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PPARγ Activation Primes Human Monocytes into Alternative M2 Macrophages with Anti-inflammatory Properties

M. Amine Bouhlel,[1,2,3] Bruno Derudas,[1,2,3] Elena Rigamonti,[1,2,3] Rébecca Dièvart,[1,2,3] John Brozek,[4] Stéphan Haulon,[5] Christophe Zawadzki,[6] Brigitte Jude,[6] Gérard Torpier,[1,2,3] Nikolaus Marx,[7] Bart Staels,[1,2,3,*] and Giulia Chinetti-Gbaguidi[1,2,3]

[1] Institut Pasteur de Lille, F-59019 Lille, France
[2] Inserm, U545, F-59019 Lille, France
[3] Université de Lille 2, Faculté des Sciences Pharmaceutiques et Biologiques et Faculté de Médecine, F-59006 Lille, France
[4] Genfit, F-59120 Loos, France
[5] Clinique de Chirurgie Cardiovasculaire, Centre Hospitalier Régional et Universitaire, F-59037 Lille, France
[6] INSERM ERI-9 and Equipe d'Accueil 2693, IFR114, Université de Lille, F-59037 Lille, France
[7] Department of Internal Medicine II, Cardiology, University of Ulm, D-89081 Ulm, Germany
*Correspondence: bart.staels@pasteur-lille.fr
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SUMMARY

Th1 cytokines promote monocyte differentiation into proatherogenic M1 macrophages, while Th2 cytokines lead to an "alternative" anti-inflammatory M2 macrophage phenotype. Here we show that in human atherosclerotic lesions, the expression of M2 markers and PPARγ, a nuclear receptor controlling macrophage inflammation, correlate positively. Moreover, PPARγ activation primes primary human monocytes into M2 differentiation, resulting in a more pronounced anti-inflammatory activity in M1 macrophages. However, PPARγ activation does not influence M2 marker expression in resting or M1 macrophages, nor does PPARγ agonist treatment influence the expression of M2 markers in atherosclerotic lesions, indicating that only native monocytes can be primed by PPARγ activation to an enhanced M2 phenotype. Furthermore, PPARγ activation significantly increases expression of the M2 marker MR in circulating peripheral blood mononuclear cells. These data demonstrate that PPARγ activation skews human monocytes toward an anti-inflammatory M2 phenotype.

INTRODUCTION

A crucial step in atherogenesis consists of the infiltration of the subendothelial space of large arteries by monocytes, where they differentiate into macrophages (Ross, 1999). Macrophages are a heterogeneous cell population that adapt and respond to a large variety of microenvironmental signals (Van Ginderachter et al., 2006). The activation state and functions of mononuclear phagocytes are profoundly affected by different cytokines and microbial products. While Th1 cytokines, such as interferon γ (IFNγ), interleukin-1β (IL-1β), and lipopolysaccharide (LPS), induce a "classical" activation profile (M1), Th2 cytokines, such as IL-4 and IL-13, induce an "alternative" activation program (M2) in macrophages. Moreover, macrophages are plastic cells because they can switch from an activated M1 state back to M2, and vice versa, upon specific signals (Porcheray et al., 2005). M1 macrophages are potent effector cells that kill microorganisms and produce primarily proinflammatory cytokines, such as tumor necrosis factor α (TNF-α), IL-6, and IL-12 (Gordon, 2003). In contrast, M2 macrophages dampen these inflammatory and adaptive Th1 responses by producing anti-inflammatory factors (IL-10, transforming growth factor β [TGF-β], and IL-1 receptor antagonist [IL-1Ra]), scavenging debris, and promoting angiogenesis, tissue remodeling, and repair (Gordon, 2003; Mantovani et al., 2001). Thus, inflammatory diseases, such as atherosclerosis, may be caused not only by a sustained proinflammatory reaction but also by failure of anti-inflammatory control mechanisms.

Peroxisome proliferator-activated receptor γ (PPARγ) is a ligand-activated nuclear receptor with potent anti-inflammatory properties that modulates the immune inflammatory response (Chinetti et al., 2003). Transcriptional repression of inflammatory response genes occurs by negative interference of PPARγ with the nuclear factor κB (NF-κB), signal transducer and activator of transcription (STAT), and activating protein 1 (AP-1) signaling pathways (Chinetti et al., 2000a). In activated M1 macrophages, the PPARγ-dependent transrepression pathway is initiated by sumoylation of the liganded PPARγ ligand-binding domain maintaining the corepressor complex on NF-κB response elements (Pascual et al., 2005). PPARγ is activated by natural (15-deoxy-D12;14-prostaglandin J2 [15d-PGJ2]) (Kliewer et al., 1995) and synthetic ligands, including the antidiabetic thiazolidinediones (TZDs) (such

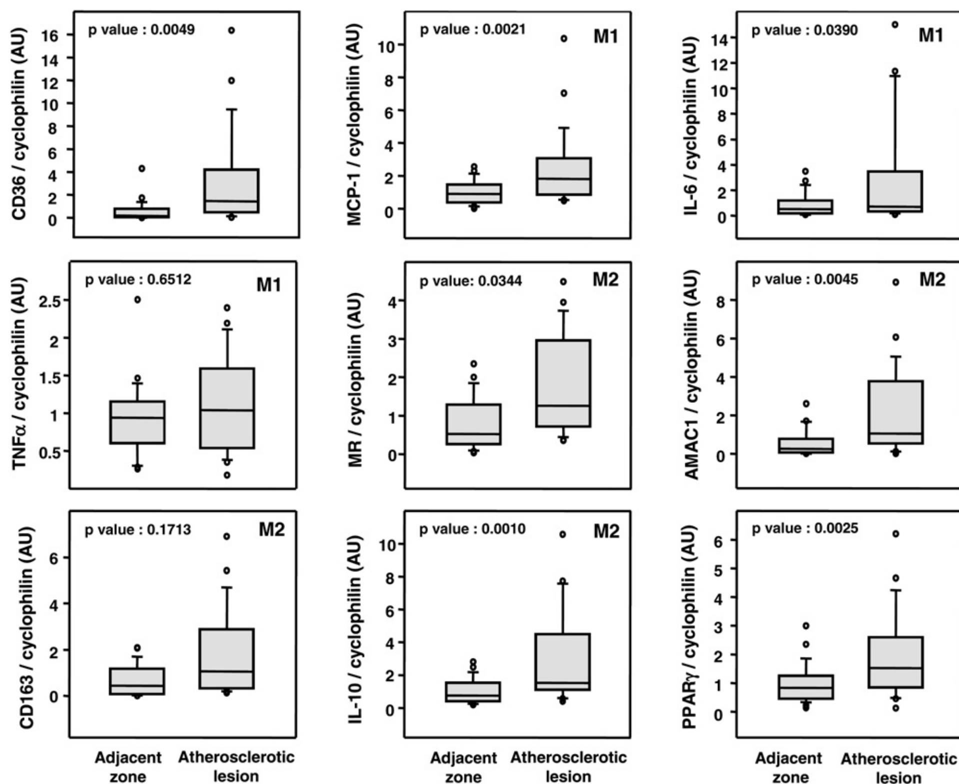

Figure 1. M1 and M2 Macrophage Markers Are Expressed in Human Carotid Atherosclerotic Lesions
RNA was extracted from human carotid atherosclerotic lesions and adjacent zones. mRNA levels were measured by qPCR and are represented in box plots indicating the median and the lower and upper quartiles. Statistically significant differences are indicated (paired Student's t test; $p < 0.05$).

as rosiglitazone and pioglitazone) and the GW1929 compound (Willson et al., 2000). PPARγ is abundantly expressed in macrophages, where its expression is rapidly induced upon differentiation of monocytes into macrophages (Chinetti et al., 1998). In atherosclerotic lesion macrophages, PPARγ modulates cholesterol homeostasis and the inflammatory response (Chinetti et al., 2000b, 2003). Moreover various leukocyte populations, including lymphocytes and dendritic cells, also express PPARγ, suggesting a role for this receptor in the regulation of the immune response (Standiford et al., 2005).

In the present study, we evaluated whether PPARγ, in addition to its well-described anti-inflammatory action in M1 macrophages, could also exert anti-inflammatory activities by modulating the M1/M2 differentiation of monocytes into macrophages.

RESULTS

M2 Macrophage Markers Are Present in Human Carotid Atherosclerotic Lesions and Correlate Positively with PPARγ Expression Levels

Previous studies have identified the presence and role of activated M1 macrophages in atherosclerosis (Ross, 1999). However, it was unknown whether M2 macrophages are present in atherosclerotic lesions. Therefore, we performed M1 and M2 marker RNA analysis on carotid plaques isolated by endarterectomy from 27 patients. Interestingly, atherosclerotic lesions, which are enriched in macrophages as demonstrated by the high expression level of CD36 when compared to the adjacent zone (Figure 1), contain both macrophage subpopulations. Both markers of the M1 state, such as *MCP-1*, *IL-6*, and *TNF-α*, as well as markers of M2 activation, such as *CD163*, *MR* (mannose receptor; also called *CD206*), *AMAC1* (alternative macrophage activation-associated CC chemokine 1; also called chemokine [C-C motif] ligand 18 [*CCL-18*]), and *IL-10*, are more abundantly expressed in the pathological tissue compared to the adjacent zone (Figure 1).

Moreover, the staining pattern of MR in serial sections of human atherosclerotic plaques showed that this M2 marker is expressed in the same infiltration area of CD68-positive macrophages (Figure 2A). Interestingly, the macrophage population expressing MR displays a distinct tissue distribution profile compared to foam cells (lipid-laden macrophages). Furthermore, staining with anti-MCP-1 antibody, an M1 marker, indicated that MR-positive cells and MCP-1-positive cells present a distinct tissue localization pattern (Figure 2A). These results show the presence of at least two macrophage populations in human atherosclerotic plaques.

As previously shown (Chinetti et al., 2000b, 2003), *PPARγ* is also abundantly expressed in lesion

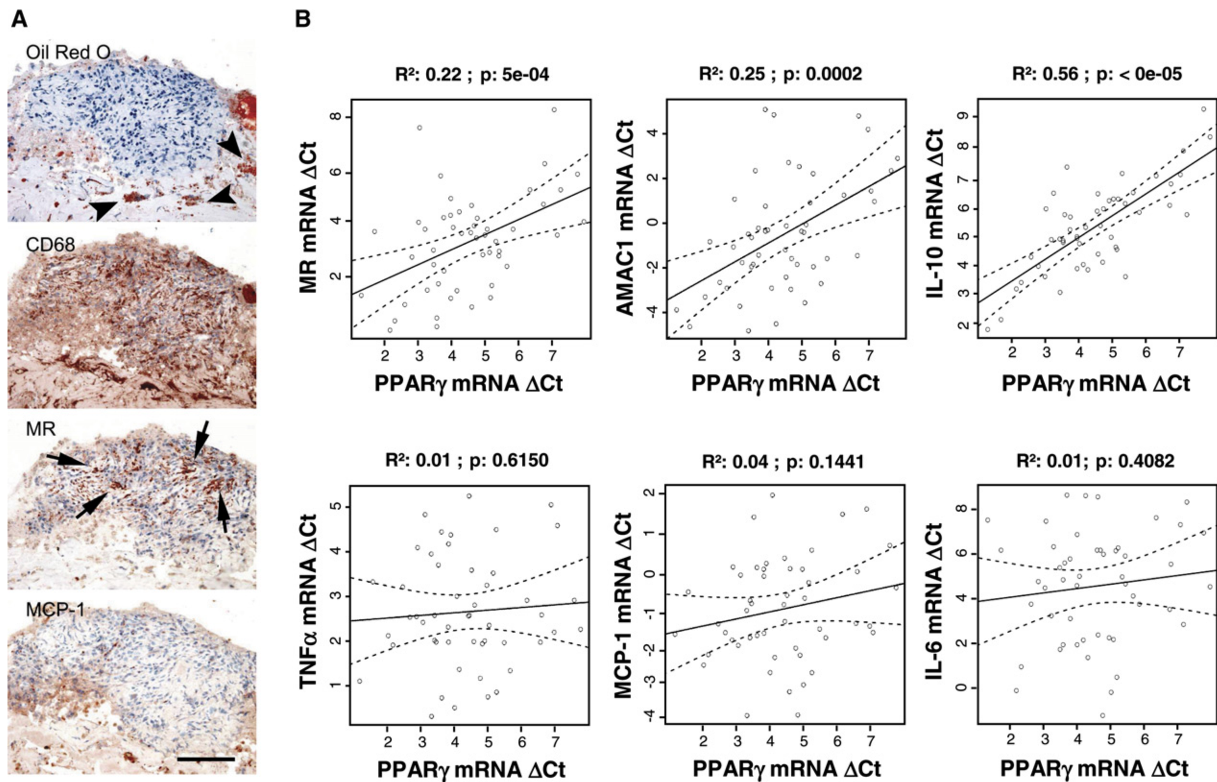

Figure 2. MR Protein Is Present in Human Carotid Atherosclerotic Lesions Where M2 Macrophage Markers Correlate Positively with PPARγ Expression Levels

(A) Representative staining pattern for Oil red O, CD68, MR, and MCP-1 in adjacent cryosections of a human complicated carotid atherosclerotic lesion with lipid-rich cores. Infiltrated MR- (arrows) or MCP-1-positive cells within a macrophage-rich area surrounded by CD68-positive foam cells (arrowheads) are indicated. Scale bar = 100 μm.

(B) Pearson correlation coefficients (r) and multiple regression analysis were calculated from qPCR ΔCt data. Confidence intervals of 90% are indicated by the dashed arcs. Statistically significant differences are indicated (t test; $p < 0.05$).

macrophages of atherosclerotic plaques (Figure 1). Moreover, its expression correlates with the expression of *CD36*, a scavenger receptor marker of macrophages and known PPARγ target gene (data not shown; Tontonoz et al., 1998). In addition, *PPARγ* expression levels correlate positively with the expression of M2 markers, such as *MR*, *AMAC1*, and *IL-10* (Figure 2B). By contrast, no correlation was observed between *PPARγ* and the M1 markers *TNF-α*, *MCP-1*, and *IL-6* (Figure 2B). Taken together, these results suggest a potential functional relationship between PPARγ and the M2 macrophage subpopulation.

PPARγ Agonists Enhance the Alternative Differentiation of Human Monocytes into Macrophages In Vitro

To determine whether PPARγ plays a role in M2 differentiation, primary human monocytes were differentiated in vitro into alternative macrophages with recombinant IL-4 in the absence (M2) or in the presence (M2γ) of the PPARγ agonists GW1929 (600 nM) or rosiglitazone (100 nM) added only at the beginning of the differentiation process. As expected (Stein et al., 1992), the M2 macrophage marker *MR* was strongly induced by IL-4 (Figure 3A). Interestingly, this effect was amplified by both PPARγ agonists (Figure 3A). By contrast, the expression of *CD163* was significantly reduced after IL-4 stimulation (Figure 3A), in line with previous reports (Porcheray et al., 2005; Van den Heuvel et al., 1999). The presence of both PPARγ agonists enhanced the IL-4-induced decrease of *CD163* expression in M2γ macrophages (Figure 3A). A similar regulation of *MR* and *CD163* expression by the PPARγ ligands was observed in monocytes differentiated into M2 macrophages in the presence of IL-13 or an IL-4/IL-13 combination (see Figure S1 in the Supplemental Data available with this article online). As previously reported in resting macrophages (RM), *PPARγ* expression was also induced by IL-4 (Huang et al., 1999) and/or IL-13 in M2 differentiating macrophages (data not shown).

Flow cytometry analysis of RM, M2, and M2γ macrophages demonstrated, as expected, higher MR protein levels in M2 compared to RM. Moreover, MR expression was further enhanced in M2γ (Figure 3B). On the other hand, CD163 expression was lower in M2 compared to RM, and its expression was further reduced in M2γ (data not shown). By contrast, MR and CD163 protein levels

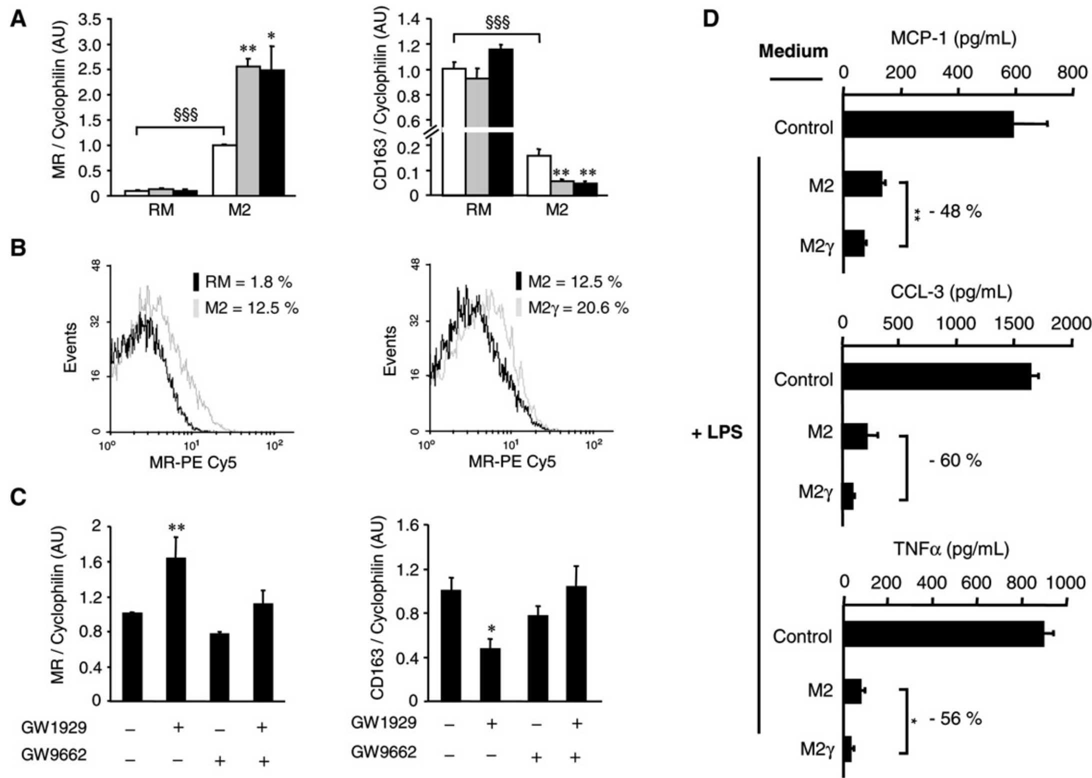

Figure 3. PPARγ Activation Promotes the Acquisition of an M2 Phenotype in Primary Human Monocytes and Enhances the Anti-inflammatory Properties of M2 Macrophages

(A) *MR* and *CD163* mRNA was measured in RM and M2 macrophages treated with GW1929 (gray columns, 600 nM), rosiglitazone (black columns, 100 nM), or vehicle (white columns). Each bar is the mean value ± SD of triplicate determinations, representative of five independent experiments. Statistically significant differences are indicated (t test; §§§p < 0.001 for RM versus M2, **p < 0.01 and *p < 0.05 for vehicle versus GW1929 or rosiglitazone in M2).

(B) MR protein expression was analyzed by flow cytometry in RM, M2, and M2γ cells. Values represent the percentage of positive cells. The percentage of autofluorescence is 0.39%.

(C) Primary human monocytes were differentiated to M2 in the presence or absence of GW1929 (600 nM) and/or GW9662 (1 μM) added only at the beginning of the differentiation process. *MR* and *CD163* mRNA levels were analyzed; each bar is the mean value ± SD of triplicate determinations, representative of three independent experiments. Statistically significant differences are indicated (t test; **p < 0.01 and *p < 0.05).

(D) MCP-1, TNF-α, and CCL-3 were quantified in macrophage supernatants (see Supplemental Data). Each bar is the mean value ± SD of triplicate determinations, representative of two independent experiments. Statistically significant differences are indicated (t test; ***p < 0.001, **p < 0.01, and *p < 0.05).

were not affected by PPARγ activation upon addition of the agonists to monocytes differentiating into RM (data not shown), indicating that PPARγ activation only modulates M2 markers in monocytes polarized to M2 with IL-4 and/or IL-13.

Finally, coincubation of monocytes undergoing M2γ differentiation with the PPARγ antagonist GW9662 completely abolished the regulation of *MR* and *CD163* expression by the PPARγ agonists, indicating that the observed effects are PPARγ dependent (Figure 3C).

M2 Macrophages Exert Paracrine Anti-inflammatory Effects on M1 Macrophages

To examine whether PPARγ-agonist-primed M2 macrophages can influence the inflammatory status of activated M1 macrophages, indirect coculture experiments were performed. Differentiated RM were incubated for 24 hr in medium supplemented with M2 or M2γ culture supernatant and subsequently stimulated with LPS to induce an M1 phenotype. Cytokines and chemokines released by M1 macrophages were then quantified by enzyme-linked immunosorbent assay (ELISA) (Figure S2). As expected, incubation of M1 macrophages with M2-derived culture supernatant resulted in a pronounced inhibition of the secretion of proinflammatory molecules, such as CCL-3, TNF-α, and MCP-1 (Figure 3D). Interestingly, this inhibitory effect was significantly enhanced when M1 macrophages were incubated with medium from M2 primed in the presence of the PPARγ agonist GW1929 (Figure 3D). These results confirm that M2 macrophages dampen the inflammatory status of surrounding M1 macrophages and that priming toward M2 differentiation by PPARγ activation enhances the anti-inflammatory properties of M2 macrophages.

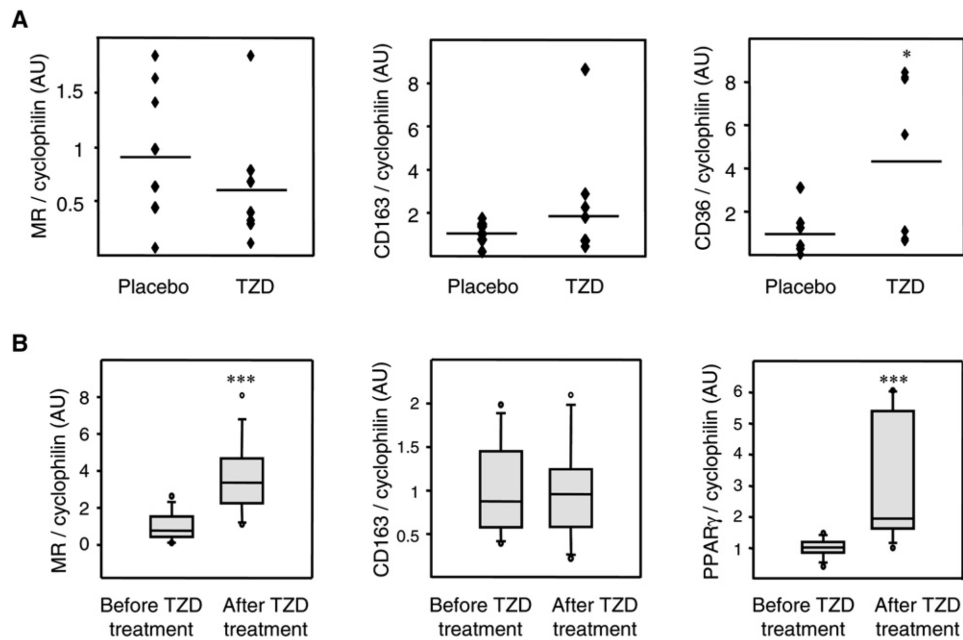

Figure 4. PPARγ Activation Induces M2 Markers in Human Blood Mononuclear Cells In Vivo

(A) RNA was extracted from human carotid atherosclerotic lesions isolated from 7 placebo- and 8 rosiglitazone (TZD)-treated subjects. Each point represents mRNA expression analysis of a single patient, and the bar indicates the mean. Statistically significant differences are indicated (Mann-Whitney rank-sum test; $^*p < 0.05$).

(B) RNA was extracted from peripheral blood mononuclear cells isolated from 15 patients before and after 2 months of 45 mg/day pioglitazone (TZD) treatment. mRNA levels are represented in box plots in which the median is surrounded by the lower and upper quartile. Statistically significant differences are indicated (t test; $^{***}p < 0.001$).

PPARγ Activation Does Not Switch RM, M1, or Foam Cells into an M2 Phenotype In Vitro

Previous studies have demonstrated that macrophages are plastic cells because they can switch from an activated M1 state back to M2, and vice versa, upon specific signals (Porcheray et al., 2005). Hence, we tested whether the incubation of RM at the end of differentiation with IL-4 and/or PPARγ ligands could revert the M1 phenotype to M2. In the absence of IL-4, PPARγ activation did not influence the expression of the M2 markers *MR*, *AMAC1*, and *CD163* in RM (Figure S3A). Treatment of fully differentiated RM with IL-4 induced a switch to an M2 phenotype, but this effect was not further enhanced by PPARγ activation, except for *AMAC1*, which was slightly (albeit not significantly) repressed in the presence of PPARγ ligands (Figure S3B). To mimic the resident macrophages in atherosclerotic lesions, which appear predominantly as M1 macrophages or foam cells, experiments were performed on RM incubated with different proinflammatory stimuli (IL-1β, TNF-α, or LPS) to induce an M1 status or with AcLDL to induce lipid accumulation, in the absence or in the presence of the PPARγ agonists GW1929 (600 nM) or rosiglitazone (100 nM). However, PPARγ activation was not able to redirect M1 or foam-cell macrophages toward an M2 phenotype, as indicated by the absence of M2 marker regulation (Figures S3C and S3D). Taken together, these data suggest that PPARγ activation promotes M2 phenotype enhancement only in monocytes, and not in differentiated RM or M1 macrophages.

PPARγ Activation Directs Human Blood Precursor Cells toward an M2 Phenotype In Vivo

To determine whether these in vitro observations also occur in vivo in humans, macrophage markers were measured in RNA isolated from atherosclerotic lesions from placebo- or rosiglitazone (TZD)-treated individuals. Interestingly, rosiglitazone treatment did not modify the expression of the M2 markers *MR* and *CD163*. By contrast, the expression of *CD36*, a known PPARγ target gene in macrophages (Tontonoz et al., 1998), was significantly increased in plaques of TZD-treated patients (Figure 4A). These results are in line with those obtained in vitro in M1 and foam cells, indicating that PPARγ is unable to influence the expression of M2 markers in established atherosclerotic lesion macrophages.

Since PPARγ agonists enhance monocyte differentiation toward an M2 phenotype in vitro, it was investigated whether this regulation also occurs in vivo. Peripheral blood mononuclear cells (PBMC), a cell population including circulating monocytes, were isolated from patients before and after pioglitazone administration (Balmforth et al., 2007). Interestingly, TZD treatment significantly increased the expression of the M2 marker *MR* in PBMC (Figure 4B). Surprisingly, the expression of *PPARγ* in PBMC was also increased by TZD treatment. Taken together, these results suggest that PPARγ activation could also be able to program precursor mononuclear cells toward an M2 phenotype in vivo.

DISCUSSION

Monocytes are precursors of macrophages, which are prominent cells in the host response to lipid accumulation in large arteries that contribute to the development of atherosclerosis and its complications. Very recently, a monocyte subpopulation characterized by high levels of the LY-6C marker antigen has been shown to dominate hypercholesterolemia-associated monocytosis in mice and to give rise to the macrophages in atherosclerotic lesions (Swirski et al., 2007; Tacke et al., 2007). LY-6C^{hi} monocytes, which correspond to human CD14^{hi}CD16^{-} monocytes, preferentially migrate to lesions with high inflammatory activity and populate sites of experimentally induced inflammation (Swirski et al., 2007; Tacke et al., 2007). This observation indicates that cell heterogeneity of circulating monocytes influences plaque formation and phenotype. Moreover, mature tissue macrophages also exhibit a level of heterogeneity reflecting the plasticity and versatility of these cells in response to microenvironmental signals (Gordon, 2007). Microbial stimuli, such as LPS as well as Th1 cytokines, activate macrophages into an M1 inflammatory state, while Th2 cytokines, such as IL-4, polarize cells to an M2 phenotype with immunoregulatory, repairing, and anti-inflammatory functions (Gordon, 2003). However, whether the cytokine tissue environment recruits and/or activates specific monocyte subsets or rather polarizes already accumulated subsets of particular macrophages is not known at the moment.

Until now, the presence of M2 macrophages in human atherosclerotic lesions had not been demonstrated. Here we provide evidence for macrophage heterogeneity in atherosclerosis, since both M1 and M2 macrophage markers are detectable in human atherosclerotic lesions. Immunohistological analysis shows that M2 macrophages positively stained for CD68 and MR are present at locations distant from the lipid core in more stable zones of the plaque. Interestingly, in human atherosclerotic plaques, expression of *PPARγ*, a transcription factor with anti-inflammatory properties in macrophages, correlates positively with the expression of M2 markers. These observations prompted us to investigate whether PPARγ plays a role in the differentiation program of monocytes into the M2 phenotype in vitro. Our results show that PPARγ activation primes monocytes into an enhanced M2 phenotype. Moreover, these PPARγ-primed M2 macrophages exert more pronounced anti-inflammatory properties on M1 macrophages. One well-established pathway via which PPARγ controls the inflammatory response is by negatively interfering with proinflammatory signaling pathways such as AP-1, NF-κB, or STAT-3 in activated M1 macrophages (Chinetti et al., 2003). More recently, it has been shown that the PPARγ-dependent transrepression pathway is initiated by sumoylation of the liganded PPARγ ligand-binding domain maintaining the corepressor complex on NF-κB response elements, thus preventing inflammatory response genes from being activated (Pascual et al., 2005). Our observations provide evidence for an additional mechanism by which PPARγ can exert anti-inflammatory activities in macrophages. This mechanism is mainly operative in native monocytes that, in the presence of an appropriate M2 stimulus such as IL-4, can be primed by PPARγ ligands to an enhanced M2 phenotype. By contrast, PPARγ activation does not influence the expression of M2 markers in already differentiated RM and M1 programmed macrophages or foam cells, an effect that is associated in vivo with the absence of M2 marker regulation by rosiglitazone in established atherosclerotic lesions isolated from treated patients. However, TZD administration reduces lesion inflammation and increases collagen content and plaque stability in the same subjects (Meisner et al., 2006). These observations indicate that, in established atherosclerotic lesion macrophages, the anti-inflammatory actions of TZD are due to a reduction of M1 macrophage activation state, mirrored, for example, by decreasing the expression of matrix-degrading metalloproteinases (Meisner et al., 2006). Interestingly, TZD treatment significantly increased expression of *MR* in PBMC, a heterogeneous cell population including circulating monocytes, thus suggesting that PPARγ activation can program mononuclear precursor cells toward an M2 phenotype in vivo. This would lead to generation of a macrophage population with enhanced anti-inflammatory properties. Our data highlight the action of PPARγ ligands and suggest that TZD may be particularly efficient in the prevention of cardiovascular complications of type 2 diabetes.

EXPERIMENTAL PROCEDURES

Endarterectomy Tissue Samples

Human atherosclerotic plaques were removed from 27 patients eligible for surgical carotid endarterectomy (Zawadzki et al., 2006). Carotid plaques were obtained from 15 nondiabetic patients with symptomatic carotid artery stenosis randomized to 4 weeks placebo or rosiglitazone before surgery (Meisner et al., 2006).

Immunohistochemical Analysis

Serial cryosections from endarterectomy specimens were stained with Oil red O, anti-human CD68 (DakoCytomation), anti-human MR, or anti-human MCP-1 (Santa Cruz Biotechnology).

Cell Culture

Primary human monocyte differentiation in resting macrophages (RM) occurred after 7 days of culture. Alternatively differentiated macrophages (M2) were obtained by stimulating monocytes with human IL-4, IL-13 (15 ng/ml), or a combination of both (10 ng/ml each). The PPARγ agonists GW1929 (600 nM) or rosiglitazone (100 nM) or the PPARγ antagonist GW9662 (1 μM) was added during differentiation. RM were activated to M1 macrophages by IL-1β (5 ng/ml), TNF-α (5 ng/ml), or LPS (100 ng/ml) or were transformed to foam cells by acetylated LDL.

Indirect M1/M2 Coculture Experiments

M2 or M2γ (M2 differentiated in the presence of PPARγ ligands) culture medium was added to RM 24 hr before LPS (100 ng/ml) stimulation. After 4 hr, M1 macrophages were washed and cultured for 24 hr in fresh medium. The resulting media were assayed for cytokine and chemokine secretion by ELISA (PeproTech).

RNA Extraction and Analysis

Total RNA isolated from human carotid plaques, macrophages, and PBMC isolated from subjects enrolled in the pioglitazone and ultrasound evaluation study before and after 2 months of pioglitazone administration (Balmforth et al., 2007) was reverse transcribed. cDNAs were quantified by qPCR using specific primers.

Flow Cytometry Analysis

Protein analysis was performed using a FACStar Plus with PE-Cy5-conjugated anti-MR or PE-conjugated anti-CD163 human antibodies (BD Biosciences PharMingen).

Statistical Analysis

Pearson correlation coefficients (r) and multiple regression analysis were calculated from qPCR ΔCt data using R statistical software (http://www.r-project.org/). Statistical differences between groups were analyzed by Student's t test and Mann Whitney rank-sum test and were considered significant at $p < 0.05$.

For more details, see Supplemental Experimental Procedures.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, Supplemental References, one table, and three figures and can be found with this article online at http://www.cellmetabolism.org/cgi/content/full/6/2/137/DC1/.

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