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Regulation of peroxisome proliferator-activated receptor γ in macrophages during inflammatory processes

Dissertation

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„Man merkt nie, was schon getan wurde;

man sieht immer nur das,

was noch zu tun bleibt.“

Marie Curie

Index

List of figures

List of tables

Abbreviations

$(MgCO_3)_4$ x $Mg(OH)_2$ x 5 H_2O	Magnesium carbonate hydroxide
A	Alanine
aa	amino acids
AA	Arachidonic acid
AC	Apoptotic cells
ACAMP	Apoptotic cell-associated molecular pattern
AF	Activation function
Ago	Argonaute protein
ANOVA	Analysis of variance
AP-1	Activating protein-1
APS	Ammonium persulfate
ARE	AU-rich element
ATP	Adenosine triphosphate
AUF1	AU-binding factor 1
Brf	B-related factor
BSA	Bovine serum albumin
Ca^{2+}	Calcium
CAF	CCR4-associated factor
CBP	cAMP response element binding protein
CCR4	Carbon catabolite repressor factor 4
CD	Cluster of differentiation
CHX	Cycloheximide
CLP	Cecal ligation and puncture
COX	Cyclooxygenase
CrkII	CT10 regulator of kinase II
D	Aspartate
d/n	dominant/negative
DAG	Diacylglycerol
DBD	DNA binding domain
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide
DRB	5–6 dichloro-1-β-ribofuranosylbenzimidazole
DTT	Dithiothreitol
E	Glutamaic acid
EAE	Experimental allergic encephalomyelitis
EDTA	Ethylene diamine tetra acetate
EGFP	Enhanced green fluorescent protein
EGTA	Ethylene glycol tetra acetate
ELMO1	Engulfment and cell motility 1
EMAP II	Endothelial-monocyte-activating polypeptide II
EMSA	Electrophoretic mobility shift assay
Erk	Extracellular signal-regulated kinase

Ets-1	erythroblastosis virus E26 oncogene homolog 1
FCS	Fetal calf serum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GM-CSF	Granulocyte macrophage-colony stimulating factor
15-HETE	15-hydroxyeicosatetraenoic acid
9- or 15-HODE	9- or 15-hydroxy-octadecadienoic acid
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HO-1	Heme oxygenase-1
HuR	Human antigen R
IFN	Interferon
IκB	Inhibitor of κB
IKK	IκB kinase
IL	Interleukin
iNOS	Inducible NO-Synthase
IRAK	IL-1 receptor associated kinase
Jnk	Jun N-terminal kinase
K	Lysine
κB-RE	κB-response element
KCl	Potassium chloride
kD	Kilodalton
KH_2PO_4	Potassium hydrogen phosphate
$KHCO_3$	Potassium hydrogen carbonate
KSRP	KH-type splicing regulatory protein
L	Leucine
LBD	Ligand-binding domain
LDL	Low density lipoprotein
LMB	Leptomycin B
LPC	Lysophosphatidylcholine
LPS	Lipopolysaccharide
LXR	Liver X receptor
MAPK	Mitogen-activated protein kinases
M-CSF	Macrophage colony-stimulating factor
MD2	myeloid differentiation 2
MFG-E8	Milk-fat globule epidermal growth factor 8
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulfate
$MgSO_4$ x 7 H_2O	Magnesium sulfate 7-hydrate
miRNA	micro ribonucleic acid
MMP	Matrix metalloproteinase
MR	Mannose receptor
mRNA	messenger ribonucleic acid
MS	Multiple sclerosis
Mt	Mutant

mTOR	mammalian target of rapamycin
Na_2HPO_4	Sodium hydrogen phosphate
NaCl	Sodium chloride
NaF	Sodium fluoride
NCoR	Nuclear receptor co-repressor
NFAT	Nuclear factor of activated T cells
NFκB	Nuclear factor κB
NH_4Cl	Ammonium chloride
NO	Nitric oxide
NOT	negative on-TATA-less
oxLDL	oxidized low-density lipoprotein
PAF	Platelet-activating factor
PAMP	Pathogen-associated molecular pattern
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PG	Prostaglandin
PI3K	Phosphatidylinositol-3 kinase
PIAS1	Protein inhibitor of activated STAT1
PK	Protein kinase
PMSF	Phenylmethylsulphonylfluoride
PPAR	Peroxisome proliferator-activated receptor
PS	Phosphatidylserine
qPCR	quantitative polymerase chain reaction
R	Arginine
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	rounds per minute
RPMI	Roswell park memorial institute
RT	Room temperature
RXR	Retinoid X receptor
S	Serine
S1P	Sphingosine-1-phosphate
SDS	Sodium dodecyl sulfate
SE	Standard error
SMRT	Silencing mediator of retinoid and thyroid hormone receptors
SphK	Sphingosine kinase
SR A	Scavenger receptor A
Src-1	Steroid receptor co-activator-1
STAT	Signal transducer and activator of transcription
SUMO	small-ubiquitin related modifier
TAB	TAK1-binding protein
TAK	TGFβ-activated kinase
TAM	Tumor-associated macrophages

TBL1	Transducin-beta like protein 1
TBLR-1	Transducin-beta like protein 1-related protein
TE	Trypsin-EDTA
TEMED	Tetraethylendiamine
TGF	Transforming growth factor
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TPA	12-Tetradecanoyl-phorbol-13-acetate
TRAF	TNF-receptor-associated factor
TSA	Trichostatin A
TTP	Tristetraprolin
TZD	Thiazolidinedione
UC	Ulcerative colitis
UTR	Untranslated region
V	Volt
VEGF	Vascular endothelial growth factor
VnR	Vitronectin receptor
WB	Western blot analysis

1 Summary

The peroxisome proliferator activated receptor γ (PPARγ) plays an eminent role during alternative activation of macrophages and resolution of inflammation. As an anti-inflammatory signaling molecule, it seems likely that it is tightly regulated dependent on the state of the immune response. There is growing evidence that PPARγ expression is reduced during inflammation, whereas molecular mechanisms are ill-defined. Even though, its role in immunosuppression is getting more definite. Apoptotic cells (AC) provoke an active repression of pro-inflammatory responses *inter alia* by the inhibition of pro-inflammatory cytokine expression or attenuated generation of reactive oxygen species (ROS). The reduced formation of ROS was attributed to PPARγ activation, while mechanisms behind the reduced cytokine expression remained unclear. Therefore, my Ph.D. thesis addressed the role of PPARγ during inhibited cytokine synthesis in response to AC and the regulation of PPARγ expression during an inflammatory response, which was initiated by lipopolysaccharide (LPS) exposure.

In the first part of the thesis, I investigated the role of PPARγ in coordinating the attenuation of pro-inflammatory cytokine expression in response to AC. Exposing murine RAW264.7 macrophages to AC prior to LPS-stimulation, reduced NFκB transactivation and lowered target gene expression of e.g. TNFα and IL-6 compared to controls. In macrophages over-expressing a dominant negative (d/n) mutant of PPARγ, NFκB transactivation in response to LPS was restored, while using macrophages from myeloid lineage-specific conditional PPARγ knock-out mice proved that PPARγ transmitted the anti-inflammatory response delivered by AC. Domain analysis revealed that amino acids 32-250 are essential for inhibition of NFκB. Mutation of a SUMOylation (SUMO: small-ubiquitin related modifier) site in this region (K77R) and interfering SUMOylation by silencing the SUMO E3 ligase PIAS1 (protein inhibitor of activated Stat1) eliminated AC-provoked NFκB inhibition and concomitant TNFα expression. Chromatin-immunoprecipitation assays demonstrated that AC prevented the LPS-induced removal of nuclear receptor co-repressor (NCoR) from the κB response element within the TNFα promoter. I concluded that AC induce PPARγ SUMOylation to attenuate the removal of NCoR, thereby blocking transactivation of

NFκB. This contributes to an anti-inflammatory phenotype shift in macrophages in response to AC, by lowering pro-inflammatory cytokine production.

The second part addressed molecular mechanisms responsible for reduced PPARγ expression upon LPS exposure. PPARγ gained considerable interest as a therapeutic target during chronic inflammatory diseases. Remarkably, the pathogenesis of diseases such as multiple sclerosis or Alzheimer's disease is associated with impaired PPARγ expression. Initiation of an inflammatory response by exposing primary human macrophages to LPS revealed a rapid decline of PPARγ1 expression. PPARγ1 mRNA decrease was prevented by inhibition of NFκB and also after pre-treatment with the PPARγ agonist rosiglitazone, suggesting a NFκB-dependent pathway, because activated PPARγ is known to inhibit NFκB transactivation. Since promoter activities were not affected by LPS, I focused on mRNA stability and noticed a decreased PPARγ1 mRNA half-life. RNA stability is often regulated via 3' untranslated regions (UTRs). Therefore, I analyzed the impact of the PPARγ-3'UTR by luciferase assays. LPS significantly reduced luciferase activity of pGL3-PPARγ-3'UTR, suggesting that PPARγ1 mRNA is destabilized. Deletion of a potential miR-27a/b binding site within the 3'UTR completely restored luciferase activity. Moreover, inhibition of miR-27b, which was induced upon LPS-exposure, partially reversed PPARγ1 mRNA decay, whereas the mature miR-27 mimicked the effect of LPS. MiR-27b was at least partially induced by NFκB, thus correlating with NFκB-dependent PPARγ1 mRNA decrease. Since deletion of the miR-27 site also containing an AU-rich element (ARE) completely abrogated LPS-induced reduction but inhibition of miR-27b only partially restored PPARγ1 mRNA expression, I suggested an additional implication of an ARE-binding protein.

I provide evidence that LPS induces miR-27b, which in turn destabilizes PPARγ1 mRNA. Understanding the molecular mechanism of PPARγ mRNA destabilization, might help to rationalize inflammatory diseases associated with impaired PPARγ expression. Even though, further experiments are needed to clarify the potential involvement of ARE-binding proteins.

2 Zusammenfassung

Chronische Entzündungskrankheiten entstehen häufig in Folge einer unkontrollierten Entzündungsreaktion und damit verbundenen irreversiblen Schäden des umliegenden Gewebes. Die Ausbildung eines anti-inflammatorischen Makrophagen-Phänotyps ist ein wichtiger Bestandteil zur Beendigung von Entzündungen. Charakteristisch für diesen Phänotyp ist eine verminderte Synthese pro-inflammatorischer Zytokine, welche teilweise auf die Aktivierung des Transkriptionsfaktors PPARγ (‚peroxisome proliferator activated receptor γ') zurückzuführen ist. Daher ist die Regulation der Aktivierung als auch der Expression von PPARγ entscheidend für die Immunantwort von Makrophagen.

Es konnte bereits gezeigt werden, dass durch die Phagozytose apoptotischer Zellen (AZ) zum einen PPARγ aktiviert und zum anderen die Zytokinexpression durch eine Hemmung von NFκB (‚nuclear factor κB') vermindert wird. Daher untersuchte ich im ersten Teil meiner Arbeit die Rolle von PPARγ bei der Inhibition von NFκB nach Interaktion mit AZ. Die Stimulation von RAW264.7-Makrophagen mit AZ führte zu einer Hemmung der NFκB-Aktivität, welche durch Überexpression einer dominant-negativen Mutante von PPARγ reduziert war. Weiterhin konnte in primären PPARγ-knock-out Makrophagen keine Hemmung der TNFα-Expression, als klassisches NFκB-Zielgen, festgestellt werden. Analysen der PPARγ-Protein Domänen zeigten, dass die Aminosäuren 32-250 essentiell für die NFκB-Inhibition sind. Mutation der in diesem Bereich liegenden SUMOylierungsstelle K77 (SUMO: „small-ubiquitin related modifier") als auch das Ausschalten der essentiellen SUMO-E3-Ligase PIAS1 („protein inhibitor of activated Stat1") verhinderte die Hemmung von NFκB und bestätigte die SUMOylierung von PPARγ als zugrunde liegenden Mechanismus. Als verantwortlichen Repressor identifizierte ich NCoR („nuclear receptor co-repressor"), welcher im Ruhezustand konstitutiv an NFκB-Bindestellen verschiedener pro-inflammatorischer Promotoren gebunden ist. Nach TLR4-Aktivierung dissoziiert dieser von der Promotorregion und wird abgebaut. Durch Chromatin-Immunpräzipitationen konnte ich zeigen, dass vermutlich SUMOyliertes PPARγ nach Interaktion mit AZ die Dissoziation von NCoR und damit die Zielgen-Expression verhindert. Die Aufklärung dieses Mechanismus trägt damit zum weiteren Verständnis bei, wie AZ einen anti-

inflammatorischen Makrophagen-Phänotyp hervorrufen und damit zur Eindämmung einer Entzündungsreaktion beitragen.

Bei verschiedenen Entzündungskrankheiten wie Alzheimer oder auch Multipler Sklerose konnte eine Verringerung der PPARγ-Expression nachgewiesen werden. Da der Mechanismus dieser Reduktion jedoch weitgehend unbekannt ist, beschäftigte ich mich im zweiten Teil meiner Arbeit mit der Expressionsregulation von PPARγ in Makrophagen. Die Stimulation von primären humanen Makrophagen mit LPS verringerte den PPARγ1 mRNA-Gehalt. Diese mRNA-Reduktion konnte durch Hemmung von NFκB als auch durch Vorstimulation mit dem PPARγ-Agonisten Rosiglitazone verhindert werden, was auf einen NFκB-abhängigen Mechanismus hinwies. Durch Promotor-Reporteranalysen konnte eine Reduktion der PPARγ1 mRNA auf transkriptioneller Ebene ausgeschlossen werden. LPS führte vielmehr zu einer 3‘-UTR (‚untranslated region‘)-abhängigen Destabilisierung der PPARγ1 mRNA. Aufgrund einer potentiellen Bindestelle für microRNA-27a/b (miR-27a/b), untersuchte ich deren Expression. LPS führte - zum Teil NFκB abhängig - zur Induktion von miR-27a und b. Eine Depletion der miR-27 Bindestelle innerhalb der PPARγ-3’UTR verhinderte vollständig den destabilisierenden Effekt von LPS. Weiterhin führte die Inhibition von miR-27b, nicht aber von miR-27a, zur teilweisen Aufhebung der LPS-induzierten Reduktion. Die Destabilisierung von PPARγ konnte außerdem durch Transfektion mit miR-27b simuliert werden, wobei die additive Zugabe von LPS den Effekt nur wenig verstärkte.

Meine Daten beweisen, dass LPS-induzierte miR-27b zur Destabilisierung der PPARγ1 mRNA führt. Die Aufklärung des vorliegenden molekularen Mechanismus könnte dazu beitragen, das Verständnis und damit verbundene Behandlungsmethoden von Entzündungskrankheiten, welche eine reduzierte PPARγ-Expression zeigen, zu erweitern.

3 Introduction

3.1 Regulation of gene expression

Gene expression depicts a complex process where the information from a gene is translated in a gene product, most often a protein but also non-coding ribonucleic acids (RNAs) like ribosomal or micro RNAs (miRNAs or miRs). This complex machinery enables cells or organisms to accomplish the challenge of life, i.e. simple survival by regulating energy supply and metabolism, but also proliferation, differentiation or adaption to environmental changes such as infection or injury. Gene expression is controlled at different levels including transcription, RNA splicing, post-transcriptional regulation, translation and post-translational modifications. In the following paragraphs I briefly introduce regulation at transcriptional and post-transcriptional level.

3.1.1 Transcription

Transcription describes the synthesis of messenger RNA (mRNA) under the direction of a deoxyribonucleic acid (DNA) sequence. RNA is generated by the RNA polymerase II, which requires a core promoter essential for binding of the pre-initiation complex recognizing the so called TATA-box. Only a small, basal subset of genes is transcribed by this pre-initiation complex, whereas initiation of transcription mostly requires transcription factors. These proteins facilitate a coordinated induction of genes dependent on environmental changes, often along with co-activators or co-repressors. Transcription factors bind to specific DNA consensus sequences within promoter regions assisting or blocking the recruitment of the RNA polymerase complex. Initiation by transcription factors often needs the help of co-regulators, which bind to transcription factors. They facilitate recruitment or control accessibility of DNA to the RNA polymerase complex (1, 2).

Unlike prokaryotic DNA, eukaryotic DNA is packed in a highly organized structure. The double helix is wound around histone proteins, while again 8 histone/DNA complexes form a nucleosome, building a 10 nm-fiber, the so called ‘beads-on-a-string’. These fibers are packed with the help of Histone 1 to a complex chromatin structure. Chromatin structure strongly influences gene transcription simply by the accessibility

of DNA to transcription factors and the RNA polymerase complex. Thus, chromatin forms the first barrier for gene transcription and can be modified by altering the acetylation status of histones. This is accomplished among others by histone acetyltransferases (HATs) and histone deacetylases (HDACs). Histones are normally positively charged and therewith bind to the negatively charged DNA thereby decreasing accessibility. Acetylation by HATs changes amines to amides thus neutralizing positive charges and expanding chromatin. Subsequently, the transcription machinery is able to bind and initiate gene expression. HDACs remove acetyl groups and thereby increase positive charges, which concomitantly prevents transcription. Co-activators and co-repressor often exhibit an intrinsic HAT or HDAC activity or they recruit HATs or HDACs to achieve their function (1, 2). Two well established co-activators with intrinsic HAT activity are cAMP response element (CREB)-binding protein (CBP), participating in the activity of hundreds of transcription factors including nuclear factor κB (NFκB) and activation protein-1 (AP-1) (3).

3.1.2 Post-transcriptional regulation

Besides transcription, gene expression is also controlled by post-transcriptional regulation, i.e. by altering mRNA stability. In mammalian cells, mRNA half-life ranges from several minutes to days, suggesting tightly controlled and specific regulation. Altering mRNA half-life enables cells to rapidly react to environmental changes and is a common feature during immune responses. In most cases *cis*-acting elements within 3' untranslated regions (UTRs) are targeted by *trans*-acting RNA-binding proteins facilitating mRNA degradation (4). Moreover miRNAs, a new class of small non-coding RNAs have been identified as *trans*-acting molecules, also binding to *cis*-acting elements within the 3'UTR, mediating rapid mRNA decay or inhibiting translation (5).

3.1.2.1 ARE-binding proteins

Trans-acting RNA-binding proteins targeting mRNA for degradation mostly interact with AU-rich elements (AREs) within the 3'UTR of an individual transcript. AREs have first been described for various cytokine transcripts. Thus, the first ARE-mediated mRNA decay was identified by Shaw and Kamen, who inserted the ARE of GM-CSF (granulocyte-macrophage-colony stimulating factor) transcript into the 3'UTR of a β-globin reporter construct and observed rapid degradation of the β-globin mRNA (6).

Contrarily, deleting the 3'UTR of TNFα (tumor necrosis factor α) or Interleukin (IL) -3 enhanced mRNA stability and therewith gene expression (7, 8). Besides targeting mRNAs for their degradation, ARE-binding proteins can also affect translation efficiency. They were originally believed to require several copies of an AUUUA pentamer, whereas also AU-rich sequences can be target for *cis*-*trans*-interactions. AREs have been recently categorized into four classes ('W' stands for 'U' or 'A'): ARE1: AUUUA, ARE2: UUAUUUAWW, ARE3: WWWUAUUUAUWWW, and ARE4: 12-mer A/U with maximal one mismatch (9). Degradation of RNAs is in general achieved by the removal of the poly (A)-tail and following exonucleolytic decay in 5'-3' or 3'-5' direction. In 3' – 5' direction, mRNA is degraded by the exosome, which is suggested to occur during ARE-mediated mRNA decay (10).

A broad range of ARE-binding proteins with regulatory functions are known so far. One of the best described ARE-binding proteins is tristetraprolin (TTP), which is predominantly expressed in macrophages and T-cells. Studies of TTP deficient mice showed inflammatory symptoms due to enhanced stability of TNFα, GM-CSF and IL-2 mRNA (11). Besides TTP, also AUF1 (AU-binding factor 1), Brf1/2 (B-related factor 1/2) and KSRP (KH-type splicing regulatory protein) are described to destabilize, whereas HuR (human antigen R) rather stabilizes mRNAs. The combination of stabilizing and destabilizing factors enables the cell to coordinate a specific and adapted (immune) response. For instance, iNOS (inducible nitric oxide synthase) mRNA is destabilized by TTP and also KSRP, whereas HuR stabilizes the iNOS transcript, the latter ones both competing for the same ARE site. Thus, dependent on the microenvironment, macrophage iNOS mRNA is degraded or stabilized. Moreover, also ARE-binding proteins and miRNAs can act in concert, as it was first described for miR-16 and TTP. Jing *et al.* observed that miR-16-mediated TNFα mRNA decay also requires TTP, both sharing the ARE 'AUUUAUAA'. TTP did not directly bind to miR-16, but associated with Argonaute (Ago) family members and assists in targeting mRNA (12).

3.1.2.2 MicroRNAs

MiRNAs present a large family of small non-coding RNAs with a length of about ~22 nucleotides. They are transcribed as primary miRNAs (pri-miRNA), which then undergo a two-step processing. First, the pri-miRNA is cleaved by the double-strand endonuclease Drosha generating the precursor miRNA (pre-miRNA), which is exported

to the cytosol by exportin 5. There, it is further processed by Dicer resulting in the mature miRNA and its antisense strand (reviewed in (13)). The mature miRNA is incorporated into the RNA induced silencing complex (RISC), whose core components are members of the Ago family. The miRNA targets the complex to its target mRNA via base pairing between the mRNA-3'UTR and the 5' end of the miRNA (positions 2-7), termed the seed region. The seed region match seems to be critical for mRNA degradation, whereas perfect seed pairing alone was found to be insufficient to predict functional targets. Thus, the 3' end of the miRNA especially positions 12-17 and also the relative position to the mRNA stop codon might also influence silencing efficiency (14). Finally mRNA:miRNA interaction results in down-regulation of protein expression by translational repression, mRNA cleavage or promotion of mRNA decay. Perfect complementary mRNAs are cleaved by the endonuclease activity of Ago proteins and subsequent degradation by the exosome (13).

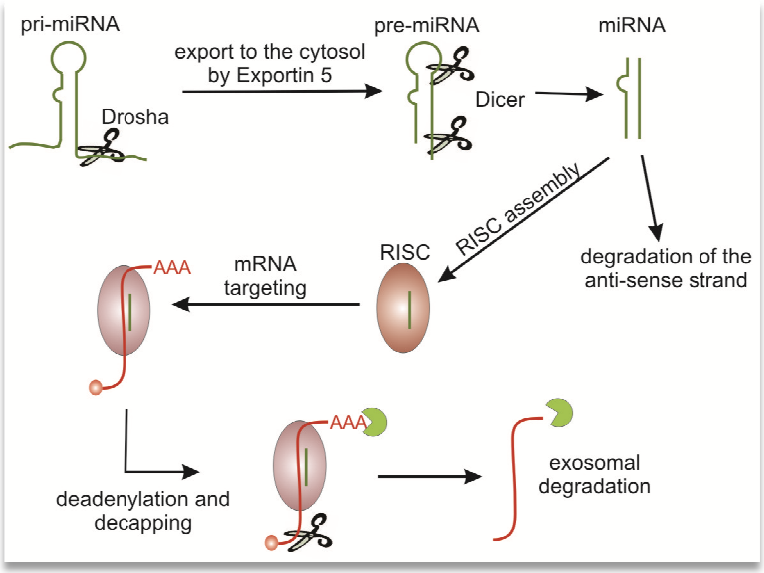

Figure 3.1 miRNA processing and mRNA degradation.
MiRNAs are transcribed as primary miRNAs (pri-miRNAs), which undergo a two-step processing. Drosha cleaves the pri-miRNA to the precursor-miRNA (pre-miRNA), which is exported by Exportin 5 to the cytosol. There, it is further processed by Dicer, resulting in the double-strand miRNA, while the antisense-strang is degraded. The mature miRNA is incorporated into the RISC (RNA induced silencing complex) and targets to specific mRNAs. Targeted transcripts are finally deadenylated and decapped following exosomal degradation.

Transcripts being imperfect complementary to miRNAs are degraded upon recruitment of several proteins such as the CAF:CCR4:NOT (CCR4-associated factor:carbon

catabolite repressor factor 4:negative on-TATA-less) deadenylase complex, decapping enzymes and activators and following exosomal degradation (reviewed in (15)) (Figure 3.1). Even though the decay is believed to occur in so called P-bodies (cellular structures enriched with mRNA catabolising enzymes), it was recently demonstrated that P-body localization is not required for transcript silencing. However, localization is mediated by the P-body component GW182, which interacts with Ago1, marking mRNA for degradation. Mutation of GW182 and Ago1 revealed that interaction is essential for mRNA degradation, showing the importance of the C-terminal region of GW182 for mRNA decay (16). Moreover, depletion of Ago1 and also GW182 led to an up-regulation of many transcripts, underlining their crucial role in miRNA-mediated mRNA degradation (17).

MiRNAs play an eminent role during development and function of immune cells and are associated with several inflammatory diseases (18). MiR-155 and miR-146 gained special interest and are both induced upon various pro-inflammatory stimuli such as the toll-like receptor 4 (TLR4)-ligand lipopolysaccharide (LPS), IL-1 or TNFα (19). MiR-146 is induced in a NFκB-dependent manner and negatively regulates TLR-signaling via repression of IRAK1 (IL-1 receptor-associated kinase 1) and TRAF6 (TNF-receptor-associated factor 6) (19). However, TLR4 activation also triggers induction of miRNAs such as miR-21 or miR-132 (18). In contrast, miR-125b, which targets TNFα for post-transcriptional repression, is reduced upon LPS exposure facilitating a proper TNFα production (20). Many miRNAs have been associated with cancer, whereas more recently, the role of miRNAs during chronic inflammation has been investigated. The first evidence for an involvement of miRNAs in inflammatory diseases came from Sonkoly *et al*.. Microarray analysis of patients with psoriasis, an inflammatory skin disorder, revealed among others elevated expression of miR-146, suggesting failure in TNFα signaling pathways (21). In rheumatoid arthritis (RA) miR-146a and miR-155 were up-regulated in comparison to healthy individuals or patients with osteoarthritis. It was proposed that miR-155 might control expression of matrix metalloproteinase-1 (MMP-1) and -3 in synovial fibroblasts, both enzymes capable of degrading extracellular matrix proteins and are involved in the pathogenesis of RA (22, 23). Still, the identification of miRNA functions in inflammatory diseases needs further investigation.

3.2 Inflammation and macrophages

Inflammation is a complex biological response to noxious conditions such as infection or injury and is characterized by its cardinal signs *rubor* (redness), *calor* (heat), *tumor* (swelling), *dolor* (pain) and *functio laesa* (loss of function), the first four being already described in the first century, a. D..

The innate immune system, consisting mainly of epithelial barrier, phagocytes, natural killer cells and the complement system, provides the early lines of defense against pathogens. After breaching the epithelial barrier, invading microbes are recognized by neutrophils and macrophages via structures that are characteristic for microbial pathogens (pathogen-associated molecular pattern (PAMP)). Identification of PAMPs enables cells to distinguish self from non-self. PAMPs such as LPS or double-stranded RNA are in general sensed by pattern recognition receptors such as TLRs. After recognition, macrophages secret cytokines, e.g. IL-1 and TNFα, which in turn stimulate endothelial cells to recruit further leukocytes. Thus, inflammation is accompanied by rapid influx of neutrophils and monocytes that differentiate into macrophages following migration into the inflamed tissue. After recognition of pathogens, macrophages and also neutrophils ingest microbes, which are then killed in part by reactive oxygen species (ROS) or nitric oxide (NO) produced by the phagocytes. Activated macrophages furthermore release a variety of inflammatory mediators such as chemokines and cytokines to attract other leukocytes to the site of infection. Macrophages also recruit and subsequently activate lymphocytes by antigen-presentation, thereby linking the innate and adaptive immune system. Activated B- and T-cells themselves enhance antimicrobial activities of macrophages, e.g. by secreting interferon γ (IFNγ), but also fight against different types of microbes by marking pathogens for elimination or by killing infected cells to abolish reservoirs of infection (24).

Besides building the first line of defense and activating lymphocytes, macrophages are also important for the resolution of inflammation and wound healing. Thus, exhibiting various functions also suggest various macrophage phenotypes.

3.2.1 Distinct macrophage phenotypes

Since Mackaness *et al.* first described classically activated macrophages in the 1960s as major immune effector cells (25), considerable knowledge was gained concerning

distinct macrophage phenotypes with diverse functional roles. Prevailing views display a remarkable plasticity of macrophages with diverse phenotypes and functions that are classified towards two extremes: M1 and M2 macrophages.

The term classical activated macrophage (or M1) defines a phenotype produced in response to infection or injury. It is characterized by a high bactericidal competence i.e. the production of ROS, NO as well as pro-inflammatory cytokines such as TNFα and IL-1β, IL-6 and IL-12, partly accomplished by activation of NFκB (26).

M2 macrophages alone comprise a broad spectrum of phenotypes with different biochemistry and function. To appreciate the functional repertoire of macrophages, taking into consideration the reports on intermediate or hybrid activation states, Mosser and Edwards avoided the term M2 macrophages and used the operatively useful discrimination between regulatory and wound-healing macrophages, considering their implication in immune regulation and wound healing (27). Originally, IL-4 and IL-13 were described as alternative activators of macrophages, characterized by attenuating the production of pro-inflammatory mediators, i.e. pro-inflammatory cytokines, NO and ROS. At the same time alternative macrophage activation enhances the expression of IL-10, arginase 1/2 and the mannose receptor (MR). This macrophage phenotype was classified as a M2a phenotype (28) or more recently as wound-healing macrophages. The latter nomenclature refers to the secretion of extracellular matrix components, thus fostering wound-healing (27).

Regulatory or M2c macrophages are generated by environmental stimuli such as IL-10, glucocorticoids, prostaglandins and also apoptotic cells (AC). Compared to wound-healing macrophages they do not produce extracellular matrix, rather secreting high levels of IL-10 and transforming growth factor β (TGFβ), and thus showing a pronounced immune regulatory function. M2b macrophages also exert immunoregulatory functions and are generated by combined exposure to immune complexes (by Fc γ receptor binding) and TLR or IL-1 receptor ligands. Similar to M2a and M2c, they show increased IL-10 levels, reduced IL-12 secretion but still produce low levels of pro-inflammatory cytokines such as TNF, IL-6 and IL-1. Because of their strong induction of Th2 responses, they were originally determined as type II macrophages (27, 29).

Even though these classifications tend to specifically describe macrophage subtypes, they still display only extremes of a continuum, especially considering that hybrid-type cells exist (extensively reviewed in (27, 28)).

3.2.2 NFκB

The NFκB/Rel family of transcription factors plays a crucial role in the coordination of both innate and adaptive immune responses. Thus, in macrophages as well as in lymphocytes, NFκB is activated upon a wide variety of stimuli including pathogens, pro-inflammatory cytokines such TNFα or IL-1 and also activation of the T- and accordingly B-cell receptors.

The NFκB family consists of five members: p65 (RelA), p50, p52, c-Rel and RelB, which can form homodimers or heterodimers with each other. The transcription activation domain is only present in p65, c-Rel and RelB, hence the homodimer p50/p50 acts as a transcriptional repressor, while binding to DNA but not transactivating. The main activated form of NFκB is the p65/p50 homodimer. In its inactivated state, cytosolic NFκB is associated to IκB (inhibitor of NFκB), preventing its translocalization to the nucleus and subsequent transactivation. Upon several stimuli, IKKα and β (IκB kinases α/β) are activated, in turn phosphorylating IκB, which is then ubiquitinated and degraded by the proteasome. Dissociation from IκB initiates translocalization of NFκB to the nucleus followed by binding to specific site within promoters of its target genes (κB-response element (κB-RE)) (reviewed in(30)).

Since I predominantly used LPS to initiate inflammation, I further introduce TLR4-dependent NFκB transactivation. LPS ligation to the TLR4:CD14:MD2 (TLR4:cluster of differentiation 14:myeloid differentiation 2) complex leads to association of MyD88 and IRAK, which in turn recruits and phosphorylates TRAF6. TRAF6 triggers activation of the TAB2 (TAK1-binding protein 2)-TAK1 (TGFβ-activated kinase 1)-TAB1 (TAK1-binding protein 1) complex in turn activating the IKK complex and following transactivation (Figure 3.2).

Activated NFκB induces the transcription of chemokines, cytokines, stress-response proteins and also enzymes with high bactericidal competence such as components of the NADPH oxidase complex or iNOS. Gene knock-out studies underscored the importance of NFκB and indicated distinct roles for the regulation of the innate and adaptive immunity. Lack of the p65 subunit is embryonically lethal due to liver

degeneration, whereas knock-out of each of the other four members resulted in immunodeficiency showing deficient leukocyte activation and development (30, 31).

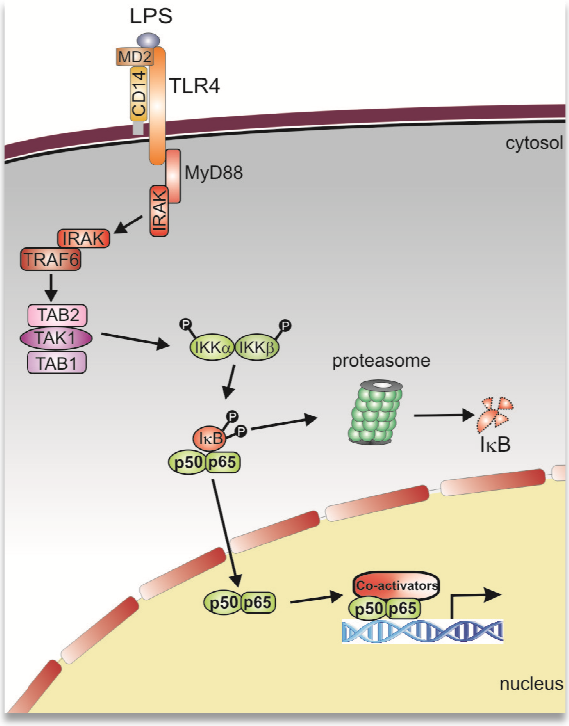

Figure 3.2 NFκB signaling pathway upon TLR4 activation.
Binding of LPS to the extracellular region of the TLR4:CD14:MD2 complex initiates recruitment of MyD88 and IRAK to the cytosolic component of TLR4, which in turn activates TRAF6. Activation of the TAB2-TAK1-TAB1 complex triggers IKK complex activation followed by phosphorylation and degradation of IκB. NFκB is released, translocates to the nucleus, where it induces target gene expression. Abbreviations: CD: cluster of differentiation, IκB: inhibitor of NFκB, IRAK: IL-1 receptor-associated kinase, MD2: myeloid differentiation 2, NFκB: nuclear factor κB, TAB: TAK1 binding protein, TAK: TGFβ-activated kinase 1, TLR: Toll-like receptor, TRAF6: TNF-receptor-associated factor 6.

Besides inducing inflammatory target genes, NFκB also shows pro-survival and anti-apoptotic functions by triggering the expression of anti-apoptotic proteins such as Bcl-xL or cFlip.

3.2.3 Resolution of inflammation

A controlled inflammatory response is in general beneficial, for example by protection against bacterial infections. The release of toxic agents such as ROS to kill pathogens is very competent but does not discriminate between microbial and host targets, thus tissue damage is unavoidable. Still, an acute inflammatory response normally ends

with resolution of inflammation and wound repair, whereas both are predominantly mediated by macrophages. Mechanisms of dampening inflammation have long been elusive, while emerging evidence now suggests an active coordinated program. Apoptosis of leukocytes especially of neutrophils and concomitant clearance by phagocytes is a relevant event during resolution. As described below, phagocytosis of AC by itself exhibits a shift towards an anti-inflammatory phenotype of macrophages (32). As switching off inflammation is an intrinsic feature of inflammation, the pro-inflammatory prostaglandins (PG) E_2 and D_2 trigger the generation of lipoxins and resolvins, in turn repressing cytokine expression and stimulating engulfment of AC. Moreover, anti-inflammatory cytokines such as IL-10 or TGFβ are also induced during the initial phase of an immune response, again suppressing inflammation by e.g. negatively regulating TLR signaling (33, 34).

Beside IL-10 and TGFβ, peroxisome proliferator-activated receptor γ (PPARγ) has been implicated in macrophage polarization towards an anti-inflammatory phenotype and therewith contributes to resolution of inflammation.

3.3 Peroxisome proliferator–activated receptor γ (PPARγ)

PPARs belong to the nuclear receptor superfamily of ligand-dependent transcription factors. There are three isoforms described: α, γ and δ. PPARα was described in 1990 as a factor responding to several compounds inducing peroxisome proliferation in rodents, where this subfamily was named after (35). Indeed, PPARα mediates peroxisome proliferation, whereas this function is not shared by the other two isoforms. PPARγ was first identified as a crucial regulator of adipogenesis and glucose metabolism, whereas it later also emerges as an anti-inflammatory mediator.

3.3.1 Structure

As nearly all nuclear receptors, PPARγ can be divided into four domains (Figure 3.3). The C-terminal ligand-binding domain (LBD) containing the activation function 2 (AF2), is required for ligand-binding and dimerization with retinoid X receptors (RXRs). Beside a hinge and a DNA binding domain (DBD), PPARγ also contains the so called activation function 1 (AF1). This region has ligand-independent transcriptional activity (36), whereas deletion of the N-termini also showed inhibitory effects on ligand-dependent transactivation, which was due to phosphorylation of S82 (112 in PPARγ2) by members

of the MAPK (mitogen-activated protein kinase) family (37, 38). Different opinions about the length of PPARγ exist (475 and 477 amino acids (aa)), which is due to two possible start codons, whereas the first one is suggested to belong to the so called Kozak sequence (ATGACC). Accordingly, S82 might also be defined as S84 for example. All positions used in the following text relate to a length of 475 aa.

However, alternate promoter usage results in two PPARγ isoforms with additional 30 aa at the N-terminus of PPARγ2 (Figure 3.3). The latter one is mainly expressed in adipocytes (39, 40).

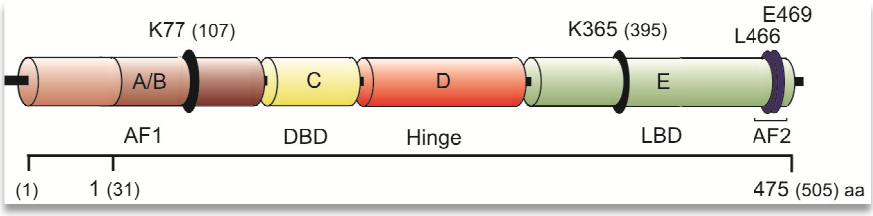

Figure 3.3 PPARγ domain structure.
PPARγ consist of 475 amino acids (aa) and is divided in 4 domains (A/B, C, D and E) characteristic for nuclear receptors: a ligand-independent domain comprising a C-terminal activation function 1 (AF1), a DNA binding domain (DBD), a hinge region and a ligand-binding domain (LBD) with the activation function 2 (AF2). PPARγ1 contains two potential SUMOylation (SUMO: small-ubiquitin related modifier) sites at K77 and K365. L466 and E469 are essential for transactivation and are substituted by alanine in a dominant-negative mutant. Numbers in brackets indicate positions within the PPARγ2 protein.

Alternate transcription is accomplished by the use of three established promoters, whereas transcript variant 1 and 3 code for protein PPARγ1 and transcript variant 2 codes for PPARγ2 (39, 41). Beside this three, another 5 transcript variants have been identified in the last decade (42, 43), while it remains elusive whether they have additional and particular function.

3.3.2 Transactivation and function

PPARγ is bound and activated by several natural and synthetic ligands. A variety of natural compounds have been suggested to bind PPARγ including 15d-PGJ_2 (44, 45), components of oxidized low-density lipoprotein (oxLDL) such as 9- and 13-hydroxy-octadecadienoic acid (9-HODE, 13-HODE) or 15-hydroxyeicosa-tetraenoic acid (15-HETE) (46), lysophosphatidic acid (47) and nitrolinoleic acid (48). Thiazolidinediones (TZDs) are synthetic ligands, which were discovered in 1994 as drugs reducing glucose

levels in rodents. One year later, it was found that TZDs are direct ligands for PPARγ (49), resulting in an improved insulin sensitivity and concomitant reduction of glucose levels (reviewed in (50)). Two prominent TZDs are pioglitazone and rosiglitazone, which are in widespread use for the treatment of type-2 diabetes mellitus.

The role of PPARγ during adipogenesis and glucose metabolism predominantly relies on PPARγ-dependent gene induction of e.g. adipocyte protein 2, CD36, lipoprotein lipase or glucose transporter 4 (50). Gene induction requires transactivation by ligand-binding and heterodimerization with the ligand-dependent transcription factor RXR, which also belongs to the nuclear hormone receptors. PPARγ-ligand binding is not essential for interaction with RXR. However, under basal conditions PPARγ is halted in an inactive state by the co-repressor complex NCoR/SMRT (nuclear receptor co-repressor/silencing mediator of retinoid and thyroid hormone) (51, 52). Ligand binding triggers conformational changes or stabilizes dynamical changes which in turn initiate dissociation of NCoR/SMRT and favour co-activator recruitment of e.g. steroid receptor co-activator-1 (Src-1) and CBP/p300 (53).

3.3.3 Anti-inflammatory properties

Beside regulation of adipogenesis and glucose metabolism, PPARγ is well described for its anti-inflammatory properties in T-, B-cells, monocytes, and macrophages. During differentiation of monocytes into macrophages PPARγ is strongly induced mostly via the promoter 3 but also via promoter 1 (54), whereas it seems not to be required for differentiation (55, 56).

Immunosuppressive action of PPARγ results either from the induction of anti-inflammatory or the inhibition of pro-inflammatory mediators. Especially in alternatively activated macrophages, IL-4 induced expression of arginase 1 and the mannose receptor was eliminated in macrophages from macrophage-specific PPARγ knock-out mice (57). IL-4 activates PPARγ by induction of the 15-lipoxygenase which in turn generates the ligands 13-HODE and 15-HETE through metabolizing arachidonic and linoleic acid (58). Moreover, Odegaard *et al.* gave evidence that the reduced IL-6 expression in response to IL-4 is also PPARγ-dependent. Furthermore, activated PPARγ represses the expression of TNFα, IL-1β, IL-12, MMP-9 and iNOS (59), whereas several mechanisms of transrepression are described (Figure 3.4 A-E):

(A) Interaction with co-activators,

(B) Interaction with transcription factors,

(C) Inhibition of the MAPK cascade,

(D) SUMOylation-dependent prevention of co-repressor clearance (SUMO: small-ubiquitin related modifier)

(E) Inhibition of protein kinase Cα (PKCα) by direct interaction.

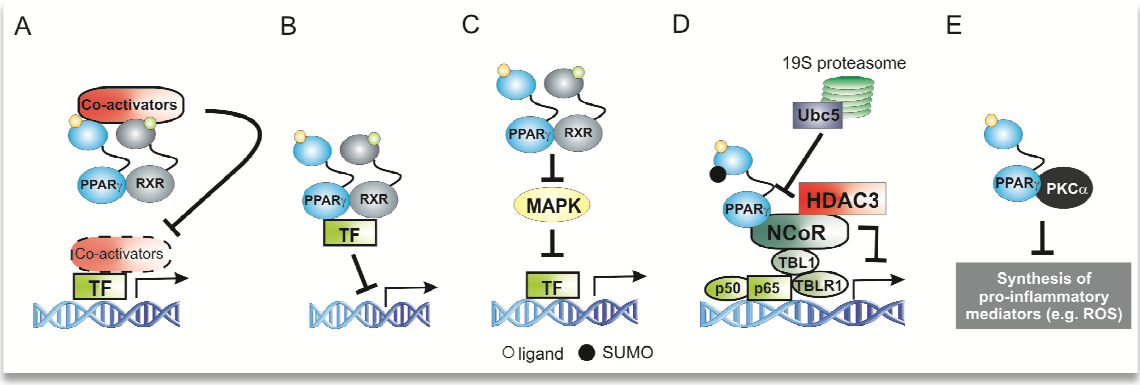

Figure 3.4 Transrepression mechanisms of PPARγ.
Several mechanisms of transrepression are described resulting in a reduced expression of pro-inflammatory mediators, e.g. iNOS or TNFα. (A) Interaction of PPARγ with co-activators e.g. p300. (B) Direct interaction with transcription factors e.g. p65. (C) Inhibition of the MAPK signaling cascade. (D) Prevention of co-repressor clearance (e.g. NCoR/HDAC3 co-repressor complex) by SUMOylated PPARγ. (E) Inhibition of PKCα by direct interaction. Abbreviations: HDAC3: histone deacetylase 3, MAPK: mitogen-activated protein kinase, NCoR: nuclear receptor co-repressor, PKCα: protein kinase C α, PPARγ: peroxisome proliferator-activated receptor γ, RXR: retinoid X receptor, SUMO: small-ubiquitin related modifier, TF: transcription factor

(A) Interaction with co-activators

Target gene induction by transcription factors often requires interaction with co-activators, while CBP and p300 are well described for regulating NFκB and AP-1 (3). Inhibition of AP-1 was attributed to the competition for limiting amounts of CBP/p300 in cells by PPARγ. This was confirmed by Li *et al.* for PPARγ-dependent iNOS promoter repression. In this regard, mutation of the amino acids E469 and K299 flanking a hydrophobic surface required for co-activator interaction (CBP/p300 and Src-1) abolished association with co-activators and concomitant transactivation as well as transrepression activity (60, 61). In addition, a two-hybrid system and GST-pull down assays showed that PPARγ also interacts with the co-activators 'transcriptional

intermediary factor 2', 'amplified in breast cancer-1' and 'thyroid hormone receptor-associated protein 220 subunit'/'vitamin D receptor-interacting protein 205` in a ligand-type specific manner (Figure 3.4 A) (62).

(B) Direct interaction with transcription factors

Several reports suggested that PPARγ/RXR heterodimers inhibit expression of pro-inflammatory cytokines by direct binding of transcription factors and therewith repress activity of NFκB, signal transducer and activator of transcription 1 (STAT1) or AP-1 (Figure 3.4 B) (reviewed in (63)). OxLDL, which is well known to activate PPARγ, reduces LPS-induced IL-12p40 expression in macrophages by inhibiting NFκB transactivation. GST-pull down assays revealed that PPARγ directly binds to p65 and to a lower extent to p50, whereas the interaction was independent of ligand-binding and RXR (64). Moreover, immunoprecipitation of nuclear factor of activated T-cells (NFAT) revealed that inhibition of NFAT in human peripheral blood T lymphocytes is also due to direct binding by PPARγ, resulting in a reduced IL-2 expression (65).

(C) Inhibition of the MAP kinase cascade

MAPKs are serine/threonine-specific kinases which respond to extracellular stimuli (mitogens) and regulate various cellular processes such as proliferation, differentiation, apoptosis and inflammation. Beside the classical MAPK Erk1/2 (extracellular signal-regulated kinase), a lot of others have been identified such as p38 or Jnk (c-jun N-terminal kinase). The latter ones are also known as stress-activated protein kinases. In general, the MAPK cascade includes several kinases activating each other and results in the transactivation of transcription factors (66).

PPARγ was found to control the cascade at different levels. In the colon, PPARγ ligands reduced activity of the Jnk and p38 MAPK resulting in a decrease of TNFα and IL-1β production (67). Furthermore, inhibition of LPS-induced p38 phosphorylation by pioglitazone was recently observed in microglia-enriched cultures (68). Goetze *et al.* noticed that activation of PPARγ by 15d-PGJ_2, rosiglitazone and trogliglitazone inhibited vascular smooth muscle cell migration downstream of the Erk/MAPK pathway (69). While ligands did not affect MAPK activity, a later study revealed that PPARγ rather inhibits activation of the transcription factor Ets-1 (erythroblastosis virus E26 oncogene homolog 1) by MAPK (70). However, in rat peritoneal macrophages, 15d-PGJ_2 significantly attenuated LPS-induced Erk1/2 activation and concomitant IκBα

degradation (71). Moreover, PPARγ ligands also inhibited vascular endothelial growth factor (VEGF)-induced Akt phosphorylation in endothelial cells (72). A recent study might provide explanation how PPARγ interferes with MAPK signaling even though in endothelial cells, flow rather induced activation of Erk5. However, immunoprecipitation experiments revealed a direct interaction between PPARγ-hinge helix 1 region and Erk5, resulting in an anti-inflammatory phenotype (73). Still, underlying molecular mechanisms of MAPK inhibition remain unclear and it has to be clarified if PPARγ ligands act independently of PPARγ or not (Figure 3.4 C).

Even though, PPARγ/RXR heterodimers are postulated to facilitate interaction with transcription factors, co-activators and inhibition of the MAPK cascade, evidence for the action by the PPARγ/RXR heterodimer is missing. Especially co-immunoprecipitations were done with PPARγ and not RXR. Thus, it might be that PPARγ alone facilitates repression by the indicated mechanisms.

(D) SUMOylation-dependent prevention of co-repressor clearance

Co-activator/co-repressor exchange is a common mechanism controlling the switch from gene repression to gene activation and *vice versa* (74). Recent studies proposed that PPARγ prevents signal-mediated clearance of co-repressors and concomitant gene induction of a subset of pro-inflammatory mediators (Figure 3.4 D) (75). Pascual *et al.* observed that under basal conditions the co-repressor complex containing NCoR, HDAC3, transducin beta-like protein-1 (TBL1) and TBL-1 related protein (TBLR1), is associated to the κB-RE within the iNOS promoter. Upon LPS exposure NCoR and HDAC3 were cleared from the promoter by ubiquitination and subsequent degradation by the 19S proteasome. In response to rosiglitazone PPARγ was SUMOylated and targeted to NCoR thereby preventing recruitment of the ubiquitination/19S proteasome machinery. PPARγ contains two potential SUMOylation sites at K77 and K365 (Figure 3.3). Pascual *et al.* identified K365 by point mutation (K365R), which abolished the interaction of PPARγ with the NCoR complex. Conjugation of SUMO1 to PPARγ was facilitated by SUMO E2 ligase Ubc9 and the SUMO E3 ligase PIAS1 (protein inhibitor of activated STAT1), originally identified to inhibit interferon-dependent transcription (76). Silencing of Ubc9 and PIAS1 respectively restored LPS-induced NFκB transactivation despite treatment with rosiglitazone (77). Prevention of co-repressor

clearance was recently extended to the nuclear receptors liver X receptor (LXR) α and β, assuming gene- and signal-specific regulation of NCoR. Thus, they gave evidence that TLR3- and 4-dependent iNOS expression is repressed by LXR and PPARγ, while TNFα but not IL1-β synthesis is inhibited by PPARγ. For explanation they found that TAB2, another component of the co-repressor complex, is associated to the iNOS and TNFα promoter but not to the IL-1β promoter under basal conditions. Knock-down of TAB2 reduced LPS-induced iNOS expression, which was further inhibited by LXR but not PPARγ (75). Thus, SUMOylation-dependent transrepression by nuclear receptors seems to be a general but very specific mechanism of negative regulation of gene expression.

(E) Inhibition of cytosolic PKCα activation by direct interaction

Besides repression of gene induction, ligand-binding of PPARγ also reduces the oxidative burst, which was in part attributed to an inhibited PKCα signaling required for assembly and activation of the NADPH oxidase complex (78, 79). PKCα belongs to the classical PKCs and is physiologically activated by diacylglycerol (DAG) and Ca^{2+}. PPARγ dependent inhibition was attributed to an increased expression of the DAG kinase α, which lowers the amount of DAG and therewith attenuates PKCα activation (80). Since oxidative burst is attenuated within minutes and protein expression requires longer time periods, a new mechanism was recently proposed based on direct protein-protein interaction. PKCα activation is accompanied by membrane translocation, which was inhibited upon pre-treatment with rosiglitazone. The use of a dominant-negative mutant of PPARγ supported the notion that PKCα inhibition by ligands was PPARγ-dependent and not due to receptor-independent effects. Co-immunoprecipitation assays and the two-hybrid system pointed to a direct interaction of PPARγ and PKCα, whereas the hinge helix 1 domain of PPARγ seemed to be responsible for binding (81). Since PPARγ was assumed to be exclusively located in the nucleus and PKCα is restricted to the cytosol, von Knethen *et al.* further gave evidence that a portion of PPARγ is actively transported to the cytosol (Figure 3.4 E) (81).

3.3.4 PPARγ in diseases

The finding that the antidiabetic drugs TZDs are direct ligands of PPARγ, established the receptor as a potent target for the treatment of type-2 diabetes. TZDs were found

to improve insulin action and to lower blood glucose levels via activating PPARγ. Type-2 diabetes is associated with increased plasma levels of free fatty acids concomitantly causing insulin resistance. The beneficial effect of PPARγ is attributed to different functions. First, the activation of PPARγ results in an increase of adipocytes, which is due to enhanced adipogenesis and concomitantly higher capability of fat storage. Furthermore, PPARγ induces adiponectin, a multimeric plasma protein correlating with improved glucose uptake and repressed hepatic glucose output (50).

Besides type-2 diabetes, TZDs are under investigation for their potential beneficial effects on chronic inflammatory diseases including inflammatory bowel diseases, rheumatoid arthritis or multiple sclerosis (MS).

Inflammatory bowel diseases i.e. ulcerative colitis (UC) and Crohn's disease are both chronic inflammatory diseases where the interplay between genetic and environmental factors leads to an abnormal immune response of the gut affecting the intestinal mucosa. Treatment of mice with rosiglitazone decreased symptoms of induced colitis in a murine model. Moreover, heterozygous PPARγ$^{+/-}$ mice were more susceptible to induced colitis and showed higher levels of TNFα and IL-1β (67, 82). In a phase II clinical trial, Lewis *et al.* observed in patients treated with rosiglitazone clinical remission concluding that rosiglitazone treatment was efficacious in the treatment of mild to moderately active UC (83). Remarkably, patients with UC displayed reduced PPARγ expression in epithelial cells, whereas colon epithelial cells of mice showed rather increased expression levels (84).

MS is an autoimmune disease which is characterized by inflammation of the central nervous system resulting in axon demyelination (85). Similar to UC, peripheral blood mononuclear cells (PBMCs) from patients with MS showed decreased PPARγ expression, whereas microglia and astrocytes from a MS mouse model (experimental allergic encephalomyelitis (EAE)) rather showed enhance expression. However, several studies have shown beneficial effects of PPARγ agonists on clinical and histopathological features of EAE (86). Treating PBMCs from MS patients with PPARγ agonists suppressed proliferation and cytokine expression and prevented PPARγ down-regulation (87).

PPARγ was also implicated in Alzheimer's disease, rheumatoid arthritis and psoriasis, all sharing a hyper-inflammatory response, thus suggesting that treatment with PPARγ ligands might be beneficial (88-90).

3.4 The impact of apoptotic cells on macrophages

3.4.1 Apoptosis

A wide spectrum of different types of cell death has been described by now including apoptosis, autophagy, cornification and necrosis (91). Still, apoptosis is considered to be the predominant way of dying and is an important feature to maintain tissue homeostasis in multicellular organisms. Apoptosis is a tightly regulated, energy-dependent process, therewith also called programmed cell death. It is characterized by morphological changes such as nuclear condensation and fragmentation, cell shrinkage and controlled cell disintegration through the formation of membrane vesicles, known as 'apoptotic bodies', whose membrane integrity is maintained (92, 93). The whole process is immunologically silent, primarily because apoptotic cells and debris provide signals for a rapid clearance by phagocytes, ensuring termination of the apoptotic program. In contrast to apoptosis, necrosis is characterized by cell swelling, membrane disrupture and following release of cell contents, which finally provokes an inflammatory response.

3.4.2 Engulfment of apoptotic cells - efferocytosis

Clearance of AC by professional phagocytes such as dendritic cells and macrophages is crucial for tissue homeostasis and resolution of inflammation. The uptake of AC – termed as efferocytosis – avoids secondary necrosis but also actively shifts macrophages towards an anti-inflammatory phenotype. The clearance of AC is a tightly regulated process, which can be divided into three steps:

A) sensing of AC, B) specific recognition of AC and C) the engulfment of AC by phagocytes (94).

A) Sensing of AC by phagocytes

Similar to inflammation, where immune cells are recruited to the sites of infection, so called 'find-me' signals attract phagocytes to apoptotic sites, since they may not be in close proximity to the dying cells. Several 'find-me' signals are described so far. During

apoptosis, the inactive enzyme tyrosyl-tRNA synthetase is cleaved and two fragments are released. The C-terminal fragment is an endothelial-monocyte-activating polypeptide II (EMAP II) –like cytokine and shows like EMAP II itself, chemotactic activity (95). Furthermore, apoptotic cells secrete lysophosphatidylcholine (LPC) (96). During apoptosis, caspase-3 activates the calcium-independent phospholipase A2, which hydrolyses the membrane phospholipid phosphatidylcholine to arachidonic acid and LPC. LPC triggers a phagocyte chemotactic response by binding to the G-protein coupled receptor G2A (97). Recruiting macrophages to damaged sites initiates an immunosuppressive phenotype in macrophages, thus implicating anti-inflammatory properties of LPC. Even though, LPC was originally seen to stimulate pro-inflammatory cytokine production and was linked to atherosclerosis (98). Recently, another lipid was implicated in sensing AC. Sphingosine-1-phosphate (S1P) is released from apoptotic cells (99) and stimulates chemotaxis of primary monocytes and macrophages *in vitro* (100). Besides phagocyte attraction, S1P also exerts anti-inflammatory properties on macrophages, which will be described in more detail below.

B) Recognition of AC by phagocytes

After attraction towards AC, phagocytes need to distinguish between pathogens, apoptotic, necrotic and living cells. Therefore, they recognize AC-associated molecular patterns (ACAMPs) or so called ‘eat-me’ signals at the cell surface, which are specific for apoptotic cell death. ACAMPs are recognized via specific receptors, often coupled to soluble bridging molecules, which strengthen their recognition. The most prominent and most discussed ‘eat-me’ signal is phosphatidylserine (PS). As a component of the cell membrane, PS is actively stored at the inner leaflet by the aminophospholipid translocase in living cells (101, 102). During apoptosis, PS is exposed at the outer leaflet of the membrane by the activation of the scramblase and concomitant loss of aminophospholipid translocase activity (103, 104). Masking of PS almost completely abrogated apoptotic cell uptake, suggesting a key role of PS during efferocytosis (105). Various receptors and bridging molecules are known and will be described briefly. The bridging molecules Gas6 and milk-fat globule epidermal growth factor 8 (MFG-E8) bind PS and in turn are recognized by their receptors Mer tyrosine kinase receptor (MerTK) and vitronectin receptor (VnR). Since the first identified PS receptor acting without the help of bridging molecules (106) was subsequently demonstrated to be located in the

nucleus and dispensable for phagocytosis (107), three novel and more promising PS receptors at the surface of phagocytes (Tim4, BAI1 and Stabilin2) were discovered (108-110).

Beside PS, apoptotic cells carry oxLDL-like sites, which are recognized by different scavenger receptors (SR), such as SR A, lectin-like oxLDL receptor 1 or CD36. The latter one also links AC to phagocytes via thrombospondin-1 binding (111). Single blocking of those receptors often results in a partial reduction of AC uptake, suggesting that different receptors and pathways act in concert.

C) Engulfment of apoptotic cells

Signaling pathways triggering the engulfment of AC emerge from the interactions of 'eat me' signals with their receptors regulating the GTPases Rho and Rac. Activation of Rac and Rho is accompanied by cytoskeletal re-organization (112) and following ingestion in a 'zipper-like' process. Genes involved in the engulfment machinery were identified in the model organism *Caenorhabditis elegans* (113). The proteins have mammalian homologues, whose functional properties in phagocytosis of AC are now being confirmed (111). A complex containing CrkII (CT10 regulator of kinase II), ELMO1 (engulfment and cell motility 1) and DOCK180 has been identified to function upstream of Rac. Upon AC recognition by VnR (114) or Mer (112), the complex is recruited to the plasma membrane triggering nucleotide exchange and activation of Rac. ELMO1 is an adapter molecule, whereas DOCK180 contains a guanine exchange function, the so called Docker domain. Silencing DOCK180 by siRNA in J774 macrophages abolished their phagocytic activity. Moreover, association of DOCK180 and ELMO1 seems to be required for Rac activation. CrkII also associates to DOCK180 and ELMO1, whereas its function remains unknown (94).

Besides the mentioned receptors Mer and VnR, also CD36 (115), annexin1 (116) and stabilin-2 seem to regulate AC uptake (108), even though detailed mechanisms remain unclear. Recently, a Rac-independent signal pathway was postulated, showing that ATP-binding cassette transporter A7 and low-density lipoprotein receptor-related protein 1 enhance clearance of AC in an Erk-dependent manner (117). However, mechanistic studies are obscure.

3.4.3 Immunological consequences of phagocytosis of apoptotic cells

In the late 1990's Voll *et al.* observed that LPS-induced TNFα and IL-1β expression in monocytes was reduced after co-culture with apoptotic lymphocytes, whereas IL-10 was up-regulated (118). In the meantime these early studies were supported by many others, corroborating the ability of AC to repress macrophage inflammatory responses (Figure 3.5). AC provide signals to directly influence the phenotype of their 'captors', thereby dampening inflammation. Obviously, suppression of inflammation is an intrinsic feature of apoptotic cell death, even though phagocytosis *per se* is not required. Studies in CD14 deficient mice showed impaired phagocytosis of AC but elevated TGFβ and reduced TNFα secretion (119). This was supported by Lucas *et al.* using CD36 and $\alpha_v\beta_3$ integrin deficient mice. CD36 and $\alpha_v\beta_3$ integrin contributed to AC clearance, but were not essential for immunosuppression e.g. attenuating TNFα production (115).

Although alternative activation of macrophages by the interaction with AC is widely accepted, the type of macrophage/AC interaction as well as underlying molecular signaling circuits are ill-defined. Some of the inhibitory effects were attributed to PS, but generalized effects and details are controversial as outlined in the following paragraphs.

3.4.3.1 Attenuated ROS and NO formation

Bactericidal capacity of macrophages is in part mediated by the production of ROS (120) and NO. In response to LPS and/or IFNγ, macrophages up-regulate iNOS, which catalyzes the oxidation of L-arginine to L-citrulline and NO (121). While it is accepted that AC reduce NO production, several explanations are discussed. It is commonly believed that reduced NO formation results from increased arginase expression. Two isoforms, arginase 1 and 2, metabolize L-arginine to urea and ornithine, thus competing with iNOS for the same substrate (122). It is proposed that AC up-regulate arginase 1, at the same time down-regulating iNOS in a TGFβ- and PS-dependent manner (123). Others reported cell-cell-contact- and TGFβ-independent up-regulation of arginase 2 triggered by soluble factors secreted by AC (124). Although reduced NO formation was linked to the TGFβ/arginase 1 axis (125), it was questioned whether TGFβ alone induces arginase 1 expression in RAW264.7 macrophages (126). In addition, expression of arginase 1 occurred late compared to NO inhibition, which

argues for additional inhibitory mechanisms. Likely, the rapid induction of arginase 2 seen with AC contributes to reduced NO formation in alternatively activated macrophages with secondary and late inhibition being, at least in part, TGFβ-mediated.

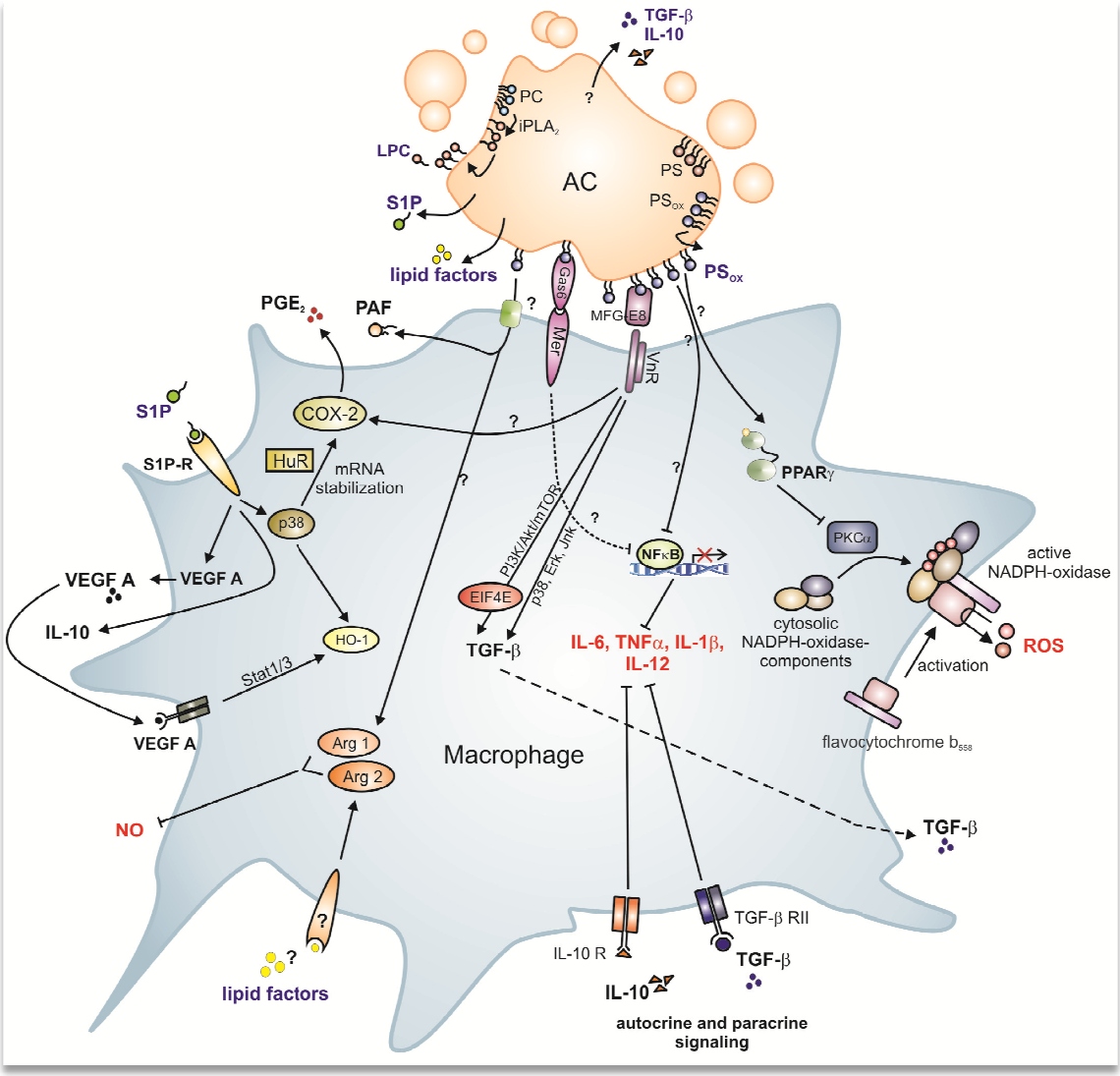

Figure 3.5 Immunological consequences of AC on macrophages.
AC provide signals (blue) that shift macrophages towards a 'regulatory' phenotype, which is characterized by inhibition of pro-inflammatory mediators (red) as well as induction of anti-inflammatory mediators (black). stimulation; ⊣ inhibition; for detailed information see text. Abbreviations: AC: apoptotic cells, Arg1, 2: arginase 1 or 2, COX-2: cyclooxygenase 2, HO-1: heme oxygenase 1, LPC: lysophosphatidylcholine, NCoR: nuclear receptor co-repressor, PAF: platelet-activating factor, PPARγ: peroxisome proliferator activated receptor γ, PS: phosphatidylserine, ROS: reactive oxygen species, S1P: sphingosine-1-phosphate, VnR: vitronectin receptor, MFG-E8: milk-fat globule epidermal growth factor 8.

Another feature of macrophage cytotoxicity is the production of ROS. The oxidative burst is initiated by PKCα-dependent phosphorylation of $p47^{phox}$ and assembly of the

NADPH oxidase complex (78, 79). ROS formation in macrophages was inhibited by apoptotic Jurkat cells as well as PS-enriched viable Jurkat cells, whereas oxidized PS was even more effective (127). Transwell-experiments pointed to the requirement for cell-cell-contacts and possibly a PS-dependent pathway. Mechanistically, Johann *et al.* suggested PPARγ, activated by AC by a so far unknown mechanism, to account for ROS inhibition (128). Activation of PPARγ with synthetic agonists induced a direct protein interaction with PKCα, thereby interfering with translocation of the kinase from the cytosol to the membrane, which is a prerequisite for oxidative burst activation (81).

3.4.3.2 Modulation of cytokine expression

Besides repressing ROS and NO formation, AC strongly suppress pro-inflammatory cytokine expression of e.g. IL-1β, TNFα, IL-6 and IL-12, subsequent to LPS stimulation. Different explanations are plausible and reduction was tied to autocrine signaling of TGFβ, platelet-activating factor (PAF) and PGE_2, all of which are released from macrophages in response to AC in a PS-dependent manner. The reduction in IL-1β, IL-8 and TNFα, seen with incubation periods of more than 18 h, was abolished by a neutralizing TGFβ-antibody, blocking cyclooxygenase (COX) with indomethacin or a PAF receptor antagonist (129).

A prominent cytokine being up-regulated by AC is TGFβ. Supportive argumentation for the PS-dependence came from experiments using human monomyelocyte cells, which do not expose PS at their surface during UV-induced apoptosis. These cells failed to promote TGFβ1 secretion, while TGFβ release was seen with PS-exposing apoptotic Jurkat cells (130). Moreover, masking PS on irradiated tumor cells by annexin V averted TGFβ production in macrophages (131). A recently reported PS-receptor, Stabilin-2, mediated AC engulfment and anti-Stabilin-2 antibodies induced TGFβ, supporting the indirect notion that PS-receptor activation triggers TGFβ expression (108). Enhanced TGFβ secretion in response to AC is facilitated by transcriptional and translational regulation. TGFβ mRNA transcription was sensitive to p38 MAPK, Erk and Jnk inhibition, while translation demands Rho kinase, followed by phosphorylation of PI3K/Akt and mTOR (mammalian target of rapamycin) and subsequent activation of eukaryotic initiation factor 4E (132).

3.4.3.3 Inhibition of NFκB

Even though TGFβ is a known anti-inflammatory mediator (133), reduced cytokine expression is primarily associated with inhibition of NFκB. Identifying Mer tyrosine kinase as a potential candidate to block NFκB, strengthens a role for PS in this process. Over-expression of a Mer kinase-dead mutant relieved NFκB inhibition (134). In dendritic cells the Mer kinase receptor was associated with NFκB inhibition in response to AC by using Mer receptor knock-down cells and blocking antibodies (135). Future studies need to provide mechanistic details, especially as a direct role for inhibition of pro-inflammatory cytokine formation via this receptor was still missing.

Despite a potential role of the Mer kinase or TGFβ in down-regulating pro-inflammatory mediators, the macrophage shift towards an anti-inflammatory phenotype also occurred independently of PS and TGFβ, especially at earlier times, i.e. below 18 h. Cvetanovic *et al.* provided evidence that NFκB, although being inactive binds to the DNA, implying that co-activators/co-repressors are involved (136). But still the mechanism remained so far elusive. For tumor-associated macrophages, showing a similar phenotype as macrophages, which interacted with AC, it has been proposed that p50 homodimer formation accounts for NFκB inhibition (137). Although not formally shown for macrophages polarized by AC, it opens the possibility that, depending on the polarization signal distinct but qualitatively equivalent mechanisms interfere with NFκB activation.

3.4.3.4 S1P and IL-10 in regulatory macrophage polarization

There is evidence that soluble factors released from AC polarize macrophages. Examples comprise IL-10 (138), TGFβ (139) and more recently the lipid mediator S1P (99). During a co-culture of tumor cells with primary macrophages, apoptosis in tumor cells occurred and in turn macrophages were alternatively activated. Interrupting apoptosis by over-expressing Bcl-2 in tumor cells or using an apoptosis-resistant tumor cell line failed to induce the accompanying anti-inflammatory macrophage phenotype shift. Interestingly, conditioned medium from AC also polarized macrophages, which indisputably argues for a soluble factor transmitting information to macrophages. S1P, described for its anti-inflammatory (140, 141) and anti-apoptotic effects (142), emerged as a potential candidate. Indeed, S1P is released from AC, protected

macrophages against apoptosis, reduced TNFα and IL-12p70 secretion but enhanced the expression of IL-10 and IL-8 (143).

Expression of macrophage IL-10 in response to AC was first noticed in the seminal paper by Voll *et al.* (118). IL-10 emerged as a key regulator in suppressing Th1 responses. Strikingly, IL-10 deficient mice developed chronic intestinal inflammation, as they apparently could not restrict inflammation (144). Especially in macrophages, which express high levels of IL-10R, the immunosuppressive role of IL-10 was intensively characterized. IL-10 activates STAT3 homodimer formation and DNA binding, with the consequence of suppressing pro-inflammatory mediator transcription (26). Regarding the question how IL-10 is induced by AC, it is tempting to attribute this to COX-2 expression (145). COX-2 is rate limiting in converting arachidonate to PGE_2, with the further conversion of PGE_2 towards anti-inflammatory mediators such as the PPARγ agonist 15d-PGJ_2 or resolvins (33). COX-2 expression in macrophages by AC is either facilitated via the VnR (146), which requires cell-cell contacts between AC and macrophages, or by AC-derived S1P and HuR-mediated COX-2 mRNA stabilization (147). Strikingly, efferocytosis-dependent PGE_2 production was shown to markedly impair pathogen clearance by lung alveolar macrophages *in vivo*, consistent with elevated levels of IL-10 (148).

A knock-down of sphingosine kinase 2 in tumor cells reduced S1P levels in the supernatant of AC, at the same time interrupting to a great extent the immunosuppressive properties of AC supernatants. S1P not only induced COX-2, but also up-regulated heme oxygenase-1 (HO-1) in a biphasic manner. Early expression of HO-1 after 6 h was transmitted by activating the S1P receptor 1, whereas a second wave of expression was attributed to autocrine signaling of VEGF A (149). HO-1 catalyzes the rate limiting step in the degradation of heme to biliverdin, ferrous iron and carbon monoxide and is known for its anti-apoptotic and anti-inflammatory properties (150-152). HO-1 was causatively involved in up-regulation of Bcl-2 and Bcl-$X_{L,}$ explaining the mechanism behind S1P-mediated protection from apoptosis (149).

3.5 Aims of the study

PPARγ plays an eminent role during alternative activation of macrophages and resolution of inflammation. As an anti-inflammatory signaling molecule, it seems likely that it is tightly regulated dependent on the state of the immune response. There is growing evidence that PPARγ expression is reduced during inflammation, whereas molecular mechanisms are ill-defined. Even though, its role in immunosuppression is getting more definite. AC provoke an active repression of pro-inflammatory responses *inter alia* by the inhibition of pro-inflammatory cytokine expression or attenuated generation of reactive oxygen species. The reduced oxidative burst was attributed to PPARγ activation, while mechanisms behind the reduced cytokine expression remained unclear.

The first part of my thesis dealt with the question whether AC-provoked NFκB inhibition in macrophages depends on PPARγ. Furthermore, I was intrigued to investigate underlying mechanisms repressing NFκB transactivation. Thus, this study should allow to further understand how AC effect macrophage plasticity associated with decreased pro-inflammatory cytokine production.

The second part of my Ph.D. thesis addressed regulation mechanisms of PPARγ expression during the inflammatory response, which was initiated by LPS treatment. Special interest was set on the question, if altered mRNA was due to transcriptional or post-transcriptional changes, subsequently investigating detailed mechanisms. Clarification of pathway decreasing PPARγ expression might help to understand disease conditions thus providing options for new therapeutic approaches during chronic inflammation.

4 Material and Methods

4.1 Material

4.1.1 Cells

Jurkat T cells

Jurkat T cells are derived from a 14-year-old boy with acute lymphatic leukemia. This T cell line was established in 1977 (153).

Murine splenocytes

Primary murine splenocytes were isolated from C57BL/6 PPARγ$^{fl/fl}$ or LysMCre$^{+/+}$/PPARγ$^{fl/fl}$ mice. All procedures performed on these mice followed the guidelines of the Hessian animal care and use committee.

Primary human monocytes

Primary human monocytes were isolated from buffy coats, which were obtained from DRK-Blutspendedienst Baden-Württemberg-Hessen, Frankfurt.

RAW 264.7 cells

RAW 264.7 mouse monocytes/macrophages were established from ascites of a tumor induced in a male mouse by intraperitoneal injection of Abselon Leukaemia Virus (A-MuLV) in 1977 (154).

THP-1 cells

THP-1 human monocytes were obtained from the peripheral blood of a 1-year-old boy with acute monocytic leukemia at relapse in 1978 (155).

4.1.2 Bacteria

Competent bacteria strains were provided by Stratagene GmbH (Amsterdam, The Netherlands). XL1-Blue® supercompetent cells were generally used for amplification. Vectors mutated with QuikChange XL II site directed mutagenesis kit were transformed in XL10-Gold® ultracompetent cells. For generation of unmethylated DNA, vectors were transformed in the *Dam*$^{-}$ bacteria strain SCS110®. Genotypes are described in Table 4.1, while the listed genes signify mutant alleles.

Table 4.1: Bacteria strains

Bacteria strain	Genotype
XL1-Blue	recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F´ proAB lacIqZ.M15 Tn10(Tetr)].
XL10-Gold	TetrD(mcrA)183 D(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac Hte [F´ proAB lacIqZDM15 Tn10 (Tetr) Amy Camr].
SCS110	rpsL (Str^r) thr leu endA thi-1 lacY galK galT ara tonA tsx dam dcm supE44 Δ(lac-proAB) [F´ traD36 proAB lacIqZΔM15]

4.1.3 Chemicals and reagents

All chemicals were of highest grade of purity, commercially available and usually purchased from Sigma-Aldrich GmbH (Deisenhofen), Roth GmbH (Karlsruhe) or Merck Eurolab GmbH (Darmstadt). Cell culture media and supplements came from PAA (Cölbe). Special reagents and kits are listed in the table below.

Table 4.2: Special reagents and kits

Chemical/Kit	Provider
12-Tetradecanoyl-phorbol-13-acetate (TPA)	Sigma-Aldrich GmbH (Deisenhofen)
Absolute™ qPCR SYBR® Green Fluorescein Mix	ABgene (Hamburg)
Allstars negative control siRNA	Qiagen GmbH (Hilden)
Amaxa® Nucleofector® Kits (V and Human Macrophages)	Lonza Cologne AG (Köln)
Bay 11-7082	Sigma-Aldrich GmbH (Deisenhofen)
CD11b Microbeads (mouse)	Miltenyi Biotec GmbH (Bergisch-Gladbach)
Chelex® 100	BioRad GmbH (Munich)
Cycloheximide	Sigma-Aldrich GmbH (Deisenhofen)
Deoxynucleotide Solution Mix (dNTPs)	New England Biolabs (Frankfurt)
D-luciferine	AppliChem GmbH (Darmstadt)
DRB (5–6 dichloro-1-β- ribofuranosyl-benzimidazole)	Sigma-Aldrich GmbH (Deisenhofen)
GW9662	Alexis Biochemicals (Lausen, Switzerland)
HiSpeed Plasmid Maxi Kit	Qiagen GmbH (Hilden)
In-Fusion™ Dry-Down PCR Cloning Kit	Clontech-Takara (Saint-Germain-en-Laye, France)

Interferon γ (human)	Roche Diagnostics GmbH (Mannheim)
iScript™ cDNA Synthesis Kit	BioRad GmbH (Munich)
JetPEI™ transfection reagent	Polyplus transfection (Illkirch, France)
Leptomycin B	Sigma-Aldrich GmbH (Deisenhofen)
Lipopolysaccharide (from *Escherichia coli,* serotype 0127:B8)	Sigma-Aldrich GmbH (Deisenhofen)
Luminol (3-Aminophtalhydrazide)	Acros Organics (Geel, Belgium)
Lymphocyte separation medium (Ficoll)	PAA Laboratories GmbH (Cölbe)
Anti-miR™ - miRNA Inhibitors (neg. control, miR-27a, miR-27b)	Applied Biosystems/Ambion, Austin, TX, USA
miR-27a and b mimic	Qiagen GmbH (Hilden)
miScript Reverse Transcription Kit	Qiagen GmbH (Hilden)
miScript SYBR® Green PCR Kit	Qiagen GmbH (Hilden)
Normal rabbit IgG	Millipore/Upstate (Billerica, MA, USA)
ON-TARGETplus SMARTpool siRNA (murine NCoR1 and PIAS1)	ThermoScientific GmbH (Lafayette, CO, USA)
peqGOLD RNAPure™	PeqLab Biotechnologie GmbH (Erlangen)
Proteinase K	New England Biolabs (Frankfurt)
Protein G-Agarose	Roche Diagnostics GmbH (Mannheim)
QuikChange® XL II site directed mutagenesis kit	Stratagene (Amsterdam, The Netherlands)
Restriction enzymes (*XbaI, Bpu10I* and *NcoI*)	New England Biolabs (Frankfurt)
Rosiglitazone	Alexis Biochemicals (Lörrach)
SB203580 (p38 inhibitor)	Sigma-Aldrich GmbH (Deisenhofen)
Standard DC Protein Assay Kit	BioRad GmbH (Munich)
Taq DNA Polymerase, recombinant	Invitrogen GmbH (Karlsruhe)
TNFα (human, recombinant)	Roche Diagnostics GmbH (Mannheim)
Trichostatin A	Sigma-Aldrich GmbH (Deisenhofen)

4.1.4 Antibodies

Secondary antibodies (IRDye800-labelled anti-mouse, IRDye800-labelled anti-rabbit) were obtained from Li-COR Biosciences GmbH (Bad Homburg). Primary antibodies are listed in Table 4.3.

Table 4.3: Primary antibodies

Antibody	Provider	Dilution used for WB analysis
Anti-actin	Sigma-Aldrich GmbH (Deisenhofen)	1:2000
Anti-tubulin	Sigma-Aldrich GmbH (Deisenhofen)	1:1000
Anti-NCoR	Affinity Bioreagents (Golden, CO, USA)	
Anti-PPARγ (H-100X)	Santa Cruz Biotechnology, Inc. (Heidelberg)	1:2000

4.1.5 Plasmids

Used plasmids are listed in the tables below (Table 4.4 and Table 4.5).

Table 4.4: Reporter plasmids

Plasmid	Information	Provider
pGL3-control	Contains a SV40 promoter upstream of the luciferase encoding region, ampicillin resistance	Promega (Mannheim)
pGL3-basic	Contains a minimal promoter upstream of the luciferase encoding region, ampicillin resistance	Promega (Mannheim)
pNFκB-Luc	Contains three κB sites and a minimal promoter upstream of the luciferase encoding region	(156)
pAOX-TK	Contains the promoter of the acyl CoA oxidase (AOX) upstream of the luciferase encoding region	(157)
pGL3γ1p3000, pGL3γ3p800	Contains the different PPARγ promoters upstream of the luciferase encoding region	(39, 41)

Table 4.5 Expression plasmids

Plasmid	Description	Provider
pcDNA3-PPARγ1 and pcDNA3-PPARγ1-AF2 mt	codes for human PPARγ1 protein, the mutant carries the mutations L466A/E469A, ampicillin resistance	V.K.K. Chatterjee, University of Cambridge, UK
pDsRed-PPARγ1 and deletion constructs	codes for DsRed-PPARγ1 fusion protein, parts of PPARγ1 are deleted for structure analysis	(81)

4.1.6 Oligonucleotides

Oligonucleotides were purchased from Biomers.net GmbH (Ulm) and described in Table 4.6. Changed nucleotides of primers used for point mutation with QuikChange are underlined. PPARγ1, actin and PIAS1 were determined using QuantiTect Primer Assay from Qiagen GmbH (Hilden).

Table 4.6: Oligonucleotides

Primer	Forward	reverse	annealing temperature
18S rRNA (human)	5'-GTAACCCGTTGAACCCCATT-3'	5'-CCATCCAATCGGTAGTAGCG-3'	55°C
Actin (human)	5´-TGACGGGGTCAC CCACACTGTGCCCATCTA-3´	5´-CTAGAAGCATTT GCGGTGGACGATGGAGGG-3´	55°C
ChIP-TNFα (mouse)	5´-GGCTTGTGAGGTCCGTGAAT-3´	5´-GAAAGCTGGGTGCATAAGGG-3´	56°C
DsRed	5´-GAGGTGCAGCAGGACTCCTC-3´	5´-TGGCCTTGTACACGTCTTG-3´	55°C
GAPDH (mouse)	5´-CTCATGACCACAGTCCATGC-3´	5´-TTCAGCTCTGGGATGACCTT-3´	55°C
IL-6 (mouse)	5´-GAACAACGATGATGCACTTGC-3´	5´-TCTCTGAA GGACTCTGGCTTTG-3´	55°C
K77R (QuikChange)	5´-GAGTACCAAA GTGCAATC<u>AGA</u>GTGGAGCCTGC-3´	5´-GCAGGCTCCA C<u>TCT</u>GATTGCACTTTGGTACTC-3´	68°C
PPARγ-UTR (In-Fusion)	5´-GCCGTGTAATTC TAGCAGAGAGTCCTGAGCC-3´	5´-CCGCCCCGACTC TAGTTCATAATATGGTAATTTTTA-3´	40°C
PPARγ-UTR-ΔmiR-27 (QuikChange)	5´-GGGAAAATCTGACACCTAAAA AGCATTTTAAAAAGAAAAGG-3´	5´-CCTTTTCTTTTTAAAATCGTTT TTAGGT GTCAGATTTTCCC-3´	68°C
PPARγ transcript variant 1	5'-GGCCCAGCGCACTCGGA-3'		60°C
PPARγ transcript variant 3	5'-GCTGGTGA CCAGAAGCCTGCAT-3'		60°C
PPARγ exon 1		5'-GGCCAGAA TGGCATCTCTGTGT-3'.	60°C
PPRE (EMSA) - IRD700-labeled	5'-CAAAACTA GGTCAA**AGGTCA**TCAA-3'	5'-TTGA**TGAC** **CT**TTGACCTAGTTTTG-3'	
PPRE mt (EMSA) - IRD700-labeled	5'-CAAAACTA GCACAAAGCACATCAA-3'	5'-TTGATGTG CTTTGTGCTAGTTTTG-3'	

NCoR (mouse)	5´-GCAAAAGT AGAGCAGCAGATCC-3´	5´-GGGCCAAG ACCTTCAAATATTT-3´	55°C
S82A (QuikChange)	5´-GTGGAGCCTGCA TCGCCACCTTATTATTCTGAG-3´	5´-CTCAGAATAATA AGGTGGCGATGCAGGCTCCAC-3´	68°C
TNFα (mouse)	5´-CCATTCCT GAGTTCTGCAAAGG-3´	5´-AAGTAGGA AGGCCTGAGATCTTATC-3´	55°C

4.1.7 Instruments and Software

Used instruments and software are listed in Table 4.7 and Table 4.8.

Table 4.7: Instruments

Instruments	Provider
AIDA Image Analyzer	Raytest GmbH (Straubenhardt)
Autoclave HV 85	BPW GmbH (Süssen)
AutoMACS™ Seperator	Miltenyi Biotec GmbH (Bergisch-Gladbach)
B250 Sonifier	Branson Ultrasonics (Danbury, USA)
Bacteria clean bench Hera guard	Heraeus GmbH (Hanau)
Bacteria incubator B5042	Heraeus GmbH (Hanau)
Bacteria incubator Innova®44	New Brunswick Scientific GmbH (Nürtingen)
CASY®	Schärfe System (Reutlingen)
Centrifuge 5415 R and 5810 R	Eppendorf GmbH (Hamburg)
Hera cell 150 (Lamina)	Thermo Fisher Scientific Inc. (Waltham, MA, USA)
LabLine Orbit Shaker	Uniequip GmbH (Martinsried)
Magnetic stirrer Combimag RCH	IKA Labortechnik GmbH & Co. KG (Staufen)
Mastercycler®	Eppendorf GmbH (Hamburg)
Mini-PROTEAN 3 System	Bio-Rad Laboratories GmbH (Munich)
Mithras LB940 multimode reader	Berthold Technologies (Bad Wildbad)
MyiQ iCycler system	Bio-Rad Laboratories GmbH (Munich)
NanoDrop ND-1000	Peqlab Biotechnologie GmbH (Erlangen)
Nucleofector	Amaxa AG (Cologne)
Odyssey infrared imaging system	Li-COR Biosciences GmbH (Bad Homburg)

Pure water system Purelab Plus	ELGA LabWater GmbH (Siershahn)
Sub-Cell® GT electrophoresis system	Bio-Rad Laboratories GmbH (Munich)
Thermomixer 5436	Eppendorf GmbH (Hamburg)
Trans-Blot SD blotting machine	Bio-Rad Laboratories GmbH (Munich)
Ultrasonic bath Sonorex	Bandelin electronic GmbH (Berlin)
UV-Transilluminator gel documentation system	Raytest GmbH (Straubenhardt)

Table 4.8: Software

Software	Provider
AIDA Image Analyzer	Raytest GmbH (Straubenhardt)
Clone Manager	SciEd Software (Cary, NC, USA)
MikroWin 2000	Berthold Technologies (Bad Wildbad)
MiQ	Bio-Rad Laboratories GmbH (Munich)

4.2 Methods

4.2.1 Cell biology

4.2.1.1 Cell culture

Jurkat T cells, RAW264.7 mouse macrophages, RAW264.7 dominant/negative (d/n) PPARγ macrophages and THP-1 human monocytes were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium. Media were supplemented with 10% heat-inactivated fetal calf serum (FCS), 5 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were kept in a humidified atmosphere of 5% CO_2 in air at 37°C and were transferred twice a week. Cell numbers were determined using the cell counter system Casy®.

4.2.1.2 Isolation and culture of human monocytes

Human monocytes were isolated from buffy coats (obtained from DRK-Blutspendedienst Baden-Württemberg-Hessen, Institut für Transfusionsmedizin und Immunhämatologie Frankfurt am Main, Frankfurt) using Ficoll-Hypaque gradients as described previously (158). In brief, 50 ml Leukosep® tubes were layered with 15 ml

lymphocyte separation media, blood cells were added, followed by density gradient centrifugation (440 x g, 35 min, RT). PBMCs were collected, washed twice with leukocyte washing buffer and were allowed to adhere to culture dishes (Primaria 3072, BD Becton Dickinson GmbH, Heidelberg) for 1 h at 37°C. Non-adherent cells were removed. Monocytes were then differentiated into macrophages with RPMI 1640 containing 10% AB-positive human serum, 5 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin for 7 days.

4.2.1.3 Isolation of murine CD11b+ splenocytes and differentiation into macrophages

All isolation steps were performed at 4°C or on ice, respectively. The spleen was placed into PBS in a 6 well-plate and homogenized between the frosted ends of two glass slides, which were sterilized with ethanol before. The homogenized spleen (in PBS) was passed through a 70 µm Filcon syringe to generate a single-cell suspension and transferred into a 15 ml-Falcon tube. The filtered suspension was incubated with 5 ml erythrocyte lysis buffer (see Appendix) for 5 min and centrifuged at 500 x g for 5 min. Splenocytes were washed with PBS and cell number was determined using Casy®. Up to $1*10^8$ splenocytes were used for magnetic cell sorting (see 4.2.1.4). For differentiation of the $CD11b^+$ splenocytes into macrophages, cells were cultured in the presence of 25 ng/ml macrophage-colony stimulating factor (M-CSF) for 5 d (159) in RPMI 1640 supplemented with 10% FCS, non-essential amino acids, pyruvate, 5 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin.

4.2.1.4 Magnetic cell sorting

$CD11b^+$ splenocytes were sorted using the autoMACS separator. According to the manufacturer's protocol, $1*10^8$ cells were washed with PBS and suspended in 800 µl (80 µl/$1*10^7$ cells) leukocyte running buffer (see Appendix). For magnetic labelling 200 µl (20 µl/$1*10^7$ cells) CD11b microbeads were added and incubated on ice for 20 min. Cells were washed with 1 ml leukocyte running buffer, centrifuge at 300 x g for 10 min and re-suspended in 500 µl running buffer. Cells were sorted using the autoMACS program "positive selection" (<possel>). The eluted fraction was centrifuged at 300 x g for 10 min and supernatant was discarded. Then, cells were re-suspended in the appropriate amount of medium and seeded on 6 well-plates.

4.2.1.5 Differentiation of THP-1 monocytes

For differentiation of THP-1 monocytes into macrophages, cells were seeded in the appropriate cell number and treated with 50 nM TPA over night. The next day the medium was changed and cells were cultured for another 24 h in fresh medium prior to experiments.

4.2.1.6 Generation of apoptotic Jurkat cells

To generate apoptotic Jurkat cells, they were cultured in FCS-free medium and stimulated with 0.5 µg/ml staurosporine for 3 h provoking roughly 80% apoptotic cell death as described previously (99). Afterwards cells were washed twice with medium to remove staurosporine. For co-culture, AC were re-suspended in FCS-containing RPMI 1640 medium and added to macrophages at a ratio of 5:1. Following experiments but prior to sample preparation non-ingested AC were removed and macrophages washed twice with PBS excluding variations on results by AC as described previously (128).

4.2.1.7 Transient transfection

Transient transfection with several over-expression vectors and reporter plasmids was performed using the JetPEI™ transfection reagent. For reporter analysis $1*10^5$ THP-1 cells were seeded in 24-well plates and differentiated with 50 nM TPA over night. Medium was changed the next day and cells were transfected after another 24 h. For transfection of RAW264.7 macrophages, $5*10^4$ cells per well were seeded in 24-well plates and also transfected 24 h later. Both cell lines were transfected with 1 µg DNA as described by the manufacturer. In brief, 1 µg DNA and 2 µl JetPEI™ transfection reagent were mixed each with 25 µl 150 mM NaCl per well and vortexed briefly. The JetPEI mixture was added to the DNA mixture, vortexed, spinned down and incubated for 30 min at room temperature (RT). Afterwards 50 µl/well of the mixture were added to the cells, incubated for 4 h and then cultured in fresh medium for another 24 h prior to experiments.

Transient transfection of siRNA was performed using the Nucleofector® technology from Amaxa biosystems. According to the manufacturer's protocol, $2*10^6$ RAW264.7 macrophages or $1.5*10^6$ primary human macrophages, which were detached from plates by incubating them with 1 x trypsin-EDTA for 2 h, were centrifuged for 10 min at

90 x g and re-suspended in 100 µl Nucleofector® Solution (Nucleofector® solution V for RAW264.7 and Human Macrophage Nucleofector® Solution for primary human macrophages). After addition of 3 µg siRNA, 300 pmol miR-inhibitors or 20 pmol of the miR-27 mimic respectively, cells were electroporized using program "D-032" for RAW264.7 or "Y-010" for primary human macrophages. Transfected cells were immediately transferred in pre-warmed medium, seeded in 3 or 6 cm plates an cultured for another 48 h.

4.2.2 Biochemistry

4.2.2.1 Protein determination (Lowry)

The protein content of cell lysates was determined using the DC Protein Assay Kit, based on the Lowry method (160). Briefly, a standard dilution series of bovine serum albumin (BSA) in protein lysis buffer (see Appendix) was prepared (0.625 to 10 mg/ml). 2 µl of the samples as well as of the standard dilution were pipetted in duplicates into a 96-well plate, 20 µl solution A were added, and then the colorimetric reaction was started by adding 160 µl of solution B. After incubation for 15 min (RT with shaking), extinction was measured at 750 nm using the Mithras LB 940 multimode reader.

4.2.2.2 Nuclear protein extraction

3 x 10^6 RAW264.7 macrophages were seeded in 10 cm plates and stimulated with AC for 3 h the next day. Cells were washed with ice-cold PBS and lysed with 200 µl ice cold hypotonic cell lysis buffer (see Appendix) for 10 min on ice. Nuclei were sedimented by centrifugation at 12,000 x g for 1 min and cytosolic fraction was transferred in a new tube. Subsequently, sedimented nuclei were lysed with 50 µl nuclear lysis buffer (see Appendix), incubated for 30 min on ice and centrifuged at 12,000 x g for 10 min. Supernatant was transferred in a new tube and protein content was determined using Lowry method (see 4.2.2.1).

4.2.2.3 SDS-PAGE/Western blot (WB) analysis

For Western analysis, 2-4*10^6 cells were treated as indicated, scraped off, lysed with 200 µl protein lysis buffer and sonicated for 8 x 1 s. Subsequently, lysates were incubated on ice for 30 min and vortexed every 5 min. After centrifugation (4°C, 16,000 x g), supernatants were transferred into a new tube. Protein concentrations

were determined as described above (see 4.2.2.1). 80 µg of protein were mixed with 4 x SDS (sodium dodecyl sulfate) sample buffer and denatured at 95°C for 5 min. Proteins were separated on 10% SDS-polyacrylamide gels using 1 x SDS-running buffer and the Mini-PROTEAN 3 system Proteins were transferred onto a nitrocellulose membrane by semi-dry blotting. To prohibit unspecific binding, membranes were blocked with 5% milk/TTBS for 1 h at RT. Afterwards membranes were incubated with antibodies in 5% milk/TTBS at 4°C over night at indicated concentrations (see Table 4.3). For protein detection, membrane was washed 3 times with TTBS for 7 min and incubated with IRDye secondary antibodies (anti-rabbit or anti-mouse; 1:10,000) in 5% milk/TTBS for 1 h at RT. The membrane was washed again 3 times for 7 min and proteins were detected and densitometrically analysed using the Odyssey infrared imaging system. Buffers are described in the appendix.

4.2.3 Molecular biology

4.2.3.1 Reporter assay

All reporter assays were performed in duplicate. For reporter analysis cells were re-suspended in 60 µl reporter lysis buffer (see Appendix) and incubated for 15 min under shaking at RT. Lysates were cleared by centrifugation at 4,000 x g for 5 min. Reporter activity was measured using a Mithras LB 940 multimode reader. Therefore, 20 µl of the lysate were transferred in a 96-well plate, 50 µl reporter assay reagent were added automatically, plates were shaked for 2 s, and each well measured for 10 s.

Luciferase activity was normalized to protein concentration of each sample. To control transfection efficiency concerning over-expression of the different pDsRed-PPARγ1 constructs, mRNA levels of DsRed were determined by quantitative PCR.

4.2.3.2 Chromatin immunoprecipitation (ChIP) assay

$3*10^6$ RAW264.7 cells were seeded in 10 cm plates and cultured over night. Before cross-linking, cells were pre-treated with AC (90 min) followed by 1 µg/ml LPS (60 min) afterwards. ChIP assays were performed as described by Nelson *et al.* (161). In brief, cells were cross-linked by adding 45 µl 37 % formaldehyde/ml medium, shaked at 200 rpm for 5 min at RT. Afterwards, 80 µl 1 M glycine/ml medium was added and incubated for another 5 min with shaking. Cells were scraped off, washed with PBS and re-suspended in 1 ml IP buffer directly followed by centrifugation for 1 min at 4°C and

12,000 x g. Sedimented nuclei were re-suspended in 400 µl IP buffer and incubated for 15 min on ice. To shredder genomic DNA, glass beads were added to the lysates and sonicated thrice with 15 x 8 pulses. Probes from 2-3 plates were combined before precipitation. To determine DNA fragmentation and for input control, DNA was extracted and analysed by agarose gel electrophoresis.

NCoR was precipitated using 1 µg of anti-NCoR from Affinity Bioreagents (Golden, CO, USA), an established ChIP assay antibody (77). For mock-IP, lysates were incubated with 1 µg of normal rabbit IgG from Millipore/Upstate (Billerica, MA, USA). 120 µl of each lysate were incubated with antibody or IgG for 15 min at 4°C in an ultrasonic bath and cleared afterwards by centrifugation (12,000 x g, 10 min, 4°C). Subsequently, 100 µl of each probe were transferred to a new tube, 30 µl protein G agarose was added and incubated for 1-2 h. To wash away non-bound proteins, agarose beads were washed 5 times with 1 ml IP buffer and following centrifugation for 1 min at 2,000 x g. Afterwards, 100 µl 10% Chelex 100 were added, briefly vortexed and boiled at 99°C for 10 min. To digest proteins, 1 µl of proteinase K (20 µg/µl) was added and incubated at 37°C for 1 h followed by another 10 min at 99°C for another 10 min. Tubes were then centrifuged for 1 min at 12,000 x g. To finally isolate the DNA, 100 µl distilled H_2O were added, solution was vortexed for 10 s and centrifuged at 12,000 x g for 1 min. Approximately 80 µl of the DNA-containing supernatant were transferred in a new tube and isolation step was repeated, resulting in a final volume of 160 µl DNA. A 211 bp fragment of the TNFα promoter, spanning an established κB-RE, was amplified using up to 5 µl of DNA and the ChIP-TNFα primer (see Table 4.6). For input controls, 15% DNA of each probe was used. Resulting fragments were separated at a 1.5% agarose gel in 0.5 x TBE. Finally, gels were stained in an ethidium bromide solution (0.5 mg/l) for 20 min, destained in H_2O and visualized by UV excitation.

4.2.3.3 RNA isolation

Total RNA was isolated using a PeqGold RNAPure™ Kit as described by the manufacturer. For mRNA isolation of adherent cells, 1 ml of PeqGold RNAPure™ per 1-5*10^6 cells was added to the plate, lysed for 5 min and transferred to a 1.5 ml tube. After addition of 200 µl chloroform, samples were vortexed thoroughly and incubated at RT for another 5 min. Subsequently, samples were centrifuged at 12,000 x g for 5 min to separate the RNA containing water-phase from the phenol-phase and

interphase. To precipitate RNA, 0.5 ml isopropanol were added to the collected water-phase, incubated for 15 min at RT and centrifuged (12,000 x g, 15 min, 4°C). Sedimented RNA was washed twice with 75% ethanol in DEPC-treated H_2O, dried and finally solved in 10-50 µl DEPC-treated distilled H_2O by incubation at 56°C for 20 min. RNA concentration was determined using optical density (OD) at 260 nm. An OD_{260} of 1 is equivalent to a RNA concentration of 40 µg/ml.

4.2.3.4 Reverse transcription (RT)

Reverse transcription for determination of RNA content was performed according to the provided manual using iScript™ cDNA Synthesis Kit. In brief, 1 µg of isolated mRNA were mixed with 4 µl 5 x reaction buffer, 1 µl iScript reverse transcriptase and filled up with nuclease-free H_2O to 20 µl. The reaction mix was incubated for 5 min at 25°C, 30 min at 42°C and finally 5 min at 85°C for inactivation of the enzyme. The resulting cDNA was diluted 1:5.

To analyze miRNA expression, RNA was transcribed using the miScript Reverse Transcription Kit. Therefore, 1 µg of RNA were mixed with 4 µl 5x reaction buffer, 1 µl of miScript Reverse Transcriptase Mix and filled up with RNAse-free water to 20 µl. The mixture was incubated at 37°C for 60 min followed by an inactivation step for 5 min at 95°C. The resulting cDNA was diluted 1:2.

4.2.3.5 Polymerase chain reaction (PCR)

For amplification of DNA, conventional PCR was performed using recombinant *Taq* DNA polymerase according to the manufacturer's protocol. Briefly, 3 µl forward and reverse primer (5 µM) each, 1 µl dNTP mix (10 µM each), 1.5 µl $MgCl_2$, 5 µl 10 x reaction buffer were mixed with template DNA, filled up with distilled H_2O to 49.5 µl and finally 0.5 µl *Taq* polymerase were added. PCRs were performed according to the following profile, while annealing temperature was dependent on the used primer pairs.

	Initial denaturation	95°C	1 min
30-35 cycles	Denaturation	95°C	50 s
	Annealing	50-65°C	30 s
	Extension	72°C	90 s
	Final extension	72°C	7 min

4.2.3.6 Quantitative PCR (qPCR)

For quantitative PCR (qPCR), 2-4 µl of cDNA (see 4.2.3.4) were used and mixed with either 2 µl QuantiTect Primer Assay or 0.5 µl forward and reverse primer (5 µM) each, 10 µl Absolute™ qPCR SYBR ® Green Fluorescein Mix and filled up with distilled H_2O to 20 µl. Determination of miRNA expression was performed using miScript SYBR Green and additionally 2 µl of 10 x Universal Primer. The mixtures were transferred to a 96-well plate, briefly spinned down and the plate sealed with an optical adhesive seal sheet. QPCR was performed using the MyiQ Single-Colour Real-time PCR Detection System and the following thermal cycling program.

	Enzyme activation	95°C	15 min
35-45 cycles	Denaturation	95°C	15 s
	Annealing	55-60°C	30 s
	Extension	72°C	30 s

To confirm the specificity of the reaction, a melt curve was created using the following program:

	Denaturation	95°C	30 s
	Starting temperature	60°C	30 s
80 cycles	Melting step	60°C	10 s
		+ 0.5°C per cycle	

4.2.3.7 Electrophoretic mobility shift assay (EMSA)

EMSA was performed as previously described (128). 10 µg nuclear protein (see 4.2.2.2) were incubated with 2 µg poly(dIdC), 2 µl buffer D, 4 µl buffer F (see Appendix), and 250 fmol 5'-IRD700-labelled oligonucleotide in a final volume of 20 µl and incubated for 30 min at RT. DNA-protein complexes were resolved at 80 V for approximately 1 h using native 4% polyacrylamide gels and visualized with Odyssey infrared imaging system.

4.2.3.8 Construction of the pGL3-PPARγ-3´-UTR

To assess the impact of PPARγ mRNA stability, its 3´-UTR was cloned into the pGL3-control vector showing constitutive luciferase expression. Therefore, the 3´-UTR was

amplified by PCR and introduced downstream of the luciferase encoding region of the pGL3-control vector using In-Fusion™ Dry-Down PCR cloning Kit. The method is based on a recombination process performed by the In-Fusion enzyme.

Amplification of the insert

The 3´-UTR was amplified by PCR (see 4.2.3.5) using the primer pair PPARγ-UTR listed in Table 4.6. The primer pair contains 15 bp extensions on both sides, which are complementary to the flanking regions of the *XbaI* restriction site of the vector. The amplification was performed using 5 µl of cDNA from differentiated THP-1 cells. The fragment size and DNA content was verified by agarose gel electrophoresis (1.5% agarose in 0.5 x TBE).

Insertion of the PCR fragment into the vector

The vector pGL3-control was transformed in SCS110 bacteria to generate unmethylated DNA, which was linearized with the restriction enzyme *XbaI*. Therefore 1 µg of unmethylated pGL3-control were mixed with 10 U of *XbaI*, 2.5 µl 10 x reaction buffer (NEBuffer 2), filled up to 25 µl with distilled H_2O and incubated at 37°C for 1 h. For inactivation of the endonuclease, incubation was continued at 65°C for 20 min. Subsequently, linearization of the vector was checked by agarose gel electrophoresis (1% agarose in 0.5 x TBE). Finally, the pellet of the In-Fusion™ Dry-Down PCR cloning Kit was solved with 10 µl distilled H_2O containing 1 µl of the restriction probe and 2 µl of PCR probe and incubated at 42°C for 30 min. Subsequently, 1 µl of the solution was transformed into XL-1 Blue competent bacteria by heat shock method (see 4.2.4.1). From potential positive clones plasmids were extracted and digested with *Bpn10I* and *NcoI* to check insertion of the UTR fragment. Correct insertion was verified by sequencing (Agowa GmbH, Berlin).

4.2.3.9 Site directed mutagenesis for generation of point mutations or deletions

Site-directed mutagenesis for generation of point mutations or deletions was performed using the QuikChange XL II site-directed mutagenesis kit. For point mutation of K77 and S82 within PPARγ1, pDsRed-PPARγ1 was used as a template. For deletion of the miR-27 binding site within the PPARγ-3'-UTR, pGL3-PPARγ-3'-UTR provided the basis. Oligonucleotides were designed according to the manufacturer's specifications and listed in Table 4.6. 10 ng of the template DNA, 125 ng of each

primer, 5 µl 10 x reaction buffer, 1 µl dNTP mix, 3 µl QuikSolution reagent and 1 µl PfuUltra HF DNA polymerase were mixed together and filled up with distilled H_2O to 50 µl. An initial denaturation step was performed at 95°C for 1 min, followed by 18 cycles of denaturation at 95°C for 50 s and annealing and extension at 68°C for 8 min. A final extension phase was performed at 68°C for 10 min. Next, the non-mutated parental DNA template was digested with 10 U *DpnI* for 2 h at 37°C and plasmids were transformed into XL10-Gold ultracompetent bacteria using the heat shock method (see 4.2.4.1). From potential positive clones plasmids were prepared and verified by digestion and finally sequencing (Agowa GmbH, Berlin).

4.2.4 Microbiology

4.2.4.1 Transformation of bacteria by heat-shock

Bacteria were transformed with plasmid DNA by heat-shock. Therefore, 50 µl of bacteria glycerol stocks were thawed on ice, 50 ng plasmid DNA were added and incubated for 30 min on ice. After a heat-shock for 45 s at 42°C, bacteria were incubated for another 2 min on ice. For initial growth, 450 µl of SOC medium (see Appendix) were added followed by an incubation period for 45 min at 37°C with shaking at 250 rpm. Depending on the prior experiment, 100-500 µl were inoculated on a LB agar plate containing the appropriate antibiotic (100 µg/ml ampicillin or 50 µg/ml kanamycin) and incubated over night at 37°C to select positive, plasmid carrying bacteria clones.

4.2.4.2 Bacterial culture and plasmid preparation

For preparation of plasmids a single clone from the LB agar plate was picked, transferred into 3 ml LB medium with the appropriate antibiotic and cultured over night at 37°C with shaking (250 rpm). The next day, the culture was transferred into 200 ml LB medium containing the appropriate antibiotic and again shaked over night at 37°C. Isolation of plasmids was performed according to the manufacturer's protocol using the HiSpeed Plasmid Maxi Kit. DNA content was measured with the NanoDrop ND-1000.

4.2.5 Statistical analysis

Each experiment was performed at least three times and statistical analysis was done either with one- or two-way-ANOVA modified with Bonferroni's multiple comparison test or unpaired and paired Student's t-test, respectively. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

5 Results

5.1 PPARγ contributes to macrophage polarization towards an anti-inflammatory phenotype in response to AC

AC shift macrophages towards an anti-inflammatory phenotype, which is characterized by the expression of anti-inflammatory mediators and a reduced production of pro-inflammatory mediators. This reduction is mainly due to an inhibited NFκB transactivation, whereas the underlying mechanism remains elusive. PPARγ attenuates ROS formation in response to AC and is in general known for its anti-inflammatory properties especially by inhibition of NFκB. Therefore, I hypothesized a PPARγ-dependent inhibition of NFκB and concomitantly attenuated cytokine expression in response to AC.

5.1.1 Activation of PPARγ in response to AC

Since AC were described to activate PPARγ (128), I first analyzed PPARγ activation in response to AC by EMSA and reporter assay in RAW264.7 macrophages. To identify the specific PPRE band, a mutated PPRE oligonucleotide was used.

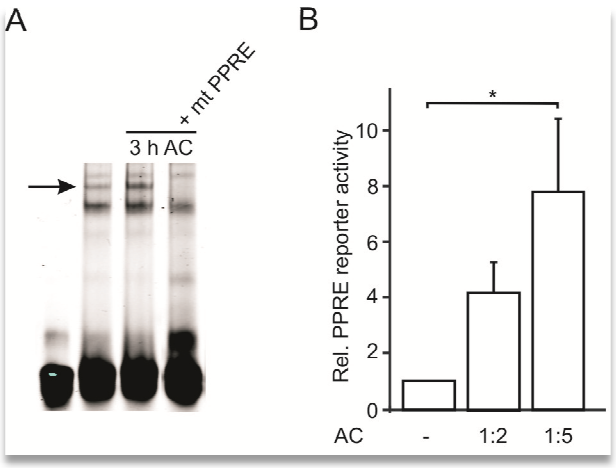

Figure 5.1 Transactivation of PPARγ in response to AC.
(A) RAW264.7 macrophages were co-cultured with AC (1:5) for 3 h and transactivation of PPARγ was analyzed by EMSA. The arrow marks the PPRE band identified by using a mutated oligonucleotide (mt PPRE). (B) RAW264.7 macrophages were incubated with AC for 5 h in the indicated ratios and PPRE luciferase activity was measured. Controls were set to 1. Columns present mean values ± SE (n ≥ 3). Statistical analysis was performed using one-way-ANOVA.

In line with previous reports (128), co-culturing RAW264.7 macrophages with AC for 3 h increased protein binding to the PPRE consensus sequence in EMSA (Figure 5.1 A), suggesting transactivation of PPAR in response to AC. Activation of PPARγ by AC was further analyzed by reporter assay. Therefore, RAW264.7 macrophages were transfected with the PPRE reporter plasmid pAOX-TK and stimulated with AC for 5 h in the indicated ratios the following day. AC increased reporter activity up to 8-fold in a concentration-dependent manner (Figure 5.1 B), confirming AC-provoked PPAR activation.

To address reports, that RAW264.7 macrophages do not express PPARγ (162) and also to exclude an altered protein expression responsible for the increased PPARγ activity, protein expression levels were determined by Western analysis. Therefore, RAW264.7 macrophages were incubated with AC for 90 min following LPS (1 µg/ml) treatment for another 60 min. The RAW264.7 clone I used, expressed PPARγ and treating macrophages with AC and/or LPS, did not alter PPARγ protein expression (Figure 5.2). It should be noted, that LPS treatment for 1 h did not change basal PPARγ expression as well (Figure 5.2).

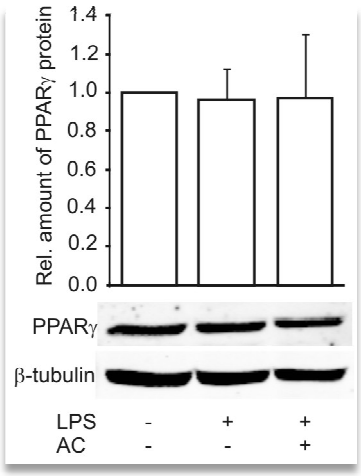

Figure 5.2 Unaltered PPARγ expression in response to AC.
Macrophages were co-incubated with AC for 90 min and treated with 1 µg/ml LPS for 60 min afterwards. Protein expression was analyzed by Western blot. Columns present mean values ± SE and the blot is representative of three independent experiments. Statistics were analyzed with one-way-ANOVA modified with Bonferroni's multiple comparison test.

5.1.2 PPARγ attenuates NFκB transactivation and target gene expression

As Cvetanovic *et al.* observed attenuated NFκB activity in response to AC, I repeated experiments in my system. Therefore, I performed NFκB reporter assay in RAW264.7 cells. Pre-incubating macrophages with AC for different time periods and following LPS treatment for 5 h revealed that co-culture of AC with macrophages for ≥ 90 min reduced NFκB reporter activity to approximately 50% (Figure 5.3). Removing AC after 30 min and following LPS exposure for 5 h did not affect LPS-induced NFκB activity, whereas co-culturing AC and macrophages for 30 min, followed by LPS stimulation without removing non-ingested cells reduced luciferase activity to approximately 75% (Figure 5.3). A constant interaction without removing cells seems to be necessary for inhibition. Since 90 min pre-incubation with AC revealed the most significant inhibitory effect on NFκB transactivation, further experiments were performed using this experimental set up.

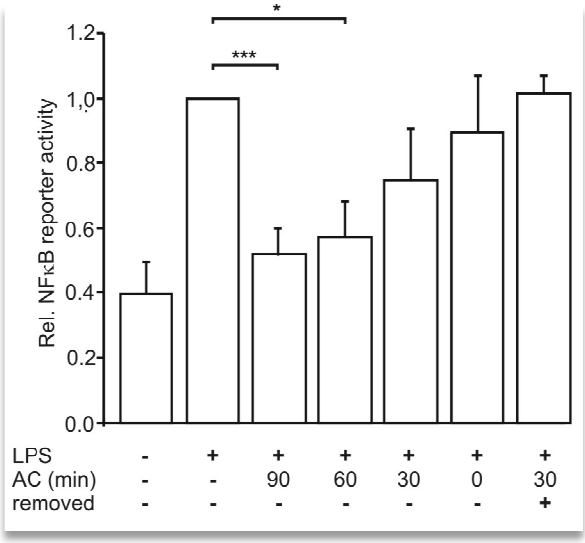

Figure 5.3 Time-dependent inhibition of NFκB activity in response to AC.
NFκB reporter activity was measured after co-culturing RAW264.7 macrophages with AC for the indicated time periods and following LPS treatment (1 µg/ml) for 5 h. Non-ingested cells were removed as indicated. Data are mean values ± SE (n ≥ 3) and statistics were analyzed using one-way-ANOVA.

Addressing the question whether PPARγ blocks NFκB transactivation in response to AC, I performed NFκB reporter assays in RAW264.7 macrophages compared to RAW264.7 macrophages expressing a d/n PPARγ mutant (128). The mutant is characterized by two amino acid substitutions (L466A/E469A), which impair ligand-dependent PPARγ

transactivation and interaction with co-activators such as p300 (163). Macrophages were co-incubated with AC at a ratio of 1:5 for 90 min and then stimulated with 1 µg/ml LPS for 5 h. LPS stimulation caused an approximately 2-3-fold induction of reporter activity compared to resting cells (Figure 5.4 A). Following the interaction with AC, NFκB-dependent transactivation in RAW264.7 macrophages was reduced by roughly 50% compared to LPS stimulation (Figure 5.4 A).

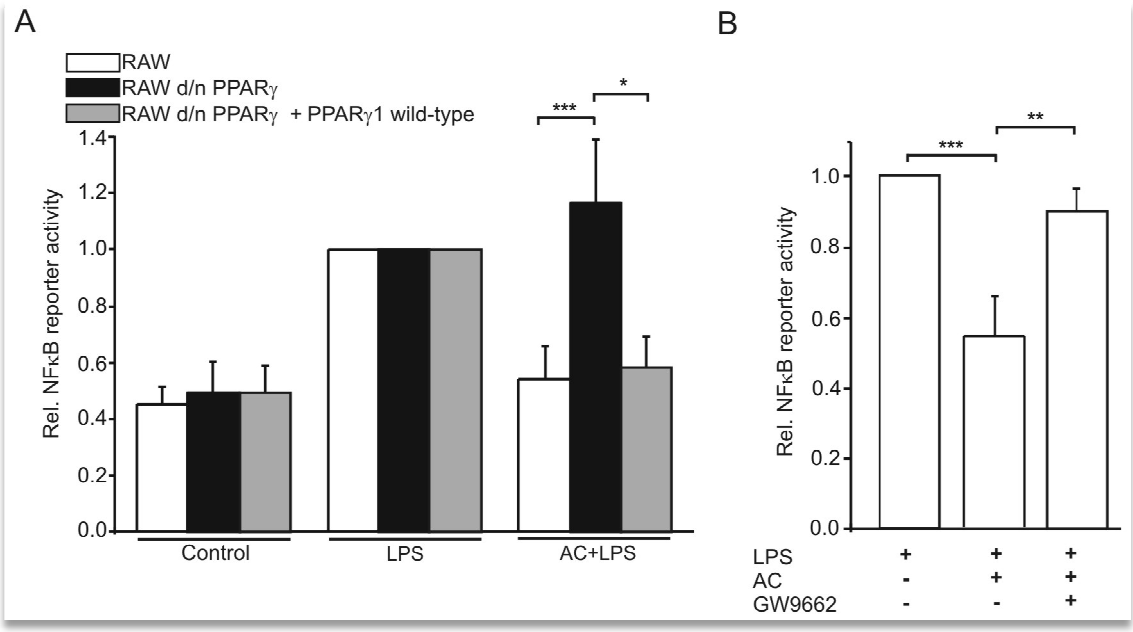

Figure 5.4 NFκB reporter activity is restored in RAW264.7 d/n PPARγ macrophages.
Cells were incubated for 90 min with AC (ratio 1:5), followed by stimulation with LPS (1 µg/ml, 5 h) in (A) RAW264.7, RAW264.7 d/n PPARγ macrophages and RAW264.7 d/n PPARγ cells over-expressing PPARγ1 wild-type and in (B) RAW264.7 macrophages pre-treated with 1 µM GW9662 for 3 h. NFκB reporter activity in control macrophages, stimulated with LPS alone was set to 1. Statistics in (A) were analyzed with two-way-ANOVA and in (B) with one-way-ANOVA, both modified with Bonferroni's multiple comparison test. Each column represents mean values of duplicate determinations of a minimum of four independent experiments.

Inhibition was completely reversed in RAW264.7 d/n PPARγ expressing cells, suggesting a causative role of PPARγ in reducing NFκB activation. To prove the importance of PPARγ, I over-expressed PPARγ1 wild-type in RAW264.7 d/n PPARγ macrophages to restore its functionality. This was achieved by transfecting RAW264.7 d/n PPARγ macrophages with the NFκB reporter plasmid in combination with a PPARγ1 wild-type encoding vector.

As expected, over-expression of PPARγ1 wild-type in d/n PPARγ macrophages restored the inhibitory potential of AC on NFκB transactivation, which was comparable regarding to control cells (Figure 5.4 A).

To further strengthen the role of PPARγ, RAW264.7 cells were pre-stimulated for 3 h with 1 µM GW9662, a specific PPARγ antagonist (164). Thereafter, macrophages were exposed to AC followed by LPS stimulation as described above. GW9662 completely abrogated the ability of AC to attenuate NFκB reporter activity (Figure 5.4 B), suggesting a PPARγ-dependent blocking of NFκB transactivation in response to AC.

To elucidate the functional consequence of NFκB inhibition, I analyzed the expression of pro-inflammatory, established NFκB target genes such as TNFα (165) and IL-6 (166) in macrophages treated with AC and LPS. Therefore, I determined IL-6 and TNFα mRNA amount by qPCR in RAW264.7 and RAW264.7 d/n PPARγ macrophages. Cells were co-incubated with AC for 90 min and treated with 1 µg/ml LPS for 3 h afterwards. This time period was chosen, because mRNA should appear earlier than luciferase protein.

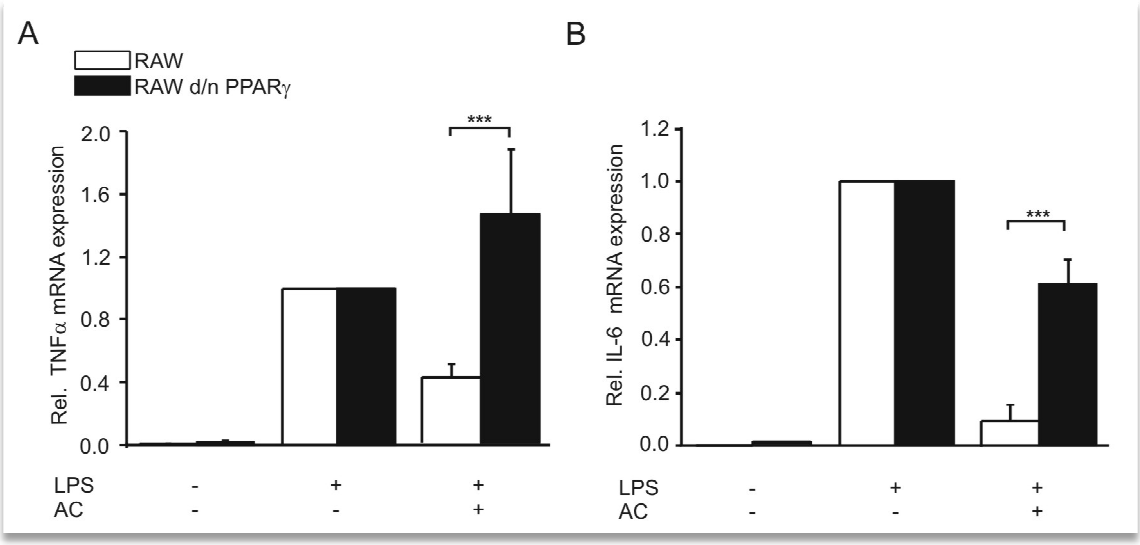

Figure 5.5 PPARγ-dependent reduction of cytokine expression.

(A) TNFα and (B) IL-6 mRNA expression were measured by qPCR in RAW264.7 and RAW264.7 d/n PPARγ cells. Macrophages were co-incubated with AC for 90 min (ratio 1:5) and treated with 1 µg/ml LPS for 3 h afterwards. As a control, macrophages were stimulated with LPS alone and relative mRNA expression was set to 1. Data were normalized to GAPDH mRNA levels. Columns present mean values ± SE (n ≥ 5) and statistics were analyzed with two-way-ANOVA modified with Bonferroni's multiple comparison test.

In response to LPS, mRNA expression of TNFα and IL-6 was at least 50-fold increased compared to unstimulated cells and this response was set to a relative mRNA increase of 1 (Figure 5.5). Recognition of AC by RAW264.7 macrophages prior to LPS stimulation reduced TNFα expression by roughly 60% (Figure 5.5 A), while IL-6 mRNA expression was diminished by 90% (Figure 5.5 B).

To substantiate a role of PPARγ, experiments were also performed in RAW264.7 d/n PPARγ macrophages. Pre-treating cells with AC, followed by LPS stimulation restored TNFα expression (Figure 5.5 A) and largely reversed suppressed formation of IL-6 (Figure 5.5 B), thus underscoring the impact of PPARγ on NFκB target gene expression.

To verify the physiological significance of PPARγ during the anti-inflammatory response, I analyzed TNFα mRNA levels in primary murine macrophages from PPARγ$^{fl/fl}$ (Control) and myeloid lineage-specific conditional PPARγ knock-out mice (MΦ-PPARγ KO). Enriched CD11b^{+} splenocytes were differentiated with 25 ng M-CSF for 5 d. Knock-out of PPARγ was proven by quantifying PPARγ-exon 2 mRNA amount, which was reduced in PPARγ-deficient macrophages by 90% (Figure 5.6 A). Following their differentiation, macrophages were treated with AC and LPS as described before.

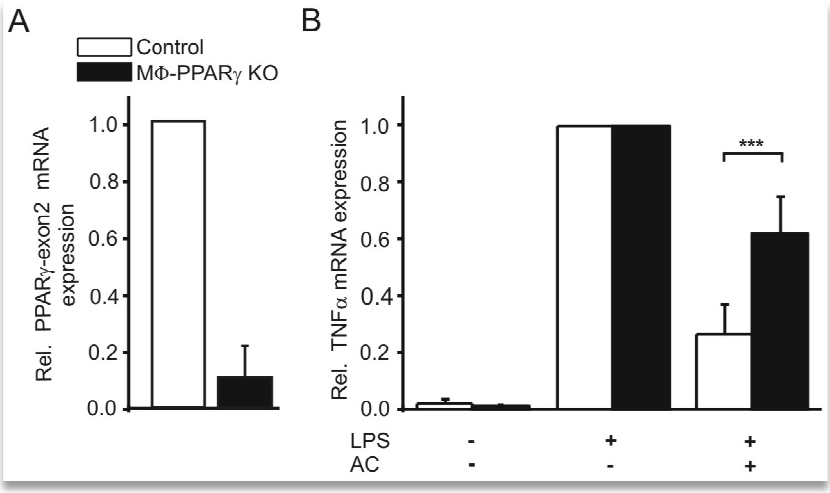

Figure 5.6 PPARγ-deficient macrophages impaired attenuated TNFα mRNA expression.
(A) PPARγ-exon 2 mRNA expression and (B) TNFα mRNA expression in primary murine macrophages from PPARγ$^{fl/fl}$ (Control) and conditional PPARγ knock-out mice (MΦ-PPARγ KO) were measured by qPCR. Cells were stimulated with AC and LPS as before. Data are mean values ± SE (n = 5) and statistics were analyzed with two-way-ANOVA modified with Bonferroni's multiple comparison test.

Recognition of AC diminished LPS-induced TNFα expression by roughly 80%. This reduction was significantly mitigated in PPARγ knock-out macrophages (Figure 5.6 A), giving evidence for a PPARγ-dependent anti-inflammatory phenotype switch in response to AC.

5.1.3 Identification of PPARγ domains required for NFκB inhibition

Next, I was interested in elucidating the underlying molecular mechanism. This was accomplished by NFκB reporter assays in RAW264.7 d/n PPARγ macrophages over-expressing various deletion constructs of DsRed-tagged PPARγ1, that were previously verified for their expression by Western analysis (81 and unpublished data). The DsRed-PPARγ1 wild-type encoding vector was included as a control. As expected, over-expression of DsRed-PPARγ1 wild-type restored NFκB inhibition in response to AC in comparison to RAW264.7 d/n PPARγ macrophages (Figure 5.7).

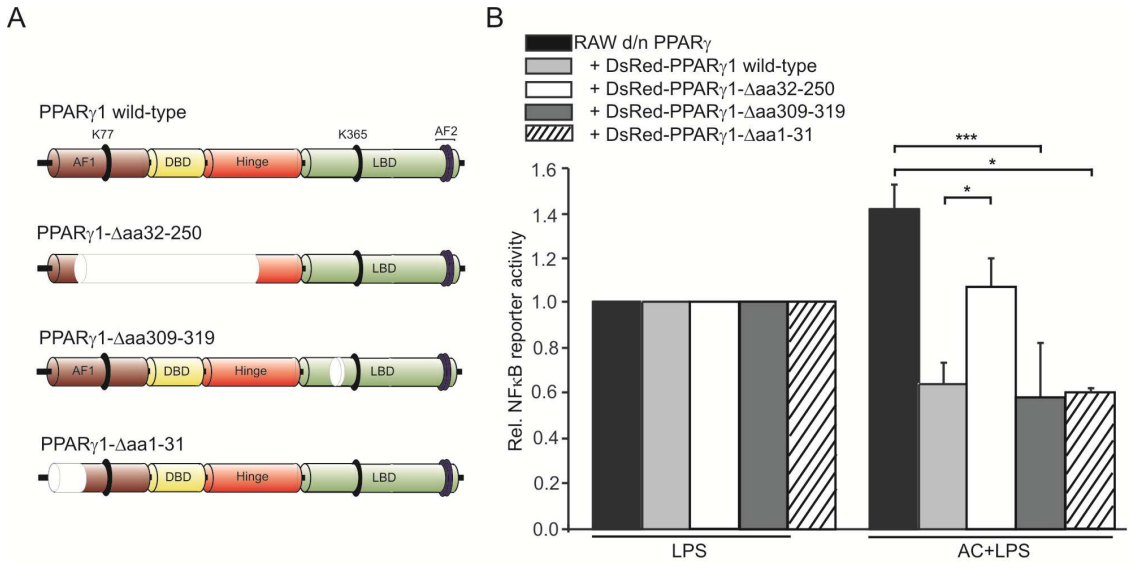

Figure 5.7 Domain analysis of PPARγ.
(A) Scheme of the used deletion constructs. (B) NFκB reporter activity was measured in RAW264.7 d/n PPARγ cells over-expressing deletion constructs of pDsRed-PPARγ1 as indicated. Cells were co-transfected with pNFκB-Luc. Reporter activity was measured after co-incubation with AC for 90 min followed by stimulation with 1 µg/ml LPS for 5 h. As a control, cells were stimulated with LPS alone and values set to 1. Columns present mean values ± SE, n ≥ 3. Statistics were analyzed with two-way-ANOVA modified with Bonferroni's multiple comparison test.

Over-expression of DsRed-PPARγ1-Δaa32-250 failed to restore the ability of AC to inhibit NFκB transactivation, comparable to the situation seen in RAW264.7 d/n PPARγ

cells. Amino acids 32-250 of PPARγ span a region of the ligand-independent activation domain AF1, the DNA binding domain and a part of the hinge domain. From this results I concluded that besides the AF2 domain, responsible for ligand-binding, amino acids within the region 32-250 are required for NFκB inhibition.

Next I observed that over-expression of DsRed-PPARγ1-Δaa309-319, a PPARγ deletion construct lacking a region that appears important for co-factor binding (167, 168), restored NFκB inhibition (Figure 5.7). These data suggest that amino acids 309-319, within the ligand-binding domain, are dispensable for blocking NFκB transactivation in response to AC. Moreover, deletion of the aa1-31 also failed to restore NFκB activity, assuming that they are not implicated in the AC-provoked repression, whereas amino acids 32-250 seem to play a role. Considering that SUMOylation of PPARγ and concomitant prevention of NCoR removal is a postulated mechanism for transrepression, I analyzed protein motives *in silico* and noticed a possible SUMOylation site (KXE) at K77 within the AF1 domain. Therefore, I reasoned that SUMOylation of PPARγ may contribute to NFκB inhibition.

5.1.4 SUMOylation of PPARγ prevents co-repressor removal

Preventing removal of NCoR from promoter regions of different pro-inflammatory genes such as iNOS has been described as a mechanism for PPARγ-mediated transrepression that occurs after SUMOylation of PPARγ (77). Among other proteins, HDAC3 is associated to the NCoR co-repressor complex and is crucial for transcriptional repression (169).

In a first approach, I inhibited HDAC3 by treatment with trichostatin A (TSA) to see whether the NCoR/HDAC3 complex might be involved in blocking NFκB activity. RAW264.7 macrophages were pre-treated with 10 nM TSA 1 h prior to AC addition, followed by LPS stimulation and subsequent determination of NFκB reporter activity. In the presence of TSA, NFκB was not any longer inhibited by AC, whereas TSA alone did not alter the LPS response (Figure 5.8).

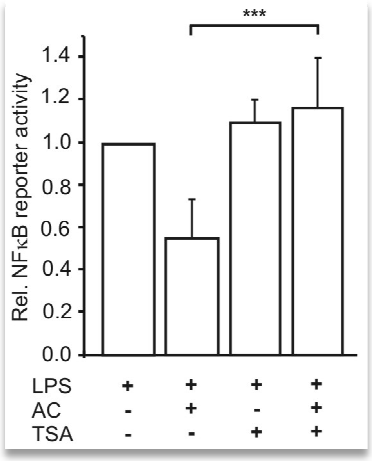

Figure 5.8 Trichostatin A reverses AC-provoked inhibition of NFκB transactivation.
NFκB reporter activity was measured in RAW264.7 macrophages after pre-treatment with 10 nM trichostatin A (TSA) for 1 h, followed by exposure to AC for 90 min and subsequent addition of 1 µg/ml LPS for 5 h. LPS-elicited values were set to 1. Columns present mean values ± SE, n = 4. Statistics were analyzed with one-way-ANOVA modified with Bonferroni's multiple comparison test.

Although this experiment suggests a HDAC-mediated NFκB inhibition, it remains unclear whether PPARγ is SUMOylated and concomitantly retains NCoR bound at the promoter. Taking into consideration that amino acids 32-250 are involved (Figure 5.7), I reasoned K77 to be SUMOylated.

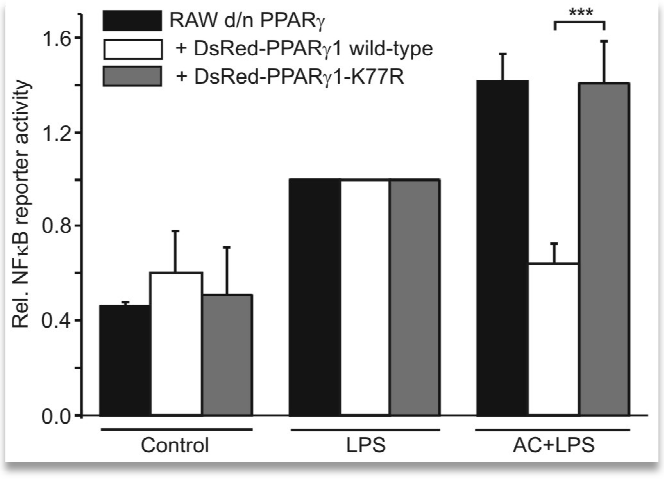

Figure 5.9 Interfering with SUMOylation of PPARγ restored NFκB inhibition.
NFκB reporter activity was measured in RAW264.7 d/n PPARγ cells over-expressing pDsRed-PPARγ1 wild-type or pDsRed-PPARγ1-K77R as indicated. Cells were co-transfected with pNFκB-Luc. Reporter activity was measured after co-incubation with AC for 90 min followed by stimulation with 1 µg/ml LPS for 5 h. As a control, cells were stimulated with LPS alone and values set to 1. Columns present mean values ± SE, n ≥ 4. Statistics were analyzed with two-way-ANOVA modified with Bonferroni's multiple comparison test.

To approach this possibility, I mutated the SUMOylation site (K77R) in the pDsRed-PPARγ1 wild-type encoding vector. Experimentally, I performed NFκB reporter assays in RAW264.7 d/n PPARγ macrophages and over-expressed DsRed-PPARγ1-K77R and DsRed-PPARγ1 wild-type. Along with my expectations, the K77R-mutated protein was unable to restore NFκB inhibition compared to DsRed-PPARγ1 wild-type protein (Figure 5.9).

Since PIAS1 mediates PPARγ SUMOylation (77), I knocked down PIAS1 by siRNA to abrogate PPARγ SUMOylation and analyzed its relevance in attenuating TNFα expression in response to AC. Two days after transfection, RAW264.7 cells were exposed to AC and LPS as described above and TNFα as well as PIAS1 mRNA levels were determined by qPCR. AC reduced LPS-induced TNFα formation in RAW264.7 macrophages transfected with control siRNA. In comparison, PIAS1 knock-down by approximately 50% at mRNA level (Figure 5.10 A) significantly reduced the ability of AC to attenuate TNFα mRNA expression (Figure 5.10 B).

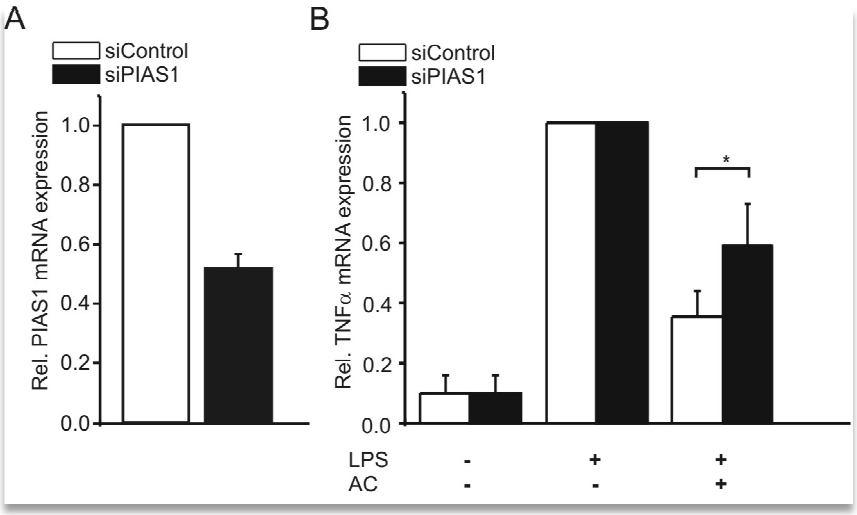

Figure 5.10 Impact of PIAS1 on TNFα expression.
Two days after transfection of RAW264.7 macrophages with PIAS1 siRNA or siControl, cells were co-incubated with AC for 90 min, followed by LPS stimulation (1 µg/ml LPS, 3 h). (A) PIAS1 and (B) TNFα mRNA levels were measured by qPCR, the LPS response was set to 1. Columns present mean values ± SE, n ≥ 3. Statistics were analyzed with two-way-ANOVA modified with Bonferroni's multiple comparison test.

Silencing PIAS1 did not affect LPS-induced TNFα expression compared to basal level, whereas lowering PPARγ SUMOylation by silencing PIAS1 attenuates an AC-provoked anti-inflammatory phenotype shift.

Since SUMOylation of PPARγ by PIAS1 was shown to prevent removal of the co-repressor NCoR from pro-inflammatory promoters (75, 77), I proved its impact by siRNA knock-down and analyzed TNFα mRNA expression. In comparison to cells transfected with control siRNA, knock-down of NCoR already reduced the extension of LPS-induced TNFα expression compared to basal level. In control siRNA transfected cells interaction with AC reduced LPS-induced TNFα expression by approximately 50%. Knock-down of NCoR by roughly 50%, as determined by qPCR (Figure 5.11 A), significantly reverted TNFα expression (Figure 5.11 B), arguing for the relevance of NCoR during inhibition of NFκB-dependent cytokine expression.

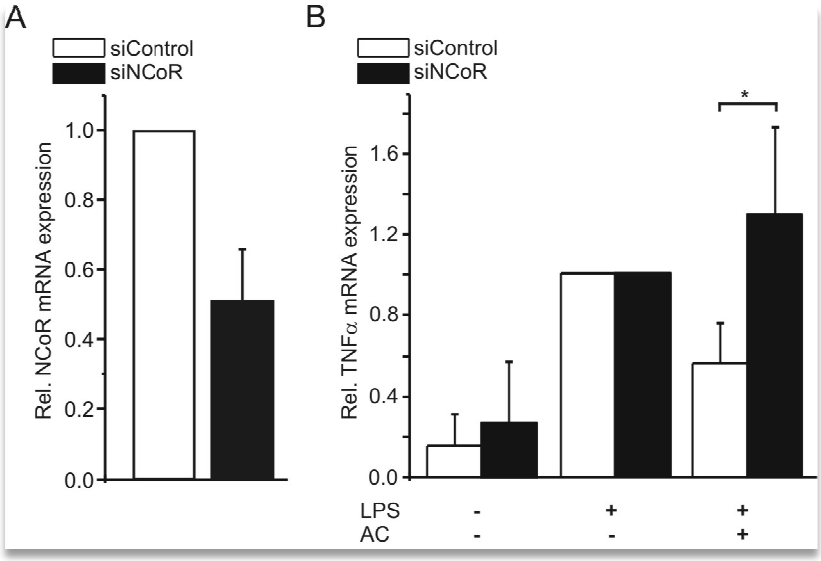

Figure 5.11 Impact of NCoR on TNFα expression.
Two days after transfection of RAW264.7 macrophages with NCoR siRNA or siControl, cells were co-incubated with AC for 90 min, followed by stimulation with 1 µg/ml LPS for 3 h. (A) NCoR and (B) TNFα mRNA levels were measured by qPCR, the LPS response was set to 1. Data are mean values ± SE, n ≥ 4. Statistics were analyzed with two-way-ANOVA modified with Bonferroni's multiple comparison test.

To finally verify that SUMOylated PPARγ affects the occupancy of NFκB sites by NCoR, I examined the association of NCoR within the TNFα promoter by ChIP analysis. RAW264.7 and RAW264.7 d/n PPARγ macrophages were pre-treated with AC for 90 min and stimulated with 1 µg/ml LPS for 1 h afterwards or remained as controls. Under control conditions NCoR associated with the NFκB site of the TNFα promoter, whereas it was removed from that promoter region in response to LPS in wild-type as well as d/n PPARγ over-expressing macrophages (Figure 5.12). In RAW264.7

macrophages, NCoR remained bound to the promoter after recognition of AC despite LPS-stimulation (Figure 5.12 A), whereas in RAW264.7 d/n PPARγ macrophages NCoR was cleared from the promoter even in response to AC, followed by LPS stimulation (Figure 5.12 B). Densitometric analysis using AIDA Image analyzer proved significance for the described effect on NCoR association (Figure 5.12 C).

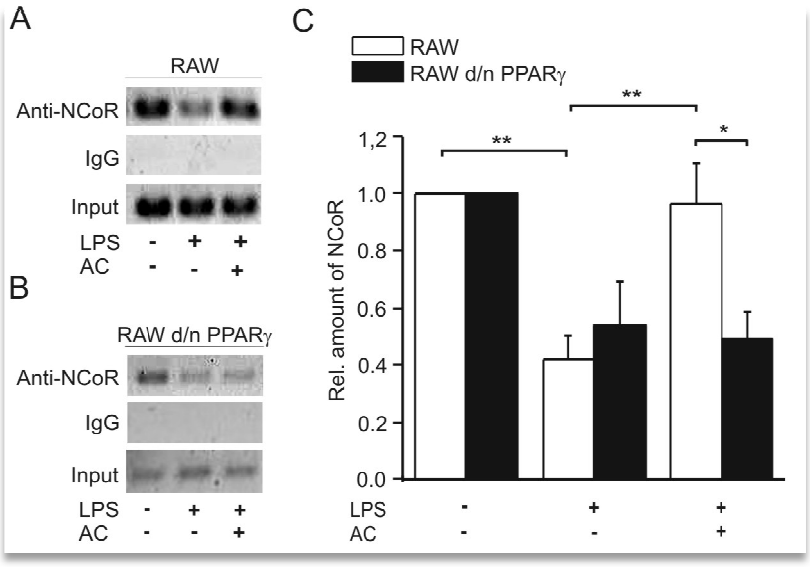

Figure 5.12 PPARγ antagonizes the removal of NCoR.
Association of NCoR with the κB response element within the TNFα promoter was analyzed by ChIP analysis. (A) RAW264.7 and (B) RAW264.7 d/n PPARγ macrophages were incubated with AC for 90 min and 1 µg/ml LPS for 1 h afterwards. Data are representative for three independent experiments. (C) Statistical evaluation of ChIP assays showing the relative amount of NCoR vs. input controls. Statistics were analyzed with two-way-ANOVA modified with Bonferroni's multiple comparison test.

Even though I could elucidate the underlying molecular mechanism of NFκB repression, it remained so far unclear how PPARγ is SUMOylated. It was previously described that SUMOylation of PPARγ2 in adipocytes is accompanied with subsequent phosphorylation of a serine residue next to the SUMOylation site (170). For this reason, I pre-treated RAW264.7 macrophages with 10 µM of the p38 inhibitor SB203580 (171) for 1 h followed by stimulation with AC (90 min) and LPS (5 h) as described above and determined NFκB reporter activity.

And indeed, inhibition of p38 reversed the potential of AC to repress NFκB activation, whereas the LPS response was not significantly altered (Figure 5.13 A). To investigate whether p38 phosphorylates PPARγ, I performed a reporter assay in RAW264.7 d/n

PPARγ macrophages over-expressing PPARγ with a mutated S82 (S82A). In comparison to DsRed-PPARγ1 wild-type, over-expression of DsRed-PPARγ1-S82A also restored NFκB inhibition in response to AC, excluding phosphorylation of PPARγ at S82 during the AC-provoked anti-inflammatory response (Figure 5.13 B).

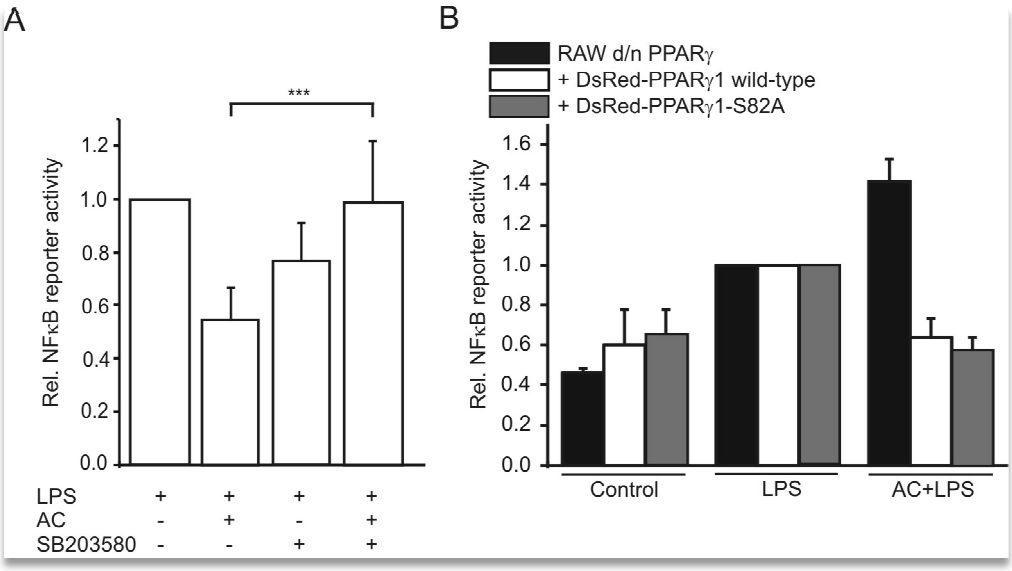

Figure 5.13 p38-dependent NFκB inhibition in response to AC.
(A) RAW264.7 cells were treated with 10 µM SB203580 for 1 h followed by AC (90 min) and LPS (1 µg/ml, 5 h). NFκB reporter activity was measured. (B) NFκB reporter activity was determined in RAW264.7 d/n PPARγ cells over-expressing pDsRed-PPARγ1 wild-type or pDsRed-PPARγ1-S82A. After transfection, cells were treated with AC and LPS as before. LPS values were set to 1. Data present mean values ± SE, n ≥ 4. Statistics were analyzed with one-way-ANOVA.

On account of this, I concluded that PPARγ SUMOylation in response to AC regulates the activity of NFκB via NCoR, which contributes to immune modulation of macrophages. Moreover, p38 seems to be involved during inhibition of NFκB, whereas detailed signaling remains unclear.

5.2 Regulation of PPARγ expression during the inflammatory response

My data of the first part corroborate the eminent role of PPARγ during polarization of macrophages and resolution of inflammation. As an anti-inflammatory signaling molecule it seems likely that it is tightly regulated dependent on the state of the immune response. There is growing evidence that PPARγ expression is reduced during inflammation and remarkably, several chronic inflammatory diseases were associated with an impaired PPARγ expression. Still, signaling pathways reducing PPARγ

expression are ill-defined. Therefore, I was interested in elucidating the underlying mechanisms of PPARγ reduction in response to the classical pro-inflammatory stimulus LPS.

5.2.1 PPARγ1 expression during monocyte differentiation and upon LPS exposure

In the last years, the number of described promoters for different PPARγ transcript variants was nearly doubled, even though three transcript variants are well established (39, 41). Macrophages predominantly express PPARγ1, which was postulated to be under the control of promoter 1 and 3 (54). As a start, I investigated the expression levels of the different transcript variants during differentiation of primary human monocytes. Therefore mRNA was extracted from monocytes/macrophages at 1 h, 1 d, 3 d and 7 d after isolation from buffy coats, which were additionally stimulated with 1 µg/ml LPS for 3 h at each time point. Expression of transcript variants 1 and 3 was determined by qPCR using specific primer pairs (see Table 4.6, forward: PPARγ transcript variant 1 or 3, reverse: PPARγ exon 1).

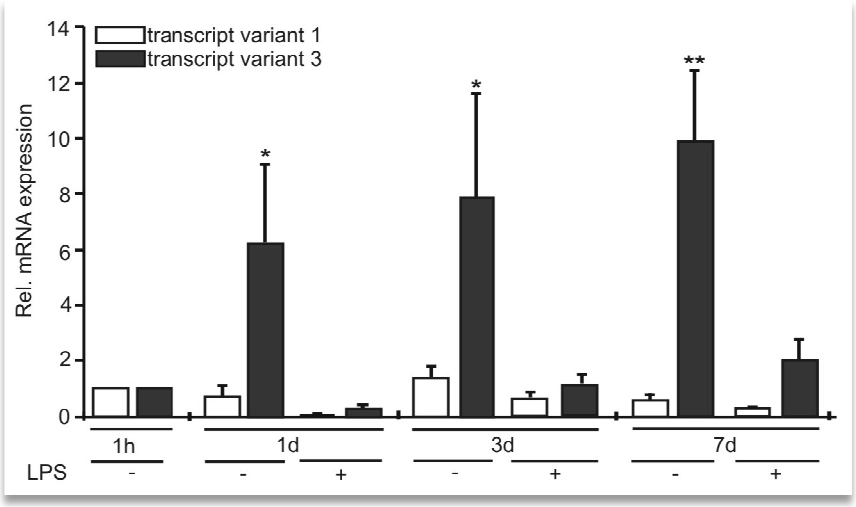

Figure 5.14 Differential expression of PPARγ during monocyte/macrophage differentiation. Primary human monocytes were isolated from buffy coats and cultured for increasing time periods for differentiation into macrophages. At each time point, monocytes/macrophages were stimulated with 1 µg/ml LPS or left untreated. RNA levels of the different PPARγ transcript variants were determined by qPCR. Data are mean values ± SE, n = 3. Statistics were analyzed using unpaired Student's t-test.

I predominantly observed induction of the PPARγ transcript variant 3 (10-fold at day 7), and a minor induction of variant 1 after 3 d (1.3-fold), which was even less (0.58-fold at day 7) after full differentiation. Upon LPS exposure both variants were down-regulated (Figure 5.14).

The reduction of both transcript variants upon LPS exposure pointed to a common regulation mechanism. Therefore, I further determined total PPARγ1 mRNA (transcript variant 1 and 3). Time-dependent expression analysis of PPARγ1 mRNA in response to 1 µg/ml LPS revealed a slight increase after 30 min followed by a rapid decrease with the lowest PPARγ1 mRNA amounts after 6 h (Figure 5.15 A). Extended incubation periods recovered PPARγ1 mRNA content nearly reaching control levels after 24 h of LPS treatment (Figure 5.15 A). The same pattern was observed in differentiated THP-1 macrophages. As seen in Figure 5.15 B, 3 h of LPS exposure significantly decreased PPARγ1 mRNA to approximately 20% in comparison to unstimulated cells.

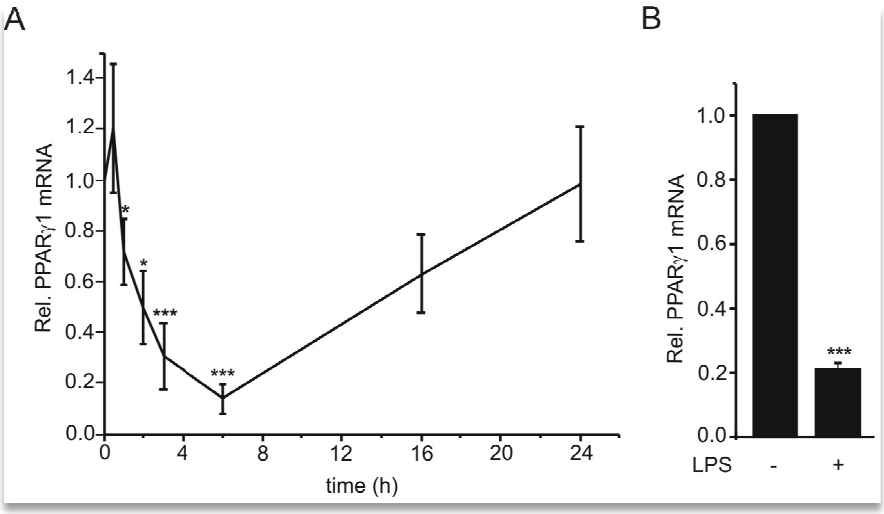

Figure 5.15 Time-dependent reduction of PPARγ1 mRNA in macrophages.
(A) Primary human macrophages were treated with 1 µg/ml LPS for different time periods and PPARγ1 mRNA levels were determined by qPCR. (B) Differentiated THP-1 macrophages were treated with 1 µg/ml LPS for 3 h and PPARγ1 mRNA was measured. Data present mean values ± SE, n ≥ 5. Statistics were performed using unpaired Student's t-test.

Since inhibition of NFκB with several inhibitors prevented LPS-mediated PPARγ decrease in RAW264.7 macrophages (172), I pre-treated primary human macrophages with 10 µM of the NFκB inhibitor Bay11-7082 for 1 h followed by LPS exposure for 3 h. Inhibition of NFκB significantly reversed PPARγ1 mRNA decrease (Figure 5.16). Moreover, pre-stimulating macrophages with 1 µM rosiglitazone for 16 h prior to 3 h

of LPS also restored PPARγ1 mRNA expression, suggesting that this is due to inhibited NFκB activity as well. Alternatively to LPS, I further treated cells with the pro-inflammatory cytokines TNFα (5 ng/ml) or IFNγ (10 U/ml) for 3 h and determined PPARγ1 mRNA expression.

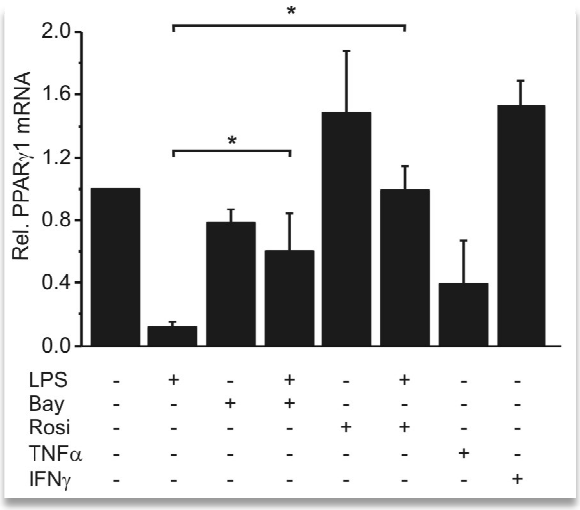

Figure 5.16 NFκB-dependent PPARγ1 mRNA decrease.
Primary human macrophages were pre-treated with 10 µM Bay11-7082 for 1 h or 1 µM rosiglitazone for 16 h followed by 3 h of LPS (1 µg/ml). Alternatively to LPS cells were stimulated with 5 ng/ml TNFα or 10 U/ml IFNγ. PPARγ1 mRNA amount was determined by qPCR. Columns present mean values ± SE, n ≥ 5. Statistics were performed using unpaired Student's t-test.

TNFα also attenuated PPARγ1 mRNA even though to a lower extent than LPS. IFNγ rather induced mRNA expression (Figure 5.16). Since TNFα but not IFNγ also triggers NFκB activation, I suggested an analogous molecular mechanism and focused on the investigation of mRNA decay upon LPS exposure.

To check whether the LPS-mediated decrease of mRNA is reflected at protein level, I analyzed protein expression and PPARγ activity by reporter assay. Western analysis showed a time-dependent reduction of protein expression with a minimum at 8 h, again increasing afterwards (Figure 5.17 A). To determine PPARγ activity, I transfected differentiated THP-1 macrophages with the PPRE reporter plasmid pAOX-TK and pre-treated cells the next day with 1 µg/ml LPS for 6 h followed by stimulation with 5 µM rosiglitazone for 4 h. Rosiglitazone, a well described synthetic PPARγ agonist (173), induced luciferase expression, whereas pre-stimulation with LPS prevented

transactivation of PPARγ by rosiglitazone. LPS alone did not alter basal luciferase activity (Figure 5.17 B).

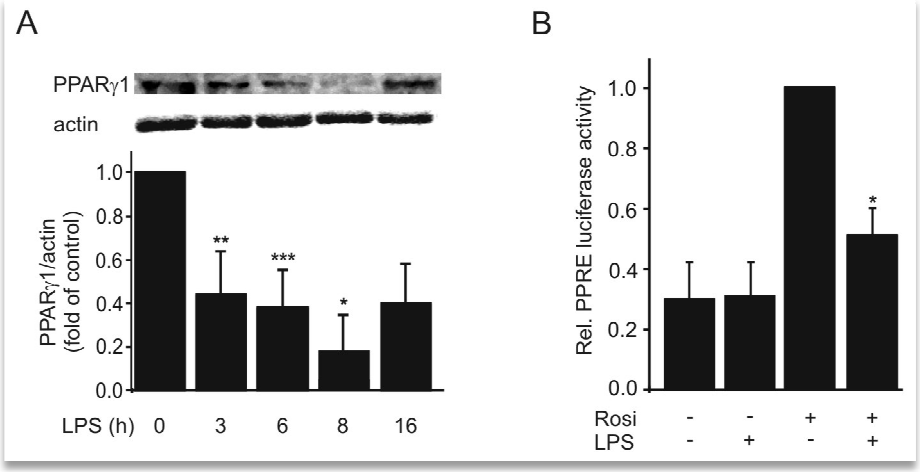

Figure 5.17 Time-dependent decrease of PPARγ protein in response to LPS
(A) PPARγ protein was determined by Western analysis after treating primary human macrophages with 1 µg/ml LPS for the indicated time points. (B) PPRE reporter activity was measured in differentiated THP-1 macrophages after pre-treatment with LPS for 6 h followed by 5 µM rosiglitazone for 4 h. Columns present mean values ± SE, n ≥ 4. Statistics were analyzed with unpaired Student's t-test.

As described before, mRNA decrease can either result from transcriptional or post-transcriptional regulation. To determine whether PPARγ1 mRNA decrease was due to reduced transcription, I performed luciferase reporter assays using PPARγ promoter 1 (pGL3-γ1p3000) and 3 (pGL3-γ3p800) constructs, containing the individual promoters upstream of the luciferase encoding region.

Therefore, THP1-macrophages were transfected with pGL3-γ1p3000 or pGL3-γ3p800 and stimulated with 1 µg/ml LPS for 3 and 6 h the following day. LPS exposure lowered PPARγ promoter 1 luciferase activity to approximately 60% after 6 h, whereas luciferase activity of the PPARγ promoter 3 construct was not significantly reduced (Figure 5.18). Taking into consideration that during differentiation of macrophages promoter 1 was only slightly induced (see Figure 5.14), I assumed that reduction of the promoter 1 activity to 60% is negligible for the LPS-induced PPARγ1 mRNA decrease. Thus, I hypothesized that the mRNA decrease is rather due to post-transcriptional regulation.

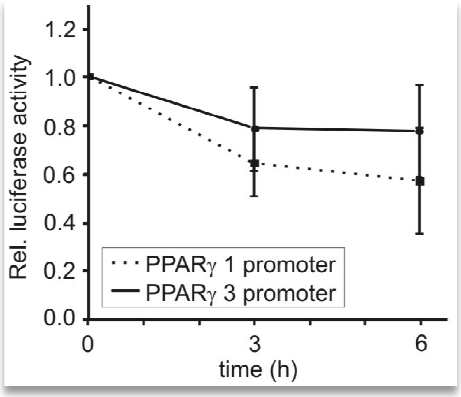

Figure 5.18 Impact of LPS on PPARγ promoter activity.
THP-1 macrophages were transfected with PPARγ 1 and 3 promoter constructs, stimulated with 1 µg/ml LPS for 3 and 6 h. Luciferase activity was normalized to protein concentration. Each measuring point represents the mean value of duplicate determinations of a minimum of three independent experiments. Statistics were analyzed using unpaired Student's t-test, whereas reduction was only significant (p = 0.03) for PPARγ 1 promoter activity.

5.2.2 Post-transcriptional regulation of PPARγ1 mRNA

Altered mRNA half-life is a common mechanism regulating gene expression and is responsible for reduced mRNA amounts. For this reason, I determined mRNA stability by exposing cells to LPS and/or the transcription inhibitor 5–6 dichloro-1-β-ribofuranosylbenzimidazole (DRB) (174). DRB treatment reduced PPARγ1 mRNA expression to approximately 60% after 3 h, whereas a DRB plus LPS exposure decreased mRNA to 26% in comparison to untreated cells (Figure 5.19).

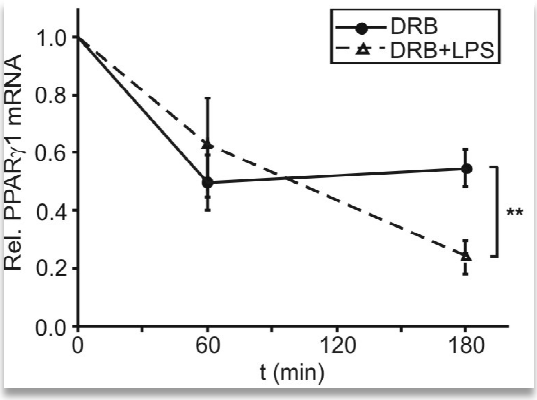

Figure 5.19 Altered mRNA half-life upon LPS exposure.
Primary human macrophages were exposed to 10 µM 5–6 dichloro-1-β-ribofuranosyl-benzimidazole (DRB, squares and black solid line) or DRB plus 1 µg/ml LPS (triangle and dashed trend line) for 1 and 3 h and PPARγ1 mRNA was determined by qPCR. Data present mean values ± SE, n ≥ 4. Statistics were analyzed with unpaired Student's t-test.

Calculating the half-life (t ½) of PPARγ1 by extrapolation revealed a mRNA half-life of 3.0 h. Interrupted transcription by DRB plus LPS reduced t ½ to 1.93 h.

Destabilization is mostly due to AU-rich 3'UTRs, which targets mRNAs for cytoplasmic degradation. Hence, I analyzed the PPARγ1 mRNA sequence and noticed an AU-rich 3'UTR with various ARE sites and a miR-27 binding site. Referring to Shavora *et al*. the PPARγ-3'UTR contains three ARE1 sites (AUUUA) and one ARE4 site (12-mer A/U with maximal one mismatch) (Figure 5.20).

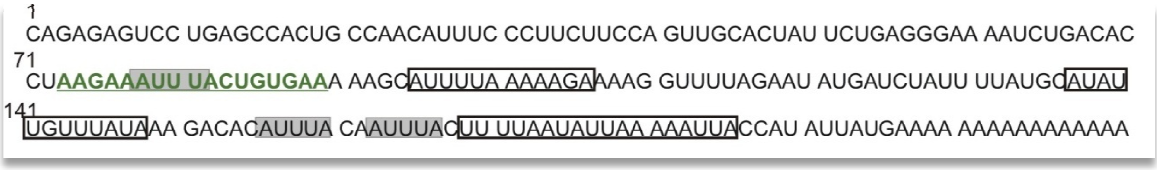

Figure 5.20 Sequence of the AU-rich PPARγ-3'UTR.
The miR-27 binding site is displayed in a green underlined font, whereas ARE1 (AUUUA) and ARE4 (12-mer A/U with max. one mismatch) sites are marked with boxes.

To prove the hypothesis that PPARγ1 mRNA decrease is due to destabilization, I investigated the effect of the PPARγ-3'UTR on mRNA stability by reporter assay. Therefore, the 3'UTR was inserted downstream of the luciferase encoding region within the pGL3-control vector, which contains a SV40-promoter upstream of the luciferase encoding region. Therewith, cells transfected with pGL3-control constitutively express luciferase.

Differentiated THP-1 cells were transfected with pGL3-control and pGL3-PPARγ-3'UTR respectively, and stimulated with LPS for 3 and 6 h the following day. LPS significantly reduced luciferase activity of pGL3-PPARγ-3'UTR to approximately 65% after 3 h and to 50% after 6 h in comparison to pGL3-control. Figure 5.21 displays the ratio of the luciferase activity of pGL3-PPARγ-3'UTR vs. pGL3-control, demonstrating the importance of the PPARγ-3'UTR for mRNA stability.

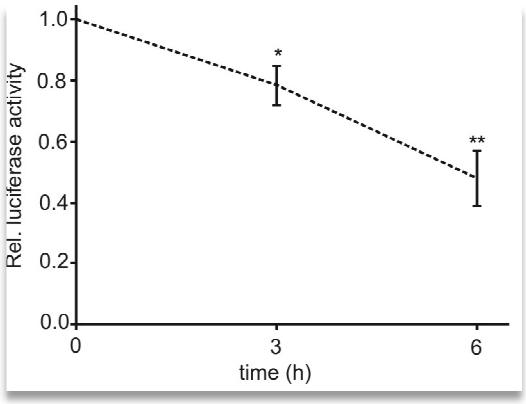

Figure 5.21 PPARγ-3'UTR-dependent reduction of luciferase expression.
Differentiated THP-1 macrophages were transfected with pGL3-control or pGL3-PPARγ-3'UTR and stimulated with LPS for 3 and 6 h. Luciferase activity was measured and normalized to protein concentration. The ratio of pGL3-PPARγ-3'UTR/pGL3-control is displayed. Each measuring point represents mean values of duplicate determinations of five independent experiments. Statistics were analyzed using unpaired Student's t-test.

5.2.3 miR-27b destabilizes PPARγ1 mRNA

In silico analysis revealed a miR-27 binding site within the 3'UTR (Figure 5.20), showing perfect match to the miR-27a and b seed (positions 1-8) and additional complementary nucleotides between positions 12-17 (Figure 5.22 A).

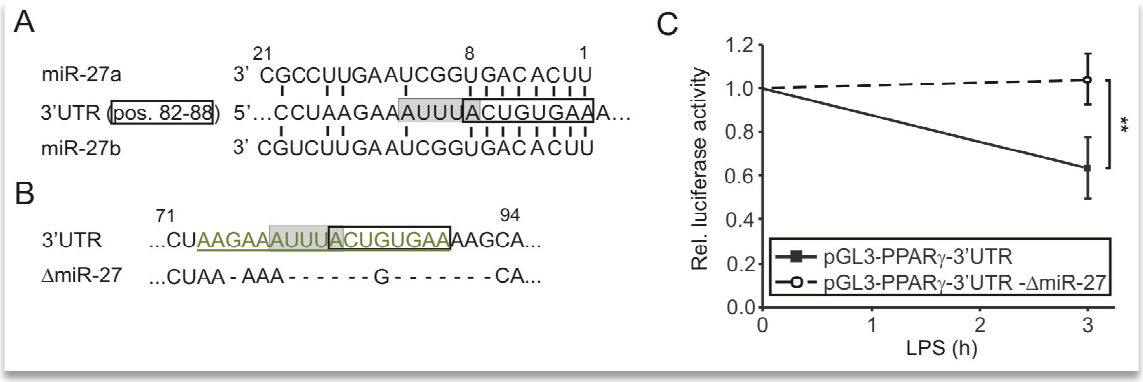

Figure 5.22 Impact of the miR-27 binding site within the 3'UTR.
(A) Alignment of the miR-27a/b sequences with the PPARγ-3'UTR. (B) Alignment of the deletion construct pGL3-PPARγ-UTR-ΔmiR-27 with the PPARγ-3'UTR. In (A) and (B), the seed region is marked with a box, the ARE1 site is highlighted in grey, while the miR-27 binding site is displayed in a green underlined font (C) Differentiated THP-1 cells were transfected with pGL3-control, pGL3-PPARγ-3'UTR or pGL3-PPARγ-3'UTR-ΔmiR-27 and luciferase expression was measured after stimulation with 1 µg/ml LPS for 3 h and displayed in comparison to pGL3-control transfected cells. Basal activity was set to 1. Columns present mean values ± SE, n ≥ 4. Statistics were analyzed with paired Student's t-test.

As a first approach, I deleted the miR-27 sequence within the pGL3-PPARγ-3'UTR vector (Figure 5.22 B) and again measured luciferase activity in transiently transfected THP-1 macrophages. Deletion of the miR-27 site completely reversed LPS-dependent reduction of luciferase activity (Figure 5.22 C).

As deletion of the miR-27 site within the 3'UTR points to an involvement of miR-27 in the LPS-mediated decay, I analyzed miR-27a and b expression in macrophages upon LPS exposure. Therefore, cells were stimulated with 1 µg/ml LPS for 2 h, since a strong PPARγ1 mRNA reduction was already seen after 3 h.

Expression levels were determined by qPCR. I observed a 2-fold induction of miR-27b and a 1.6-fold induction of miR-27a (Figure 5.23 A). Moreover, induction of miR-27a was significantly and miR-27b at least in part prevented by Bay11-7082 in comparison to LPS alone (Figure 5.23). Both induction of miR-27a/b and at least partial NFκB-dependence pointed to the involvement of miR-27 in PPARγ1 mRNA destabilization, since decay was also seen to be NFκB-dependent.

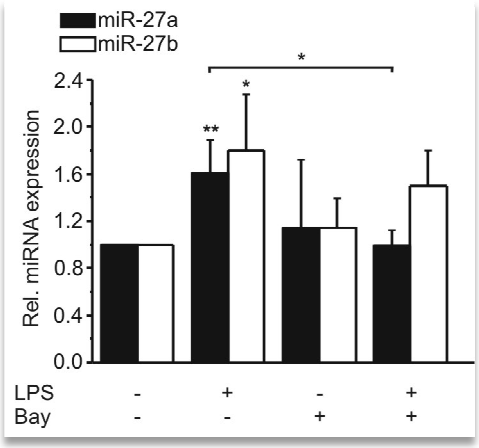

Figure 5.23 MiR-27 expression and NFκB dependence.
(A) MiR-27a and b expression was measured in primary human macrophages in response to LPS (1 µg/ml, 2 h) by qPCR. To investigate possible a NFκB-dependence, cells were additionally pre-stimulated with 10 µM Bay11-7082 for 1 h. Basal expression was set to 1. Columns present mean values ± SE, n = 4. Statistics were analyzed with unpaired Student's t-test.

To prove this, primary macrophages were transfected with either an anti-miR-27a or b miRNA-inhibitor or a miR-27a or b mimic, respectively. 48 h after transfection, cells were stimulated with 1 µg/ml LPS for 3 h and PPARγ1 mRNA was determined by qPCR. In cells transfected with anti-miR-27b, LPS-dependent mRNA reduction was partially reversed in comparison to the negative control (Figure 5.24 A).

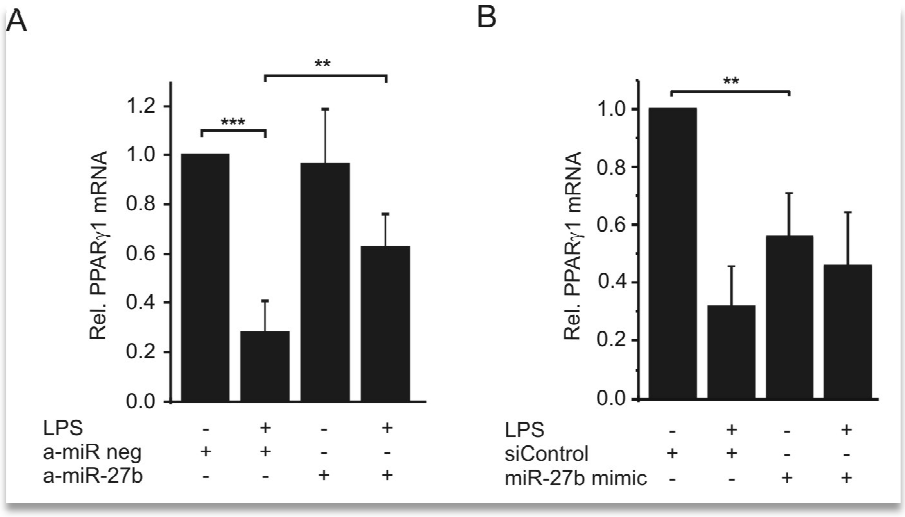

Figure 5.24 Effect of miR-27b on PPARγ1 mRNA decay.
Primary human macrophages were transfected with (A) anti-miR-27b or a negative control or (B) miR-27b mimic or siControl. After transfection, cells were stimulated with 1 µg/ml LPS for 3 h and PPARγ1 mRNA level was determined by qPCR. Columns present mean values ± SE, n ≥ 4. Statistics were analyzed with paired Student's t-test.

Consequently, transfection with a miR-27b mimic reduced PPARγ1 mRNA, which was not significantly enhanced by further addition of LPS (Figure 5.24 B).

In contrast, inhibition of miR-27a did not affect PPARγ1 mRNA reduction upon LPS exposure (Figure 5.25 A).

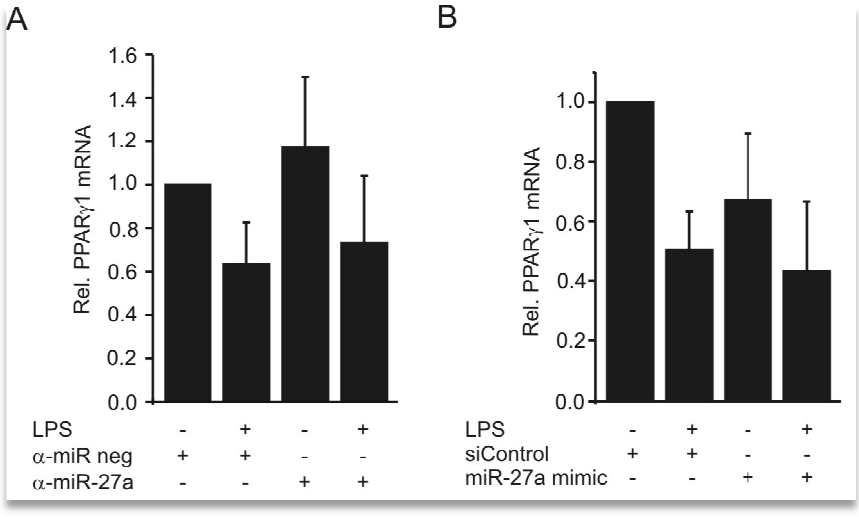

Figure 5.25 Effect of miR-27a on PPARγ1 mRNA decay.
Primary human macrophages were transfected with (A) anti-miR-27a or a negative control or (B) miR-27a mimic or siControl. After transfection, cells were stimulated with 1 µg/ml LPS for 3 h and PPARγ1 mRNA level was determined by qPCR. Columns present mean values ± SE, n ≥ 4.

Transfection with the miR-27a mimic also reduced PPARγ mRNA, which moreover was enforced by the further addition of LPS (Figure 5.25 B), suggesting a miR-27b- but not miR-27a-dependent destabilization of PPARγ1 mRNA. Inhibition of miR-27b only partially reversed PPARγ1 mRNA decay, but deletion of the miR-27 site completely restored luciferase activity. Since the deleted sequence also contained an ARE1 site with one nucleotide overlap to the miR-27 binding site (Figure 5.22 B), I assumed that an ARE-binding protein might be involved additionally to miR-27b. Exposing macrophages to several inhibitors might support this assumption. Since ARE-dependent mRNA decay can depend on translation, I inhibited translation using cycloheximide (CHX). Pre-treating cells with 10 mg/ml CHX for 1 h significantly induced PPARγ1 mRNA, whereas the LPS-dependent decrease seems not to be affected (Figure 5.26). Moreover, several ARE-binding proteins are regulated by the control of cellular localization. Therefore, I blocked nuclear export with leptomycin B (LMB). LMB inhibits the export receptor Crm1 (chromosome maintenance 1) or exportin 1, which is essential for nuclear export signal-dependent transport (175, 176). Pre-stimulation with 50 nM LMB reduced basal expression level and showed a slighter reduction of PPARγ1 mRNA upon LPS exposure in comparison to control cells, thus suggesting at least partial involvement of protein export (Figure 5.26).

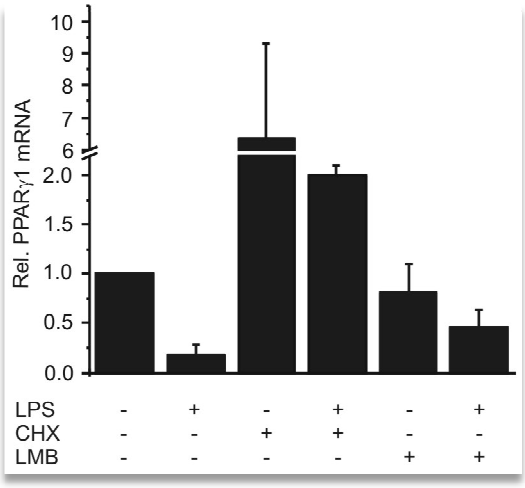

Figure 5.26 Impact of translation and nuclear export on PPARγ1 mRNA decay.
Primary human macrophages were pre-treated with 10 mg/ml cycloheximide (CHX) or 50 nM leptomycin B (LMB) for 1 h followed by stimulation with 1 µg/ml LPS for 3 h. PPARγ1 mRNA was determined by qPCR. Columns present mean values ± SE, n ≥ 3.

My data support the assumption that LPS destabilizes PPARγ1 mRNA partially by the NFκB-dependent induction of miR-27b. The ARE1 site proximate to the miR-27b-binding site as well as a protein export-dependent mRNA decay point to a further involvement of ARE-binding proteins, whereas *de-novo* synthesis of an ARE-binding protein can be excluded.

6 Discussion

Macrophages play a fundamental role during initiation as well as resolution of inflammation, which is due to distinct macrophage phenotypes. Deficient initiation of inflammation is as detrimental as deficient resolution, while the latter one often causes the development of chronic inflammatory diseases. On account of this, coordination of macrophage phenotypes during whole inflammatory processes is crucial for tissue homeostasis. In the resolution phase determination of macrophage phenotypes is among others dependent on phagocytosis of AC. Removal of AC avoids secondary necrosis and actively shifts macrophages towards a regulatory phenotype. One characteristic of this anti-inflammatory phenotype is the attenuation of NFκB activity. Underlying mechanisms blocking NFκB transactivation are poorly described, thus the aim of my studies was to investigate pathways leading to NFκB inhibition focusing on the role of PPARγ in this context.

PPARγ is well described to suppress release of pro-inflammatory cytokines as well as mediators such as NO and ROS in macrophages. During initiation of inflammation, activation of PPARγ would rather be harmful by preventing a proper immune response whereas during resolution it helps to dampen inflammation. For this reason, it seems obvious that PPARγ, as an anti-inflammatory mediator, is tightly regulated during inflammation. Since, TZDs are already approved for use in treatment of type-2 diabetes, PPARγ gained special interest as a target for chronic inflammatory diseases. These diseases are often associated with an impaired PPARγ expression, whereas underlying mechanisms for reduction of PPARγ expression are poorly understood. Thus, the second part of my thesis addressed the clarification of underlying mechanisms responsible for PPARγ decrease during inflammation.

6.1 PPARγ contributes to macrophage polarization in response to AC

PPARγ is well described for its anti-inflammatory properties by inhibiting MAPK cascades, PKCα activation or by repressing several transcription factors such as NFκB, AP-1 or Stat1 (63). Even though, many of these effects were discovered rather by treatment with synthetic PPARγ agonists (TZDs) than in physiological processes.

Recently, PPARγ was implicated in macrophage polarization provoked by IL-4, the classical stimulus for alternative activation of macrophages (or wound-healing

macrophages). Using macrophages from macrophage-specific PPARγ knock-out mice revealed that the reduced production of pro-inflammatory cytokines such as IL-6 and the enhanced expression of the alternative phenotype markers arginase I and mannose receptor are mediated by either PPARγ-dependent suppression or gene induction (57). Besides IL-4, AC induce a switch of macrophages towards an anti-inflammatory phenotype, although underlying mechanisms are insufficiently clear. I provide evidence that PPARγ gets activated and most likely SUMOylated in response to AC, which is essential for blocking NFκB transactivation. My basic observation that AC attenuate LPS-induced NFκB transactivation is in line with the work of Cvetanovic *et al*., demonstrating a diminished NFκB activity in response to AC (136). The notion that PPARγ is activated by AC (128) and well established for inhibition of NFκB (64, 162, 177), stimulated my interest in identifying underlying molecular mechanisms. I followed a molecular and pharmacological approach to establish the contributing role of PPARγ by using cells that express a dominant negative (d/n) mutant of PPARγ. This mutant carries two amino acid substitutions in the AF2 domain (L466A/E469A) of the protein, which impair ligand-dependent PPARγ transactivation and the interaction with co-activators, e.g. p300 (163). In RAW264.7 d/n PPARγ macrophages, inhibition of NFκB in response to AC was completely relieved. As a prove of concept, functionality in RAW264.7 d/n PPARγ macrophages was restored by over-expressing PPARγ1 wild-type, which again suppressed NFκB reporter activity in response to AC. Pharmacologically, the impact of PPARγ was further corroborated by using GW9662 to antagonize PPARγ, which restored NFκB reporter activity after adding AC to macrophages. During these studies, AC were co-cultured with macrophages for 6.5 h, which comprises a 90 min lasting pre-incubation period with AC, followed by LPS stimulation for 5 h. During the entire incubation period, AC remained in the medium, without removing non-ingested cells. However, removing non-phagocytosed cells after 30 min followed by LPS stimulation failed to block NFκB activity in my system. Variations in the stimulation regimes of macrophages with AC may affect macrophage plasticity. Majai *et al.* observed that a treatment of cells with LPS for 30 min, followed by incubations with AC for 25 min and subsequent by removal of non-ingested cells, lowered the amount of the NFκB target gene TNFα when measured 18-24 h later (178). This response was not antagonized by GW9662. Likely, pro-inflammatory stimuli given

to macrophages prior to confronting them with AC might activate distinct pathways, e.g. receptor desensitization or PPARγ decrease. These pathways contribute to the diversity of anti-inflammatory responses, with the further possibility that short vs. long incubation periods differ towards the involvement of PPARγ.

To verify the inhibitory role of PPARγ in my system, I not only followed NFκB reporter activity, but also searched for the expression of NFκB downstream target genes, i.e. TNFα and IL-6. Their transcriptional expression was reduced in response to AC in RAW264.7 and primary murine macrophages. Furthermore, cytokine formation was partially restored in the case of IL-6 and fully restored in the case of TNFα, when exposing d/n PPARγ cells to AC. Supporting evidence for PPARγ-mediated suppression came from experiments in PPARγ knock-out macrophages, where the inhibitory effect of AC on NFκB activity was abolished. These data support conclusions by Odegaard *et al.* using macrophages from PPARγ knock-out mice, showing that PPARγ is required for attenuation of IL-6 expression by IL-4 (57). In addition, the role of PPARγ for macrophage polarization was further corroborated by Bouhlel *et al.*, reporting that IL-4 promotes an anti-inflammatory macrophage phenotype by activating PPARγ (179). Alternative activation of macrophages by IL-13 also activated PPARγ, in turn generating an anti-inflammatory phenotype (180).

Despite increasing evidence for a role of PPARγ in macrophage polarization, molecular mechanisms explaining repression of NFκB, one crucial transcription factor regulating the inflammatory repertoire of macrophages by AC are ill-defined. Proposed strategies how PPARγ represses NFκB comprise competition with co-activators or inhibition of co-repressor clearance (63). I analyzed domains of PPARγ being involved in blocking NFκB transactivation. DsRed-PPARγ1 wild-type, DsRed-PPARγ1-Δaa1-31 as well as DsRed-PPARγ1-Δaa309-319 attenuated NFκB activity in response to AC. Amino acids 1-31 regulate cytosolic translocalization of PPARγ and concomitant PKCα inhibition (unpublished data). Thus, missing prevention of NFκB inhibition points to mechanisms occurring in the nucleus and hence PKCα-independent. Moreover, considering that amino acids 309-319 are required for binding transcriptional co-activators (167, 168) ruled out a simple co-activator scavenging of e.g. p300, to explain inhibition of NFκB. Cvetanovic *et al.* postulated that reduced availability of p300 is responsible for inhibited NFκB transactivation, which was due to restored NFκB activity after over-

expressing p300 (136). Over-expression of co-activators in general might not reflect the specificity of transrepression observed for endogenous occurring co-activator abundance. Moreover, enhanced availability of required co-activators might overcome repressive effects occurring by different mechanisms.

Interestingly, over-expression of DsRed-PPARγ1-Δaa32-250 restored NFκB transactivation, compared to the action of DsRed-PPARγ1 wild-type. Deleted amino acids in PPARγ1-Δaa32-250 span a part of the AF1 domain, the DNA binding domain, a part of the hinge domain and thus, contain a predicted SUMOylation site at K77. SUMOylation of PPARγ was shown to attenuate NFκB target gene expression by preventing NCoR removal from NFκB binding sites in various promoter regions of target genes such as iNOS (77). NCoR is a component of a co-repressor complex, containing TBL1, TBLR1 and HDAC3, the latter one mediating transcriptional repression (169). A potential role for the NCoR-associated HDAC3 was proposed when the HDAC inhibitor TSA reversed PPARγ-dependent repression of iNOS (77). In analogy, TSA reversed inhibition of NFκB by AC, suggesting that a similar mechanism might operate in response to AC. ChIP analysis confirmed that NCoR is cleared from the NFκB site within the TNFα promoter after LPS stimulation, but remained bound when macrophages were pre-stimulated with AC. Pascual *et al.* noticed that SUMOylated PPARγ suppressed the NFκB target gene iNOS (77). The model predicts that NCoR/HDAC3 associates with NFκB binding sites along with TBL1 and TBLR1, which are required for ubiquitination of NCoR in response to pro-inflammatory stimuli (74). Following SUMOylation, PPARγ binds to NCoR/HDAC3 and prevents the recruitment of the ubiquitination/19S proteasome machinery that normally degrades the co-repressor complex. This scenario requires ligand-dependent PPARγ activation and K365 SUMOylation (77). PPARγ contains two possible SUMOylation sites at K77 and K365 and my experiments with the PPARγ aa32-250 deletion fragment pointed to the involvement of K77 rather than K365. Indeed, over-expression of DsRed-PPARγ1-K77R in RAW264.7 d/n PPARγ cells failed to restore NFκB repression, indicating that SUMOylation of PPARγ at K77 represses NFκB transactivation. Moreover, this also demonstrates that beside SUMOylation at K77, also ligand-binding is required, since the use of the d/n mutant failed to repress NFκB. My studies do not rule out the possibility that PPARγ is also SUMOylated at K365, but at least this would not to be

sufficient for NFκB inhibition under my experimental conditions. Furthermore, knock-down of the SUMO E3 ligase PIAS1, which mediates PPARγ SUMOylation (77), reversed the inhibitory ability of AC. Even though I could not directly show PPARγ SUMOylation, it seems very likely, since mutation of the SUMOylation site as well as knock-down of the SUMO E3 ligase PIAS1 abrogated NFκB inhibition or TNFα reduction in response to AC.

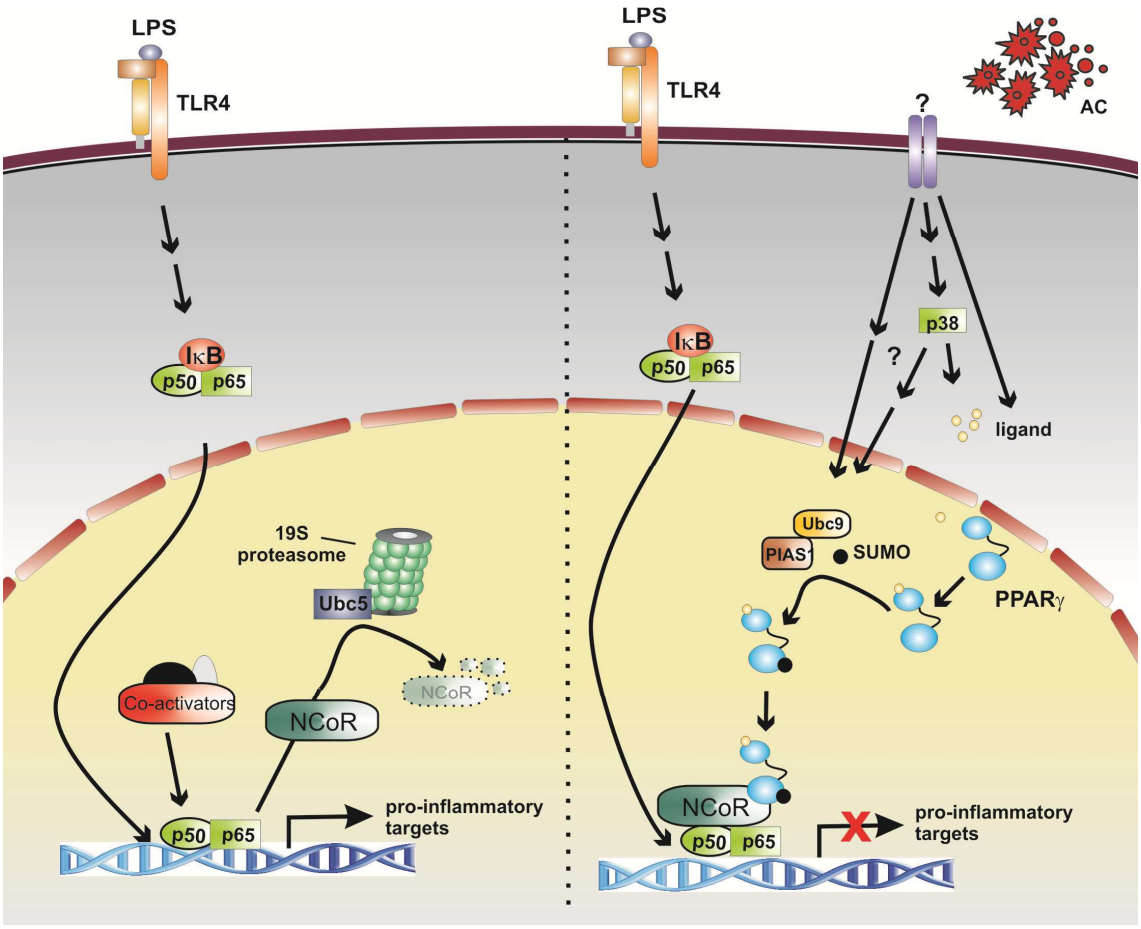

Figure 6.1. SUMOylation of PPARγ prevents NCoR removal and concomitant NFκB activation. Left panel: LPS binding to the TLR4 complex induces IκB degradation and translocalization of NFκB to the nucleus. Upon LPS, NCoR is ubiquitinated and degraded, co-activators can bind and induce target gene transcription. Right panel: Recognition of AC activates p38 and Ubc9/PIAS1, inducing SUMOylation of PPARγ. SUMOylated PPARγ is targeted to NCoR, prevents its removal and therewith represses NFκB transactivation. Abbreviations: AC: apoptotic cells, IκB: inhibitor of κB, LPS: lipopolysaccharide, NCoR: nuclear receptor co-repressor, TLR4: toll-like receptor 4, PIAS1: protein inhibitor of activated Stat 1, PPARγ: peroxisome proliferator-activated receptor γ, SUMO: small-ubiquitin related modifier

However, aiming at molecular mechanisms triggering PPARγ SUMOylation, I found that NFκB inhibition was dependent on p38 activation, since pharmacological inhibition of

p38 restored NFκB activity. Yamashita *et al*. demonstrated that PPARγ2 SUMOylation at residue K107, negatively regulating PPARγ transactivation in adipocytes, was promoted by phosphorylation at S112 (170). As a possible mechanism, I interfered with phosphorylation at S82 by point mutation, which turned out to be not involved in NFκB inhibition. A tentative explanation might be inherent in the p38-dependent stabilization and induced gene expression of PIAS1. TGFβ-mediated activation of Smad4 was due to SUMOylation by PIAS1, the essential SUMO E3 ligase. PIAS1 protein amount was increased by gene induction and protein stabilization in a p38-dependent manner (181). Moreover, PIAS1 can also be phosphorylated by IKKα, which in turn can be activated by p38. This might explain the role of p38 on attenuated NFκB transactivation, although it is highly speculative and needs further investigation (Figure 6.1).

Even though, p38 is involved in the AC-induced NFκB repression, whereas receptors activating p38 and initiating SUMOylation remain elusive. NFκB-inhibition was shown to occur in a PS-independent manner (136), thus ruling out many of the described recognition receptors. Receptors sensing oxLDL-like sites might facilitate PPARγ SUMOylation. Scavenger receptor A was recently shown to mediate p38 activation in dendritic cells, even though it induced a pro-inflammatory response in this system (182). Further investigations will be required to identify mechanisms, which facilitate SUMOylation of PPARγ in response to AC.

There is increasing evidence that PPARγ essentially contributes to a macrophage phenotype shift. My data suggest that this signaling circuit operates under conditions when AC re-program immune functions of macrophages, exemplified by an altered NFκB-mediated target gene expression profile. I propose that SUMOylated PPARγ attenuates NFκB transactivation in response to AC by preventing NCoR co-repressor displacement. My data reinforce the importance of PPARγ during resolution of inflammation and help to understand how AC affect the remarkable plasticity of macrophages associated with decreased pro-inflammatory cytokine production.

6.2 Regulation of PPARγ during the inflammatory response

As an anti-inflammatory mediator, the potential therapeutic role of PPARγ emerges not only for type-2 diabetes but also for acute and chronic inflammatory diseases (84, 86, 88, 183). TZDs already entered phase III clinical trial for the treatment of

Alzheimer's disease and phase II trials for ulcerative colitis showing clinical improvement (83, 88). Remarkably, the outcome and development of several diseases are accompanied with decreased PPARγ protein level. However, mechanisms impairing PPARγ expression are ill-defined. For this reason I elucidated pathways decreasing PPARγ expression during the onset of inflammation.

LPS, a classical pro-inflammatory stimulus time-dependently reduced PPARγ mRNA and protein amounts in macrophages. In response to LPS the maximum reduction was seen after 6 h, which is in line with the work of Necela *et al.*, who investigated reduction of PPARγ mRNA in RAW264.7 macrophages (172). Prolonged LPS exposure allowed to recover mRNA levels to almost basal value after 24 h. Accordingly, treating macrophages with LPS for 24 h (184) or LPS and IFNγ for 15 h even provoked PPARγ transactivation (185). It seems likely, that prolonged inflammation, in this case prolonged stimulation with LPS, restores and activates PPARγ, helping to resolve an immune response by facilitating negative regulation of pro-inflammatory transcription factors. This late increase might be dysregulated during chronic inflammation.

However, elucidating underlying mechanisms, Necela *et al*. already proposed a NFκB-dependence during PPARγ mRNA down-regulation, which I could corroborate. Moreover, pre-treatment of macrophages with rosiglitazone also prevented mRNA decay. Interestingly, Klotz *et al*. observed reduced PPARγ expression in PBMCs of MS patients and demonstrated that pre-treatment of PBMCs from healthy individuals *in vitro* or long-term oral medication with pioglitazone prior to PHA prevented PHA-induced PPARγ decrease (87), whereas explanations therefore were left open. I suggested that restoration of PPARγ expression following agonist treatment is also due to abrogated NFκB activation.

Recently, Zhou *et al*. observed reduced PPARγ expression in a sepsis model, which they contributed to increased TNFα release. Hepatic tissue and Kupffer cells from septic rats, subjected to cecal ligation and puncture (CLP) to initiate sepsis, revealed attenuated PPARγ protein expression. Inhibiting LPS signaling by polymyxin B did not prevent PPARγ expression at 20 h after CLP, whereas administration of TNFα neutralizing antibodies before the onset of sepsis prevented down-regulation of PPARγ in Kupffer cells (186). I assumed that a poly-microbial sepsis model initiated by CLP triggers NFκB activation despite blocking LPS signaling. Moreover, short vs. long

stimulation periods might trigger different pathways contributing to PPARγ regulation. Exposure of primary human macrophages to TNFα for 3 h also reduced PPARγ1 mRNA even to a lower extent than LPS. In contrast, stimulation of macrophages with IFNγ slightly induced PPARγ1 mRNA expression, probably because of a missing NFκB activation. Beside LPS and TNFα, PPARγ down-regulation was also observed upon TLR1/2 and 5 activation (19). I suggested that NFκB-activating inflammatory signals in general such as oxidative stress, inflammatory mediators and pathogens provoke PPARγ mRNA decrease. This hypothesis is supported by the evidence that NFκB is implicated in disease conditions such as inflammatory bowel diseases or rheumatoid arthritis associated with impaired PPARγ expression.

However, to further elucidate underlying mechanisms, I checked whether the PPARγ mRNA decrease results from an altered rate of transcription. Promoter reporter assays revealed a reduction of the promoter 1 activity to 60%, which seemed to be negligible, since promoter 1 was not induced in differentiated macrophages. Moreover, a not significant reduction of the promoter 3 activity after 6 h of LPS exposure unlikely would be sufficient to explain a 90% decrease of mRNA. Thus, I rather suggested mRNA destabilization as a post-transcriptional regulatory mechanism.

Therefore, I determined PPARγ mRNA stability by treating macrophages with the transcription inhibitor DRB and noticed a reduced mRNA half-life upon LPS exposure. DRB was used, because actinomycin D affects mRNA stability by controlling ARE-binding proteins. Actinomycin D activated HuR by inducing its translocalization to the cytosol (187), which is a major regulatory step for activation of several ARE binding proteins, e.g. AUF1 and tristetraprolin (188-190). This might explain why Necela *et al.* observed no effect of LPS on mRNA stability when using actinomycin D to block transcription (172). Moreover, estimated half-lives can differ depending on the inhibitor used. Determination of the Ig κ light chain mRNA half-life varied considerably depending on the inhibitor, ranging from a high value of 5.9 h following DRB to a low value of 2.4 h following actinomycin D treatment (191). The use of 3'UTR reporter constructs is an established method to verify potential destabilization mechanisms (6-8), since mRNA stability is often regulated via AU-rich 3'UTRs. Luciferase assays with a generated pGL3-PPARγ-3'UTR construct demonstrated the importance of the PPARγ-3'UTR, since LPS significantly reduced luciferase activity. *In silico* analysis showed

several ARE1 (AUUUA) and ARE4 (12-mer A/U, max. one mismatch) sites (9) and a potential miR-27a/b binding site (TargetScanHuman 5.1, http://www.targetscan.org). As deletion of the miR-27 site within the PPARγ-3'UTR reporter construct completely restored luciferase activity, I also checked miR-27a and b expression. I observed a 1.6-fold increase of miR-27a and a nearly 2-fold increase of miR-27b in response to LPS, which is comparable to the induction of miR-146a after 2 h of LPS exposure in THP-1 cells. MiR-146 is well described for its function in macrophages, where it negatively regulates TLR signaling (19). In addition, RNA from mouse lung extracts showed an increase of miR-27 a and b expression after 2-3 h of LPS exposure (192).

Several diseases are associated with dysregulated miRNA expression. MiR-146a and miR-155 have been implicated in the development of rheumatoid arthritis, supposably by regulating components of the inflammatory response (22, 193). These miRNAs are induced upon NFκB transactivation (19, 20, 194). I also observed that induction of miR-27b was at least partially NFκB-dependent, correlating with a NFκB-dependent decrease of PPARγ mRNA. On account of this, I concluded that the NFκB-dependent PPARγ mRNA decrease results at least in part from the NFκB-dependent induction of miR-27b upon LPS exposure. Since transfection with anti-miR-27b restored PPARγ1 mRNA, a relative low induction of miR-27b upon LPS (2-fold) seems to be sufficient for PPARγ1 mRNA decay. The impact of miR-27b on PPARγ decay was proven by transfecting cells with a specific miR-27b inhibitor, which partially restored PPARγ expression. PPARγ mRNA decrease mediated by miR-27 was corroborated by Lin *et al.* (195). During adipogenic differentiation of 3T3-L1 cells, microarray analysis revealed a reduced expression of miR-27a and b, which was associated with an increase of PPARγ mRNA. Moreover, transfection of cells with miR-27a and b resulted in the decay of PPARγ mRNA (195).

However, taking into consideration that inhibition of miR-27b not completely restored PPARγ1 mRNA level, but reporter assays with the deletion construct pGL3-PPARγ-UTR-ΔmiR-27 completely reversed LPS-mediated attenuation of luciferase activity, I assumed additional regulatory mechanisms. I speculated that the ARE1 site (AUUUA) within the deleted region might as well be essential for destabilization, possibly by an ARE-binding protein. Several mechanisms are proposed to contribute to activation of ARE-binding proteins including simple gene induction but also co-translational

degradation, phosphorylation and control of cellular localization by nucleocytosolic shuttling. Inhibiting translation with CHX significantly induced PPARγ1 mRNA levels under basal conditions, while showing no effect on mRNA decay, excluding co-translational regulation mechanisms. Blocking exportin1-sensitive nuclear export with LMB partially reversed PPARγ1 decrease. On account of this, I proposed that LPS induces export of an ARE-binding protein concomitantly facilitating PPARγ1 mRNA decay in concert with miR-27b (Figure 6.2).

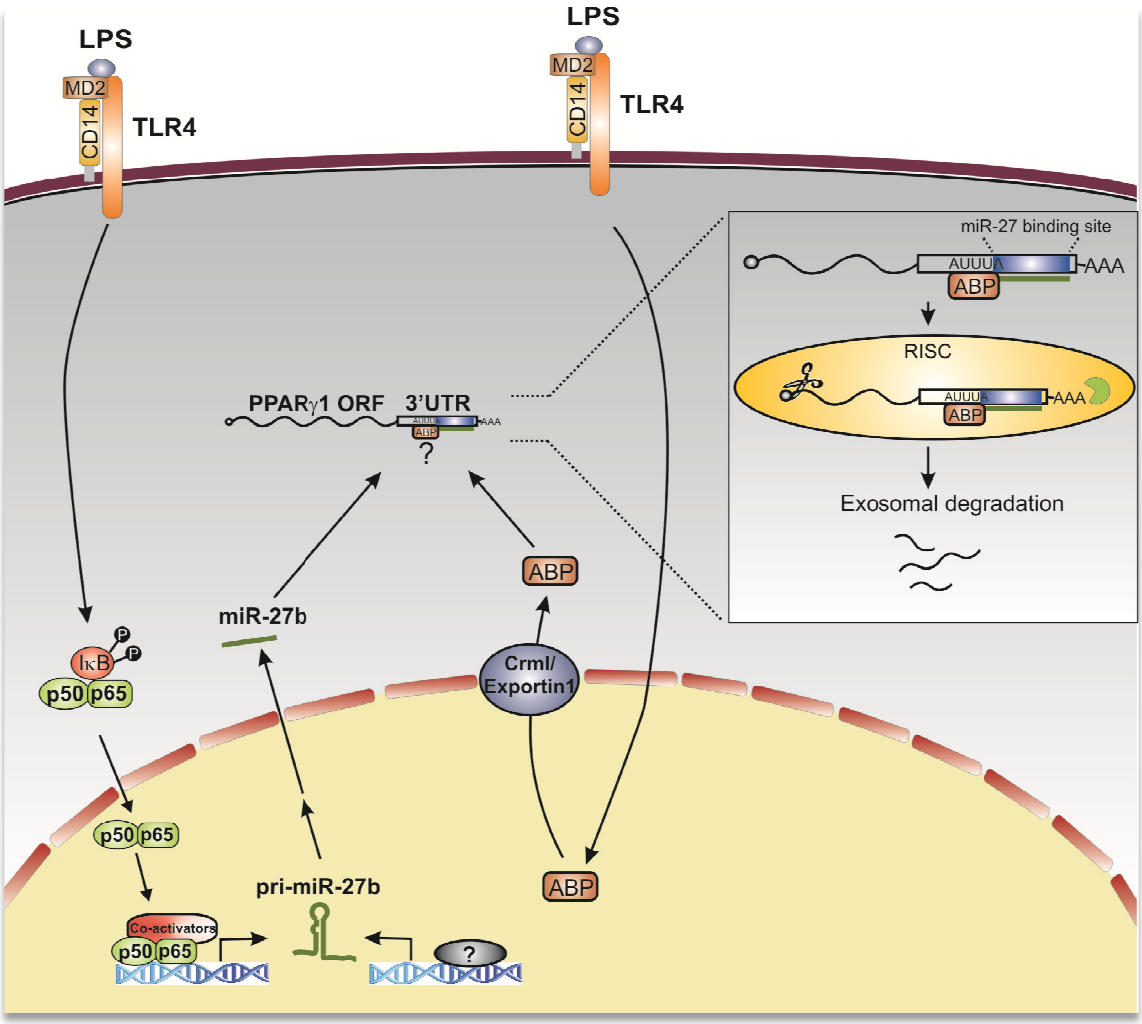

Figure 6.2 LPS-mediated induction of miR-27b destabilizes PPARγ1 mRNA.
LPS induces in part by NFκB the transcription of pri-miR-27b, which binds to the miR-27 binding site within the PPARγ-3'UTR. Moreover, LPS induces the export of a so far unknown ARE-binding protein facilitating in concert with miR-27b PPARγ1 mRNA degradation. Abbreviations: ABP: ARE-binding protein, TLR4: Toll-like receptor 4, ORF: open reading frame, PPARγ: peroxisome proliferator-activated receptor γ, RISC: RNA induced silencing complex

However, many autoimmune and chronic inflammatory diseases have been linked to impaired PPARγ expression. For example, patients with MS exhibit enhanced expression of inflammatory cytokines such as TNFα and show reduced PPARγ expression in PBMCs in comparison to healthy individuals (87). Besides MS, also patients with ulcerative colitis (196), inflammatory skin disorders (89) and Alzheimer's disease (197) exhibit attenuated PPARγ expression, suggesting a link between down-regulated PPARγ and chronic inflammatory diseases. PPARγ is well established for its anti-inflammatory effects in attenuating the production of pro-inflammatory mediators. One might speculate that decreased PPARγ expression prolongs inflammation and thus interferes with resolution of inflammation. Therefore, understanding molecular mechanisms that attenuate PPARγ expression may provide options for new therapeutic approaches during chronic inflammation.

6.3 Concluding remarks

Many diseases are due to dysregulated inflammation, while an insufficient immune response is as detrimental as insufficient resolution. Macrophages coordinate initiation as well as resolution of inflammation. PPARγ is well described for its anti-inflammatory properties, accounting for an alternative or regulatory macrophage phenotype in response to IL-4, IL-13 and also AC. Thus, dependent on environmental conditions a defined regulation of PPARγ is fundamental. My studies addressed both pro-inflammatory as well as anti-inflammatory conditions in macrophages. Phagocytosis of AC is an important feature of macrophages during resolution of inflammation. I could show that AC provoke activation and SUMOylation of PPARγ followed by inhibition of NFκB transactivation and concomitant target gene expression. In contrast, inflammatory conditions provoke a rapid decrease of PPARγ expression, therewith facilitating a proper immune response. I could demonstrate that activation of NFκB induces miR-27b concomitantly destabilizing PPARγ mRNA, while pre-treatment with TZDs prevented PPARγ decay. In conclusion, regulation of PPARγ function depends on the order of events. Hence, an up-coming infection down-regulates PPARγ expression in a NFκB-dependent manner, while activation of PPARγ before the onset of inflammation inhibits NFκB transactivation and therewith PPARγ decay.

Understanding regulatory mechanisms of PPARγ, rather during pro- or anti-inflammatory conditions might help to understand dysregulated immune responses.

Especially the knowledge of mechanisms reducing PPARγ expression might provide options for new therapeutic approaches for chronic inflammatory diseases, since many have been associated with impaired PPARγ expression.

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8 Appendix

Buffers and solutions

Buffers for cell biology

Erythrocyte lysis buffer

NH_4Cl	155 mM
$KHCO_3$	10 mM
EDTA	0.1 mM

Leukocyte running buffer

EDTA	2 mM
BSA	0.5% (w/v)

→ in PBS

Leukcoyte washing buffer

EDTA	2 mM

→ in PBS

Phosphate buffered saline (PBS)

NaCl	137 mM
KCl	2.7 mM
Na_2HPO_4	8.1 mM
KH_2PO_4	1.5 mM

→ Adjust pH to 7.4

Buffers and solutions for protein analysis

Blotting buffer

Tris-HCl	25 mM
Glycine	192 mM
Methanol	20% (v/v)

→ Check pH to be 8.3

Hypotonic cell lysis buffer

HEPES	10 mM
$MgCl_2$	2 mM
EDTA	100 µM
KCl	10 mM

→ Adjust pH to 7.9

Freshly added prior to use:

DTT	1 mM
PMSF	0.5 mM
Protease inhibitor mix	1 x

Lower tris buffer (4 x)

Tris/HCl	1.5 M

→ Adjust pH to 8.8

Nuclear lysis buffer

HEPES	50 mM
KCl	50 mM
NaCl	300 mM
EDTA	100 µM
Glycerol	10% (v/v)

→ Adjust pH to 7.9

Freshly added prior to use:

DTT	1 mM
PMSF	0.5 mM
Protease inhibitor mix	1 x

Protein lysis buffer

Tris/HCl	50 mM
EDTA	5 mM
NaCl	150 mM
Nonidet P-40	0.5% (v/v)

→ Adjust pH to 8.0

Freshly added prior to use:

PMSF	0.5 mM
DTT	1 mM
Protease inhibitor mix	1 x

SDS-running buffer

Tris/HCl	25 mM
Glycine	192 mM
SDS	0.7 mM

→ Adjust pH to 8.3

SDS sample buffer (4 x)

Tris/HCl	125 mM
SDS	2% (v/v)
Glycerol	20% (v/v)
Bromophenol blue	0.002% (w/v)
DTT	5 mM

→ Adjust pH to 6.9

Sodium dodecyl sulfate (SDS)-polyacrylamide gels

	Seperating gels		Stacking gel
	12.5%	10%	4%
40% Acrylamide/Bis-acrylamide (37.5% : 1.0% w/v)	3 ml	2.5 ml	300 µl
Lower tris buffer (4 x)	2.5 ml	2.5 ml	
Upper tris buffer (4 x)			750 µl
H_2O distilled	4.4 ml	4.9 ml	1.95 ml
10% SDS	100 µl	100 µl	30 µl
TEMED	10 µl	10 µl	2.5 µl
10% (w/v) ammonium persulfate	100 µl	100 µl	25 µl

TBS (tris buffered saline)

Tris/HCl 50 mM

NaCl 140 mM

→ Adjust pH to 7.4

TTBS

Tween-20 0.06% (v/v)

→ in TBS

Upper tris buffer (4 x)

Tris/HCl 0.5 M

→ Adjust pH to 6.8

Buffers for molecular biology and microbiology

DEPC-treated water

1 ml Diethylpyrocarbonate (DEPC) in 1 l distilled H_2O

→ stir overnight and autoclave

EMSA buffer D

HEPES/KOH	20 mM
Glycerol	20% (v/v)
KCl	100 mM
EDTA	0.5 mM
Nonidet P-40	0.25% (v/v)
DTT	2 mM
PMSF	0.5 mM

→ Adjust pH to 7.9

EMSA buffer F

Ficoll	20% (v/v)
HEPES/KOH	100 mM
KCl	300 mM
DTT	10 mM
PMSF	0.5 mM

→ Adjust pH to 7.9

EMSA running buffer

Running buffer (glycerol tolerant)

(Purchased from Amersham Biosciences Europe GmbH, Freiburg)

Fractionation buffer

Tris-HCl	20 mM
EDTA	2 mM
EGTA	5 mM

→ Check pH to be 7.5

<u>Freshly added prior to use:</u>

DTT	1 mM
Protease inhibitor mix	1 x

Immunoprecipitation (IP) buffer

NaCl	150 mM
Tris/HCl (pH 8)	50 mM
EDTA	5 mM
TritonX-100	1% (v/v)
NP-40	0.5% (v/v)
Freshly added prior to use:	
NaF	10 mM
PMSF	500 µM
Protease inhibitor mix	1 x

SOC medium

Tryptone	20 g/l
Yeast extract	5 g/l
NaCl	0.5 g/l
$MgCl_2$	10 mM
$MgSO_4$	10 mM
Glucose	2 mM

TBE (tris borate EDTA buffer)

Tris/HCl	90 mM
Boric acid	90 mM
EDTA	1 mM

→ Check pH to be 8.0

Buffers for luciferase assay

Luciferase lysis buffer

Tris-H_3PO_4	125 mM
DTT	10 mM
Triton X 100	5%
Glycerol	50%

→ Adjust pH to 7.8

Luciferase assay reagent

Tricine	20 mM
$(MgCO_3)_4$ x $Mg(OH)_2$ x 5 H_2O	1.07 mM
$MgSO_4$ x 7 H_2O	2.67 mM
EDTA-K^+	100 µM
DTT	33.3 mM
ATP	530 µM
Coenzyme A lithium	0.213 mg/ml
D-luciferine	470 mM

→ Check pH to be 7.8

9 Publications

Articles

Jennewein C., von Knethen A., Schmid T., and Brüne B. (2009). Induction of miR-27b by LPS destabilizes PPARγ mRNA in human macrophages. Submitted to **J Leukoc Biol** (August 2009)

Namgaladze D., Jennewein C., Preiss S., von Knethen A., and Brüne B. (2009). Attenuated suppression of the oxidative burst by cells dying in the presence of oxidized low density lipoprotein. **J Lipid Res**. (Epub: doi:10.1194/jlr.M800615-JLR200)

Weigert A., Jennewein C., and Brüne B. (2009). The liason between macrophages and AC: the end programs the beginning. **Biol Chem**. 390 (5-6): 379-90

Jennewein C., Kuhn AM., Schmidt MV., Meilladec-Jullig V., von Knethen A., Gonzalez FJ., and Brüne B. (2008). SUMOylation of PPARγ by apoptotic cells prevents LPS-induced NCoR removal from κB binding sites mediating transrepression of pro-inflammatory cytokines. **J Immunol.** 181 (8): 5646-52

von Knethen A., Soller M., Tzieply N. Weigert A., Johann A.M., Jennewein C., Köhl R., and Brüne B. (2007). PPARγ1 attenuates cytosol to membrane translocation of PKCalpha to desensitize monocytes/macrophages. **J Cell Biol**. 176 (5): 681-94

Oral and poster presentation:

Poster and selected oral presentation at the 22nd EMDS Annual Meeting 2008 in Brescia/Italy: 'Apoptotic cells SUMOylate PPARγ thereby transrepressing NFκB'

10 Danksagung

Die letzten (fast) vier Jahre waren von vielen Höhen und Tiefen, Begeisterung aber auch Frust, begleitet. Letztendlich steht das Ende kurz bevor und ich kann zufrieden auf meine Zeit am Institut für Biochemie I zurückblicken.
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11 Curriculum vitae

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Frankfurt, den 03.09.2009

12 Erklärung

Ich erkläre, dass ich die dem Fachbereich Medizin der Goethe-Universität Frankfurt am Main zur Promotionsprüfung eingereichte Arbeit mit dem Titel

"Regulation of peroxisome proliferator-activated receptor γ in macrophages during inflammatory processes"

im Institut für Biochemie I – Pathobiochemie unter Betreuung und Anleitung von Dr. habil. Andreas von Knethen ohne sonstige Hilfe selbst durchgeführt und bei der Abfassung der Arbeit keine anderen als die in der Dissertation angeführten Hilfsmittel benutzt habe.

Ich habe bisher an keiner in- oder ausländischen Universität ein Gesuch um Zulassung zur Promotion eingereicht. Die vorliegende Arbeit wurde bisher nicht als Dissertation eingereicht.

Der erste Teil der vorliegenden Arbeit wurde in

Journal of Immunology (2008), 181: 5646-5652 veröffentlicht.

Der zweite Teil der Arbeit wurde bei Journal of Leukocyte Biology eingereicht.

Frankfurt am Main, den 03. September 2009