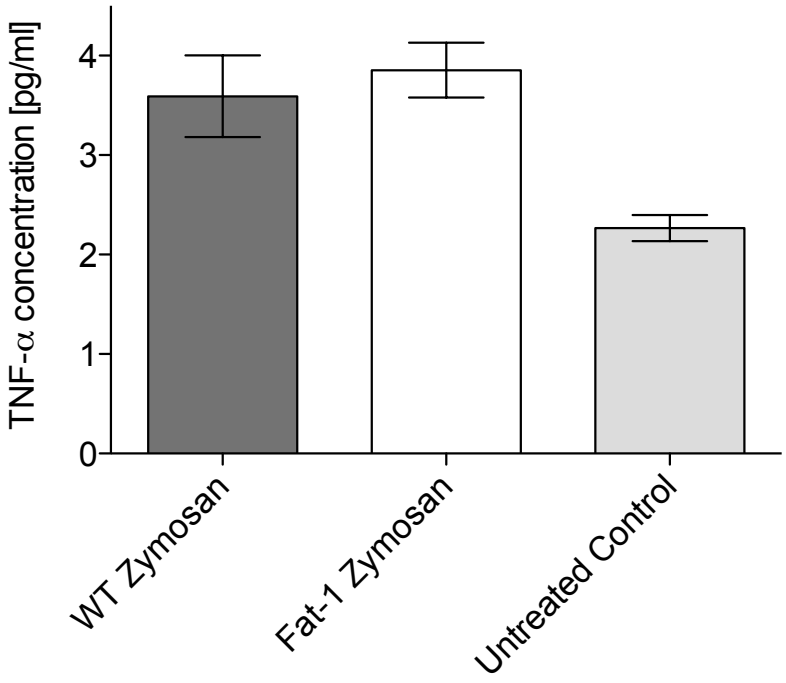

Fig. 4.2.4: *Serum levels of TNF-α*

TNF-α ELISA results of serum samples. n = 6 for WT, n = 7 for fat-1, n = 3 for control. Untreated control = untreated WT animals.

4.2.5 Liquid chromatography/tandem mass spectrometry

Beyond analysis of fatty acid profiles, we were interested in determining whether a higher proportion of n-3 PUFAs in fat-1 mice does indeed lead to a higher concentration of n-3-derived mediators. Thus, we analyzed liver tissue for concentrations of DHA-derived metabolite 17-HDHA and EPA-derived metabolite 18-HEPE as pathway markers of DHA and EPA utilization. Results are given as nanogram mediator per gram sample tissue (ng/g). We found strongly elevated levels of 18-HEPE and 17-HDHA in fat-1 mice vs. WT animals.

17-HDHA levels were significantly different for zymosan-treated WT animals (297.7 ± 22.72 ng/g) vs. control animals (151.6 ± 40.69 ng/g). Moreover, fat-1 animals (527.9 ± 59.06 ng/g) showed the highest concentration of 17-HDHA, with a statistically significant difference between zymosan-treated fat-1 and WT animals (Fig. 4.2.5 A).

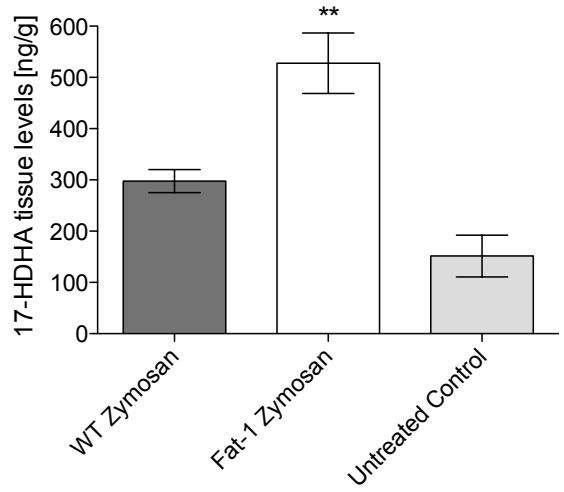

Fig. 4.2.5 A: *17-HDHA levels in liver tissue*

Results are from LC-MS/MS analysis. Fat-1 mice show significantly higher concentrations of 17-HDHA vs. WT animals. n = 7 for WT, n = 8 for fat-1, n = 5 for control. Asterisks indicate level of significance (WT zymosan vs. fat-1 zymosan), with "**" if $p < 0.01$. Untreated control = untreated WT animals.

Observations were similar for 18-HEPE. In fat-1 mice (25.24 ± 2.920 ng/g), 18-HEPE levels were ~17 times higher than in zymosan-treated WT animals (1.44 ± 0.9783 ng/g). 18-HEPE levels were undetectable in control mice liver samples (Fig. 4.2.5. B).

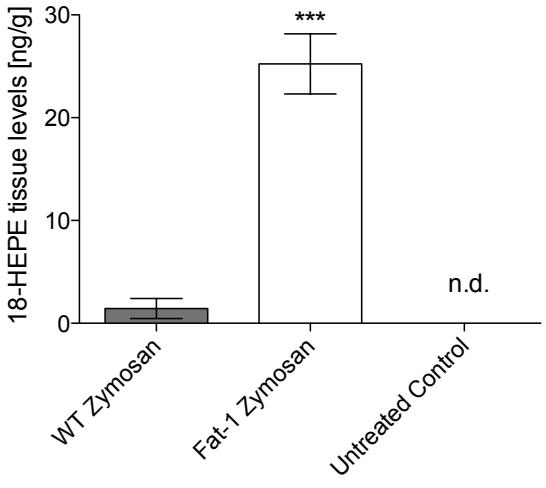

Fig. 4.2.5 B: *18-HEPE levels in liver tissue*

Results are from LC-MS/MS analysis. Fat-1 mice show significantly higher concentrations of 17-HDHA vs. WT animals. n = 7 for WT, n = 8 for fat-1, n = 5 for control. Asterisks indicate level of significance (WT zymosan vs. fat-1 zymosan), with "***" if $p < 0.001$. N.d. = not detectable. Untreated control = untreated WT animals.

5. Discussion

5.1 Summary of results

The purpose of this study was to further elucidate the mechanisms of the anti-inflammatory properties of n-3 PUFAs on a cellular level and in the context of inflammation resolution.

We first studied the effect of n-3 PUFAs DHA and EPA and their monohydroxylated metabolites 17-HDHA and 18-HEPE, which are known precursors of the newly described anti-inflammatory and pro-resolution resolving lipid mediator class in RAW 264.7 cells. For this, the RAW 264.7 murine macrophage cell line was used to establish an *in vitro* assay of resolution effects.

In one experimental setup, cells were exposed to DHA and EPA as well as 17-HDHA and 18-HEPE and TNF-α secretion after LPS stimulation was measured. While DHA and EPA had no significant effect, 17-HDHA as well as 18-HEPE showed a significant reduction of TNF-α production. This experiment therefore established a cytokine-dampening effect of these n-3 PUFA-derived metabolites.

In a second experimental setup we tested the paradigm that these fatty acids and lipid metabolites might increase the macrophages' phagocytic activity, thereby contributing to an increased clearance of inflammatory agents and debris and improving resolution. Indeed, incubation with EPA, 17-HDHA and 18-HEPE, however not DHA, led to a consistent increase in phagocytic activity of the RAW 264.7 macrophages in a phagocytosis assay using fluorescently labeled bioparticles.

Since zymosan peritonitis was used in recent studies examining the effect of n-3 PUFA-derived lipid mediators [21], these *in vitro* experiments were then followed by the examination of zymosan-induced peritonitis in the transgenic fat-1 mouse model with endogenously increased n-3 PUFA tissue levels. As expected, both fat-1 and WT animals showed an inflammatory response by influx of mainly macrophages and PMNs into the peritoneal cavity upon zymosan injection. However, there were no significant differences in cell numbers between these two groups and neither did we find any

differences in the systemic TNF-α production between the two groups, as determined by ELISA of serum samples. In contrast to these results, we did find significantly higher concentrations of lipid mediator metabolites in the fat-1 mice. 17-HDHA levels, as marker of DHA utilization, and 18-HEPE levels, as marker of EPA utilization, were significantly increased in liver tissue from the fat-1 mice with zymosan peritonitis as compared to WT animals.

5.2 Discussion of cell culture study

5.2.1 Methods of cell culture analysis

We conducted multiple TNF-α ELISA studies of cell culture supernatants. As described above, results are mean values from 4 different experiments with 3 separate samples per group in each experiment, leading to a high reliability regarding the observed results.

For analysis of phagocytosis, we used 2 separate data sets that consisted of 4 samples per group (n = 4 for each set). Phagocytosis was determined through fluorescein-labeled *Escherichia coli* K12 particles. As opposed to the ELISA study, measurements were made directly from cells, not supernatants, allowing only one measurement per sample. This circumstance makes this study more prone to measuring errors than other techniques, which allow multiple measurements from e.g. the supernatant of each sample. However, with 4 samples per group, we have measured a number high enough to assure validity of the values.

5.2.2 Results of TNF-α study

TNF-α is a well-investigated, pro-inflammatory mediator that allows conclusions about severity of inflammation and is mainly produced by macrophages.

Observations from our cell culture study showed a significant reduction of TNF-α levels for 17-HDHA and 18-HEPE. This underlines the pro-resolving potential of n-3 PUFA mediators. It is of interest to note that, in spite of leading to a slight decrease in TNF-α levels, we could not find significant differences of TNF-α production in DHA or EPA vs. positive control groups. These results support the notion that not a lack of pro-resolving properties of n-3-derived mediators, but rather a deficiency of n-3 mediator synthesis could be responsible for these observations. Moreover, EPA led to a slightly stronger decrease of TNF-α levels than DHA. This correlates with our observation of EPA-derived 18-HEPE lowering TNF-α levels more potently than DHA-derived 17-HDHA.

Regarding the effect of n-3 PUFAs on TNF-α synthesis *in vitro*, results of previous studies are inconsistent. For instance, Lo *et al.* report that murine macrophages incubated with EPA secrete less TNF-α than cells without fatty acid preincubation [64]. Similarly, Novak *et al.* and Babcock *et al.* report that incubation of murine macrophages with an emulsion of n-3 PUFAs also leads to a decrease of TNF-α production [65, 66]. In contrast, other studies have reported results that stand in disagreement with these findings. Several groups have investigated the effect of n-3 PUFAs and mediators on macrophages. Skuladottir *et al.*, for instance, report an increased TNF-α production of murine macrophages *in vitro* upon LPS stimulation when preincubated with n-3 PUFAs, both in comparison to untreated control as well as to n-6 PUFAs (arachidonic acid and linoleic acid). Factors that might lead to these contradictory results could be differences in the experimental setup. For instance, concentrations of n-3 PUFAs used in Skuladottir *et al.*`s study are more than twice as high (50 μM vs. 20 μM in our study). Moreover, incubation times differ greatly. We preincubated cells with fatty acids for 2 hours and stimulated with LPS for 4 hours. In the study of Skuladottir *et al.*, with fatty acid preincubation for 20 hours and LPS stimulation for 12 hours, incubation times were far longer. Therefore, the time point of analysis of TNF-α concentration is 6 hours in our study vs. 32 hours in the study of Skuladottir *et al.*, which constitutes a difference of 26 hours.

Interestingly, they also report an increased *in vitro* TNF-α production of LPS stimulated elicited murine macrophages of mice that had received high dietary n-3 PUFAs. While the absolute number of cells secreting TNF-α is not altered, the amount of TNF-α secreted per cell is significantly higher in mice that had been fed on a n-3 rich diet [67]. These results are consistent with other studies, which have also reported an increased *ex vivo* TNF-α production by mouse peritoneal macrophages after high dietary n-3 intake [68-75]. However, due to the differences in the experimental setting (*in vitro* vs. *in vivo* application of n-3 PUFAs), direct comparability with our study is limited.

The discrepancies of results amongst previous studies show that there are still numerous aspects regarding the effect of n-3 PUFAs on inflammation and especially TNF-α synthesis that need further investigation.

5.2.3 Results of phagocytosis assay

As illustrated above, phagocytosis is a key marker of inflammation resolution. Hence, determination of phagocytic activity is of interest when trying to quantify anti-inflammatory and pro-resolving properties of compounds like n-3 PUFAs. In our study we consistently found EPA, 17-HDHA and 18-HEPE to significantly enhance phagocytosis *in vitro*. Of two individual data sets, we only saw statistically significant effects of DHA in one, with a non-significant increase of phagocytosis in another data set. EPA, 17-HDHA and 18-HEPE significantly increased phagocytosis in both experiments. Thus, an overall augmentation of phagocytosis, particularly by 17-HDHA and 18-HEPE, is clearly recognizable in all groups.

It is important to point out that the increase of phagocytosis was equally effective for 17-HDHA and 18-HEPE at concentrations 40 times lower than DHA and EPA. This suggests a stronger pro-phagocytic potential for 17-HDHA and 18-HEPE than their precursor fatty acids.

These results are consistent with those of previous studies. Pisani *et al.* have described an increase in phagocytosis of elicited goat monocytes after *in vitro* incubation with DHA

and EPA [76]. In terms of fatty acid mediators, Schwab *et al.* have shown an increase of *in vivo* phagocytosis of apoptotic neutrophils in a murine peritonitis model through i.p. application of RvE_1 and PD_1 prior to induction of peritonitis [25]. Yet another model examined the dietary effects of fish oil, and thus a blend of various n-3 PUFAs on isolated peritoneal macrophages from rats [77]. Here, too, phagocytosis was enhanced in the fish oil group.

Our results, together with previous studies, support the understanding that n-3 PUFAs and its metabolites and mediators enhance phagocytosis and therefore possess pro-resolving effects through clearance of inflammatory stimuli as well as remnants of inflammation and thus promote the return to tissue integrity.

5.3 Discussion of animal model study

5.3.1 Intraperitoneal injection

Several publications have reported that intraperitoneal injection is prone to procedural errors and report error rates of injections up to 14% [78-80]. Various studies investigated different methods of i.p. injection. They could not find advantages of using different needle sizes [80] and report the highest number of misplaced injections in the lumen of the stomach or small bowel.

We compared two different techniques of i.p. injection, a one-man and a two-man procedure. Arioli *et al.* report a 12% error in the placement of intraperitoneal injections of mice with the one-man procedure as compared to 1.2% with a two man procedure [78]. Our results did not confirm this, however, with a success rate of 100% for the one-man procedure as to 80% for the two-man procedure.

Another study reports a high variability of properly placed injections between different investigators, ranging from 4% to 15%, suggesting the success of i.p. injection to be strongly dependent on the person conducting it [79, 80]. This might be an explanation for the difference between our results and Arioli *et al.*

5.3.2 The fat-1 model

The fat-1 model offers a unique possibility to investigate the influence of n-3 fatty acids. In order to comprehend the advantages of this model with regard to the situation in humans, it is vital to know how mankind's living situation and nutrition have evolved over the millennia. Nowadays, humans live in an environment that differs greatly from that for which our genetic constellation was selected. Our diet today has changed immensely in comparison to preagricultural humans, whose main intake was food such as lean meat, fish, green leafy vegetables, fruits, nuts, and berries, all containing sufficient amounts of n-3 fatty acids. Back then, the body's demand of n-3 PUFAs was entirely covered through our daily nutrition. However, due to the drastically increasing consumption of meat and vegetable oils derived from n-6 PUFA-rich grains, our intake of n-6 PUFAs has strongly exceeded the intake of n-3 PUFAs to an extend that has shifted our food's n-6/n-3 ratio to 15-16.7/1 [81]. Considering that our genetic background evolved at a time when this ratio is thought to have been around 1, the potential impact of this transition becomes evident. While n-6 PUFA intake has reached the magnitude that we experience today, n-3 intake has gradually declined. A ratio of 1/1 – 4/1, which is assumed by experts to be favorable, can therefore only be achieved through lowering n-6 PUFA intake and at the same time increasing n-3 PUFA intake.

Most experimental models rely upon application of n-3 fatty acids or mediators to simulate their impact. In our model, however, we tried to create a condition of high n-3 PUFA tissue content by transgenic means. Through insertion of the fat-1 gene of the roundworm *C. elegans* into C57Bl6 mice, n-6 PUFAs are converted into their n-3 counterparts. This enzyme acts on C16 to C20 fatty acids as substrate and introduces a double bond at the n-3 atom [82]. Through genetic engineering, it is thus possible to circumvent confounding factors that might arise through n-3 additives to a special diet, or through other ways of n-3 application. Furthermore, addition of n-3 fatty acids to an organism does not alter n-6 fatty acids concentrations. With the present animal model, it is possible to simulate a balanced n-6/n-3 PUFA ratio in a way that is not possible through mere n-3 PUFA addition.

A disadvantage of this study is the fact that WT mice used here were derived from an animal facility other than the one that bred fat-1 mice. WT mice were thus fed a different

diet (Purina 5L79) as compared to the fat-1 mice up to an age of 1 month and only then switched to the same diet as fat-1 mice, i.e. AIN-76A with 10% safflower oil, which they received for another 2 months before the experiment. While Purina 5L79 and the basal AIN-76A have a similar nutritional composition, with comparable calorie provision shares (Purina 5L79: protein 21%, fat 14%, carbohydrates 65%; AIN-76A: protein 19%, fat 12%, carbohydrates 69%), the n-3 PUFA content in Purina 5L79 is at 0.2%, while it is only 0.05% in AIN-76A. This could explain that – unlike in previous experiments performed with fat-1 mice and their WT littermates as control – the DHA content in the tail tissues of the fat-1 and WT group used here was not significantly different (1.97 ± 0.12% in WT vs. 2.10 ± 0.09% in fat-1 animals). Overall, however, the fatty acid profiles of the test animals showed a significantly lower n-6/n-3 ratio in fat-1 mice compared to WT animals (9.57 in WT vs. 1.51 in fat-1 animals). Moreover, lipidomic analysis showed significantly higher levels of n-3 metabolites in the livers of fat-1 animals.

Even though these results confirm high n-3 PUFA and metabolite levels in fat-1 animals, they can only be understood as representative of concentrations of fatty acids and their mediators at the site of inflammation, i.e. the peritoneal cavity. Various studies have reported PUFA concentrations and n-6/n-3 ratios that vary greatly between different tissues. Kang *et al.* found AA/(EPA+DPA+DHA) ratios to vary between 0.4 (muscle) and 2.5 (breast milk) ratio in fat-1 mouse tissues. WT mice ratios, which were consistently higher, were between 3.6 (brain) and 27.0 (erythrocytes) [61]. Unfortunately, in our case, we were not able to determine fatty acid profiles of the inflamed peritoneum. Samples that could have been used for gas chromatography, i.e. the peritoneal layer or exudate cells, were not suitable for further gas chromatographic analysis: a lipid extraction from the peritoneum is impossible to conduct since it is a mere mesothelial layer and not a parenchymatous organ, and cellular yield from a peritoneal lavage did not suffice for further processing. On the other hand, previous studies have shown that, in spite of varying PUFA levels between different tissue types, WT mice consistently show higher n-6/n-3 ratios in all examined tissues than fat-1 mice, both inflamed and not inflamed [61].

A possible disadvantage of fat-1 animals used in this study might be the circumstance that they were backcrossed four times onto a C57BL/6 background at least. Many immunological studies that employ animal models use animals that were backcrossed more often in order to minimize genetic differences.

A weakness of the experimental setup of this study is certainly the fact that we used an untreated WT control group only due to limited availability of fat-1 mice. This aspect limits comparability of results. However, in order to ensure a sufficient number of animals and thus samples in each experimental group, we were forced to refrain from using an untreated fat-1 control group as well.

5.3.3 The course of inflammation over time in comparison with previous studies

Other studies have already focused on the effect of n-3 PUFAs on zymosan-induced peritonitis. Due to the advantages this inflammation model offers, it has often been used for exploration of cytological and molecular questions. One group that has studied this model extensively is that of Serhan *et al.* [21, 25, 83]. A study of Bannenberg *et al.* [21] has investigated the effect of different n-3 PUFAs and lipid mediators on the course of peritonitis over time through direct injection of the respective substance into the peritoneal cavity at the same time as zymosan. Markers used to quantify and compare inflammation were T_{max} (point in time of maximal PMN influx), ψ_{max} (maximal number of PMNs), T_{50} (point of time where 50 percent of maximal PMN number are lost from site), R_{50} (point of time when T_{50} is reached) as well as R_i (the interval between ψ_{max} and R_{50}).

According to Bannenberg *et al.*, 24 hours after injection constitutes a point in time where the number of PMNs is roughly equal to the number of macrophages in animals that were not exposed to n-3 PUFAs or mediators. Our study confirmed this observation: PMN numbers were 8.16 ± 1.05 x 10^6 cells/ml in WT and 8.10 ± 1.12 x 10^6 cells/ml in fat-1 animals, whereas macrophages were at 9.36 ± 1.48 cells/ml in WT and 9.38 ± 0.75 cells/ml in fat-1 animals. Besides, cellularity in our experiments was approximately the same as in previous experiments with similar experimental settings [21, 25, 84], which reported PMN numbers of around 8 x 10^6 cells.

Bannenberg *et al.* report that, when injected at the same time as zymosan, lipid mediators RvE_1 or PD_1 (amongst others) led to a decrease of inflammation or enhanced resolution in a specific pattern. RvE_1 and PD_1 shifted T_{max} and T_{50} to earlier points and

reduced ψ_{max}. In addition, PD_1 also shortened R_i. Differences in exudate cellularity were only significant at 4 and 12 hours after zymosan injection, however, not at 24 hours after injection. Moreover, cytokine concentrations in exudates peaked around 4 hours and gradually normalized over time, so that levels for many cytokines had already normalized at 24 hours.

While Bannenberg *et al.* report that PMN numbers reach a maximum at 12 hours after injection (ψ_{max}), another study of Kolaczkowska *et al.* [85], which also used 1 mg zymosan per mouse to induce peritonitis, found that ψ_{max} already occurs at 6 hours after injection, with maximum PMN and macrophage numbers similar to Bannenberg *et al.* and our own (i.e. 20 x 10^6). Moreover, Bannenberg *et al.* saw a 50% PMN reduction at around 20 hours after injection (T_{50}) while Kolaczkowska *et al.* report this to occur at around 12 hours post injection.

These unequal results of Bannenberg *et al.* and Kolaczkowska *et al.* are especially interesting given the similarity of the experimental conditions: both groups used C57BL/6 mice as well as a fixed dose of 1 mg zymosan from the same manufacturer (Sigma Aldrich, St. Louis, MO, USA) in saline solution per mouse.

In a study by Schwab *et al.* [25], exudates were collected 24 hours after RvE_1 and PD_1 injection together with zymosan. Interestingly, both compounds led to an increase, however not significant, in macrophages, regardless of the time point of administration. Even though a stronger influx of leukocytes like macrophages might initially seem like a proinflammatory process, this does not necessarily have to be the case. While some publications have suggested that n-3 fatty acids decrease phagocytic activity of macrophages [86], others report that they have an increasing effect [25, 77]. As described above, n-3 lipid mediators are thought to unfold their pro-resolving effects partly by enhancing phagocytosis of apoptotic neutrophils and other debris that inflammation has caused. In this context, elevated macrophage influx can be considered a way of PMN clearance and thus a pro-resolving mechanism. Most importantly, Schwab *et al.* report a significant decrease of PMN numbers 24 hours after zymosan injection through both RvE_1 and PD_1 between 20 and 40% when compared to control animals [25].

Due to the similar setting of this study and the same time of exudate collection, i.e. 24 hours post injection, as in our experiments, these results suggest that fat-1 animals

could also show diminished PMN and possibly increased macrophage influx. Our observations show different results, however, i.e. a non-significant difference between WT and fat-1 animals in terms of overall cell as well as PMN and macrophage infiltration.

The results of Bannenberg *et al.*, Schwab *et al.* and Kolaczkowska *et al.* prove that time is an essential factor in this model. When comparing cellular infiltrates, different research groups report different courses of inflammation over time even without the addition of n-3 mediators. Moreover, both Bannenberg *et al.* and Schwab *et al.* report that individual n-3 mediators unfold a unique pattern of anti-inflammatory action that does not influence inflammation equally at every stage of inflammation but rather modulates inflammatory indexes in a time-specific manner, e.g. RvE_1 reducing T_{50} while PD_1 effectively reduces R_i as well. In our study, we analyzed inflammatory exudates 24 hours after injection only. Given the time-dependence of this model, it is possible that anti-inflammatory and pro-resolving effects might be measurable at other points, i.e. earlier or even later than 24 hours. Further studies in the fat-1 mouse model are needed to examine this.

With the different setting of the experiments described above, it is difficult to evaluate whether these results can be directly transferred to our observations. Since lipid mediators were directly introduced into the peritoneal cavity in these comparative studies, the mode of n-3 PUFA application was entirely different. Mice in our study had higher levels of n-3 PUFAs that need further enzymatic processing, whereas mice in other studies received n-3 mediators, which are distinctly more potent than their precursor molecules, directly through i.p. injection.

Another aspect in which our study differs from other studies is that we used weight-adjusted dosages of zymosan, whereas others [21, 84] used a fixed dose of 1 mg zymosan in 1 ml sterile saline. At an average weight of 20 grams, a difference of 3 grams means a 15% weight difference. Therefore, an uneven weight distribution might bias results.

Besides, alcohol served as vehicle substance for mediators in the experiment described above. Impossible to circumvent in direct mediator application, this additional substance imposes a confounding factor that is not present in our study and might thus be partly responsible for the differences in the outcome between the above studies and our own.

5.3.4 Discussion of LC-MS/MS results

By phenotyping mice through gas chromatography, we were able to distinguish fatty acid profiles in fat-1 and WT animals. It was, therefore, of great interest to determine whether higher concentrations of n-3 PUFAs also lead to higher concentrations of n-3 PUFA metabolites of the resolvin pathways.

The synthesis of n-3 mediators is a complex mechanism, which is partially dependent on factors such as the presence of microbial enzymes or COX-II aspirin acetylation (see above). Therefore, synthesis of n-3 mediators from fatty acids might constitute a factor limiting the beneficial effects of n-3 PUFAs. We questioned whether tissue from fat-1 mice shows higher levels of n-3 mediators than WT mice. Since peritoneal exudates did not yield enough material to conduct LC/MS-MS from, we used liver tissue for analysis. As central metabolic organ, the liver plays an essential role in inflammatory processes, e.g. as production site of acute phase proteins in systemic inflammation. Previous studies have found n-6/n-3 fatty acid ratios in livers to be 26.0 in WT mice vs. 2.5 in fat-1 mice [61]. With an n-6/n-3 ratio around ten times higher in WT mice, this organ ensures a sufficient supply of n-3 PUFAs in fat-1 animals. In order to capture as many n-3-derived mediators as possible, we did not try to detect single mediators, i.e. individual resolvins, but rather the precursor molecules that these compounds are synthesized from. 17-HDHA from DHA, and 18-HEPE from EPA, have been identified as markers of DHA [50] and EPA [17] metabolization. Thus, we analyzed these molecules by LC-MS/MS.

We found the highest concentrations of both 17-HDHA as well as 18-HEPE in fat-1 animals. WT zymosan-treated animals had higher concentrations than WT control animals, which is in agreement with the fact that these compounds are increasingly synthesized during acute inflammation. Previous studies have made similar observations, with elevated levels of 17-HDHA as well as RvD_3, PD_1 and RvE_1 in colon tissue of fat-1 mice in a colitis model [54]. Even though DHA levels in tail tissue measured by gas chromatography were not significantly different between fat-1 and WT mice, we found 17-HDHA levels to differ significantly between the two groups. As illustrated above, previous studies have found n-6/n-3 ratios to fluctuate greatly in different tissue types of fat-1 mice. Hence, it is likely that these results are not

contradictory, but rather that they reflect the different pattern of distribution of n-3 and n-6 PUFAs throughout the organism.

6. Abstract/Zusammenfassung

High levels of omega-3 polyunsaturated fatty acids (n-3 PUFAs) have been associated with a reduced risk of coronary heart disease. It has been found that these compounds unfold potent anti-inflammatory and pro-resolving effects as precursor molecules of a newly discovered set of lipid mediators, called resolvins and protectins/neuroprotectins. Numerous studies have reported strong attenuation of inflammation and promotion of inflammation resolution through these n-3 mediators.

In order to establish an *in vitro* model of these anti-inflammatory and pro-resolving properties of n-3 mediators, we decided to analyze the effect of n-3 PUFAs and resolvin precursor metabolites 17-hydroxydocosahexaenoic acid (17-HDHA) and 18-hydroxyeicosapentaenoic acid (18-HEPE) in the RAW 264.7 murine macrophage cell line. We found that incubation with the n-3 metabolites 17-HDHA and 18-HEPE led to a decrease of lipopolysaccharide (LPS)-induced tumor necrosis factor alpha (TNF-α) secretion, supporting the inflammation dampening effect of these compounds. Moreover, we detected enhanced phagocytic activity of these macrophages when exposed to the n-3 PUFAs docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) as well as 17-HDHA or 18-HEPE, arguing for a pro-resolution role of these compounds.

Next we carried out an animal model using transgenic fat-1 mice with endogenously high levels of n-3 PUFAs. 24 hours after induction of peritonitis with intra-peritoneal injection of zymosan A, exudates were collected by means of peritoneal lavage. Subsequently, cellular composition of exudates was analyzed in Wright-Giemsa smears, demonstrating no significant differences between fat-1 and wild type (WT) mice. This was reflected in similar systemic TNF-α levels for both groups. While these findings argue against a protective effect of n-3 PUFA in this context, several factors might have disguised a potential protective effect in this setting, such as (1) wrong timeframe of analysis, (2) dietary differences between the two groups of mice, or (3) wrong analysis parameters (e.g. no assessment of phagocytic activity of macrophages in both groups). In contrast to the unequivocal findings with regard to the cell numbers of the peritoneal exudates, we found significantly increased levels of protective n-3

PUFA metabolites 17-HDHA and 18-HEPE in livers of fat-1 mice with zymosan peritonitis as compared to the WT group, arguing for an increased formation of protective n-3 PUFA lipid mediators in fat-1 mice.

Hohe Omega-3 Fettsäure (n-3 PUFA)-Spiegel werden seit langem mit einem verminderten Risiko für eine koronare Herzerkrankung assoziiert. Erst kürzlich wurde berichtet, dass diese Verbindungen als Vorläufermoleküle von Fettsäuremediatoren, den sogenannten Resolvinen und Protektinen/Neuroprotektinen, dienen. Zahlreiche Studien haben sowohl starke anti-inflammatorische als auch die Entzündungsresolution fördernde Effekte dieser n-3 PUFA Mediatoren beschrieben.

Um ein *In-vitro*-Model dieser anti-inflammatorischen und resolutionsfördernden Effekte der n-3 Mediatoren zu etablieren haben wir die Wirkung der n-3 Fettsäuren Docosahexaensäure (DHA) und Eicosapentaensäure (EPA) sowie der Resolvin-Vorläufermoleküle 17-Hydroxydocosahexaensäure (17-HDHA) und 18-Hydroxyeicosapentaensäure (18-HEPE) in einem Zellkulturmodell mit RAW 264.7 Mausmakrophagen untersucht. Wir fanden eine Reduktion der Lipopolysaccharid (LPS)-induzierten Tumornekrosefaktor alpha (TNF-α)-Sekretion durch Präinkubation der Makrophagen mit 17-HDHA und 18-HEPE, was für einen entzündungsdämpfenden Effekt dieser Substanzen spricht. Weiterhin zeigte sich eine erhöhte Phagozytoseaktivität sowohl durch Inkubation mit den n-3 Fettsäuren DHA und EPA als auch durch 17-HDHA und 18-HEPE, was die resolutionsfördernden Eigenschaften dieser Substanzen unterstreicht.

Des Weiteren beschäftigten wir uns mit einem Tiermodell transgener fat-1 Mäuse, die hohe endogene n-3 Fettsäurespiegel aufweisen. Wir gewannen 24 Stunden nach Induktion einer Peritonitis durch intraperitoneale Injektion von Zymosan A Entzündungsexsudate durch Peritoneallavage. Die zelluläre Zusammensetzung wurde anhand lichtmikroskopischer Auswertung von Wright-Giemsa-Färbungen der Exsudat-Ausstriche analysiert. Hierbei fanden wir keine signifikanten Unterschiede zwischen fat-1 und Wildtyp (WT)-Mäusen. Dieser Befund spiegelt sich in ähnlichen systemischen TNF-α Konzentrationen in beiden Versuchsgruppen wieder. Wenngleich diese Ergebnisse gegen eine entzündungsmodulierende Wirkung dieser Substanzen sprechen, so könnten mehrere Faktoren, wie beispielsweise (1) ein falsch gewählter Analysezeitpunkt, (2) Unterschiede im Diätregime beider Gruppen oder (3) falsche Analyseparameter (z.B. keine Analyse der Phagozytoseaktivität der Peritonealmakrophagen) einen möglichen protektiven Effekt verschleiert haben. Diese Befunde stehen im Kontrast zu den Ergebnissen der Bestimmung von 17-HDHA und 18-HEPE in den Lebern der Versuchstiere. Hierbei fanden wir signifikant erhöhte

Spiegel beider Metaboliten in Zymosan-behandelten fat-1 Mäusen gegenüber WT Mäusen, was für eine erhöhte Synthese von n-3 Mediatoren in fat-1 Mäusen spricht.

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8. Statement/Selbständigkeitserklärung

„Ich, Simon Fabian Wächter erkläre, dass ich die vorgelegte Dissertation mit dem Thema: „Effects of omega-3 fatty acids docosahexaenoic acid and eicosapentaenoic acid and their metabolites in acute inflammation“ selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt, ohne die (unzulässige) Hilfe Dritter verfasst und auch in Teilen keine Kopien anderer Arbeiten dargestellt habe.“

Datum Unterschrift

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10. Curriculum vitae/Lebenslauf

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

Publikationsliste

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