Peroxisome proliferator–activated receptor γ (PPARγ) induces the gene expression of integrin α5β3 to promote macrophage M2 polarization

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Peroxisome proliferator–activated receptor γ (PPARγ) is a member of the nuclear receptor superfamily and polarizes the macrophages into an anti-inflammatory M2 state. Integrins are transmembrane receptors that drive various cellular functions, including monocyte adhesion and foam cell formation. In this study, we first reported that the expression of integrins α5 and β3 was up-regulated by PPARγ activation in RAW264.7 cells and human peripheral blood monocytes. Luciferase reporter and ChIP assay revealed that PPARγ directly bound to the potential PPAR-responsive elements sites in the 5’-flanking regions of both murine and human integrin α5, and β3 genes, respectively. In addition, we showed that PPARγ augmented the ligation of integrins α5, and β3. Knockdown of integrin α5β3 by siRNA strategy or treatment with cilegitudide, a potent inhibitor of integrin α5β3, attenuated PPARγ-induced expression of Ym1, Arg1, Fizz1, and interleukin-10 genes, respectively. In addition, we showed that PPARγ increases the expression and the ensuing ligation of integrins α5, and β3 are implicated in macrophage M2 polarization.

Macrophages are markedly heterogenous cells that are responsive to various stimuli in either acute (infection) or chronic (metabolic syndrome) state (1, 2). M1 macrophages, activated upon classical pro-inflammatory cytokines (Toll-like receptor ligands and interferon-γ), are involved in Th1 immune response (3). In the presence of Th2 cytokines (interleukin-4, interleukin-13, and colony-stimulating factor), monocytes undergo an alternative M2 activation, characterized by an increased expression of Ym1, Arg1, Fizz1, and interleukin-10 (IL-10) (4). Macrophage polarization is tightly regulated at the transcription level (5, 6). Interferon-regulatory factor 5 and signal transducer and activator of transcription 1 (STAT1) promote M1 polarization, whereas PPARγ cooperates with STAT6 to drive to a M2 phenotype (7). PPARγ is a member of the nuclear receptor superfamily and a key regulator of lipid metabolism. It has been shown to suppress inflammation by inhibiting nuclear factor κ light-chain enhancer of activated B cells, STAT, and activator protein-1 pathways (8, 9). Constitutive expression of PPARγ in macrophages leads to an anti-inflammatory M2-like phenotype in adipose tissue. In contrast, knockdown of PPARγ impairs M2 polarization and insulin signaling (10). It has been demonstrated that PPARγ-dependent monocyte differentiation into M2 macrophage is beneficial to human carotid atherosclerosis (11). PPARγ activation by pioglitazone regulates M2 macrophage infiltration and stabilizes the neovessels in the infarct border zone, leading to a better outcome for patients after stroke (12). Previous studies showed that Arg1 and IL-10 are regulated by PPARγ and involved in M2 polarization (10, 13). However, the PPARγ-target genes driving the transcriptional program toward M2 polarization remain largely unknown.

Integrins are a family of ubiquitious transmembrane receptor expressed on a variety of cell types. Mammalian integrins comprise 18 α and 8 β subunits that constitute 24 heterodimers. Ligation of α and β subunits leads to a conformational change according to the extracellular ligands presented on the cell surface (14). In macrophages, integrin ανβ5 (15), α5β2 (16), and α5β3 (17) have been implicated in phagocytosis, M1 polarization, and inflammasome activation. Integrin α5 has recently been found to modulate metabolic inflammation in obesity (18). The absence of integrin β3 favors macrophage polarization into M2a phenotype, which in turn increases fibrosis and impairs muscle regeneration (19). Mice lacking integrin β3 in macrophage lineage cells have enhanced melanoma and breast cancer growth, because of increased tumor-promoting M2 macrophages (20). In addition, ligation of integrin α5β3 prevents macrophage differentiating into foam cells (21). In this study, we identified integrins α5, and β3 as PPARγ target genes, reverse transcriptase–polymerase chain reaction; hPBMC, human peripheral blood monocyte; PPRE, PPAR-responsive element; STAT, signal transducers and activators of transcription; iNOS, inducible nitric-oxide synthase; TNF, tumor necrosis factor; RGZ, rosiglitazone.
and the heterodimer of integrin $\alpha_v\beta_5$ was involved in M2 polarization.

**Results**

**PPAR$\gamma$ regulated the expression of integrin $\alpha_v\beta_5$**

To investigate the activation of PPAR$\gamma$ on different integrins, RAW264.7 cells were treated with rosiglitazone, a PPAR$\gamma$ agonist for 24 h, the mRNA level of integrins $\alpha_{5\alpha i}$, $\alpha_v$, $\alpha_{5\alpha j}$, $\alpha_{1\beta i}$, $\alpha_{1\beta j}$, $\beta_1$, $\beta_2$, $\beta_5$, and $\beta_6$ was detected with RT–qPCR. Integrins $\alpha_v$, $\beta_5$, and $\alpha_M$ were increased upon rosiglitazone treatment, whereas integrins $\alpha_5$, $\alpha_v$, $\alpha_{1\beta i}$, and $\beta_1$ remained unchanged. Integrin $\beta_5$ was down-regulated. Integrin $\beta_5$ was undetectable (Fig. S1).

Integrins $\alpha_v$ and $\beta_5$ are the most prominently up-regulated subunits and could be potentially ligated, prompting us to evaluate their precise regulations by PPAR$\gamma$. To this end, RAW264.7 cells were treated with rosiglitazone for the indicated times or with different doses. Rosiglitazone increased the expression of integrins $\alpha_v$ and $\beta_5$ in a time- (Fig. 1, A and B) and concentration-dependent manner (Fig. 1, C and D). Moreover, they were up-regulated by pioglitazone, another agonist of PPAR$\gamma$ (Fig. 1, E and F). Selective PPAR$\gamma$ antagonists GW9662 abolished the stimulatory effect of rosiglitazone on integrin $\alpha_v$ and $\beta_5$ expression (Fig. 1, G and H), indicating that the up-regulation by rosiglitazone was PPAR$\gamma$-specific. Experiments conducted in human peripheral blood monocytes (hPBMCs) confirmed that both integrins $\alpha_v$ and $\beta_5$ could be induced by PPAR$\gamma$ activation. We then tested the expression of integrins $\alpha_v$ and $\beta_5$ in IL-4-promoted M2 macrophages. We found that both integrins $\alpha_v$ and $\beta_5$ could be induced by PPAR$\gamma$ in RAW264.7 cells (Fig. 2, A and B) and hPBMCs (Fig. 2C), demonstrating that integrins $\alpha_v$ and $\beta_5$ might be downstream molecules during M2 polarization.

The heterodimer of integrin $\alpha_M\beta_5$ was shown to mediate the inflammatory response in macrophages. To verify whether PPAR$\gamma$ activation could affect the expression of integrins $\alpha_M$ and $\beta_5$, RAW264.7 cells were treated with rosiglitazone as mentioned above. Expression of integrin $\alpha_M$ was increased in a time- (Fig. 3A) and dose-dependent manner (Fig. 3B) by rosiglitazone treatment, whereas integrin $\beta_5$ expression was significantly decreased (Fig. 3, C and D). Taken together, these results indicated that PPAR$\gamma$ was capable of regulating the expression of integrins $\alpha_M$ and $\beta_5$ in macrophages.

**Integrins $\alpha_v$ and $\beta_5$ were direct target genes of PPAR$\gamma$**

To investigate whether murine integrin $\alpha_v$ could be targeted by PPAR$\gamma$, sequence analysis was performed using PPAR GENE (22) and JASPAR Database (http://jaspar.genereg.net/). Integrin $\alpha_v$ possesses two potential PPREs within the 2300-bp region upstream of murine integrin $\alpha_v$ gene (Fig. 4A). ChIP assay was executed to examine the bindings for PPAR$\gamma$ to the promoter region of integrin $\alpha_v$. PPAR$\gamma$ could directly bind to integrin $\alpha_v$ promoter at either mPPRE-$\alpha_v$1 (−1297/−1283) or mPPRE-$\alpha_v$2 (−802/−788). The binding for mPPRE-$\alpha_v$1 was increased with the treatment of rosiglitazone, whereas the binding for mPPRE-$\alpha_v$2 remained unchanged (Fig. 4B). We then created two plasmid constructs containing different upstream regions of the integrin $\alpha_v$ promoter fused to the luciferase reporter. HEK 293 cells were treated with rosiglitazone after transfection with mLuc-$\alpha_v$ (containing mPPRE-$\alpha_v$1 and mPPRE-$\alpha_v$2) or mLuc-$\alpha_v$-$\Delta$ (containing mPPRE-$\alpha_v$2) plasmid. Luciferase activity of mLuc-$\alpha_v$, but not mLuc-$\alpha_v$-$\Delta$, was increased by rosiglitazone treatment (Fig. 4C), suggesting that mPPRE-$\alpha_v$1 could mediate the induction of integrin $\alpha_v$ gene by PPAR$\gamma$. To further confirm the role of PPAR$\gamma$ in integrin $\alpha_v$ induction, mLuc-$\alpha_v$ vector was transfected into HEK 293 cells and then treated with rosiglitazone in the presence of a PPAR$\gamma$ specific antagonist GW9662 (Fig. 4D). The increase of luciferase activity of mLuc-$\alpha_v$ by rosiglitazone were prevented by GW9662, indicating that integrin $\alpha_v$ might be a direct target gene of PPAR$\gamma$.

Similarly, the promoter region of integrin $\beta_5$ possesses three PPREs (Fig. 4E). Both mPPRE-$\beta_5$1 (−1278/−1264) and mPPRE-$\beta_5$2 (−287/−273) could be bound to PPAR$\gamma$, and these bindings were enhanced by rosiglitazone treatment. PPAR$\gamma$ did not bind to mPPRE-$\beta_5$3 (−240/−226) (Fig. 4F). Next, mLuc-$\beta_5$ (containing mPPRE-$\beta_5$1, mPPRE-$\beta_5$2, and mPPRE-$\beta_5$3), mLuc-$\beta_5$-$\Delta$ (containing mPPRE-$\beta_5$2 and mPPRE-$\beta_5$3), or mLuc-$\beta_5$-$\Delta'$ (containing PPRE-$\beta_3$) plasmids were transfected into HEK293 cells. Luciferase activity of mLuc-$\beta_5$, but not mLuc-$\beta_5$-$\Delta$ or mLuc-$\beta_5$-$\Delta'$, was increased by rosiglitazone treatment (Fig. 4G), suggesting that mPPRE-$\beta_5$1 could mediate the induction of integrin $\beta_5$ gene by rosiglitazone. Meanwhile, the increase of luciferase activity of mLuc-$\beta_5$ by rosiglitazone was prevented by GW9662 (Fig. 4H), indicating that integrin $\beta_5$ might be direct target gene of PPAR$\gamma$ as well.

In addition, we also identified cognate PPAR$\gamma$ motifs in the regulation region of the human integrin $\alpha_v$ and $\beta_5$ genes. By using ChIP assay, we confirmed that PPAR$\gamma$ could bind to the promoter regions for human integrin $\alpha_v$ at hPPRE-$\alpha_v$1 (−2109/−2095) or hPPRE-$\alpha_v$2 (−1247/−1233) (Fig. 4I). Moreover, either the binding for hPPRE-$\alpha_v$1 or for hPPRE-$\alpha_v$2 was increased by rosiglitazone (Fig. 4J). Similarly, the functionality of PPRE in the human integrin $\beta_5$ gene was also confirmed (Fig. 4K).

**Ligation of integrin $\alpha_v\beta_5$ were increased by PPAR$\gamma$ activation**

Given that integrins are obligate heterodimers, immunoprecipitation analysis was performed to examine the effect of rosiglitazone on $\alpha_v\beta_5$ ligation. Consistent with the up-regulation of integrin $\alpha_v$ and $\beta_5$ expression by rosiglitazone, their ligation was significantly enhanced in RAW264.7 cells and hPBMCs (Fig. 5, A and B). Although integrin $\alpha_M$ was slightly increased, integrin $\alpha_M\beta_5$ ligation was attenuated when treated with rosiglitazone in RAW264.7 cells and hPBMCs (Fig. 5, C and D). These results suggested that PPAR$\gamma$ activation might shift the ligation of different integrins, leading to a distinct downstream signaling pathway in macrophages.
Integrin $\alpha_\nu\beta_5$ in M2 polarization

To investigate the participation of integrin $\alpha_\nu\beta_5$ in PPAR$\gamma$-induced M2 polarization, siRNA strategy was first used following a treatment with rosiglitazone. Rosiglitazone induced mRNA and protein levels of M2 marker genes, including Ym1, Arg1, and Fizz1. Importantly, either siRNA against integrin $\alpha_\nu$ or $\beta_5$ attenuated the induction of M2 marker genes by rosiglitazone. Combination of siRNA against integrins $\alpha_\nu$ and $\beta_5$ abrogated this augmentation in RAW264.7 cells (Fig. 6, A and B) and hPBMCs (Fig. 6C). Rosiglitazone exhibited an anti-inflammatory effect in monocytes/macrophages by reducing the expression of M1 marker genes such as iNOS, TNF$\alpha$, and IL-6. Neither siRNA against integrin $\alpha_\nu$ nor $\beta_5$ nor their combination could reverse the reduced expression of iNOS, TNF$\alpha$ and IL-6 expressions (Fig. S2, A and B).

Furthermore, pharmacological blockage with cilengitide, a potent inhibitor blocking the accessibility of integrin $\alpha_\nu\beta_5$ to their ligands (23), effectively abolished rosiglitazone-induced Ym1, Arg1, and Fizz1 in RAW264.7 cells (Fig. 7, A and B) and hPBMCs (Fig. 7C). In contrast, cilengitide had no effect on rosiglitazone-decreased expression of M1 marker genes (Fig. S3, A and B). These data suggested that the heterodimer of integrin $\alpha_\nu\beta_5$ was required in PPAR$\gamma$-induced M2 polarization.

Discussion

In this present study, we demonstrated a novel mechanism by which PPAR$\gamma$ regulates macrophage polarization via integrin $\alpha_\nu\beta_5$ induction. These results also provided evidence that a specific integrin heterodimer plays an important role in M2 polarization.

Integrins are important signaling receptors that mediate the interactions of the cells with extracellular matrix (24). They are involved in multiple inflammatory responses, including coronary atherosclerosis, obesity, etc. (25, 26). However, the gene regulation of specific integrin subunits in macrophages has not been well-characterized. Here we showed that PPAR$\gamma$ transcriptionally activated integrins $\alpha_\nu$ and $\beta_5$. GW9662, an antagonist of PPAR$\gamma$, attenuated the up-regulation of integrins $\alpha_\nu$ and $\beta_5$ by rosiglitazone. It is noticed that GW9662 elevated the basal level of integrins $\alpha_\nu$ and $\beta_5$ by an unrecognized mechanism, which has been reported in the case of the other nuclear receptor antagonist (27). We identified murine PPRE-$\alpha_\nu$1 (−1297/−1283) and murine PPRE-$\beta_5$1 (−1278/−1264) as functional binding sites to trigger integrin $\alpha_\nu$ and $\beta_5$ transcription, respectively. Meanwhile, we found that the expression of inflammatory integrins $\alpha_\nu$ and $\beta_5$ was regulated by PPAR$\gamma$ as well.

Integrin is strictly heterodimer of $\alpha$ and $\beta$ subunits, which are ligated by extracellular stimuli and required for its signaling

Figure 1. Expression of integrins $\alpha_\nu$ and $\beta_5$ was increased by PPAR$\gamma$ activation. A–D, RAW264.7 cells were incubated with 10 $\mu$mol/liter rosiglitazone (RGZ) for the indicated time (A and B) or with the indicated concentrations of RGZ for 24 h (C and D). Cell lysates were analyzed for the level of integrins $\alpha_\nu$ and $\beta_5$ by using RT–qPCR or immunoblotting. *, $p < 0.05$ versus control (Ctrl; integrin $\alpha_\nu$); #, $p < 0.05$ versus Ctrl (integrin $\beta_5$). E–J, RAW264.7 cells and hPBMCs were stimulated with RGZ (10 $\mu$mol/liter), pioglitazone (PGZ, 10 $\mu$mol/liter), or both. The integrin $\alpha_\nu$ and $\beta_5$ mRNA (E and F) or protein (G and J) levels were examined by RT–qPCR or immunoblotting. G–I, cells were pretreated with or without GW9662 for 1 h and then exposed to RGZ (10 $\mu$mol/liter) for 24 h. Cell lysates were analyzed to determine the mRNA (G and K) and protein (H and L) levels of integrin $\alpha_\nu$ and $\beta_5$. *, $p < 0.05$.

Figure 2. Expression of integrins $\alpha_\nu$ and $\beta_5$ in IL-4–induced M2 macrophages. RAW264.7 cells and hPBMCs were stimulated with IL-4 (10 ng/ml) for 24 h. Cell lysates were analyzed for the levels of integrins $\alpha_\nu$ and $\beta_5$ using RT–qPCR (A and C) or immunoblotting (B). *, $p < 0.05$ versus control (Ctrl; integrin $\alpha_\nu$); #, $p < 0.05$ versus control (integrin $\beta_5$).
Integrin α\textsubscript{V}β\textsubscript{5} in M2 polarization

**Figure 3. Expression of integrins α\textsubscript{V} and β\textsubscript{5} was modulated by PPARγ activation.** RAW264.7 cells were incubated with 10 μmol/liter RGZ for the indicated times (A and C) or with indicated concentrations of RGZ for 24 h (B and D). The cell lysates were analyzed for the level of integrin α\textsubscript{V} and β\textsubscript{5} by using RT–qPCR or immunoblotting. Immunoblots of A and C were from the same representative experiment consecutively used to detect integrin α\textsubscript{V}, integrin β\textsubscript{5}, and β-actin. The results were separately presented, but the loading control (β-actin) was identical. The same situation applies to B and D. * p < 0.05 versus control (Ctrl).

Transduction (24). The mechanism of the formation of integrin heterodimer has not been well-understood. The heterodimer of integrin α\textsubscript{2}β\textsubscript{1} ligated by C1q-containing immune complexes is required for peritoneal mast cells activation during innate immunity (28). Adhesion of monocytes to the endothelium results in integrin α\textsubscript{v},β\textsubscript{3} ligation and prevents macrophage transition into foam cells (21). Here, we found that a signaling pathway initiated by integrin α\textsubscript{V}β\textsubscript{3} ligation could participate in macrophage M2 polarization. Meanwhile, ligation of integrin α\textsubscript{M}β\textsubscript{2}, which has been implicated in inflammatory response (26), was decreased upon PPARγ activation. This is the first evidence that PPARγ switches the ligation of specific integrin subunits, leading to distinct cellular functions in macrophages.

PPARγ has been shown to function as insulin sensitizers and thus improve hyperglycemia in patients with type 2 diabetes mellitus (29). Knockdown of PPARγ in immune cells reduces insulin sensitivity by decreasing the infiltration of macrophages into white adipose tissue (30). Dominant mutations in PPARγ cause insulin resistance accompanied by early onset of severe hypertension (31, 32). The aortas from troglitazone-treated mice show decreased accumulation of macrophages in atherosclerotic lesions and attenuated expression of numerous inflammatory markers such as TNFα and iNOS (33), which is consistent with our results that the expression and ligation of inflammatory integrin α\textsubscript{M}β\textsubscript{2} were decreased by PPARγ activation in macrophages. Alternation of macrophage polarization and function requires precise regulation of master factors, including cytokines (IL-4 and IL-13) and transcription factors like PPARγ. PPARγ activates anti-inflammatory gene expressions, such as Arg-1 and IL-10 through the PPREs at their promoter regions (10, 13). In this study, we found that integrins α\textsubscript{V} and β\textsubscript{5} are PPARγ target genes and necessary to PPARγ-induced M2 polarization. To the best of our knowledge, this is the first evidence implicating a specific integrin heterodimer in M2 macrophages.

Integrins are transmembrane receptors that respond to extracellular stimuli. Ligation of two integrin subunits is not sufficient to induce the downstream signaling; thus it is important to identify the ligands of integrin α\textsubscript{V}β\textsubscript{5} during M2 polarization, especially on the basis of physiopathological context. Integrin α\textsubscript{V}β\textsubscript{5} binds to a variety of extracellular matrix proteins, including osteopontin, fibronectin, vitronectin, von Willebrand factor, and thrombospondin. An *in vitro* study demonstrated that IL-10 acts synergistically with IL-18 to amplify the production of osteopontin, thereby augmenting M2 polarization of macrophage (34). The osteopontin-generated M2 macrophages exhibit a protective effect in vascular calcification of patients with hypertension (35). Although the culture of macrophage on fibronectin-coated surface does not induce a M2 phenotype (36), the production and deposition of fibronectin are common features during M2 polarization (37, 38), which is believed to govern the remodeling process after tissue damage (39). Moreover, the downstream signaling of integrin α\textsubscript{V}β\textsubscript{5} in macrophages should be implemented in our further studies. It has been previously shown that the selective inhibitor of Rok2 (Rho–associated kinase 2) decreases M2-like macrophages (40).
Integrin αvβ5 in M2 polarization

As an upstream receptor of Rho, integrin αvβ5 activation on Rok2 could explain how it participated in M2 polarization. Furthermore, because macrophages exhibit a longer shape when differentiated into M2 phenotypes (41), the effect of integrin αvβ5 on cytoskeleton rearrangement (42) could be another hypothesis to elucidate the involving mechanism. Taken together, our results suggested a novel molecular mechanism with which the induction and ligation of integrin αvβ5 participate in PPARγ-induced M2 polarization, whereas PPARγ represses the inflammation by targeting the expression and the ligation of integrin αvβ5 (Fig. 8), which may provide a potential target against inflammatory diseases.

Experimental procedures

Reagents

Rosiglitazone, GW9662, and recombinant IL-4 were from Sigma–Aldrich. Pioglitazone and cilengitide were from Selleck Chemicals (Houston, TX). Antibodies against integrins αv and β2 were from Santa Cruz Biotechnology (Dallas, TX). Antibodies against Ym1 and Fizz1 were from Abcam (Cambridge, MA). From Cell Signaling (Danvers, MA). Antibodies against Arg1, mLuc-α5, and mLuc-β5 were from Abcam (Cambridge, MA).

Cell culture, isolation, and transfection

Murine monocytic cell line RAW264.7 and HEK 293 cells were obtained from ATCC (Manassas, VA) and maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum in a humidified 5% CO2 atmosphere at 37 °C. Human PBMCs were isolated from healthy donors by Ficoll– Hypaque density centrifugation. After washing three times, hPBMCs were incubated in RPMI 1640 medium supplemented with 10% fetal bovine for 4 days. The experiments were approved by the institutional ethics review board of Xi’an Jiaotong University (approval XJTULAC2018-497) and performed in accordance with the National Institutes of Health guidelines.

RNA extraction and reverse transcriptase–PCR (RT–qPCR)

Total RNA was extracted from RAW264.7 cells and hPBMCs by using TRIzol (Invitrogen). Quantitative RT–qPCR was performed using the SYBR Green technique (Promega, Madison, WI). Primer sequences were described in Table S1. GAPDH was used as a housekeeping gene.

Plasmids and luciferase reporter assay

mLuc-α5 (−1383/+109), mLuc-α5-Δ (−831/+109), Luc-β5 (−1346/+40), mLuc-β5-Δ (−443/+40), and mLuc-β5-Δ′ were transfected into HEK293 cells incubated with RGZ (10 μmol/liter) after pretreatment with GW9662 (20 μmol/liter). Luciferase activity was measured and normalized to that of β-gal. Schematic presentation of PPREs located in the regions of human integrin αv and β5 promoters. hPBMCs were treated with RGZ (10 μmol/liter). The indicated human PPREs of integrin αv (J) or β5 (K) were analyzed by ChIP assay with the use of anti-PPARγ antibody. IgG was used as an isotype control. * p < 0.05.
(-270/+40) plasmids were made by PCR cloning into the pGL3 basic luciferase-reporter plasmid. The cells were co-transfected with a reporter gene and a β-gal plasmid. Luciferase and the β-gal activities were measured as previously described (43).

**ChIP assay**

The cells were cross-linked with 0.75% formaldehyde before harvesting. Sheared chromatin was immunoprecipitated with an anti-PPARγ antibody (or control IgG) and pulled down with protein A/G–Sepharose beads (Santa Cruz). The eluted immunoprecipitates were digested with proteinase K to reverse the cross-link between DNA and proteins. DNA was extracted and subjected to PCR experiment with specific primers flanking the putative PPARγ binding motifs. Primer sequences were described in Table S2.

**Immunoblotting and immunoprecipitation**

Proteins were extracted in RIPA buffer supplemented with protease inhibitors. Protein concentrations were measured

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**Figure 6. Integrin αvβ3 knockdown abolished rosiglitazone-promoted M2 polarization.** RAW264.7 cells and hPBMCs were transfected with murine and human si control (Ctrl), si αv, si β3 or si αv+β3, respectively. Then the cells were treated with RGZ (10 μmol/liter) for 24 h. The cell lysates were analyzed for the level of Ym1, Arg1, and Fizz1 by using RT-qPCR (A and C) or immunoblotting (B).
Integrin αvβ5 in M2 polarization

A. 

![Graph showing the level of Ym1, Arg1, and Fizz1 in RAW264.7 cells and hPBMCs.](image)

Figure 7. Cilengitide partially inhibited rosiglitazone-induced M2 polarization. RAW264.7 cells and hPBMCs were pretreated with cilengitide (Cilen, 1 μmol/liter) for 30 min and then incubated with RGZ (10 μmol/liter) for 24 h. Lysates were analyzed for the level of Ym1, Arg1, and Fizz1 by using RT-qPCR (A and C) or immunoblotting (B). Ctrl, control.

B. 

![Immunoblots showing the level of Ym1, Arg1, and Fizz1 in RAW264.7 cells.](image)

C. 

![Immunoblots showing the level of Ym1, Arg1, and Fizz1 in hPBMCs.](image)

Figure 8. Mechanism of integrin in PPARγ-induced M2 macrophage polarization. PPARγ activates the expression of integrin αv and β5 by targeting the PPREs in their promoter regions. The formation of integrin αvβ5 heterodimer is therewith increased, leading to M2 macrophages polarization. Meanwhile, PPARγ activation regulates the expression and the ligation of integrin αM and β2, leading to a reduced inflammatory phenotype in macrophages.

Using the BCA protein assay. Immunoblotting was performed with appropriate primary antibodies and horseradish peroxidase-conjugated secondary antibodies followed by ECL detection. Immunoblots shown were representative of three independent experiments.

For immunoprecipitation, cell lysates were incubated with the appropriate antibodies or control IgG at 4 °C overnight followed by incubation with protein A/G-Sepharose beads. Immunoprecipitates were washed with NETN buffer (20 mM Tris, pH 8.0, 100 mM NaCl, 1 mM EDTA, and 0.5% NP-40). Immunoblots shown were representative of three independent experiments.

siRNA and transfection

RAW264.7 cells or hPBMCs were transfected with adequate species of integrin αv, integrin β5, or scrambled siRNA (si Ctrl). Sequences were described in Table S3. Experiments using these cells were executed at 24 h after transfection.

Statistical analysis

The results are reported as means ± S.D. Comparisons within and between groups were performed using analysis of variance and Mann–Whitney U test, respectively. p < 0.05 was considered significant.
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