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Peroxisome proliferator-activated receptor α and γ agonists differently regulate classical and alternative macrophage activation

Abstract: Peroxisome proliferator-activated receptor (PPAR) agonists, fibrates and thiazolidinediones, are commonly used drugs in the treatment of dyslipidemia and diabetes. Their targets, PPARα and PPARγ, have also been shown to have a role in the regulation of inflammatory responses linking metabolism and inflammation. In the present study we investigated the effects of PPAR agonists on macrophage activation. In addition to the proinflammatory classical activation, we also focused on interleukin (IL) 4 and 13 -induced alternative activation which is a significant macrophage phenotype in tissue repairing processes and in fibrosing diseases. PPARα agonists GW7647 and fenofibrate as well as PPARγ agonist GW1929 inhibited lipopolysaccharide-induced classical macrophage activation and production of the characteristic biomarkers of this phenotype, i.e. IL-6 and nitric oxide, in murine J774 macrophages. Remarkably, the PPARα agonists also inhibited IL-4 and IL-13 –induced expression of alternative activation markers arginase-1, fizz1 and mannose receptor 1 whereas the PPARγ agonist GW1929 enhanced their expression in J774 macrophages. The PPARα agonists GW7647 and fenofibrate also attenuated the production of alternative activation markers chemokine (C-C motif) ligand 13 and platelet-derived growth factor in human THP-1 macrophages. The present findings show that PPARα and PPARγ agonists differently regulate classical and alternative macrophage phenotypes. Furthermore, PPARα activation was introduced as a novel concept to down-regulate alternative macrophage activation indicating that PPARα agonists have therapeutic potential in conditions associated with aberrant alternative macrophage activation such as fibrosing diseases.

Keywords: Macrophage polarization, peroxisome proliferator-activated receptor, fibrosis

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1 Introduction

Macrophages are important effector cells in inflammatory responses. They are involved in bacterial infections and autoimmune diseases but also in the low grade inflammation associated with obesity and metabolic syndrome. Macrophages also have homeostatic functions and they activate healing process associated with inflammation and tissue injury [1-3]. To achieve these multiform tasks, macrophages are programmed to display distinct phenotypes in response to environmental signals [2-5]. For example, Th1 cytokine interferon γ (IFNγ) and toll-like receptor 4 (TLR4) ligand lipopolysaccharide (LPS) trigger classical, i.e. type M1, activation of macrophages, which is typical for bacterial infections and autoimmune reactions [6]. On the other hand, Th2 cytokines interleukin (IL) 4 and 13 trigger alternative, i.e. type M2, activation, which is associated with wound healing but also with the aberrant immune responses associated with over-activation of fibroblasts and subsequent fibrosis [6,7].

Classical activation of macrophages leads to the release of proinflammatory and antimicrobial factors including IL-6, IL-12, tumour necrosis factor (TNF) and nitric oxide (NO) produced by inducible nitric oxide synthase (iNOS) pathway. On the other hand, alternatively activated macrophages express factors that have been associated with recognition of extracellular pathogens and/or stimulation of fibroblasts. Some of

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these factors are arginase 1, chemokine (C-C motif) ligand 13 (CCL13), platelet-derived growth factor (PDGF), mannose receptor 1 (mrc-1) and, in murine macrophages, found in inflammatory zone 1 (fizz1), for instance [4,8-10]. Likewise, also in clinical settings, alternatively activated macrophages have been linked to defence against extracellular pathogens, and to resolution of inflammation, wound healing and fibrosis [11-13].

In addition to IL-4 and IL-13, peroxisome proliferator-activated receptor γ (PPARγ) ligands have been found to polarize macrophages towards the alternative phenotype [14,15]. PPARs are nuclear receptors that have a crucial physiological role in the regulation of energy homeostasis. PPARγ regulates the import of glucose into cells, and PPARγ agonists thiazolidinediones (TZDs) are widely used as pharmacological agents in the treatment of type 2 diabetes. Another isotype of PPARs, PPARα is linked to the regulation of lipid metabolism. Synthetic ligands include fibrates, which are used to treat dyslipidemia. As compared to PPARγ agonists, less is known about the possible involvement of PPARα agonists in the regulation of immune and inflammatory responses [16-19] and their effects on alternative activation of macrophages remains unknown.

In the present study, we aimed to compare the effects of PPARα and PPARγ agonists on classical and alternative activation of macrophages. We used LPS or a combination of IL-4 and IL-13 to induce macrophage polarization to classical or alternative phenotype, respectively. The results show that PPARα and PPARγ agonists differently regulate macrophage phenotypes, and introduce PPARα activation as a novel concept to down-regulate alternative macrophage activation.

2 Materials and methods

2.1 Materials

Reagents were obtained as follows: GW1929 and GW7647 from Tocris Bioscience (Bristol, UK), rabbit polyclonal β-actin, arginase 1 and STAT6 antibodies and goat HRP-conjugated anti-rabbit and donkey HRP-conjugated anti-goat polyclonal antibodies from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). pSTAT6 (Tyr^{641}) antibody was from Calbiochem (Merck Millipore, Billerica, MA, USA) and PPARα and PPARγ antibodies from Alexis Biochemicals (Enzo Life Sciences, Lausen, Switzerland). Recombinant mouse IL-4 and IL-13 and human IL-4 were from R&D Systems (Minneapolis, MN, USA). Other reagents were from Sigma-Aldrich Co. (St. Louis, MO, USA) unless otherwise stated.

2.2 Cell culture

Murine J774A.1 macrophages (American Type Culture Collection, Manassas, VA, USA) were cultured at 37°C in 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium with Ultraglutamine 1 (Lonza Group Ltd, Basel, Switzerland) supplemented with 10% heat-inactivated foetal bovine serum (Lonza Group Ltd), 100 U/ml penicillin, 100 µg/ml streptomycin and 250 ng/ml amphotericin B (Invitrogen Co., Carlsbad, CA, USA) and harvested with trypsin-EDTA (Invitrogen Co.). Cells were seeded on 24-well plates for RNA extraction, nitrite and ELISA measurements and for preparation of cell lysates for western blotting and on 96-well plates for XTT test. Confluent cultures were exposed to fresh culture medium containing the compounds of interest. PPAR agonists were added to the medium together with LPS or a combination of IL-4 and IL-13 unless otherwise stated.

Human THP-1 promonocytes (American Type Culture Collection) were cultured at 37°C in 5% CO_2 atmosphere in RPMI 1640 (Lonza Group Ltd) adjusted to contain 2 mM L-glutamine (Lonza Group Ltd), 10 mM HEPES (Lonza Group Ltd), 4.5 g/l glucose (Sigma-Aldrich Co.) and 1.5 g/l bicarbonate (Lonza Group Ltd) and supplemented with 10% heat-inactivated foetal bovine serum (Lonza Group Ltd), 100 U/ml penicillin, 100 µg/ml streptomycin, 250 ng/ml amphotericin B (Invitrogen Co.), and 0.05 mM 2-mercaptoethanol (Sigma-Aldrich Co.). The cells were differentiated by adding phorbol ester 12-O-tetradecanoylphorbol-13-acetate (100 nM) (Sigma-Aldrich Co.) at the time of seeding on 24-well plates. Seventy-two hours after the seeding the cultures were confluent and they were exposed to fresh culture medium containing the compounds of interest. PPAR agonists were added to the medium together with LPS or a combination of IL-4 and IL-13 unless otherwise stated.

Cell viability after treatment with combinations of LPS or cytokine mixture and the tested compounds was assessed by modified XTT test (Cell proliferation Kit II, Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions.

2.3 Nitrite determination

NO production was determined by measuring the accumulation of nitrite, a stable metabolite of NO in aqueous condition, into the culture medium. The culture medium was collected at indicated time points and nitrite was measured by the Griess reaction [20].

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2.4 Preparation of cell lysates for western blotting

At indicated time points, the cells were rapidly washed with ice-cold phosphate-buffered saline and solubilized in cold lysis buffer containing 10 mM Tris-base, pH 7.4, 5 mM EDTA, 50 mM NaCl, 1% Triton X-100, 0.5 mM phenylmethylsulphonyl fluoride, 1 mM sodium orthovanadate, 20 µg/ml leupeptin, 50 µg/ml aprotinin, 5 mM NaF, 2 mM sodium pyrophosphate and 10 µM n-octyl-β-D-glucopyranoside. After incubation on ice for 15 minutes, lysates were centrifuged (13 400 *g*, 4°C, 10 min), supernatants were collected and mixed 3:1 with SDS sample buffer (62.5 mM Tris–HCl, pH 6.8, 10% glycerol, 2% SDS, 0.025% bromophenol blue and 5% β-mercaptoethanol). The samples were stored at -20°C until analysed. An aliquot of the supernatant was used to determine protein concentration by the Coomassie blue method [21].

2.5 Western blotting

Prior to western blotting, samples were boiled for 10 minutes and 20 µg of protein was loaded per lane on 8% or 10% SDS-polyacrylamide gels and separated by electrophoresis. Proteins were transferred to Hybond enhanced chemiluminescence nitrocellulose membrane (GE Healthcare, Little Chalfont, Buckinghamshire, UK). After the transfer, the membrane was blocked in TBS/T (20 mM Tris-base pH 7.6, 150 mM NaCl, 0.1% Tween-20) containing 5% of non-fat dry milk or 5% bovine serum albumin at room temperature for 1 h and incubated with the primary antibody in the blocking solution at 4 °C overnight. The membrane was washed with TBS/T, incubated with the secondary antibody in the blocking solution at room temperature for 1 hour and washed. Bound antibody was detected using SuperSignal West Pico or Dura chemiluminescent substrate (Pierce, Rockford, IL, USA) and ImageQuant LAS 4000 mini imaging system (GE Healthcare). The chemiluminescent signal was quantified with ImageQuant TL 7.0 image analysis software (GE Healthcare).

2.6 RNA extraction and quantitative real-time PCR

At the indicated time points, culture medium was removed and total RNA extraction of the cells was carried out with GenElute™ Mammalian Total RNA Miniprep kit (Sigma-Aldrich Co.). 100 ng of total RNA was reverse-transcribed to cDNA using TaqMan Reverse Transcription reagents and random hexamers (Applied Biosystems, Foster City, CA, USA). cDNA obtained from the reverse transcription reaction was diluted 1:20 with RNase-free water and was subjected to quantitative PCR using TaqMan Universal PCR Master Mix and ABI Prism 7000 sequence detection system (Applied Biosystems).

Primers and probes for arginase 1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were optimized according to the manufacturer's instructions in TaqMan Universal PCR Master Mix Protocol part number 4304449 revision C and were as follows: 5'-TCCAAGCCAAAGTCCTTAGAGATTAT-3', 5'-CGTCATACTCTGTTTCTTTAAGTTTTTCC-3', 5'-CGCCTTTCTCAAAAGGACAGCCTCGA-3' (forward and reverse primers and probe for mouse arginase 1) and 5'-GCATGGCCTTCCGTGTTC-3', 5'-GATGTCATCATACTTGGCAGGTTT-3', 5'-TCGTGGATCTGACGTGCCGCC-3' (forward and reverse primers and probe for mouse GAPDH). The expression of mouse PPARα, PPARγ, fizz1 and mrc-1 and human CCL13 and PDGF mRNA was measured by using TaqMan® Gene Expression Assay (Mm00440939_m1, Mm01184322_m1, Mm00445109_m1, Mm00485148_m1, Hs01033504_g1, Hs00966522_m1, Applied Biosystems).

PCR reaction parameters were as follows: incubation at 50°C for 2 min, incubation at 95°C for 10 min, and thereafter 40 cycles of denaturation at 95°C for 15 s and annealing and extension at 60°C for 1 min. Each sample was determined in duplicate.

The relative mRNA levels were quantified and compared using the relative standard curve method as described in Applied Biosystems User Bulletin number 2 (arginase 1) or ΔCt method (TaqMan® Gene Expression Assays).

2.7 Enzyme-Linked Immunosorbent Assay (ELISA)

Culture medium samples were kept at -20°C until assayed. The concentrations of IL-6 and CCL13 in culture medium were determined by ELISA according to the manufacturer's instructions using reagents from R&D Systems Europe (Abingdon, UK).

2.8 Statistics

Results are expressed as mean + standard error of mean (SEM). When indicated, statistical significance was calculated by analysis of variance followed by Dunnett's multiple comparisons test or by unpaired t test with Welch correction. All the statistical analyses were performed

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using GraphPad InStat version 3.10 for Windows (GraphPad Software, San Diego, CA, USA). Differences were considered significant at $p<0.05$.

Ethical approval: The conducted research is not related to either human or animals use.

3 Results

3.1 J774 macrophages express PPARs and can represent classical and alternative activation phenotypes

J774 macrophages were found to express PPARα and PPARγ as detected by western blotting and RT-qPCR (Figure 1). As shown in Figures 2 and 3, the activation of the macrophages with LPS increased the expression of classical activation markers including IL-6 and inducible nitric oxide synthase (iNOS) whereas stimulation with a combination of IL-4 and IL-13 induced the expression of alternative activation markers arginase 1, fizz1 and mrc-1. The levels of arginase 1, fizz1 and mrc-1 mRNA were highest at 36 h, 12 h and 12 - 24 h, respectively, after the onset of the interleukin stimulation (Figure 2).

3.2 PPAR agonists reduced the levels of classical activation markers after LPS-stimulation, but had differing effects on alternative activation

PPARα agonists GW7647 and fenofibrate and PPARγ agonist GW1929 attenuated classical macrophage activation as shown by reduced iNOS expression (Figure 3a) and NO and IL-6 production (Figure 3b) in J774 macrophages exposed to LPS. The agonists did not alter the levels of classical activation markers in the absence

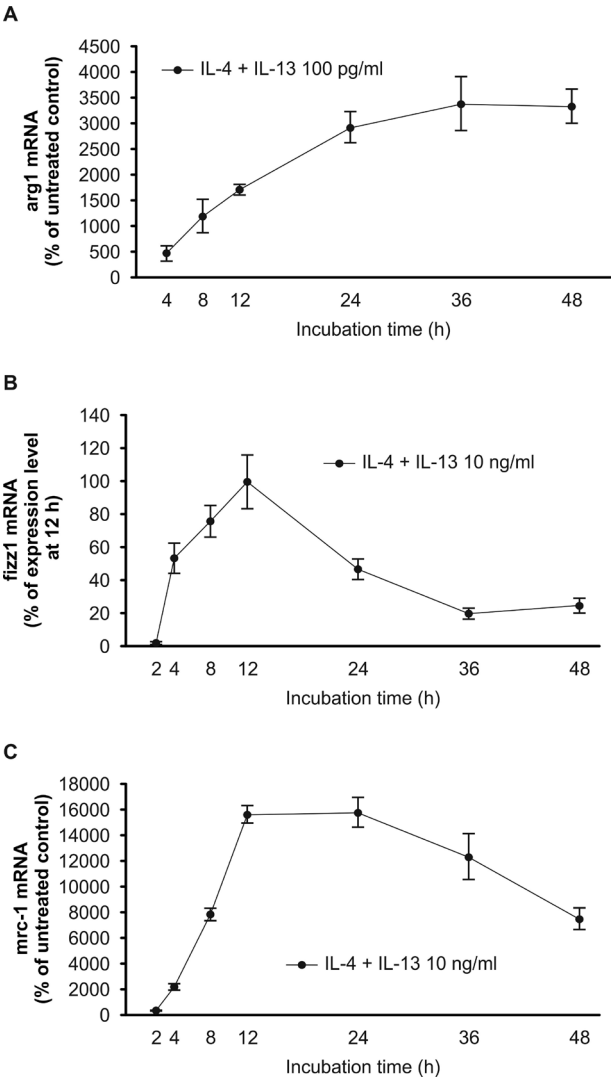

Figure 2 Time-dependent expression of alternative activation markers in J774 macrophages. The cells were stimulated with IL-4 and IL-13 and incubations were terminated at indicated time points and total RNA was extracted. Arginase 1 (a), fizz1 (b) and mrc-1 (c) mRNA were determined by RT-qPCR. The results were normalized against GAPDH mRNA. Results represent the mean ± SEM (n=4).

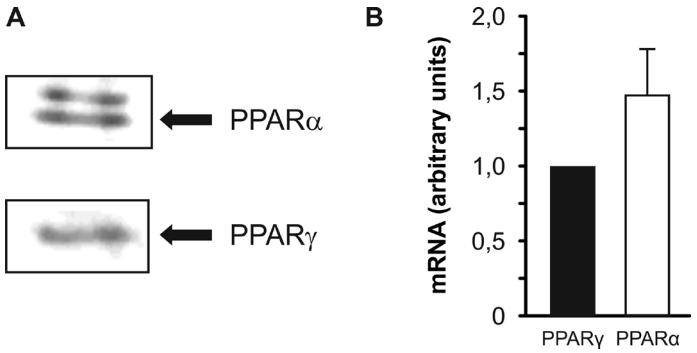

Figure 1 The expression of PPARα and PPARγ in J774 macrophages. (a) Proteins were extracted and PPARα and PPARγ protein expression was determined by western blotting. The gels shown are representatives of three others with similar results. (b) Total RNA was extracted and the levels of PPARα and PPARγ mRNA were analysed by RT-qPCR. The results were normalized against GAPDH mRNA and the level of PPARα mRNA was compared to that of PPARγ (n=6).

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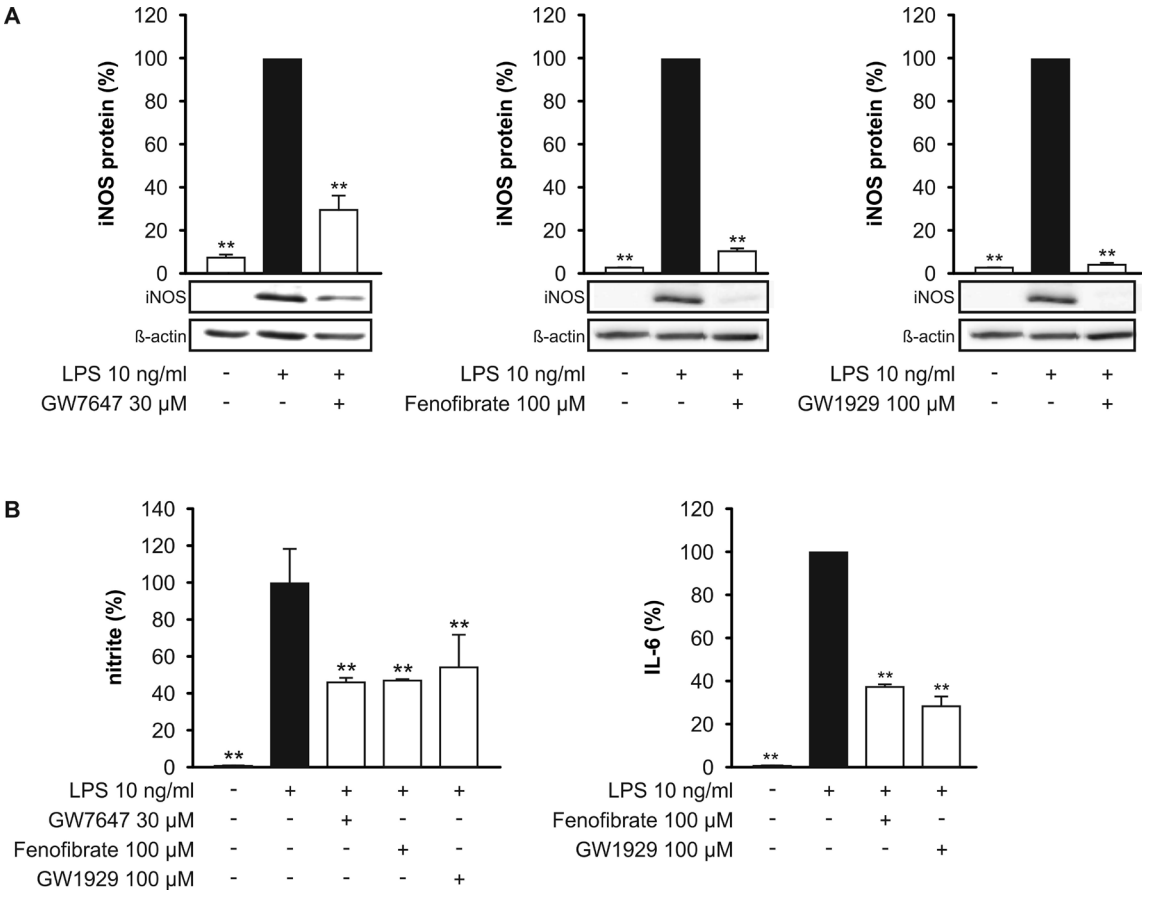

Figure 3 PPARα and PPARγ agonists suppressed classical macrophage activation. (a) Cells were treated with the PPARα agonists GW7647 or fenofibrate or with the PPARγ agonist GW1929 and stimulated with LPS. After 24 h incubation, proteins were extracted and iNOS expression was analysed by western blotting. β-actin was used as a loading control. (b) Cells were treated with the PPARα agonists GW7647 or fenofibrate or with the PPARγ agonist GW1929 and stimulated with LPS. After 24 h incubation, nitrite accumulated into the culture medium was measured by Griess reaction as a marker of NO production. IL-6 in the culture medium was measured by ELISA. Results represent the mean + SEM (n=4). ** = p<0.01 as compared to cells cultured with LPS alone.

of LPS or affect cell viability at the concentration used as determined by XTT test (data not shown).

To investigate if PPARs are able to induce alternative activation in macrophages, we tested the effects of the PPAR agonists on the expression of arginase 1. As expected, the PPARγ agonist GW1929 increased the expression of arginase 1 mRNA when measured after 24 h incubation. In contrast, the PPARα agonists GW7647 and fenofibrate decreased the levels of arginase 1 (Figure 4).

Next, we tested the effect of PPAR agonists on IL-4 + IL-13 -induced alternative phenotype in J774 cells. The selective PPARγ agonist GW1929 increased IL-4 + IL-13 -induced protein expression of arginase 1, and mRNA levels of fizz1 and mrc-1, when determined after 24 hour incubation (Figure 5). In contrast, the PPARα agonists decreased IL-4 + IL-13 -induced levels of arginase 1, fizz1 and mrc-1 mRNA, when determined after 24 hour incubation (Figure 6). In line with the results at mRNA level, GW7647 and fenofibrate also decreased the expression of arginase 1 protein (Figure 7).

3.3 PPARα agonists reduced the levels of alternative activation markers also in human THP-1 macrophages

Before evaluating the effects of PPARα agonists on alternative activation in human macrophages, we examined CCL13 and PDGF mRNA transcription following IL-4 stimulation, as markers of alternative activation [3,22]. In THP-1 macrophages IL-4-enhanced CCL13 and PDGF expression, and their mRNA levels continued to increase at least until 36 hours after the addition of IL-4 (Figure 8). GW7647 and fenofibrate clearly decreased IL-4–induced CCL13 expression as shown by reduced cellular levels of CCL13 mRNA and decreased amounts of CCL13 protein in the culture medium (Figure 9a-b). GW7647, and to a lesser

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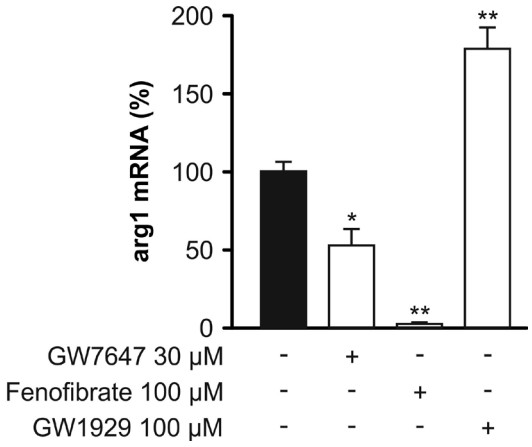

Figure 4 PPARα agonists suppressed, but PPARγ agonists enhanced the expression of the classical marker of alternative activation, i.e. arginase 1, in J774 macrophages. Cells were treated with the PPARα agonists GW7647 or fenofibrate or with the PPARγ agonist GW1929 for 24 hours. Total RNA was extracted and arginase 1 mRNA was determined by RT-qPCR. The results were normalized against GAPDH mRNA. Results represent the mean + SEM (n=4). * = p<0.05 and ** = p<0.01 as compared to untreated cells.

extend fenofibrate, also decreased PDGF mRNA levels in THP-1 cells (Figure 9c).

3.4 PPAR agonists did not affect the phosphorylation of STAT6

STAT6 has been shown to be an important cytosolic mediator for transcriptional effects of IL-4 and IL-13. Therefore we investigated the effects of PPAR agonists on STAT6 phosphorylation (i.e. activation). Costimulation with IL-4 and IL-13 induced the phosphorylation of STAT6 peaking at 30 minutes after addition of IL-4 and IL-13 in J774 macrophages (Figure 10a). As seen in Figure 10b-c, neither fenofibrate nor GW7647 modified the phosphorylation of STAT6 in J774 or THP-1 macrophages.

4 Discussion

The present findings introduce PPARα agonists as compounds able to down-regulate alternative macrophage activation, while PPARγ agonists enhanced the alternative phenotype of macrophages. Further, both PPARα and PPARγ agonists attenuated the classical macrophage activation. The alternative activation of adipose tissue macrophages has been proposed to protect lean people from insulin resistance and other obesity-linked metabolic manifestations. Thus, PPARγ agonists and other compounds that polarize macrophages towards the

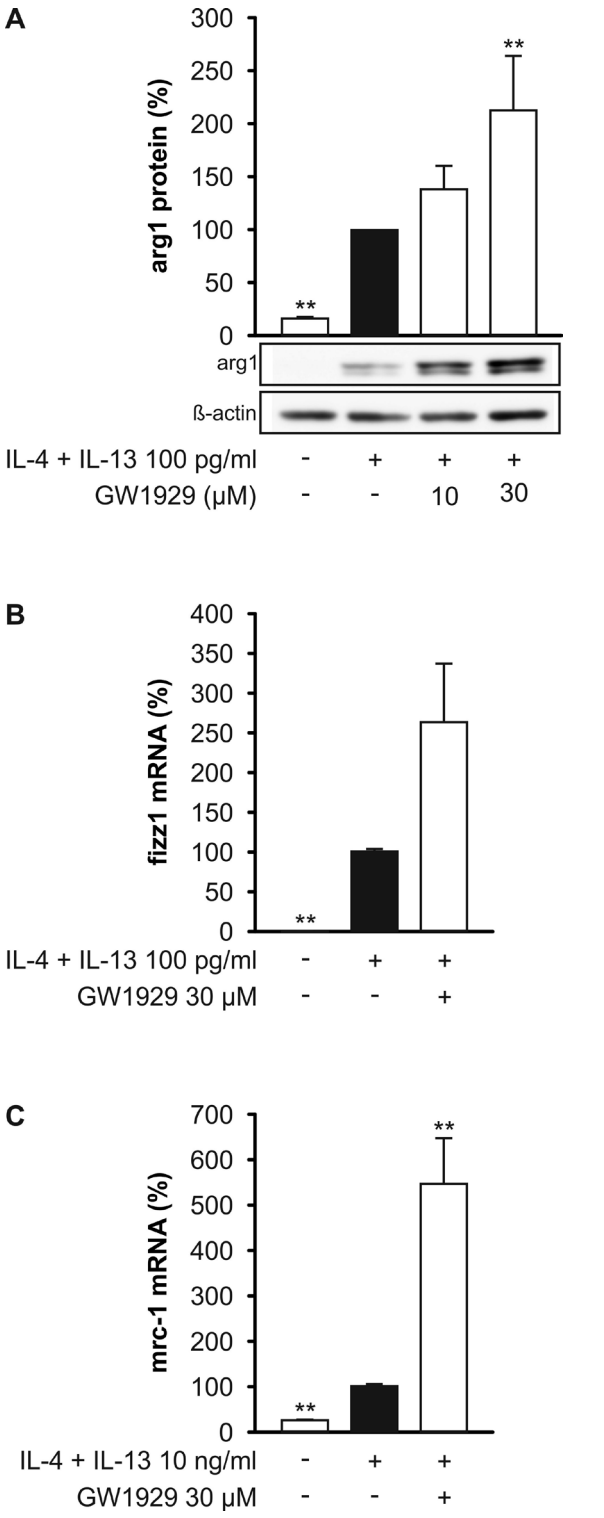

Figure 5 PPARγ agonist GW1929 enhanced the expression of alternative activation markers in J774 macrophages. Cells were treated with GW1929 and stimulated with IL-4 and IL-13 for 24 hours. (a) Proteins were extracted and the levels of arginase 1 were analysed by western blotting. β-actin was used as a loading control. (b-c) Total RNA was extracted and subjected to RT-qPCR. The results were normalized against GAPDH mRNA. Results represent the mean + SEM (n=4). ** = p<0.01 as compared to cells cultured with IL-4 and IL-13.

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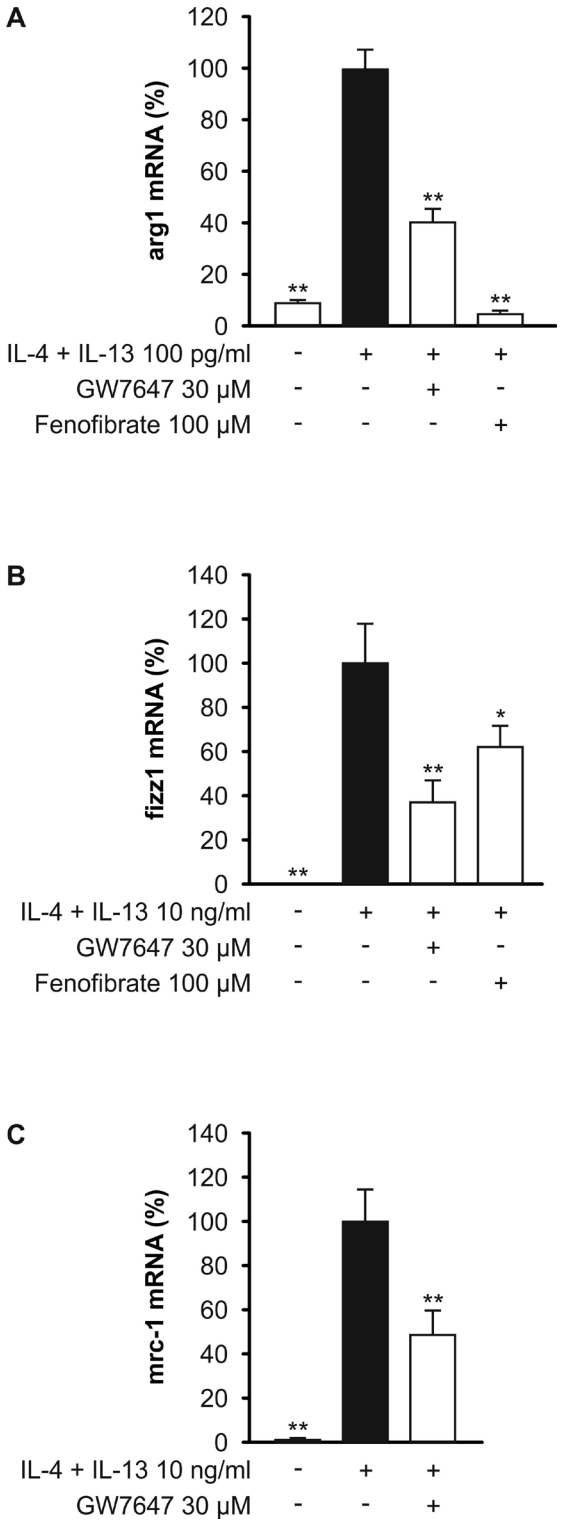

Figure 6 PPARα agonists suppressed the expression of alternative activation markers in J774 macrophages. Cells were treated with GW7647 or fenofibrate and stimulated with IL-4 and IL-13 for 24 hours. Total RNA was extracted and arginase 1 (a), fizz1 (b) and mrc-1 (c) mRNA was determined by RT-qPCR. The results were normalized against GAPDH mRNA. Results represent the mean + SEM (n=4). * = p<0.05 and ** = p<0.01 as compared to cells cultured with IL-4 and IL-13.

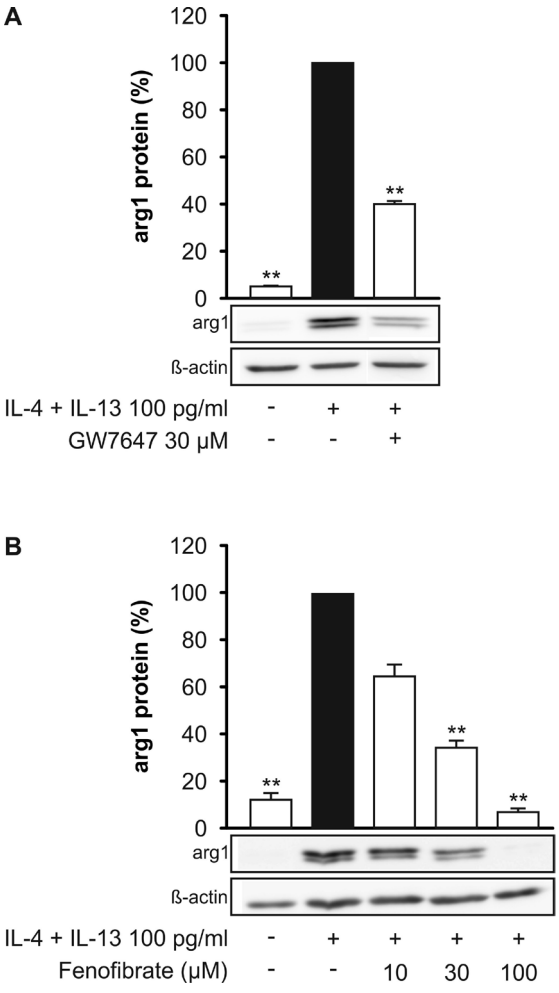

Figure 7 PPARα agonists suppressed arginase 1 protein expression in J774 macrophages. Cells were treated with GW7647 (a) or fenofibrate (b) and stimulated with IL-4 and IL-13 for 24 hours. Proteins were extracted and the levels of arginase 1 were analysed by western blotting. β-actin was used as a loading control. Results represent the mean + SEM (n=4). ** = p<0.01 as compared to cells cultured with IL-4 and IL-13.

alternative phenotype may be beneficial in obesity related metabolic disorders [23,24]. In contrast, compounds able to inhibit alternative macrophage phenotype may have a therapeutic effect in fibrosing diseases, which are complicated with aberrantly enhanced activation of alternative macrophages [25-27].

Alternatively activated macrophages activate fibroblasts to proliferate, migrate to the fibrosing area and to produce extracellular matrix components resulting in fibroblast-populated granulation tissue [28]. However, the mechanisms how this occurs are not known in detail. According to previous reports, systemic sclerosis (SSc) is an example of the diseases where macrophages induce profibrotic action of fibroblasts. Hormer and Herzog showed in 2010 that most of the macrophages in the

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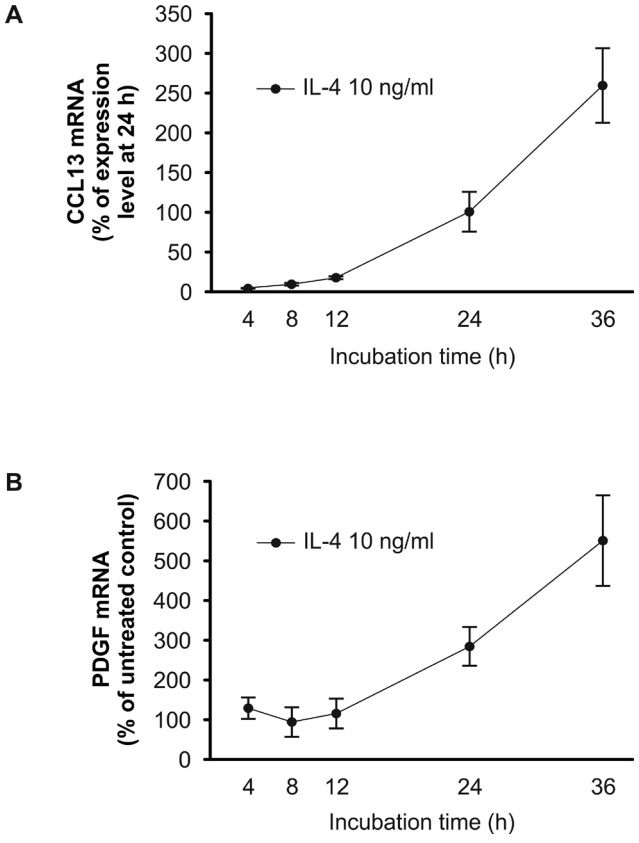

Figure 8 Time-dependent expression of alternative activation markers in THP-1 cells. The cells were stimulated with IL-4 and incubations were terminated and total RNA was extracted at indicated time points. CCL13 (a) and PDGF (b) mRNA was determined by RT-qPCR. The results were normalized against GAPDH mRNA. Results represent the mean ± SEM (n=4).

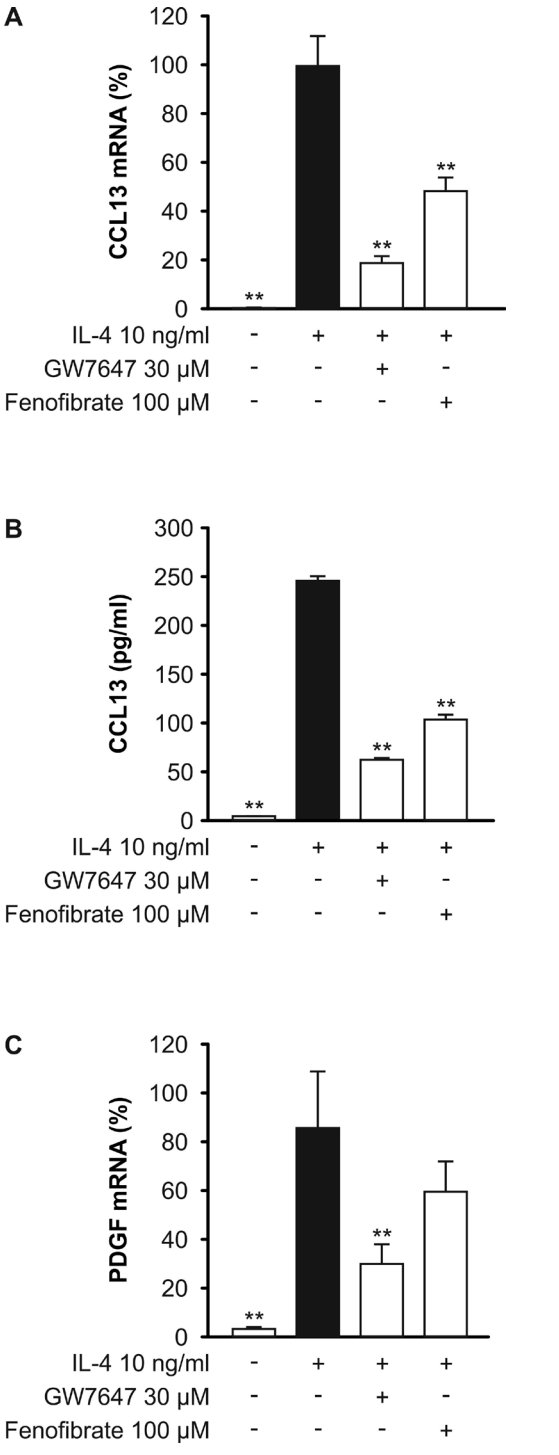

Figure 9 PPARα agonists suppressed the expression of alternative activation markers in THP-1 cells. (a, c) Cells were treated with GW7647 or fenofibrate and stimulated with IL-4 for 24 hours. Total RNA was extracted and CCL13 (a) and PDGF (c) mRNA was determined by RT-qPCR. The results were normalized against GAPDH mRNA. (b) Cells were treated with GW7647 or fenofibrate and stimulated with IL-4. After 24 h incubation, CCL13 accumulated into the culture medium was measured by ELISA. Results represent the mean + SEM (n=4). ** = p<0.01 as compared to cells cultured with IL-4.

lung tissue of patients with SSc-associated interstitial lung disease display markers of alternative macrophage activation [27]. Also in another report, mrc-1 expression was elevated in peripheral blood mononuclear cells (PBMCs) from patients with limited cutaneous SSc and pulmonary hypertension when compared to healthy controls [25]. Additionally, patients with either limited or diffuse cutaneous SSc have been reported to have elevated serum levels of alternative macrophage-related cytokines IL-4, IL-10 and IL-13 [25,26]. IL-13 receptor antagonists also attenuated skin and lung fibrosis in experimental models. Accordingly, treatment with IFN-γ (which drives macrophages towards M1 phenotype) has been shown to improve skin scores in patients with SSc [31]. Most interestingly, an arginase inhibitor, pirfenidone, has been proven to be beneficial in patients with idiopathic pulmonary fibrosis when compared to placebo in two multicentre studies [32]. Pirfenidone was approved for the treatment of idiopathic pulmonary fibrosis in Europe

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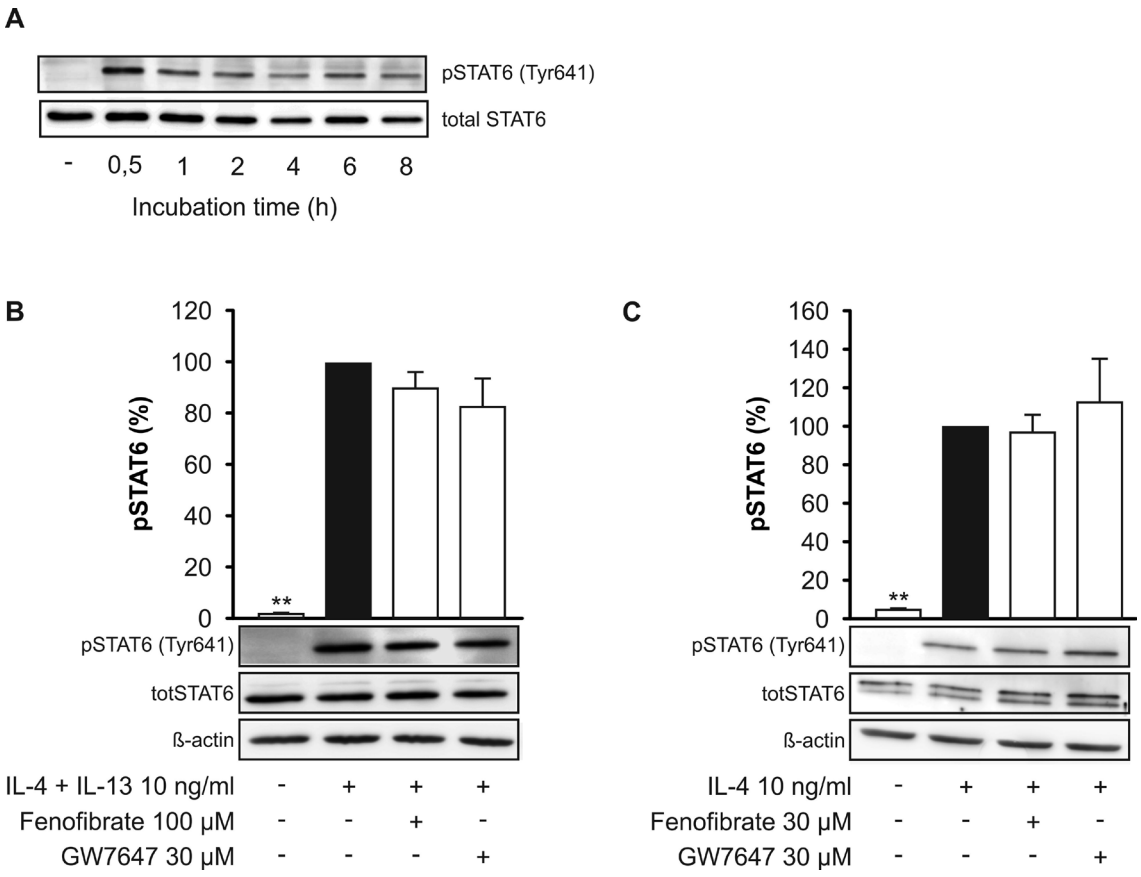

Figure 10 The effects of PPARα agonists on STAT6 phosphorylation in J774 and THP-1 cells. (a) Cells were stimulated with IL-4 and IL-13 and incubations were terminated at indicated time points. Proteins were extracted and the levels of pSTAT6 were analysed by western blotting. Total STAT6 was used as a loading control. The gel shown is a representative of three others with similar results. (b) J774 macrophages were preincubated with the PPARα agonists or the vehicle for an hour before addition of IL-4 and IL-13. After 30 min incubation with IL-4 and IL-13 proteins were extracted and the levels of pSTAT6 were analysed by western blotting. (c) THP-1 cells were preincubated with the PPARα agonists or the vehicle for an hour before addition of IL-4. After 30 min incubation with IL-4, proteins were extracted and the levels of pSTAT6 were analysed by western blotting. (b,c) Total STAT6 and β-actin were used as loading controls. Results represent the mean + SEM (n=4). ** = $p<0.01$ as compared to cells cultured with IL-4 and IL-13 (a,b) or IL-4 (c).

in 2011 and may be beneficial also in other fibrosing diseases including SSc. At the moment, there are no specific treatments targeting the pathophysiology of the fibrosing processes in SSc or related diseases but most of the therapies used act as immunomodulators, like methotrexate, cyclophosphamide and azathioprine, or vasodilators, like calcium channel blockers, prostacyclin analogs, telmisartan, phosphodiesterase inhibitors and endothelin I antagonists. However, the efficacy of these drugs is very limited and better treatment options are critically needed.

PPARα agonists fibrates and PPARγ agonists TZDs are in clinical use for the treatment of metabolic disorders. In line with the present results, both fibrates and TZDs have also shown to decrease the production of IFNγ or LPS –induced proinflammatory mediators typical for classical macrophage activation like iNOS, NO, IL-6 and TNF [17,33,34]. In addition, PPARγ agonists have been reported to shift the polarization of macrophages towards the alternative phenotype [14,15] and that was also seen in the present study. Interestingly, we report here that PPARα agonists have an opposite effect on M2 polarization. To our knowledge, the present study is the first report showing the inhibitory effects of PPARα agonists on the alternative activation of macrophages indicating that PPARα and PPARγ agonists differently regulate alternative macrophage activation.

The diversity of macrophage phenotypes has best been described in mouse macrophages. Some of the known markers of alternative activation, e.g. fizz1, are expressed only in mice [35] and some other markers, like arginase 1, are important *in vivo* but become silenced rapidly in *ex vivo* conditions in human cells [36,37]. Also as reported in this study, J774 macrophages increased

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the production of arginase 1, fizz1 and mrc-1 in response to IL-4 and IL-13 treatment showing the suitability of the model to figure alternative activation. During last years, several markers of alternative activation in human cells have been proposed. CCL13 is one of the most widely applied markers, and it was chosen also in the present study to depict alternative activation. CCL13 expression was highly elevated in response to IL-4 to induce alternative activation of macrophages, which is supported by the previous results [22]. Another human marker of alternative activation used in this study was PDGF. PDGF is an essential promoting factor for fibroblast proliferation during wound healing process and fibrosis [38]. It is also one of the direct mediators of wound healing and pro-fibrotic activity of macrophages [3] and a central pathophysiological factor in SSc [39].

Today, fibrates are used in the treatment of hyperlipidemia. The present results introduce fibrates, and PPARα agonists in general, as inhibitors of alternative macrophage activation. Considering that aberrantly enhanced alternative macrophage activation is associated with the pathogenesis of fibrosis, the present findings suggest that fibrates (and other PPARα agonists) would be beneficial also in fibrosing diseases. This hypothesis is supported by the finding reported by Diep et al. that fenofibrate attenuates the angiotensin II -induced expression of pro-fibrotic markers in murine myocardial tissue [40]. Since fibrates are an old group of drugs, the side effects are well known. According to the meta-analysis published recently, fibrates did not show increased risk of any serious drug-related adverse events when they were used as a monotherapy for the treatment of dyslipidemia [41]. So, also from the safety aspect, fibrates or other PPARα agonists hold potential for the treatment of fibrosing diseases including SSc.

5 Conclusions

The present study shows that PPARα agonists suppress alternative activation of macrophages, which is aberrantly enhanced in fibrosis. These results suggest that PPARα agonists might have anti-fibrotic effects, which offers a pharmacological option to prevent the pathological manifestations of fibrosing diseases.

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