PROJECT FINAL REPORT

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Part A Table of Contents

1. FINAL PUBLISHABLE SUMMARY REPORT

1.1. EXECUTIVE SUMMARY

We have carried out a cross-disciplinary research project (involving functional genetics, *in vivo* imaging, gene engineered animal models, renal and cardiovascular phenotyping) to facilitate the better understanding of renocardial effects of the thiazolidinedione (TZD)-type antidiabetic/antiatherogenic drugs and to find possible mechanisms to reduce their unwanted cardiovascular effects. TZDs are synthetic ligands of peroxisome proliferator activated receptor gamma (PPARγ), which belongs to the heterodimer-forming nuclear receptor superfamily of ligand-dependent transcription factors. PPARγ forms a permissive heterodimer with retinoid X receptors (RXRs) and affects various cellular processes at the level of gene expression regulation. Additionally to its insulin sensitizing effects, PPARγ regulates the expression of a range of inflammatory genes. This makes TZDs promising drugs in the treatment of inflammatory disorders, e.g. atherosclerosis, kidney disease and autoimmune inflammation. However, the immunomodulatory role of PPARγ/RXRα heterodimers is still undefined. As of medical importance, our studies show that activation of PPARγ/RXRα heterodimers in macrophages is a potential target in the resolution of autoimmune inflammation and the treatment of kidney disease. Moreover, activation of macrophage PPARγ and RXRα potentially yields medical benefits in the treatment of autoimmunity. We have also shown that macrophage RXRα and RXRβ but not PPARγ can affect the kidney-to-heart hormonal signaling (the renin-angiotensin aldosterone system) thereby establishing a link between inflammation, fluid retention and cardiac fibrosis. Our project also provides important data to the basic research. We have shown that PPARγ and RXRα are essential for the proper expression of several cell surface receptors and complement molecules in macrophages, making PPARγ/RXRα heterodimer an important regulator of the phagocytosis. Our results also confirm that uptake of apoptotic cell debris increases anti-inflammatory macrophage phenotype acquisition by mitigating inflammatory gene transcription in a PPARγ/RXRα dependent manner. This mechanism can involve the interaction of PPAR/RXR with other transcription factors. Accordingly, macrophages deficient in PPARγ or RXRα display impaired phagocytosis and consequently reduced anti-inflammatory polarization. Macrophage-specific deletion of PPARγ or RXRα in mice leads to apoptotic cell accumulation in tissues, provoking autoimmune kidney disease. These results show that PPARγ and RXRα signaling in macrophages is essential for the efficient clearance of apoptotic cells and the maintenance of immunological self-tolerance, an important element of tissue homeostasis. Our studies also indicate that macrophage RXRs are involved in the progression of cardiac fibrosis in response to the activation of the renal blood pressure regulating hormonal cascade, the renin-angiotensin-aldosetrone system. Macrophage PPARγ and RXRs are therefore candidate targets for new medication strategies in the treatment of human autoimmune renal and cardiovascular disease and heart hypertrophy. Pharmacological targeting of RXRs may allow reduce the administration of TZDs in order to reach beneficial effects without cardiac complications.

List of Keywords:

PPARγ, RXR, inflammation, autoimmunity, kidney, cardiovascular system, phagocytosis, nuclear receptors

2. A SUMMARY DESCRIPTION OF PROJECT CONTEXT AND OBJECTIVES

2.1. Research objectives

Background. TZD-type ligands of the nuclear receptor PPARγ have been introduced to the clinical practice as insulin sensitizers [1]. Further progress in the field has shown that **PPARγ is a promising target in the medication of atherosclerosis and its comorbidities** [2-3]. Atherosclerosis is a result of a pathological interaction between circulating inflammatory cytokines, lipids, hematodynamic factors and tissue macrophages. Pharmacological activation of PPARγ lowers triglyceride levels by promoting fatty acid storage and utilization by liver and muscle via the elevation of their insulin sensitivity. PPARγ activation also facilitates the removal of cholesterol from macrophages to HDL, therefore exerts antiatherogenic effects. Potential antiatherogenic consequences of activating PPARγ include the reduced expression of inflammatory response genes and chemokine receptors that promote the recruitment of macrophages into lesions and their subsequent differentiation and activation [4-5]. PPARγ activation can also inhibit the remodeling of extracellular matrix proteins in the fatty streak, which is critically involved in foam cell generation and atheroprogression [6]. Hypertension is a common comorbidity of atherosclerosis and insulin resistance and clinical observations show that TZDs effectively reduce blood pressure in type 2 diabetic patients [6-8]. TZDs also reduce peripheral resistance in several experimental models of hypertension leading to reduced blood pressure [9]. However, **pharmacological PPARγ activation by TZDs may have cardiovascular complications** [10]. Recent reports show that chronic TZD treatment leads to a cardiac lipodystrophy in animal studies and clinical trials of TZDs with cardiovascular endpoints have also questioned the clear benefits of TZDs, since they can lead to fluid retention and ischemic heart events [8, 11]. These finding most recently led to the withdrawal of rosiglitazone, a widely used TZD from the market [10, 12]. Administration of TZDs spreaded rapidly in the medication of insulin resistance and its cardiovascular/metabolic complications. Serious side effects however urge to provide the crucial missing information required to improve cardiovascular benefits of PPARγ activation and avoid the secondary cardiovascular complications [13].

Objective and hypothesis. The main objective of this project was the better understanding of the mechanisms behind the unwanted cardiovascular side effects of TZDs, in order **to find alternatives to acquire sufficient PPARγ activation with reduced doses of TZD administration**. Since **kidney is an important regulator of cardiac performance**, this project focused on the potential renal effects of PPARγ activation, which secondarily can affect blood pressure and cardiac functions. The kidneys establish a hromonal communication channel between fluid homeostasis and the cardiovascular system. This hormonal system is the renin-angiotensin-aldosterone system (RAAS). The RAAS is a cascade formed by hormone-like substances released from the kidney and the adrenal gland, and acts as a long-term regulator of systemic blood pressure, heart contractility and interstitial fluid volume. The most potent effector molecule of the RAAS is angiotensin II (Ang II), which elevates systemic blood pressure through the constriction of blood vessels and furthermore enhances aldosterone secretion, catecholamine release, sympathetic nerve activity and myocardial contractility.

Aldosterone, the other effector molecule of the RAAS, contributes to the maintenance of fluid homeostasis and blood volume through the regulation of the sodium and water resorbtion capacity of the kidneys and the intestinal epithelium [8].

The patient population using TZDs suffers form metabolic syndrome, a disease hallmarked with increased macrophage inflammatory state and is associated with a high risk to develop cardiac disease, hypertension and renal failure. TZDs are also known to target macrophages, favoring their anti-inflammatory polarization, thus mitigating inflammation [5-6]. Macrophages are immune cells, which infiltrate the majority of tissues, thus their behavior can determine the local inflammatory state in the tissues. Macrophages can maintain immunological self-tolerance, establish tissue turnover, regeneration potential; but also exacerbate impaired insulin signaling, tissue destruction and cell death. We hypothesized that **macrophages link inflammation, renal physiology and heart disease**, since RAAS activation is known to influence inflammation thus contribute not only to the development of hypertension but also to its cardiovascular complications, such as microvascular damage, kidney injury and left ventricular hypertrophy.

2.2. Report on work performed and results

Aims. The project presented in our proposal was focused on three specific aims. **Specific Aim 1.** To test the hypothesis that cardiac side effects of TZD treatment can be mainly due to fluid retention which impairs cardiac performance. **Specific Aim 2.** To understand PPARγ mediated mechanisms leading to fluid retention and ischemic heart failure. **Specific Aim 3.** To characterize the contribution of macrophage PPARγ to the development of TZD side effects. Test the hypothesis that PPARγ activation is associated with fluid retention, which impairs cardiac performance.

Changes to the original proposal. The contribution of collecting duct specific PPARγ to sodium and water retention has been addressed by reports published at the beginning of our project [11]. Thereby we turned our attention to other possible mechanisms implicated in altered fluid homeostasis and cardiac disease, such as the role of PPARγ/RXR heterodimers in RAAS and cardiac fibrosis, as described in the second and third specific aims of the original workplan.

Objective 1. First objective of the project was the understanding of PPARγ mediated mechanisms leading to fluid retention and ischemic heart failure.

Work performed:

- Measurement of plasma levels of aldosterone, sodium/chloride ratio in PPARγ, RXRα and RXRα/β deficient mice and their wild-type (WT) littermate controls.
- Analysis of renal function by determination of albumin, creatinine, urea nitrogen excretion and the glomerular filtration rate based on inulin clearance.
- Determination of the mRNA expression levels of inflammatory cytokines (IL6, IL1β, TNFα, MCP-1, F4/80) and iNOS in renal tissues.
- Analysis of cardiac performance using echocardiography and ECG.
- Analysis of the cardiac and renal response to renal artery ligation (model of severe fluid retention) and angiotensin II infusion (experimentally induced cardiac hypertrophy model).

Results:

- Macrophages are key players in mediating cardiac remodelling in response to altered fluid homeostasis and increased activation of the RAAS. Macrophage deficiency of RXRα and RXRβ leads to increased RAAS activation, reflected by increased plasma aldosterone levels.
- Lack of macrophage RXRα and RXRβ leads to cardiac hypertrophy and increases cardiac fibrosis in response to RAAS activation in mice. These effects do not require PPARγ, reflected by the lack of cardiac pathology in mice with macrophage specific ablation of PPARγ.

Specific training:

- technical training: blood sample obtaining, determination of cytokines and aldosterone by ELISA, western blotting, electron microscopy, surgical techniques: artery ligation, implantation of osmotic minipumps; echocardiography, small animal ECG.

Relevance for basic science:

Macrophage RXRα and RXRβ deficient mice display a basal phenotype characterized by increased heart muscle mass and RAAS activation. Since PPARγ deficiency does not result a similar phenotype, it is possible that RXRs act by forming heterodimers with other nuclear receptors. The underlying mechanism may be due to a proinflammatory macrophage polarization in the kidney and the heart, however this possibility should be tested by further studies.

Objective 2. The second objective of the project was to characterize the contribution of macrophage PPARγ to the development of TZD side effects.

Work performed:

- Analysis of renal function by determination of albumin, creatinine, urea nitrogen excretion and the glomerular filtration rate based on inulin clearance in PPARγ and RXRα deficient mice and their wild-type (WT) littermate controls.
- Histological, immunohistochemical and transmission electron microscopical analysis of renal morphology.
- Optical projection tomography analysis of kidney arteriolar structure.
- Determination of the mRNA expression levels of inflammatory cytokines (IL6, IL1β, TNFα, MCP-1, F4/80) and iNOS in renal tissues.
- *In vivo* and *in vitro* phagocytosis assays, apoptotic cell clearance.
- Functional genomics approaches to identify PPARγ/RXRα regulated genes. Chromatin immunoprecipitation sequencing, DNA microarray analysis, RT PCR studies
- Cardiovascular phenotyping (echocardiography, ECG)
- Development of RXRα/RXRβ deficient mouse line

Results:

- Mice lacking macrophage expression of PPARγ or RXRα develop glomerulonephritis and autoantibodies to nuclear antigens.

- In the lack of macrophage PPARγ and RXRα mice show deficiencies in phagocytosis and impaired clearance of apoptotic cells. These mice are also unable to acquire an anti-inflammatory phenotype upon feeding of apoptotic cells, which is critical for the maintenance of self-tolerance.
- Stimulation of PPARγ and RXRα in macrophages facilitates apoptotic cell engulfment, and provides a potential strategy to avoid autoimmunity against dying cells.

Specific training:

- technical training: functional genomics, western blotting, phagocytosis assays, cell culture, advanced techniques in fluorescence microscopy

Relevance for basic science:

- The results obtained will give a strong experimental basis for further medication strategies to reduce autoimmunity against apoptoti cells (e.g. in the human autoimmune disorder systemic lupus erythematosus [SLE]). Our work suggests a new therapeutic potential for PPARγ/RXRα ligands as enhancers of apoptotic-cell removal by macrophages. Therefore we anticipate that our findings will be a starting point for studies aimed at the selective modulation of macrophage PPARγ/RXRα potential targets for the treatment of renal and cardiovascular manifestation of SLE [14].

3. A DESCRIPTION OF THE MAIN S&T RESULTS/FOREGROUNDS

3.1. Targeting macrophage PPARγ/RXRα: a new strategy to reduce autoimmune disease

3.1.1. Lack of macrophage PPARγ or RXRα leads to kidney disease

At an early phase of our project we have analyzed DNA microarray data and identified a set of candidate PPARγ target genes which are associated with renal disease and involved in phagocytosis [14]. In order to explore their contribution to TZD effects, we generated conditional knockout mice lacking macrophage expression of PPARγ or RXRα by crossing mice bearing the lox-P-targeted PPARγ ($PPAR\gamma^{f/f}$) or RXRα ($RXR\alpha^{f/f}$) alleles with mice bearing the lysosyme-M Cre (LysMCre) recombinase transgene. We refer to LysM Cre-negative $PPAR\gamma^{f/f}$ and $RXR\alpha^{f/f}$ mice as wild-types (WT), and their $PPAR\gamma^{f/f}LysMCre^{+}$ or $RXR\alpha^{f/f}LysMCre^{+}$ littermates as knockout animals (PPARγ-KO and RXRα-KO) [14].

Interestingly, female mice lacking macrophage expression of PPARγ or RXRα developed nephropathy at 4-6 months of age. We analyzed further the kidney angioarchitecture by optical projection tomography, showing enlargement of renal arteriolar casts within the kidneys of PPARγ-KO and RXRα-KO animals. Kidney hypertrophy, a consequence of glomerulomegaly, also caused a significant increase in kidney weight in PPARγ-KO and RXRα-KO mice compared with WT littermates. As a next step, histological analysis and transmission electron microscopy revealed mesangial hypercellularity, mesangial matrix expansion, and basement membrane thickening in PPARγ-KO and RXRα-KO kidneys. Moreover, we also detected increased excretion of albumin and high molecular weight proteins in the urine of PPARγ-KO and RXRα-KO mice, as well as elevated plasma creatinine levels, all of which are indicators of kidney disease. To further characterize the glomerular injury, we examined the localization of inflammatory markers and mouse immunoglobulins by immunohistochemistry. Most glomeruli in the kidneys of PPARγ-KO and RXRα-KO mice contained numerous F4/80-positive inflammatory cells. These glomeruli also showed strong immunostaining for vascular endothelial growth factor-A (VEGF-A) in the mesangium surrounding the glomerular capillaries, correlating with elevated serum levels of VEGF-A in PPARγ-KO and RXRα-KO mice.

These findings indicate glomerular inflammation associated with macrophage infiltration. In addition, there was a marked deposition of IgG and IgM in the mesangial matrix of PPARγ-KO and RXRα-KO mice, and analysis of serum showed that these animals developed autoantibodies against nuclear proteins (ANA, antinuclear antibody), single-stranded DNA (anti-ssDNA) and double-stranded DNA (anti-dsDNA), suggesting an autoimmune origin of the observed renal disease. Autoimmune glomerulonephritis is a common manifestation of human systemic lupus erythematosus (SLE). Our results show that mice lacking macrophage expression of PPARγ or RXRα develop glomerulonephritis and autoantibodies to nuclear antigens, resembling the nephritis seen in SLE.

3.1.2. Macrophage PPARγ and RXRα are required for normal apoptotic cell clearance

Impaired clearance of apoptotic cells within the kidney glomeruli can contribute to autoimmune kidney disease. To test the possibility that excess tissue deposition of apoptotic cell debris can be the cause of the observed renal pathoplogy, we detected apoptotic cell nuclei by terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL). A significant increase in the number of apoptotic cells in the glomeruli of PPARγ-KO and RXRα-KO mice was found. Consistently, PPARγ-KO and RXRα-KO glomeruli were abundant in endothelial and mesangial cells containing the early apoptotic marker cleaved caspase-3. Immunostaining revealed that apoptotic cells were distributed throughout other tissues of PPARγ-KO and RXRα-KO mice and that a significantly lower proportion of splenic macrophages contained apoptotic cells in these mice, indicating a generalized defect in apoptotic cell clearance.

We believed that understanding of PPARγ to apoptotic cell clearance has a general medical impact, therefore we explored the underlying cellular and molecular mechanism. To investigate whether macrophage phagocytosis was altered in PPARγ-KO or RXRα-KO mice, we injected their peritoneal cavities with fluorescently labeled apoptotic thymocytes (ATs). Peritoneal lavage showed that ATs were still abundant 6 or 14 h after injection; in contrast, the peritoneal macrophages of similarly treated WT littermates efficiently scavenged most of the injected ATs. Phagocytosis deficit of peritoneal macrophages of PPARγ-KO and RXRα-KO mice was also reflected by their notably less phagosome content and smaller filopodia compared to WT macrophages seen by electron microscopy. These findings suggest a profound *in vivo* defect in apoptotic cell clearance in PPARγ-KO and RXRα-KO mice (Figure 1).

3.1.3. PPARγ/RXRα heterodimers regulate the expression of genes involved in the engulfment of apoptotic cells by macrophages

To understand the molecular mechanism underlying the defective apoptotic cell removal detected in our mouse models, we profiled gene expression in WT and knockout macrophages by microarray analysis. Clustering analyses revealed that knockout peritoneal macrophages are deficient for the transcription of a set of genes encoding phagocytosis-associated receptors and opsonins. Quantitative PCR analysis confirmed these differences. The digestion of apoptotic cells by macrophages leads to the accumulation of cellular components, including cholesterol and fatty acids, that could act as endogenous ligands of PPARγ and RXRα. To investigate this possibility, we analyzed the expression of phagocytosis related genes in WT, PPARγ-KO and RXRα-KO peritoneal macrophages incubated with ATs. The presence of ATs increased the expression of several phagocytosis-related genes (*Cd36, Mertk, Axl, Fcgr1, C1qa, C1qb, C1qc, Tgm2)* in WT macrophages but not in PPARγ-KO and RXRα-KO macrophages. Apoptotic cell components thus enhance the expression of macrophage receptors and opsonins required for further apoptotic cell uptake, via a PPARγ- and RXRα-dependent mechanism.

We next tested whether the expression of these genes can be modulated by pharmacological activation of PPARγ or RXRα. Peritoneal macrophages from WT, PPARγ-KO and RXRα-KO mice were cultured for 24 h in the presence of PPARγ or RXRα agonists (RSG for PPARγ, 9cRA and

LG268 for RXRα). All three agonists selectively induced the expression of phagocytosis-associated genes in WT macrophages but not in PPARγ-KO or RXRα-KO cells. Further confirmation that the phagocytosis genes *Cd36*, *Mertk*, *Axl*, *Fcgr1* and *Tgm2* are directly regulated by PPARγ was obtained by assessing genomic PPARγ binding in peritoneal macrophages by chromatin immunoprecipitation followed by high-throughput sequencing (ChIP-seq). In this analysis, *Cd36*, *Axl*, *Fcgr1*, and *Tgm2* all had high-confidence PPARγ binding regions within 100 kb of their transcription start sites. Together with their responsiveness to a PPARγ ligand, and reduced expression in PPARγ and RXRα deficient macrophages, the data strongly suggest that these genes are under the direct transcriptional control of PPARγ/RXRα [14].

3.1.4. Diminished anti-inflammatory response to apoptotic cells in macrophages lacking PPARγ or RXRα

Following the identification of key target genes of PPARγ/RXRα which are responsible for the observed kindey pathology, we asked whether the PPARγ/RXRα has role in the differentiation of macrophages to deactivated (anti-inflammatory) phenotype that is closely associated with the uptake od apoptotic cells and diminished production of inflammatory mediators. We found, that engulfment of ATs upregulates the anti-inflammatory cytokine genes in WT macrophages. These genes were not affected or only in a lower extent in PPARγ-KO and RXRα-KO macrophages. To test the possible involvement of PPARγ and RXRα in the regulation of proinflammatory cytokine production, we analyzed inflammatory gene expression in WT, PPARγ-KO and RXRα-KO macrophages stimulated with lipopolysaccharide (LPS) in the presence or absence of ATs. LPS upregulated the expression of genes encoding interleukin-12B (*Il12b*), interleukin-1β (*Il1b*), inducible nitric oxide synthase (*Nos2*) and tumor necrosis factor-α (*Tnf*). The presence of ATs significantly reduced the transcript levels of these proinflammatory markers in WT macrophages, but had no effect on PPARγ-KO and RXRα-KO macrophages. Cytokine secretion was also monitored by ELISA. As expected, the presence of ATs suppressed the release of proinflammatory cytokines by WT macrophages but had little effect on PPARγ-KO and RXRα-KO macrophages. Consistently, the kidney glomeruli of PPARγ-KO and RXRα-KO mice were infiltrated with cells expressing proinflammatory markers, such as IL1β, iNOS and TNFα. These data indicate that the observed kidney inflammation in mice lacking macrophage expression of PPARγ or RXRα is a consequence of enhanced proinflammatory macrophage activation within the kidney glomeruli [14] (Figure 1).

3.1.5. Apoptotic cell uptake is impaired in macrophages lacking PPARγ or RXRα

To determine directly whether macrophages from PPARγ-KO and RXRα-KO mice were defective in apoptotic cell uptake we tested the ability of peritoneal macrophages to ingest fluorescently-labeled ATs *in vitro*. Compared with WT cells, PPARγ-KO and RXRα-KO peritoneal macrophages were markedly deficient in the phagocytosis of fluorescently labeled ATs. Phagocytosis of ATs by WT macrophages was significantly enhanced by pharmacological activation of PPARγ or RXRα. Treatment of PPARγ-KO or RXRα-KO macrophages with RSG or 9cRA had no effect on either parameter. In conclusion, mice lacking macrophage PPARγ-KO or RXRα-KO show deficiencies in

phagocytosis and clearance of apoptotic cells, and are unable to acquire an anti-inflammatory phenotype upon feeding of apoptotic cells, which is critical for the maintenance of self-tolerance [15]. These results demonstrate that stimulation of PPARγ and RXRα in macrophages facilitates apoptotic cell engulfment, and provide a potential strategy to avoid autoimmunity against dying cells and attenuate renal outcome of SLE [14] (Figure 1).

3.2. The role of macrophage PPAR/RXR in the kidney-heart hormonal crosstalk

3.2.1. PPARs are players in the cardiac effects of RAAS

Recent research has revealed roles for the PPAR family of transcription factors in blood pressure regulation, expanding the possible therapeutic use of PPAR ligands [8]. PPARα and PPARγ modulate the RAAS, a major regulator of systemic blood pressure and interstitial fluid volume by transcriptional control of renin, angiotensinogen, angiotensin converting enzyme (ACE) and angiotensin II receptor 1 (AT-R1). Blockade of RAAS is an important therapeutic target in hypertension management and attenuates microvascular damage, glomerular inflammation and left ventricular hypertrophy in hypertensive patients and also show antidiabetic effects. The mechanisms underlying the benefits of RAAS inhibition appear to involve PPARγ-regulated pathways. Although the role of PPARs in the transcriptional control of the RAAS may allow the use of PPAR ligands in the treatment of RAAS dependent hypertension, unwanted cardiac effects of the PPARγ ligand TZDs limits the potential medical benefits. We aimed to understand whether the targeting of the PPARγ partner RXRs may be also effective in the modulation of heart-kidney hormonal crosstalk and normalize fluid homeostasis and reduce blood pressure [8].

3.2.2. Animal model: ablation of PPARγ, RXRα and RXRβ

In order to establish the contribution of macrophage PPARγ/RXRα heterodimers to the development of cardiovascular complications of fluid retention and RAAS activation, we have analyzed mouse lines with macrophage specific deletion of PPARγ and RXRα. Since our first results showed the lack of cardiac patholologies in these mouse lines (Table 1), we have generated a novel mouse model with macrophage specific ablation of both RXRα and RXRβ (RXR-KO). Conditional knockout mice were generated by using the LoxP recombinase system. Dysfunction of the RAAS, a major regulator of systemic blood pressure and interstitial fluid volume was modelled by subjecting mice to a constant angiotensin-II infusion. Osmotic minipumps were implanted into wild-type (WT) and macrophage RXR-KO mice in order to maintain a constant angiotenin-II infusion rate for 28 days. Since obesity and insulin resistance are risk factors for the development of RAAS-dependent hypertension and cardiac hypertrophy, in another set of experiment we used diet-induced obesity (DIO) model. High caloric and saturated fatty acid intake provokes insulin resistance, atherogenesis and a general inflammatory state with disturbed balance of inflammatory (M1) and anti-inflammatory (M2) macrophage phenotypes [4, 16]. In order to assess the role of macrophage M1/M2 phenotypes in RAAS dysfunction, we analyzed renal and cardiac performance.

3.2.3. Cardiac hypertrophy and fibrosis: a new niche for macrophage RXRs

Interestingly, mice lacking both macrophage RXRα and RXRβ (RXR-KO) develop spontaneous ventricular hypertrophy and accompanied with hypertension. The development of this cardiac phenotype is associated with ageing and may be a consequence of RAAS activation, suggested by the increased aldosterone levels in the plasma of RXR-KO mice. The severity and development of heart hypertrophy was estimated with *in vivo* detection systems, including electrocardiography, Doppler ultrasound system and echocardiography. Histological and electron microscopy studies also confirmed the cardiac pathology. Induction of obesity and insulin resistance in WT (WT DIO) and RXR-KO (KO DIO) mice led to severe cardiac alterations, conductance deficits, impaired heart wall motility, all of them are indicatives of ischemic heart diseas (Table 1). However, the severity of DIO-associated cardiac pathology was the same in WT DIO and KO DIO animals, suggesting that this mechanism does not require the involvement of macrophage RXRs. Surprisingly, in the other model system, in which we activated the RAAS by angiotensin II infusion, the severity of cardiac fibrosis was higher in RXR-KO animals. This phenomenon implies that in the lack of RXRs macrophages invade cardiac arterioles and increase the development of vascular inflammation. This scenario implies that cell specific RXR activation can potentially act against vascular damage and cardiac fibrosis. These cardiac alterations were not observed in mice with macrophage PPARγ deficiency. Similarly, the lack of macrophage RXRα alone is not sufficient to affect cardiac performance, suggesting that RXRβ may compensate the lack of RXRα.

3.3. Summary

The main objective of this project was to address an important clinical problem, the cardiac side effects of TZDs by providing experimental basis for improved drug design and administration. PPAR function in human physiology and pathology is a current topic of the experimental medicine, since many metabolic abnormalities are coupled to PPAR dysfunctions. TZDs also have the potential to be introduced in combined medication of cardiovascular and renal disease. This project shows that activation of PPARγ/RXR heterodimers in macrophages can exert various cardiovascular benefits, including mitigation of autoimmune kidney disease, cardiac fibrosis and RAAS-induced hypertrophy (Figure 2). The outcome of this project drives our attention to the benefits of macrophage RXR activation in controlling inflammatory programs of gene expression in macrophages. The results of our studies give the novel idea that macrophage RXR activation can reduce chronic renal inflammation and improve cardiac performance, providing an alternative of the TZD administration.

4. FIGURES

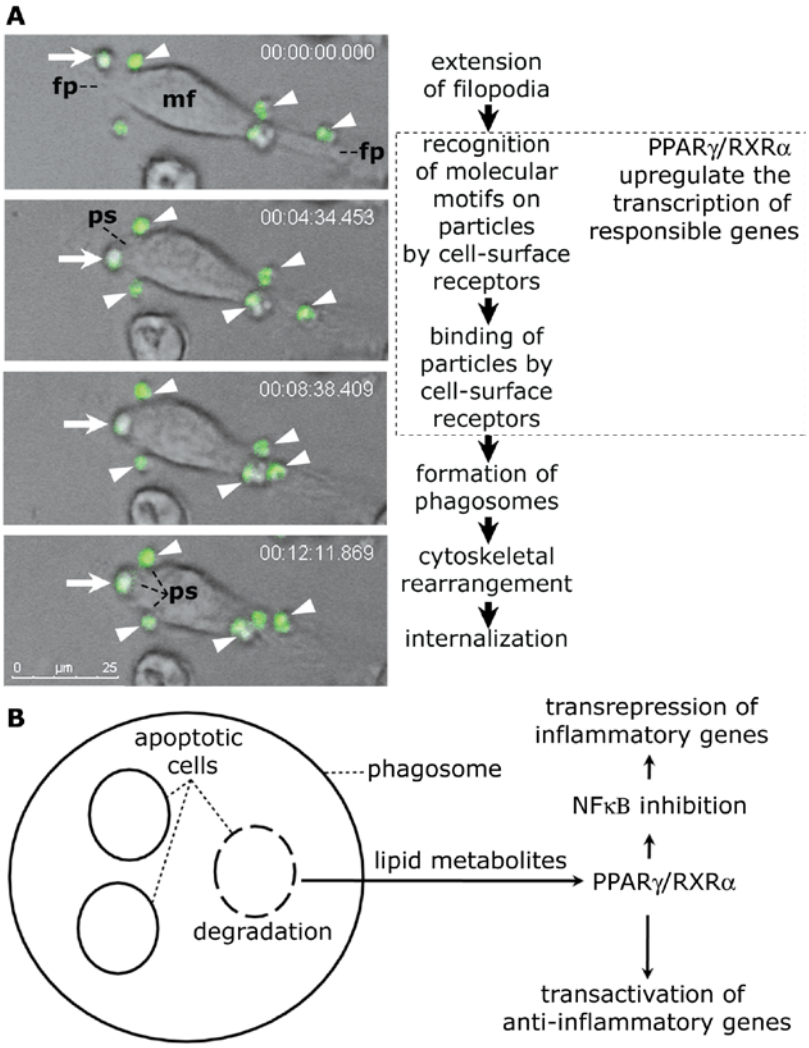

Figure 1. Dual mechanism of PPARγ/RXRα controlled gene expression in apoptotic cell uptake and immunological self-tolerance

Activation of PPARγ/RXRα is essential for the expression of a complex network of genes implicated in the recognition, binding and internalization of apoptotic cells (A). In the lack of PPARγ or RXRα macrophages are unable to engulf apoptotic debris. This clearance deficit exacerbates autoimmune disorders. Degradation of apoptotic cells by the phagosomes can provide lipid activators of PPARγ/RXRα, which leads to acquisition of anti-inflammatory macrophage phenotype and resolution of inflammation (B). Activation of either PPARγ or RXRα with synthetic ligands effectively enhance the uptake of apoptotic cells and promotes anti-inflammatory macrophage polarization, thus strengthens tissue healing and suppresses autoimmune reactions. Panel A shows a real-time acquisition of the engulfment of apoptotic thymocytes (green) by a mouse macrophage *in vitro*. mf: macrophage cell body, fp: filopodia, ps: phagosome; arrowheads: attached apoptotic cells, arrows: engulfed apoptotic cell.

Table 1. Effects of macrophage ablation of PPRγ and RXRs on herat and kidney		
Ablated nuclear receptor	**Cardiac phenotype**	**Renal phenotype**
PPARγ	has no impact on cardiac performance	autoimmune nephritis as a consequence of impaired apoptotic cell clearance
RXRα	has no impact on cardiac performance	autoimmune nephritis as a consequence of impaired apoptotic cell clearance
RXRα and RXRβ	hypertension, cardiac hypertrophy, increased cardiac fibrosis in response to RAAS activation; has no impact on cardiac alterations in DIO	increased RAAS activation, progressive decline in kidney function

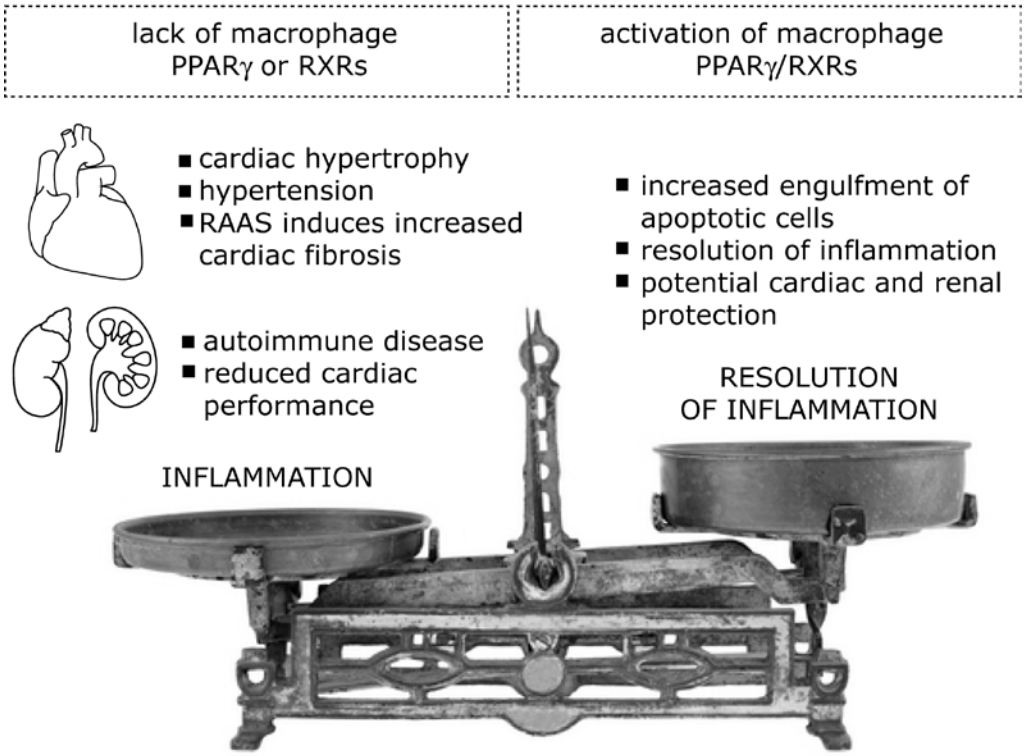

Figure 2. The macrophage balance: anti-inflammatory effects of PPARγ and RXRs can resolve cardiac and renal disease

Ligands of PPARγ modulate macrophage function and regulate the expression of a range of inflammatory genes, making them potential drugs for the treatment of inflammation and immune responses. Our studies show that macrophage-specific deletion of PPARγ or RXRs leads to a renal and cardiac disease. The overlapping effects of PPARγ and RXR ligands on macrophage-mediated inflammatory disorders raise the possibility that dual administration of PPARγ and RXR activators could allow the administration of lower doses of TZDs, reducing their side effects.

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