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The role of PPARβ/δ in human macrophages

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Index

Abbreviations

AA	Arachidonic acid
ALA	α-Linolenic acid
ANGPTL4	Angiopoietin-like 4
AP-1	Activator protein 1
APC	Antigen presenting cell
CD	Cluster of differentiation
ChIP	Chromatin immunoprecipitation
DC	Dendridic cell
DHA	Docosahexaenoic acid
EGF	Epidermal growth factor
EPA	Eicosapentaenoic acid
FA	Fatty acid
FCS	Fetal calf serum
INF-γ	Interferon γ
IL	Interleukin
IPA	Ingenuity Pathway Analysis
LA	Linoleic acid
LBD	Ligand binding domain
LC-MS/MS	Liquid chromatography–mass spectrometry/mass spectrometry
LPS	Lipopolysaccharide
MDM	Monocyte-derived macrophages
MMP	Matrix-metalloproteinase
mRNA	Messenger ribonucleic acid
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
PAMP	Pathogen-associated-molecular-pattern
PDK4	Pyruvate dehydrogenase kinase 4
PPAR	Peroxisome proliferator-activated receptor
PRR	Pattern recognition receptor
PPRE	PPAR response element
PUFA	Polyunsaturated fatty acid
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
RXR	Retinoid X receptor
STAT	Signal transducer and activator of transcription
TAM	Tumor-associated macrophage

Abbreviations

TZD	Thiazolidindion
VEGF	Vascular endothelial growth factor

1 Summary

Macrophages represent the most diverse cell type in biology. They adapt selectively to many stimuli allowing for precise functionality in any environment without harming the organism. Consequently, they monitor their surroundings carefully and react to a plethora of signals. Fatty acids and their derivatives are important signaling mediators in this context, which besides other signals impinge on the lipid-regulated nuclear receptor peroxisome proliferator-activated receptor (PPARβ/δ).

Studies conducted in mice have shown that ablation of PPARβ/δ results in the inability of adipose and liver macrophages to adopt an alternative anti-inflammatory activation state, demonstrating a prominent role of PPARβ/δ in macrophage function with implications for immune regulation. To date, however, systematic studies focusing on PPARβ/δ's role in human macrophages have not been reported.

The first part of this thesis addresses the role of PPARβ/δ in human macrophages including its transcriptional network affecting a multitude of cellular processes. A major part of this network involves cell type independent canonical regulation, which is characterized by the binding of PPARβ/δ with its obligatory dimerization partner retinoid X receptor (RXR) to specific sites in the regulatory region of established and previously unreported target genes, their induction by agonists and repression by inverse agonists. Additionally, a new set of non-canonical regulated target genes is described. These genes lack chromatin-bound PPARβ/δ complexes, are repressed by agonists (inverse regulation) and are macrophage-selective. Consistent with the prevailing opinion and the induction of an IL4-like morphological phenotype by agonists, this mode of regulation inhibits pro-inflammatory signaling. Surprisingly, anti-inflammatory genes, such as *CD32B*, *IDO1* and *CD274* (*PD-L1)* were also repressed. Consistent with these results, immune functions such as $CD8^+$ T cell activation were stimulated by these ligands. In combination, these findings point to a unique macrophage activation state induced by PPARβ/δ agonists with context dependent functions in immune regulation.

The second part describes the PPARβ/δ-regulated transcriptome for tumor-associated macrophages (TAMs) from human serous ovarian carcinoma ascites. Interestingly, most canonical PPARβ/δ target genes were found to be upregulated and refractory to synthetic agonists as compared to monocyte-derived macrophages. This was not due to a TAM specific increase in PPARβ/δ protein level or recruitment to target genes. However, the unaffected response of these genes to inverse agonists hinted at the presence of endogenous activating ligands. Lipidomic analysis of

malignancy-associated ascites indeed revealed very high concentrations of dietary polyunsaturated fatty acids (PUFAs), mainly linoleic and arachidonic acid. These PUFAs induced lipid droplet formation in macrophages which provide a potential reservoir for PPARβ/δ agonists and may serve as the causal nexus for target gene deregulation. Among the deregulated genes, *ANGPTL4* is associated with shorter relapse-free survival, illustrating the potential clinical implications of these findings.

Zusammenfassung

Makrophagen stellen den divergentesten Zelltyp dar. Sie beeinflussen und modellieren ihre Umgebung auf vielfältige Weise. Folglich müssen diese Zellen die auf sie wirkenden Umwelteinflüsse wahrnehmen und verarbeiten, um eine adäquate Adaptation zu gewährleisten. Nur so kann eine Schädigung des Organismus bei gleichzeitigem Erhalt der Funktionalität ausgeschlossen werden. Ein in diesem Kontext wichtiger Faktor ist die Verfügbarkeit und Zusammensetzung von Fettsäuren und ihren Derivaten, welche nebst anderen Signalen, auf den lipidregulierten Kernrezeptor Peroxisome Proliferator-Activated Receptor (PPARβ/δ) einwirken.

Versuche in Mäusen haben gezeigt, dass dessen genetische Ablation dazu führt, dass Fettgewebs- und Leber- Makrophagen nicht mehr befähigt sind einen alternativen anti-inflammatorischen Aktivierungszustand einzunehmen. Diese Ergebnisse unterstreichen die wichtige Rolle von PPARβ/δ in Makrophagen und der Immunregulation. Dennoch liegen bis heute keine systematischen Studien, die sich auf die Rolle von PPARβ/δ in humanen Makrophagen fokussieren, vor.

Die vorliegende Arbeit beschreibt die Rolle von PPARβ/δ in humanen Makrophagen inklusive seines transkriptionellen Netzwerks, das auf eine Vielzahl zellulärer Prozesse einwirkt. Zum einen wird dies durch die zelltypunabhängige kanonische Regulation bewirkt. Dabei bindet PPARβ/δ mit seinem obligatorischen Dimerisierungspartner Retenoid X Receptor (RXR) direkt an spezielle Stellen in den regulatorischen Regionen bereits bekannter und neubeschriebener spezifischer Zielgene, wodurch die Transkription durch Agonisten induziert und durch inverse Agonisten reprimiert wird. Zum anderen wird eine neue Klasse von nicht-kanonisch regulierten Zielgenen beschrieben. Diese Gene weisen keine chromatinassozierten PPARβ/δ Komplexe auf, werden durch Agonisten reprimiert und sind makrophagen-selektiv (inverse Regulation). Im Einklang mit der vorherrschenden Ansicht und der Induktion eines IL4-ähnlichen morphologischen Phänotyps durch Agonisten, inhibiert diese Art der Regulation pro-inflammatorische Funktionen. Überraschenderweise werden jedoch gleichzeitig auch anti-inflammatorische Gene, unter anderen *CD32B*, *IDO1* und *CD274* (*PD-L1)* reprimiert. Entsprechend konnte eine makrophagen-abhängige Stimulation der $CD8^+$ T-Zell Aktivierung durch diese Liganden beobachtet werden. In Kombination deuten diese Beobachtungen auf eine besondere Rolle von PPARβ/δ mit kontextabhängiger Funktion in der Immunregulation hin.

Der zweite Teil beschreibt das durch PPARβ/δ regulierte Transkriptom tumor-assoziierter Makrophagen (TAMs) aus dem Aszites von Patientinnen mit serösem Ovarialkarzinom. Beachtenswerterweise ist im Vergleich zu monozyten-abgeleiteten

Makrophagen die Mehrheit der PPARβ/δ Zielgene überexprimiert und refraktär gegenüber Agonisten, was weder auf ein erhöhtes Proteinlevel noch die vermehrte Rekrutierung an Zielgene zurückzuführen ist. Der Einfluss von inversen Agonisten auf TAMs war gleichzeitig unverändert, was auf die Gegenwart von endogenen aktivierenden Liganden hindeutete. Analysen von Aszitesproben hinsichtlich der Lipidzusammensetzung offenbarten tatsächlich stark erhöhte Konzentrationen mehrfachungesättigter Fettsäuren, vor allem Linolsäure und Arachidonsäure. Diese Fettsäuren verursachten die Bildung von Lipidtröpfchen in Makrophagen, welche ihrerseits ein potentielles Reservoir für PPARβ/δ Agonisten darstellen könnten, was wiederum eine Erklärung für die Deregulierung von PPARβ/δ Zielgenen bietet. Unter den deregulierten Genen findet sich *ANGPTL4*, dessen erhöhte Expression mit einem verkürzten rezidivfreien Überleben assoziiert ist und somit die potentielle klinische Bedeutung dieser Beobachtungen unterstreicht.

2 Introduction

2.1 Peroxisome Proliferator-Activated Receptors (PPARs)

Peroxisome proliferator-activated receptors were named after their potential to bind peroxisome proliferators (Issemann & Green 1990), a diverse group of chemical substances that increase size and number of peroxisomes in rodents. Peroxisomes are organelles especially associated with fatty acid α and β oxidation although exerting other functions such as the biosynthesis of ether phospholipids or the reduction of hydrogen peroxide (Wanders & Waterham 2006). These ligand-regulated transcription factors are members of the nuclear receptor superfamily. In mammals three types of PPARs with diverse tissue distribution have been characterized PPARα, PPARβ/δ and PPARγ (Dreyer et al. 1992; Braissant et al. 1996). All three subtypes are *in vivo* sensors and transcriptional effectors of dietary fatty acids and their derivatives (Forman et al. 1997). They act through the control of specific gene subsets which strongly influence metabolic functions, making these modulators intriguing pharmacologic targets.

2.1.1 PPAR subtypes: a short overview

The alpha isoform is predominantly expressed in tissue involved in lipid catabolism such as liver, brown fat, heart and intestine (Braissant et al. 1996; Rakhshandehroo et al. 2010), where its major role is regulation of lipid metabolism and energy homeostasis. Fibrates, synthetic ligands specifically activating PPARα, are therefore used to treat hypercholesterolemia since the 1930s.

PPARβ/δ is ubiquitously expressed and associated with a wide range of functions. It is involved in regulating intermediary metabolism (Desvergne et al. 2006), especially fatty acid oxidation, showing overlap with PPARα, but also glucose homeostasis (Muoio et al. 2002; Lee et al. 2006). Moreover PPARβ/δ has been reported to exert important roles in differentiation, wound healing, tumorigenesis and modulation of cell proliferation and immune function (Peters et al. 2000; Michalik et al. 2001; Müller-Brüsselbach et al. 2007; Müller, Rieck, et al. 2008; Müller, Kömhoff, et al. 2008; Kilgore & Billin 2008; Peters et al. 2015). Nonetheless these reports are due to in part deviating murine models. Efforts to study PPARβ/δ in the light of pathophysiology and immunology have been perpetuated particularly through the advent of selective ligands (Oliver Jr. et al. 2001; Sznaidman et al. 2003). These ligands permit modulation of PPARβ/δ specific transcription with potential for clarifying its role in biological

systems on one side and investigating the role as potential therapeutic target on the other. In fact the potential for GW501516, a specific PPARβ/δ agonist, in treating dyslipidemia has been evaluated in two phase-two studies.

PPARγ is found mainly in adipose tissue. Through alternative transcription start sites and splicing PPARγ has two distinct isoforms in man: PPARγ2 and the predominantly expressed PPARγ1. PPARγ1 is found in a variety of tissues including immune cells while the PPARγ2 isoform is restricted to adipose tissue (Fajas et al. 1997). PPARγ is eminently important for adipogenesis, shown by the fact that forced expression of PPARγ in fibroblasts leads to terminal adipocyte differentiation (Rosen et al. 1999; Rosen et al. 2000; Tontonoz et al. 1994). On the other hand, mice deficient in PPARγ expression fail to generate adipose tissue, even if fed a high fat diet (Jones et al. 2005). Thiazolidinedione (TZD) was revealed to be a highly specific agonistic ligand for PPARγ causing increased lipid storage into adipocytes (Lehmann et al. 1995). The reduction of free fatty acids in circulation in combination with altered adipose-derived endocrine factors results in reduced systemic insulin resistance, which is favorable for the treatment of diabetes mellitus type 2 (Evans 2004; Rangwala & Lazar 2004). In fact Troglitazone was found to improve insulin resistance and used to treat type 2 diabetes prior to the discovery of its mode of action through PPARγ in 1995 (Fujiwara et al. 1988; Suter et al. 1992; Nolan et al. 1994). Today, however, TZDs prescription to treat insulin resistance is no longer advised due to various side effects. As of 2011, Pioglitazone remains the only approved TZD on the European market, although being associated with increased risk for bladder cancer (Ferwana et al. 2013).

2.1.2 **PPAR structure**

PPARs are members of the nuclear receptor family and are thus composed of the same structurally definable domains all nuclear receptors share (Laudet et al. 1992; Kumar & Thompson 1999). At the N-terminus of the protein, there is a highly various domain that contains an activation function known to be ligand independent (Wärnmark et al. 2003). Secondly, a DNA binding domain containing two zinc-finger motifs is present. This domain binds the hormone response elements specific for each receptor. The third structure module is a flexible hinge, which is followed by the C-terminal ligand binding domain (LBD). This alpha helical domain consists of twelve helices and a four-stranded β-sheet forming not only the binding pocket for the ligand but contributing also to the dimerization and co-factor binding ability (Xu et al. 1999; Zoete et al. 2007; Schwarz et al. 2016). Moreover it harbors the ligand dependent second activation function, most important for full transcriptional activation (Wärnmark et al. 2003).

2.1.3 PPAR transcriptional activity and ligand control

PPAR transcriptional activity is regulated by ligands and depends on obligate heterodimerization with retinoid X receptor (Nolte et al. 1998). These heterodimers bind to PPAR response elements (PPRE) in the promoter region of PPAR target genes. PPAR response elements are composed of direct repeats with the consensus sequence AGGTCA, spaced by one base pair (Leid et al. 1992; Adhikary et al. 2011).
In the absence of ligand, PPARs recruit co-repressor complexes. Upon binding of an agonistic ligand the co-repressor complex is dismissed and co-activator complexes, leading to histone hyperacetylation and subsequent transcriptional activation, are recruited (Guan et al. 2005).

Specific ligands that fit the binding pocket of the LBD influence the transcriptional activity markedly. The amino acid residues comprising the surface of the ligand-binding pocket located in the LBD are 80% conserved between the three subtypes (Zoete et al. 2007). The remaining residues mediate ligand selectivity, although this pocket is by far the largest among nuclear receptors. The reason for this apparent paradox can be explained by the “mouse trap” model proposed as early as 1995 (Renaud et al. 1995). In this model, the binding of a specific ligand results in conformational changes of the LBD. In sum, this leads to a compacted structure where the highly motile helix twelve forms a lid contributing to the binding pocket surface. Consequently, this induced fit enhances the ligand enclosure and creates stable binding sites on the exterior necessary for full activation. Inverse agonists on the other hand, recruit co-repressors resulting in repressed transcriptional activity.

2.1.4 PPARs in the context of immune regulation

All PPAR subtypes have been implicated to play a role in immune regulation (Daynes & Jones 2002). The first report describing this link revealed leukotriene B4 to be a PPARα activating ligand controlling the duration of the inflammatory response (Devchand et al. 1996). Following this first publication, a number of reports found anti-inflammatory properties for PPARα mainly in murine models (Straus & Glass 2007; Bensinger & Tontonoz 2008). Since then, activation of both PPARα and PPARγ have been shown to limit pro-inflammatory cytokine production in T-cells and induce apoptosis in human macrophages (Chinetti et al. 1998; Marx et al. 2002).

PPARγ has since become the best studied subtype in the context of immune regulation. Its part in macrophage and dendritic cell (DC) function has been studied extensively (Nencioni et al. 2002; Szatmari et al. 2006). Activating ligands inhibit DC maturation as well as pro-inflamatory cytokine and chemokine production by these

cells. In combination, these effects lead to reduced antigen presentation and thereby impaired T-cell activation (Klotz et al. 2007). Adding to the complexity mechanisms by which cytokines affect the myeloid cell function via PPARγ have also been reported (Szanto et al. 2010; Schneider et al. 2014).

To date, knowledge about the precise role of PPARβ/δ in immune regulation is scarce. Studies, mostly in murine models, have however emphasized an involvement in inflammation and wound healing (Michalik et al. 2001; Tan et al. 2001; Lee et al. 2003; Welch et al. 2003; Tan et al. 2004). PPARβ/δ was soon linked to a role in macrophage function where it modulates a multitude of inflammatory pathways e.g. activator protein 1 (AP-1), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and signal transducers and activators of transcription (STATs) (Barish et al. 2008; Zingarelli et al. 2010). Other studies showed the potential of PPARβ/δ specific agonistic ligands to dampen T-cell-mediated experimental autoimmune encephalomyelitis by blocking interleukin 17 and interferon γ (IFN-γ) production (Kanakasabai et al. 2010; Dunn et al. 2010). Ablation of PPARβ/δ underlined its importance for development of an alternatively activated macrophage phenotype and led, for instance, to a reduced production of anti-inflammatory cytokines (Gallardo-Soler et al. 2008; Kang et al. 2008; Odegaard et al. 2008; Mukundan et al. 2009). The anti-inflammatory effect of the pan-PPAR agonist punicic acid, for example, was compromised in immune cell-specific *Ppard* knockout mice compared to wild type individuals, as was shown for experimental inflammatory bowel disease (Bassaganya-Riera et al. 2011). Tanaka and colleagues were able to show that such observations, at least for the gut, may also be attributed in part to the direct PPARβ/δ target *CD300a* (Tanaka et al. 2014).

2.2 **Macrophages and their role in immunology**

Macrophages are myeloid cells that have first been described in 1863 (Slavjanski 1866). They were recognized for their phagocytic activity by Élie Metchnikoff (Metchnikoff 1883; Metchnikoff 1887), who, by studying metazoan embryology, realized that phagocytosis is a fundamental function of tissue remodeling and wound healing. In his phagocytosis theory he later also advocated the phagocytes role in pathogen defense (Tauber 2003). Since Metchnikoffs insights at the end of the 19th century, the functions of macrophages have been investigated with great effort identifying, for example, their role in apoptosis (Reddien et al. 2001; Brown et al. 2002) and angiogenesis (Sunderkötter et al. 1994; Diez-Roux & Lang 1997). Nevertheless, macrophages remain best known for their essential role in immunity (Mackaness 1964;

Nathan 2012). Today, macrophages are often described as the gatekeepers of immunity. They patrol most tissues and exert their immunologic functions upon detection of pathogens. These are commonly described by a bipolar activation pattern with pathogen removal and Th1 activation on one side and suppression of inflammation through Th2 function on the other (Mills et al. 2000; Sica & Mantovani 2012). Currently this M1/M2 terminology is being reconsidered due to the diversity of macrophage activation and function (Martinez & Gordon 2014; Murray et al. 2014). This diversity is most likely due to microenvironmental features, e.g. growth factors and cytokines, which in turn can impact strongly on the immune response to the point of suppression or even reversal, as seen in autoimmunity or cancer (Crowther et al. 2001; Muñoz et al. 2010; Jager et al. 2012).

2.2.1 **Macrophages in pathogen defense**

During acute infection, macrophages present the hosts first line of defense. As mentioned above, the tissue resident cells continuously screen their environment with an array of germline-encoded receptors (Taylor et al. 2005). Among these are receptors specific for pathogens called pattern recognition receptors (PRR) directed against common molecules displayed by pathogens, e.g. lipoteichoic acid, lipopolysaccharides (LPS), flagellin or double-stranded RNA. These conserved structures are referred to as pathogen-associated-molecular-patterns (PAMPs) and are used by innate immunity to discriminate self from nonself molecules and subsequently induce effector mechanisms, e.g. iron withdrawal, increased phagocytosis or secretion of cytokines and chemokines (Janeway & Medzhitov 2002; Gordon 2002; Recalcati et al. 2010). Moreover, macrophages may engulf pathogens or internalize their associated molecules to be processed and coupled to antigen presenting complexes. These in turn will be displayed on the surface of the antigen-presenting cell to activate effector cells of adaptive immunity (Sprent & Schaefer 1990; Sprent 1995).

2.2.2 **Macrophages of the tumor microenvironment**

The aforementioned surveillance receptors expressed by macrophages help to maintain the steady state *in vivo*. Changes in the environment are sensed and reactions to any stimuli are modulated in an orchestrated manner to ensure the balance between inflammation and tissue repair, thereby protecting the organism from damage to healthy tissue as well as abnormal growth, possibly leading to cancer (Shi et al. 2001; Shankaran 2001). Consequently, factors tilting this balance may lead to neoplastic growth (Coussens & Werb 2002). Infection alone is estimated to contribute

to every sixth neoplasm worldwide (Anand et al. 2008). It has become very clear over the past decades that the microenvironment contributes strongly to cancerous disease. Immune cells, especially macrophages, are therefore linking immunity and cancer. The special role of macrophages is underlined by their enormous repertoire of secretable factors (Crowther et al. 2001). These include angiogenic factors and chemoattractants that can impact strongly on surrounding cells (Sunderkötter et al. 1994; Schoppmann et al. 2002).

Additionally, macrophages of the tumor microenvironment, often referred to as tumor-associated macrophages (TAMs), are linked closely to disease progression and outcome in various cancers (Bingle et al. 2002; Reinartz et al. 2014). A set of macrophage-secreted factors, e.g. vascular endothelial growth factor (VEGF), matrix-metalloproteinases (MMPs) or epidermal growth factor (EGF) have been shown to directly impact on tumor progression and metastasis (Schoppmann et al. 2002; Pollard 2004; Wyckoff et al. 2004). Yet, this complex interplay of environmental factors as well as autocrine and paracrine relations is difficult to grasp, particularly if the progression dependent nature of the TAM phenotype is taken into account. As outlined in a 2010 review, TAMs implement various pro-tumorigenic traits at different stages of cancer development ranging from initiation to invasion and metastasis (Qian & Pollard 2010). TAMs may therefore be a promising therapeutic target in the struggle against cancer. Thus, great effort has been put into the exploration of a possible re-education of TAMs and the tumor microenvironment in order to obtain an anti-tumor phenotype in the last years (Hagemann et al. 2008; Topalian et al. 2012; Pyonteck et al. 2013; Jiang et al. 2015). Ovarian cancer itself presents as an exceptional model for studies on tumor associated macrophages due to the fact of being frequently accompanied with peritoneal ascites. This malignant fluid accumulation often harbors large quantities of tumor and tumor-associated cells while at the same time giving insight into the actual microenvironment of these cells.

2.3 **Purpose and significance of this study**

The apparent interplay between lipid molecules, PPARβ/δ target genes, and immune regulation leads to the idea of this study. There are no sufficient studies addressing the role of PPARβ/δ in human macrophages despite the fact that the importance of this transcription factor in the context of immune regulation has been implied by murine studies (Lee et al. 2003; Kang et al. 2008; Odegaard et al. 2008). To obtain data that resembles the *in vivo* situation in humans appropriate genome wide approaches in

primary cells are necessary. This data will also establish reliable grounds for future investigation.

In the light of disease, the influence of lipid sensors and their impact on immune regulation is also of particular interest. Since very little is known about the influence of lipids on macrophage function, there is desperate need to address this issue.

This study is designed to investigate PPARβ/δs precise role in human macrophages identifying the regulated transcriptome in these cells. Thereby new insight into the functional implications will be obtained. Additional experiments in tumor-associated macrophages will also demonstrate whether PPARβ/δ takes part in their specific phenotype.

3 Results

3.1 The transcriptional PPARβ/δ network in human macrophages defines a unique agonist-induced activation state

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To investigate the PPARβ/δ ligand regulated transcriptome in primary human macrophages, peripheral blood mononuclear cells were isolated from healthy volunteering adults by density gradient centrifugation followed by positive selection of adherent cells. The obtained cluster of differentiation (CD) 14 positive cells were differentiated to monocyte-derived macrophages (MDM) in RPMI1640 medium supplemented with 10% fetal calf serum (FCS). Surface staining for macrophage markers (CD32, CD64, CD86, CD206, HLA-DR) and intracellular CD68 analysis by flow cytometry on day three and five revealed the presence of macrophage markers (Figure S3).

The transcript level of *PPARD* mRNA was assessed using reverse transcription quantitative polymerase chain reaction (RT-qPCR) at different time points during differentiation. A transcript level increase over time peaking around day five was seen (Figure 1A). The PPARβ/δ protein level, as assessed by western blot, was similarly rising over time showing the strongest signal around day six (Figure 1B, Figure S1). To verify functionality PPARβ/δ specific ligands were added to the cultured cells at different points. After one-day incubation the established target genes *PDK4* transcript level was measured by RT-qPCR. The strongest ligand-inducibility compared to solvent was detected at day six post isolation (Figure 1C). Additionally chromatin immunoprecipitation (ChIP) showed localized enrichment of PPARβ/δ and RXR at the PDK4 enhancer. Re-ChIP experiments verified PPARβ/δ and RXR complex formation at the PDK4 enhancer region (Figure S2). Taken together, day six MDMs appear as very suitable model to explore the effects of PPARβ/δ ligands on macrophage differentiation and activation.

Subsequent genome wide analysis using RNA- and ChIP- sequencing identified ligand regulated transcripts and PPARβ/δ as well as RXR enriched chromatin sites. In total 285 protein-coding putative direct target genes were upregulated by L165,041, a

PPARβ/δ specific agonist, while 388 were repressed. The inverse PPARβ/δ agonist treatment resulted in 246 downregulated genes and 174 genes with increased transcript frequency (Figure 2A and B, Table S2). A large number of genes showed inverse regulation by the ligands as opposed to the canonical concept. The increased transcript level of genes by inverse agonist and *vice versa* gave rise to the idea that an effect unrelated to PPARβ/δ might be responsible. However, data of bone marrow-derived macrophages from *Ppard* null mice in comparison to wild type rejected this theory (Figure 2F). These genes were from here on classified as inverse target genes.

ChIP-sequencing revealed 3798 and 32720 genes within 50 kb of enrichment sites for PPARβ/δ and RXR respectively (Figure 3A). 3502 genes were co-occupied and 66,4% of these showed enrichment specifically at transcription start sites, within introns or upstream locations (Figure 3C). Of the 285 L165,041 induced genes 132 showed PPARβ/δ binding, the rest showed RXR binding. Among the solely RXR occupied genes we found angiopoietin-like 4 (*ANGPTL4*) a previously described canonical target gene (Mandard et al. 2004). Genes showing RXR binding in combination with L165,041-induction are therefore regarded to be canonical PPARβ/δ targets.

The highest correlation of canonical target genes was found with lipid metabolism using Ingenuity Pathway Analysis (IPA) disease and function annotation (Figure 3E). Interestingly, other target genes were linked to cell motility or negatively correlated with systemic autoimmune disease. The aforementioned inverse PPARβ/δ targets, as analyzed by IPA upstream regulator analysis, were predominantly connected to cytokine signaling (Figure 4C). Subsequent functional annotation confirmed the link between PPARβ/δ in primary human macrophages and immune regulation (Figure 4F). Not surprisingly several immune regulatory genes were found among the inverse target genes, including chemokines, cytokines and members of the CD1 family (Table 1).

To test for PPARβ/δ participation in immune regulation morphological changes in PPARβ/δ ligand treated MDMs were addressed. MDMs treated with respective ligand for six days during differentiation were Giemsa stained and analyzed. MDMs cultured in the presence of LPS or interleukin 4 (IL-4), initiating M1 and M2 polarization respectively, were used to judge similarity. Resemblance between L165,041 and IL-4 treated MDMs was just as clear as between PT-S264, an inverse agonist, and LPS (Figure 6A-E, Figure S5A-F). A FITC-dextran uptake assay emphasized that the morphological resemblance between agonist and IL-4 treatment also correlated with an accompanying functional reduction in phagocytic/macropinocytotic activity (Figure 6F).

The reduction in the uptake as a result of agonist treatment was paralleled by a corresponding rise in T cell activation (Figure 7A). MDMs pretreated with CEFT-peptide in combination with L165,041 or solvent were co-cultured with autologous T cells. Their ability to present antigen and activate T cells, was assessed by flow cytometry, measuring the percentage of IFN-γ^+, $CD8^+$ cells.

Evidence that PPARβ/δ agonists impact on macrophage phenotype was also given by experiments focusing on the protein products of inverse target genes *CD274*, *CD32* and *IDO1* (Figure 7C-H). All of these were readily reduced by agonist treatment including the supernatant concentration of the IDO1 enzyme product kynurenine.

The functional annotation of L165,041 regulated genes predicted a positive effect on cell death of immune cells. We tested this in light of hypoxia as it resembles a stress situation commonly encountered by macrophages (Lewis et al. 1999). As figure 7I and supplementary figure 8 clearly show, PPARβ/δ agonists improve the viability while inverse agonists sensitize MDMs to hypoxic stress.

At last we compared the datasets from MDMs treated with PPARβ/δ ligands to datasets of a human breast cancer (MDA-MB-231) and a myofibroblastic cell line (WPMY-1). The results point to cell type-specific functions of PPARβ/δ. While a small fraction of target genes with PPARβ/δ peaks are mutual (n=129) and related to energy homeostasis or lipid metabolism, none of the inverse target genes are shared by all three datasets.

Author contribution to this publication: Development of methodology and acquisition of experimental data Figure 1A-C, Figure 2A-B, Figure 3A, Figure 4A, Figure 6, Figure 7B-C, I, Figure 9A-B, Table 1, Figure S1, Figure S4, Figure S5, Figure S7, Figure S8, Table S2.
Collaborating groups at Center for Tumor Biology and Immunology: PPARβ/δ selective inverse agonists were synthesized and generously provided by Philipp M. Toth and Wibke E. Diederich, Medicinal Chemistry Core Facility and Institute of Pharmaceutical Chemistry. RNA and ChIP sequencing were performed by Andrea Nist and Thorsten Stiewe, Genomics Core Facility. Kathrin Roth at Cellular Imaging Core Facility performed time-lapse video microscopy. FACS phenotyping and T cell activation assays were performed by Lara Kleinesudeik and Silke Reinartz, Clinic for Gynecology, Gynecological Oncology and Gynecological Endocrinology.

3.2 Deregulation of PPARβ/δ target genes in tumor-associated macrophages by fatty acid ligands in the ovarian cancer microenvironment

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In order to learn more about the influence of PPARβ/δ in disease, focus was set on studying its role in TAMs of serous ovarian carcinoma (Schumann et al. 2015). These cells were isolated from malignant ascites by positive selection of $CD14^+$ cells by means of magnetic cell sorting or adherent selection. TAMs kept in autologous ascites showed high transcript level of *CD163* and low levels of *MMP9* as compared to MDM cultures, which is consistent with their phenotype *in vivo* as we have described earlier (Reinartz et al. 2014) (Figure 1A). To address ligand responsiveness, TAMs and MDMs were treated with L165,041 or solvent for eight days in autologous ascites or cell culture medium respectively. As illustrated by figure 1B-E the morphological changes in response to PPARβ/δ agonist treatment was absent in TAMs while MDMs reacted as expected, and TAMs were non-responsive to exogenous agonist (Adhikary et al. 2015). To elucidate whether the TAMs' lack in responsiveness is due to a deficiency in PPARβ/δ binding to chromatin, ChIP experiments were done. As presented for the *PDK4* enhancer region monocytes, MDMs and TAMs collectively showed enrichment of RXR and PPARβ/δ, with approximately equal enrichment factors between MDMs and TAMs (Figure 2A). Subsequent comparative transcriptome analysis of TAMs and MDMs cultured in the presence of agonistic or inverse-agonistic ligand revealed that less than one third of agonist induced targets in MDMs was also upregulated in TAMs (Figure 2B). On the other hand, the number of inverse-agonist repressed genes in TAMs (50) was substantially larger compared to MDMs (18), hinting at the presence of agonistic ligands in TAMs.

Validation of these findings by RT-qPCR for *PDK4* and *ANGPTL4* using MDMs and TAMs cultured in ascites or culture medium, correlated with the transcriptome data. Moreover culture conditions only had minor effect on TAM target gene induction, questioning the immediate influence of ascites (Figure 2D). Comparing the prior defined target genes (Table S3) in freshly isolated TAMs from 10 different patients and MDMs, using the obtained transcriptome data, demonstrated that a large fraction of

targets (54) had increased transcript levels in TAMs (Figure 3A) including about half of the genes upregulated in TAMs *in vitro.* Most of these genes not only showed higher expression in TAMs but also showed impaired induction by agonist (Figure 3C, Table 1). These observations were confirmed by RT-qPCR analysis of three exemplary target genes in 12 MDM and TAM probes respectively (Figure 3D). Likewise PDK4 protein level in MDMs and TAMs cultured with and without L165,041, as illustrated by western blot, further supports the idea of deregulated and agonist insensitive PPARβ/δ target genes in TAMs (Figure 3E). In the case of ANGPTL4 this deregulation has direct implications for disease prognosis, as its secreted product is readily detectable in ovarian cancer patients' ascites (Figure 3F). Moreover a strong negative correlation of the soluble ANGPTL4 level with relapse-free survival can be determined for serous ovarian cancer (Figure 3G).

As a cause of the deregulation of PPARβ/δ targets, soluble agonists present in TAMs can be proposed. Culture of MDMs in the presence of malignancy-associated ascites followed by analysis of change in target gene transcription resulted in increased levels equivalent to L165,041 in some cases (Figure 5A). In the presence of ascites the agonist effect of L165,041 is strongly diminished (Figure 5B). This effect is clearly PPARβ/δ dependent as experiments with *Ppard* null mice compared to wild type animals show (Figure 5D). Here the induction of *Pdk4* and *Angptl4* by ascites is only present in wild type mice, which also show impaired agonist induction in the presence of ascites. The PPRE-dependence of the ascites-mediated target gene induction can also be seen in figure 5C. The three PPREs present in the enhancer region of PPARβ/δ target *PDK4* were progressively mutated showing increasing reduction in ascites-mediated induction of transcript levels (Adhikary et al. 2015).

In summation, these data strongly suggest the presence of endogenous agonistic ligands in the malignancy-associated ascites. Lipidomic analysis of 38 ascites samples by liquid chromatography–mass spectrometry/mass spectrometry (LC-MS/MS) revealed high concentrations of polyunsaturated fatty acids (PUFAs) previously described to bind PPARβ/δ (Figure 6A, Table S6). Most prominent were linoleic acid (LA) with extremely high average concentrations (180μM), arachidonic acid (AA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Evidently addition of LA, AA and DHA at a concentration of 20μM to the culture of MDMs resulted in upregulation of *PDK4* (Figure 6B). As Figure 6C illustrates *PDK4* transcript level rises dose-dependently upon LA concentration. The same holds true for the LA derivatives (9)Z, (11)E LA and (10)E, (12)Z LA. Compared to L165,041, LA exhibited comparable upregulation of PPARβ/δ targets in MDMs after 24h (Figure 6D).

Interestingly dose-dependent repression of PPARβ/δ targets by synthetic inverse-agonists was still possible in MDMs cultured in ascites. As figure 6E shows the remedial influence of the inverse agonist PTS264 is especially strong for *LRP5* and *ANGPTL4* transcripts since a repression down to basal levels, compared to RPMI1640 culture medium, can be achieved at concentrations around 2 μM (Figure 6E).

Intriguingly, TAMs seem to stagnate in a deregulated state even if cultured in absence of ascites (Figure 2D). Fluorescent Nile Red staining of freshly isolated as well as four day cultured TAMs in presence and absence of serum however elucidated the cause for this apparent discrepancy. Freshly isolated TAMs were stained brightly red, displaying large quantities of intracellular lipid droplets which remained almost unchanged even if the cells were serum starved for four days (Figure 7A and B). The presence of lipid droplets was associated with impaired target gene upregulation by L165,041 (Figure 7C). MDMs cultured in the presence of LA, at concentrations resembling those of the ascites, readily accumulated lipid droplets (Figure 7D and E). These also remained stable over a period of four days which rendered PPARβ/δ target genes refractory to agonist-treatment despite serum starvation (Figure 7F).

Author contribution to this publication: Development of methodology and acquisition of experimental data Figure 1, Figure 2B-E, Figure 3A-E, Figure 5A-B, Figure 6A-D, Figure 7, Table 1, Figure S3, Table S1, Table S3, Table S5, Table S6.
Collaborating groups at Center for Tumor Biology and Immunology: PPARβ/δ selective inverse agonists were synthesized and kindly provided by Philipp M. Toth and Wibke E. Diederich, Medicinal Chemistry Core Facility and Institute of Pharmaceutical Chemistry. Andrea Nist and Thorsten Stiewe, Genomics Core Facility, performed RNA and ChiP sequencing. Patient samples were acquired by Uwe Wagner and Silke Reinartz, Clinic for Gynecology, Gynecological Oncology and Gynecological Endocrinology.
Collaborating groups at Philipps University Marburg: Lipidomic analysis was performed at the Metabolomics Core Facility and Institute of Laboratory Medicine and Pathobiochemistry by Yvonne Schober and W. Andreas Nockher.

4 Discussion

4.1 The role of PPARβ/δ in human primary macrophages

Despite various reports hinting at a role for PPARβ/δ in the context of immune regulation especially in macrophages no study has focused on this interplay. This report underlines the important role of this transcription factor in human macrophage function.

The substantial increase in functional PPARβ/δ protein highlighting the significance of this factor in macrophage biology. In combination with the transcriptomic analyses performed, the insight into the complexity of PPARβ/δ transcriptional regulation in the context of human primary macrophages has been improved significantly. Additional to the prior known canonical target genes a new subgroup of regulated genes was identified that has apparent implications in immune regulation. Intriguingly, these targets were regulated solely in macrophages by agonistic ligands as shown by comparison with two distinct human cell lines. Further, a deviating concept of target gene regulation by PPARβ/δ emerged from the collected data. These inverse target genes are modulated by a different mechanism but also modulate in a cell type selective fashion. In case of primary macrophages mostly immune regulatory genes were modulated e.g. *ARG2, BCL3, CCL24, CD1A, IDO1, IL10*, *PD-L1*and *TNF*.

The influence of PPARβ/δ selective ligands, as illustrated by the morphological changes in differentiating macrophages, point to a fundamental link between PPARβ/δ ligands and macrophage phenotype. In the light of biological relevance, functional consequences were addressed in this study and the results clearly underline the proposed connection. Special influence in T cell activation can be inferred from the data presented namely by the involvement of PD-L1, an inhibitory protein that engages the PD-1 receptor present on T cells, consequentially leading to impaired T cell activation (Freeman et al. 2000; Francisco et al. 2010). Likewise, IDO1 by means of kynurenine production in combination with L-tryptophan consumption is a potent inhibitor of the T cell response causing functional anergy in $CD8^+$ T cells (Munn & Mellor 2013). Experimental data from macrophages treated with PPARβ/δ agonists concur as the result was an increase in $CD8^+$ T cell activity.

Furthermore, agonist treatment also increased the resilience of macrophages against hypoxic stress, which is often an accompanying factor of infection, tumor or wounds (Lewis et al. 1999). The significance of these implications *in vivo,* however, remains uncertain until further investigation. Nonetheless, these data validate the

paramount role for PPARβ/δ in macrophages and strongly underlines its contribution to immune modulation.

4.2 PPARβ/δ in the light of disease

In order to acquire further insight into the newly obtained role for PPARβ/δs impact on macrophage function in the light of disease, serous ovarian carcinoma was chosen as a model. Progression of this gynecological cancer is frequently related to the development of malignancy-associated ascites. This fluid accumulation often harbors vast amounts of tumor and tumor-associated cells and allows investigation of the tumor microenvironment.

TAMs obtained from patients' ascites showed the expected and previously described phenotype (Reinartz et al. 2014). Despite the presence of functional PPARβ/δ, these cells were non-responsive to ligands with respect to morphologic changes. Subsequent transcriptome analysis and further experiments revealed obvious differences between MDMs and TAMs. We established that a strong influence was caused by the microenvironment and went on to focus on ascites composition with special respect to ascites-borne PPARβ/δ ligands. Since PPARβ/δ is a known lipid sensor and activation leads to intracellular lipid accumulation (Vosper et al. 2001), binding fatty acids and derivatives thereof, lipidomic analyses were undertaken. The results of 38 ascites samples for 97 compounds confirmed the theory of ascites harboring substantial amounts of PPARβ/δ ligands. First and foremost polyunsaturated fatty acids stood out with extremely high concentrations, the highest of which was measured for linoleic acid, averaging at ~180 µM. Secondly, arachidonic acid and docosahexaenoic acid at average concentrations around 30 µ M were found. Noticeably, these concentrations far exceeded the determined range for sufficient PPARβ/δ binding (Xu et al. 1999). Moreover, these PUFAs were able to induce PPARβ/δ target genes in MDM cultures readily at a concentration of 20 µM . Most intriguing, LA in several cases reached the activation level achieved by synthetic ligand. Eicosapentaenoic acid and α-linolenic acid (ALA), which also have the propensity to bind PPARβ/δ as possible ligands had no significant effect on the transcript level of PPARβ/δ target *PDK4*, although the concentration used exceeded the detected level of EPA in ascites (ALA was not detected). The potential as agonistic ligand appears to be shared among the common ω-6 FA but does not extend to the ω-3 fraction of dietary FAs.

When examining *ex vivo* TAMs large accumulation of lipid droplets emerged, thus providing an intracellular reservoir of accumulated lipids (Guijas et al. 2012). The

data of serum starved TAM and MDM cultures suggest this intracellular pool may provide previously stored endogenous ligands possibly for quite long periods, as a four day culture period only had minimal effects on the lipid stores and deregulated target gene levels.

Interestingly, synthetic inverse agonists, such as PT-S264, were able to abrogate the effects seen with the ω-6 PUFAs in a dose-dependent manner. These ligands were able to restore the basal state of MDMs even in the presence of ascites. Inferring from these data, inverse agonist may be used to alleviate the effects of PUFAs on macrophages. This has exceptional implications as our previous study and functional annotations of PPARβ/δ targets showed their involvement in cell survival, cell migration and inflammation (Adhikary et al. 2015). The tumor microenvironment may therefore at least in part mitigate the phenotype observed for TAMs, which is associated with poor prognosis, exemplified in this study by the PPARβ/δ target *ANGPTL4*.

To date, there are numerous reports stating the negative implications of *de novo* fatty acid synthesis in cancer (Kuhajda 2006). In the center of these reports is the fatty acid synthase, which is overexpressed in a variety of cancers. This enzyme allows mammals to synthesize FAs from acetyl- and malonyl-CoA, which in cancer is correlated with aggressive tumors, enhanced growth and survival. Consequently, this pathway is being pharmacologically targeted to improve outcome in cancer. Scarce effort has been put into elucidating the role of dietary fatty acids in this context.

Several studies have earlier associated fatty acids with the clinical outcome of ovarian cancer (Tania et al. 2010). In the background of diet, it has been postulated that high levels of LA are correlated with higher membrane fluidity, enhanced cell motility and increased metastasis (Quinn 1983). A study has also shown that in ovarian cancer patients the percentage of LA in peripheral adipose tissue and omentum is decreased as result of FA mobilization from these sources (Yam et al. 1997). In combination with a report of controlled experiments performed on mice, advocating the negative influence of ω-6 FAs in a prostate cancer model, the implications are devastating (Berquin et al. 2007). Validation of this theory has already been proposed by an animal study demonstrating the negative influence of direct lipid transfer from adipocytes of the omentum to tumor cells (Nieman et al. 2011). As a result ovarian cancer metastasis and tumor growth were increased.

This report clearly connects the tumor promoting phenotype of TAMs to the lipid composition of the tumor microenvironment, with special emphasis on PUFA level and composition. Although the presented data were obtained solely from ovarian cancer

patients, implications reach beyond this model, highlighting the importance of the fatty acid sensor PPARβ/δ not only in macrophage regulation but also in tumor biology. Yet, this work is only the basis for urgently needed further investigation elucidating the relation of lipid sensors and immune regulation in detail.

Along this direction, our current investigations are addressing the molecular basis for FAs impacting on signaling pathways in human macrophages. Intriguingly we have observed that inverse regulation by PPARβ/δ affects to a large part STAT1 and NF-κB target genes. This places FAs at a central point in inflammation control and thereby cancer therapy. We hope to acquire detailed information on the interplay of PUFAs and macrophage function, hopefully leading to a better understanding and possibly an intervening strategy for cancer therapy.

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5.1 **Verzeichnis akademischer Lehrer**

Meine akademischen Lehrer waren die Damen und Herren in Würzburg:
Ache, Beier, Benavente, Berberich, Bodem, Buchberger, Dabauvalle, Dandekar, Dyakonov, Fischer, Floren, Geissler, Gross, Heidrich, Heisenberg, Herrmann, Hünig, Jakob, Kerkau, Kirschner, Kreft, Krone, Linsenmair, Lutz, Marten, Müller, Nagel, Pradel, Rdest, Riederer, Rössler, Rudel, Schulz, Schäfer, Tacke, Wolf.