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Loomans, C.J.M.

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Chapter 7

The PPAR γ agonist pioglitazone is a potent transcriptional trans-repressor in both monocytes and endothelial progenitor cells of patients with type 2 Diabetes

*Cindy J.M. Loomans¹, Fabrice, M.A.C. Martens², Frank L.J. Visseren²,
Joost B. Vos¹, Jacques .M.G.J. Duijs¹, Eelco J.P. de Koning¹, F.J.T.
Staal³, Anton-Jan van Zonneveld¹ and Ton J. Rabelink¹*

¹Department of Nephrology, Leiden University Medical Center, Leiden, The Netherlands.

²Department of Vascular Medicine, Utrecht Medical Center, Utrecht, The Netherlands.

³Department of Immunology, Erasmus MC, Rotterdam, The Netherlands.

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Abstract.

Myeloid cells have been identified as key players in the development of atherosclerosis and organ damage. While cells from myeloid origin have the potential to repair the vessel wall by differentiation into endothelial cells and by stimulating angiogenesis and arteriogenesis, they may also cause injury when differentiation into inflammatory phenotypes such as macrophages or dendritic cells occurs. It appears that inflammatory and metabolic stimuli determine the fate of myeloid cells. As PPAR agonists have been shown to have potent anti-inflammatory effects, we investigated whether short-term treatment with the PPAR γ agonist pioglitazone modulates the inflammatory signature of circulating myeloid cells as well as the capacity to differentiate into an endothelial phenotype (EPC) in patients with type-2 diabetes. Transcriptional profiles of myeloid cells, committed to the monocytic lineage, as well as EPC were analyzed from patients treated for four weeks with pioglitazone and compared with patients who received placebo. A marked overall transcriptional repression was observed in differentially expressed genes of both monocytes (83.3%) and EPC (91.9%). Validation experiments with real-time PCR could further demonstrate that EPC's, in comparison to monocytes, show characteristics of immature immune cells with high C1q expression and low expression of the complement propagation factor properdin. Pioglitazone treatment shifted this profile in monocytes towards that of immature immune cells. Despite the transrepression, no difference in the ability of myeloid progenitor cells to differentiate towards endothelial cells was observed. This study provides the first *in vivo* evidence of transcriptional transrepression in myeloid cells in diabetes and points to a new therapeutic mode of action of thiazolidinediones.

Introduction

Over the last years myeloid cells have been identified as key players in the pathobiology of atherosclerosis and target organ damage. Circulating myeloid cells may differentiate into monocytes that subsequently can develop into foam cells when recruited into the vessel wall. Moreover myeloid cells also are a source of dendritic cells in the vessel wall¹. This results in antigen presentation and local adaptive immune responses. Apart from these pro-inflammatory effects of myeloid cells, we and others recently found that myeloid progenitors may also differentiate into an endothelial phenotype and restore endothelial integrity^{2,3}. Moreover, myeloid cells have been involved in lymphangiogenesis and clearance of inflamed tissue⁴. Inflammatory stimuli and cytokines seem to influence the fate and plasticity of such myeloid cells⁵. For example, in the presence of cytokines such as GM-CSF and interleukin-4 the formation of dendritic will be promoted⁶. The presence of VEGF and shear stress may facilitate endothelial differentiation^{7,8} while VEGF-C and its receptor VEGF-3 have been shown to be a stimuli for formation of new lymph endothelium from these cells⁴. We previously demonstrated that diabetes is associated with a reduced capacity to form endothelial progenitor cells (EPC) from peripheral-blood mononuclear cells (PB-MNC) while function of these EPC was hampered as well⁹. In a recent study, we demonstrated that in mice hyperglycemia affects myeloid differentiation in the bone marrow resulting in a reduced differentiation of myeloid progenitor cells into EPC and a concomitant increase in the differentiation of myeloid progenitor progenitor cells into macrophages (in submission). Prevention of cellular cytokine responses and the transcription of inflammatory genes may therefore be a target to modulate the balance of myeloid cells towards repair and less to inflammatory phenotypes.

In the setting of diabetes, the synthetic PPAR γ agonists are of particular interest in this respect. These drugs were designed and developed to induce PPAR γ -dependent transcription in adipocytes, thus influencing differentiation and metabolic function of adipocytes¹⁰. This results in improved free fatty acid metabolism and enhanced glucose sensitivity¹¹. However, more recently PPAR γ agonists have also been shown to negatively regulate inflammatory gene expression¹²⁻¹⁴. The PPAR ligands exert these anti-inflammatory effects by inhibiting various transcription factors including NF κ B¹⁵.

In the current study we assessed whether the PPAR γ agonist pioglitazone modulates transcriptional activation of myeloid cells as well as the capacity to differentiate into endothelial phenotype. For this purpose mononuclear cells that were committed to the

monocytic lineage and to the endothelial lineage, as well as late outgrowth endothelial progenitor derived cells, were characterized for their transcriptional signature. Subsequently, the transcriptomes of these cells were investigated in patients with type II diabetes, comparing pioglitazone treatment to placebo.

Subjects and methods

Patient characteristics.

Ten male, non-smoking patients with type 2 diabetes were recruited into this study. All patients were treated with oral antihyperglycemic agents that were continued during the study. Subjects with poor glycemic control (HbA1c > 9%) were excluded. Other relevant exclusion criteria were presence of macro- or microvascular disease and use of vasoactive medication (eg, [beta]-blockers, calcium entry blockers, ACE inhibitors, angiotensin type-1 receptor blockers, statins, aspirin, or non-steroidal inflammatory drugs). The ethical review board of the University Medical Center Utrecht (UMCU) approved the protocol. All subjects gave written informed consent. Measurements were carried out in accordance with local institutional guidelines in a Good Clinical Practice-certified unit¹⁶.

Study design

The study was designed as a prospective, randomized, crossover, placebo-controlled, double blind trial. Patients eligible to take part in the study were randomized to receive pioglitazone 30 mg once daily (Eli Lilly) or placebo for 4 weeks in addition to their oral antihyperglycemic agents. These 4 weeks were followed by a washout period of 6 weeks. Peripheral blood was drawn at the end of each 4-week treatment period (placebo and pioglitazone). Patients were instructed to fast for at least 10 hours before the tests. No study medication or other medication was used on the morning of the study days.

Laboratory assessment

Fasting peripheral blood was drawn, and plasma was frozen at -20°C until further analysis. Glucose, creatinine, total cholesterol, triglycerides, and high-density lipoprotein cholesterol (HDL-C) were measured by standard enzymatic laboratory methods (Vitros 250; Johnson & Johnson). Low-density lipoprotein cholesterol (LDL-C) was calculated with the Friedewald formula. HbA1c and free fatty acids (FFA) were photometrically performed (Hitachi 911;

Roche). Insulin levels were determined with an immunologic method (Immulite 2000; Diagnostic Products Corp). Measurements of plasma adiponectin, interleukin-6 (IL-6), and high-sensitivity C-reactive protein (hs-CRP) were performed with a commercially available kit (ELISA; R&D Systems Inc).

EPC isolation and characterization

Peripheral blood was obtained in blood collection tubes containing EDTA (Venoject). EPC were cultured as described⁹. Briefly, PB-MNC were isolated from 100 ml whole blood by density gradient centrifugation (Histopaque 1077). 50×10^6 PB-MNC were plated at a density of 1×10^6 cells per cm^2 on 6-well culture plates coated with 2% gelatin (Sigma) in M199 medium supplemented with 20% FBS (Invitrogen), 0.05 mg/ml Bovine Pituitary Extract (Invitrogen), antibiotics and 10 U/ml heparin (Leo Pharma BV). After four days of culture, EPC characteristics were confirmed on the basis of morphology and by fluorescent confocal immunohistochemistry using Ulex europaeus agglutinin (UEA-1; Vector), a CD31 antibody (DAKO Diagnostics) and DiI-labeled acetylated LDL (Molecular Probes). EPC were isolated, counted and stored at -80°C as cell pellets until use.

To culture late-outgrowth endothelial cells with a cobblestone like morphology (CLC), MNC fractions of cord blood were isolated and cultured as short-term EPC (see above). After 7 days medium was replaced with EBM endothelial cell medium supplemented with bulletkits (Cambrex). Cells were cultured for over 4 weeks, until colonies with cobblestone morphology appeared. When they reached confluence, the cells were passaged for maximal 4 times in gelatin coated culture flasks. Before use, the CLC were analyzed by immunofluorescence techniques staining for mature EC characteristics with vWF (DakoCytomation), Endoglin (BD, transduction Laboratories) and eNOS (Pharmingen) antibodies.

Human Umbilical cord Vein Endothelial Cells (HUVEC) were also cultured in EGM medium on gelatin-coated culture flasks up to four passages. Total RNA was isolated using an RNeasy kit (Qiagen) and the integrity of RNA preparations was validated before use.

Monocyte (CD14⁺) isolation by magnetic cell sorting

CD14⁺ cells were purified from PB-MNC (see above) by positive selection with magnetic beads conjugated to anti-CD14 antibodies (Miltenyi) using an autoMACS system and according to the manufacturers protocol. CD14⁺ monocyte fractions were reproducibly over

95% pure as determined by flowcytometric analyses (FACScan) and were stored at -80°C for protein (whole cell lysate) and/or total RNA isolations (Rneasy kit, Qiagen).

In vitro angiogenesis assay.

To evaluate CLC cells for their capacity to form tube-like structures, 0.7×10^5 cells/cm² passage 4 CLC were seeded on human fibrin matrices and cultured in M199 medium supplemented with 10% human serum, 10% newborn calf serum, penicillin/streptomycin, basic fibroblast growth factor (bFGF) and tumor necrosis factor- (TNF-) α . Fresh medium was added every second day and the appearance of invading cells and tubular structures was evaluated by phase-contrast microscopy 7 days after seeding.

Microarray analyses

For gene expression profiling of CD14⁺ cells, EPC cultured for 4 days, late-outgrowth EC (CLC) and HUVEC, total RNA preparations were obtained and analyzed using Affymetrix high-density HG-U95Av2 oligonucleotide microarrays interrogating 12,600 transcripts, according to the manufactures protocol. To reduce individual-specific variation in gene expression, CD14⁺ and EPC RNA samples were derived from a pool of equal amounts of total RNA from 5 (healthy) volunteers. Also CLC and HUVEC RNA was derived from 5 donors and pooled.

For gene expression profiling of CD14⁺ and four-day EPC cultures obtained from patients with type-2 diabetes, messenger RNA profiles were analyzed using Affymetrix high-density HG-U133A oligonucleotide microarrays interrogating 18,400 transcripts. Equal amounts of total RNA from 5 patients with either placebo or pioglitazone treatment were pooled.

All the scanned images were analyzed using Affymetrix Microarray Software (MAS) and significant differentially expressed genes (placebo as background signal) were further analyzed. Scaling factor, background, noise and 3'/5' GAPDH ratios (always less than 1.2) were such that valid comparisons between similar arrays could be made. For further analyses commercially available programs Matlab and Spotfire were used.

Quantitative rt-PCR

For validation of the microarray analyses, mRNA expression levels of C1q, Factor P, CDKN1A and S100A9 and two normalization genes (Actin and GAPDH) were measured using quantitative RT-PCR. cDNA was synthesized from total RNA samples (same as used for profiling) using standard cDNA synthesis reagents and a 1:1 mixture of oligo dT(12-18)

primers and random hexamer primers (Invitrogen). Quantitative analyses of the synthesized cDNA were performed with use of SYBR green I (Molecular Probes) in optimised real-time PCR (Amplitaq Gold, Applied Biosystems), using a iCycler Thermal cycler (Biorad). Gene specific primer combinations were assembled with Oligo Explorer (Gene link). Primer sets of the various genes used were from 5' to 3': C1q forward: tcaccaaccaggaagaaccg, reverse: atgggaagatgaggaagccg. Factor P (Properdin) forward: cctaactctacccgtgcc, reverse: cttctgccctgaccttc. Cyclin-dependent kinase inhibitor-1A (CDKN1A) forward: gattagcagcggaacaagg, reverse: caacgttagtgccaggaaag. S100A9 forward: gctggaacgcaacatagag, reverse: ggtcctcatgatgtgttc. For normalization, GAPDH forward: ttccaggagcgagatccct, reverse: caccatgacgaacatggg and actin forward: tgcgtgacattaaggagaag and reverse: tgaaggtagttctgtgtagt. Samples were analyzed in triplicate and threshold cycle numbers and their SD were calculated using iCycler v3.0a analysis software (Biorad) and further used to calculate expression ratio's of the different samples in relation to both normalization genes.

Results

Patient characteristics

Ten subjects were included in this study. No carryover effects between the 2 treatment periods were observed for any parameter¹⁶. When treatments of either placebo or pioglitazone were compared (table 1) a significant reduction in FFA was observed (640 ± 38 versus 504 ± 34 $\mu\text{mol/L}$, $P=0.04$; respectively). A significant increase in adiponectin plasma levels was obtained during pioglitazone treatment compared with placebo after only 4 weeks (7421 ± 147 versus 4264 ± 856 ng/mL , $P=0.005$ respectively). No further significant differences were seen in the measured values.

CD14⁺ monocyte isolation and EPC characterization

To assess the myeloid properties and the purity of the sorted CD14⁺ cells the cells were stained with an anti-CD14 FITC-labeled antibody and analyzed by flowcytometric analysis. Figure 1A shows a representative dot plot of the sorted CD14⁺ fraction that was reproducibly over 95% pure ($96.5\% \pm 2.4\%$, $n=10$). After 4 days of cultivation under EC differentiation conditions, attached cells were analyzed for EC characteristics. EPC appeared with a typical spindle-shaped morphology (figure 1B, upper right) and over 90%

	Placebo (n=10)	Pioglitazone (n=10)	P-value
Weight (kg)	99.3 ± 3.0	98.6 ± 3.9	0.71
SBP (mm Hg)	137 ± 7.0	130 ± 3.0	0.44
DBP (mm Hg)	73 ± 2.0	74 ± 3.0	0.90
Fasting glucose (mmol/L)	7.2 ± 0.5	6.6 ± 0.6	0.25
Insulin (mU/L)	21.0 ± 4.2	17.1 ± 3.3	0.33
HbA1C %	6.7 ± 0.2	6.4 ± 0.2	0.08
Total cholesterol (mmol/L)	4.8 ± 0.2	4.9 ± 0.3	0.34
HDL cholesterol (mmol/L)	0.9 ± 0.05	0.9 ± 0.1	0.92
LDL cholesterol (mmol/L)	3.0 ± 0.2	3.1 ± 0.3	0.57
Triglycerides (mmol/L)	1.8 ± 0.2	1.8 ± 0.2	0.72
Free fatty acids (μmol/L)	640 ± 38	504 ± 34.0	0.04 *
Adiponectin (ng/ml)	4264 ± 856	7421 ± 1147	0.005 *
Creatine (μmol/L)	71 ± 3.0	69 ± 2.0	0.11
CRP (mg/L)	3.9 ± 0.8	2.7 ± 0.6	0.11
Interleukine-6 (pg/ml)	2.0 ± 0.3	1.7 ± 0.2	0.17

Values are mean ± SEM.
P-values calculated Placebo vs. Pioglitazone.
*P<0.05

Table 1

Clinical characteristics of the patients after treatment

of the attaching cells showed uptake of DiI-labeled acLDL (red) and could bind an EC specific lectin UEA-1 (figure 1B, upper left). Further characterizations show that EPC comprise more EC properties as they have a high CD31 surface expression and they express low levels of eNOS. Von Willebrand factor was also detected at low levels in the EPC although no Weibel Palade body-like structures could be observed (data not shown). Typical clonal cell expansion is seen in the late-outgrowth cultures (CLC) after 4 weeks of culture (figure 1B, middle left). These colonies were further cultured until they formed a monolayer of cells with cobblestone morphology. Endothelial characteristics of these cells were analysed and confirmed using immunofluorescent staining of vWF (in Weibel Palade bodies, figure 1B, middle right), CD31 (figure 1B, lower right), eNOS (mostly perinuclear staining) and Endoglin (data not shown). To assess whether the CLC also display functional

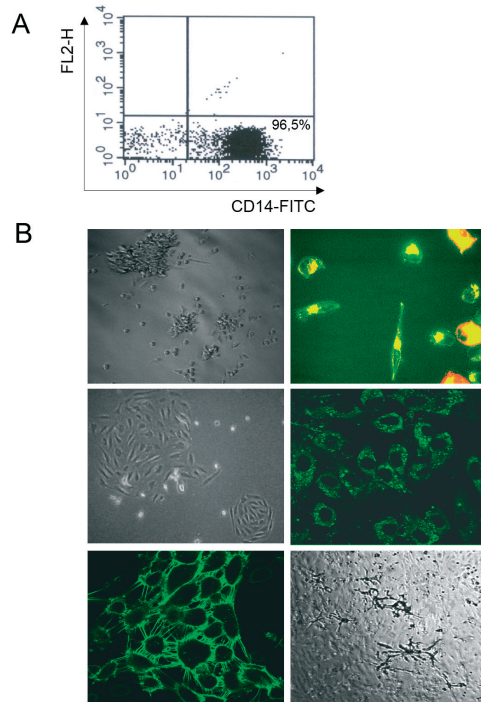


Figure 1: Morphological, phenotypic and functional analyses of the different cell populations.

(A) Flowcytometric analyses of the purity of CD14⁺ cells in the positive cell fraction after magnetic cell sorting.

(B) Morphology of cell clusters of EPC appearing after 4 days (upper left). Upper right shows uptake of Di-labeled acLDL (in red) by EPC cultured for 4 days as well as binding of lectin UEA-1 (in green). Almost all cultured cells are double positive for both EC markers. When EPC are cultured further as CLC they start out as colonies with cobble-stone appearance (middle left panel). CLC grown as monolayer all show vWF staining in Weibel pallade bodies (middle right panel) and CD31 membrane staining (lower right) similar to mature EC. Furthermore, CLC are able to form 3-D vessel structures in *in vitro* angiogenesis assays (lower right panel).

EC characteristics, the ability of these cells to form vessel like structures in an *in vitro* angiogenesis assay was examined. Indeed, CLC readily formed capillary-like structures to the same extent as mature EC upon stimulation with angiogenic factors like bFGF (figure 1B, lower right).

To demonstrate the myeloid lineage properties of the isolated CD14⁺ cells and the myeloid / EC character of the cultured EPC, mRNA profiles were analyzed for monocyte and endothelial cell specific gene expression (table 2). Genes that are associated with monocytes are shown at the top of the table, while genes known to be EC specific are depicted at the bottom. The middle represents a transition phase, as monocytes and EC are known to share some antigens. The table validates the myeloid character the sorted CD14⁺ cells reveals that EPC share many genes with these myeloid CD14⁺ cells and have only upregulated some EC specific genes (e.g. endoglin) at a low level. These data further support the concept that short term cultured EPC are derived from the myeloid lineage. Consistent with the phenotypic and functional analyses of the CLC, profiles of the late-outgrowth EPC (CLC) show a high similarity in gene expression with mature EC (HUVEC).

	CD14	EPC	CLC	HUVEC
Monocytic				
HLA-DR	192897	328905	1656	1629
MHC-class II	118788	238188	5152	6090
CD14	137451	152529	1003	-6
Endothelin-1	53935	117382	-1400	11
L-Selectin(CD62L)	38116	1850	67	735
CD33	12777	4547	825	1413
CD11b	10805	223	-316	-1126
CD11a/LFAa	10771	4860	791	759
CD49d (VLA-4)	5763	527	7	871
CD86	3954	6972	-68	879
CD68	2896	2960	566	-1502
VEGFR-1(Flt-1)	649	308	2231	487
LDL receptor	1084	2104	4562	944
ACE	54	6488	1033	1032
VCAM-1	238	121	-31	1279
eNOS	-1335	705	989	2302
VEGF-B precursor	565	6638	4253	3967
ELAM-1 (CD62E)	625	988	1150	3980
multimerin	-53	-1089	4665	5297
CD31(PECAM-1)	2620	1621	4036	6722
CD71 (VAP-2)	6295	60205	15344	9802
CD19	761	710	15608	10958
VEGFR-2 (KDR)	711	269	9636	13398
Tie (1,2)	244	1662	22716	25925
CD34	2538	2532	14300	35073
Endoglin	5503	21117	56852	44098
ICAM-2	7600	2169	59870	44527
IGFBP	-359	2852	28015	45982
vWF	93	377	42634	73754
angiomodulin (ANG,TAF)	3488	614	69365	78028
CTGF	-139	-477	61268	80972
VE-Cadherin	-556	-813	43922	83299
PAI-1	-3279	-2206	172704	138238
Endothelial				

Signal intensity:

- 330000-100000
- 100000-50000
- 50000-10000
- 10000-low detection
- no detection

Table 2

Myeloid/Endothelial characteristics of CD14⁺ cells, EPC, and CLC. Micro array analyses were screened for monocytic and endothelial genes and expression (laser signals) of these genes are depicted as high (dark green), intermediate (light green) and low or below detection (orange).

Pioglitazone treatment does not induce differences in the number of monocytes or EPC

To investigate whether pioglitazone treatment did affect the number of circulating CD14⁺ cells, the purified CD14⁺ fractions derived from equal amounts of MNC fractions were quantified. As shown in figure 2A, no significant difference in CD14⁺ cells numbers were observed. Pioglitazone treatment did also not have an effect on the differentiation of myeloid cells into the EC lineage, as the number of attaching EPC cells (4 day cultures) derived from 50x10⁶ MNC was similar compared to the cultures from placebo treated patients (figure 2B).

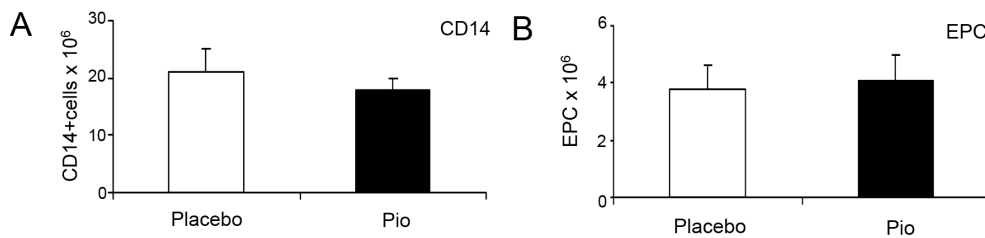


Figure 2: Effect of pioglitazone treatment on number of cells.

The treatment of the patients with pioglitazone (Pio) had no significant effects on the total number of isolated CD14⁺ cells (A) and the total number of cultured EPC at day 4 (B) when compared to placebo treatments.

Transrepression of differentially expressed genes by pioglitazone treatment.

As the overall average expression of the raw data signals of all microarrays were comparable (data not shown), the effects of different treatments on the two different cell types could be compared using MAS microarray analyses. Significantly differentially expressed genes (1.5x cut-off value) in CD14⁺ cells and in EPC were extracted from the panel and their Signal Log Ratio's were plotted (figure 3). There were more significant differentially expressed genes in the EPC panel when compared to the CD14⁺ cell panel (1178 genes vs. 514 respectively). Strikingly, almost all differentially expressed genes were down regulated in both CD14⁺ cells and EPC (83.3% and 91.9% respectively), suggesting a highly dominant transrepressive effect of pioglitazone treatment in subsets of myeloid cells derived from diabetes patients.

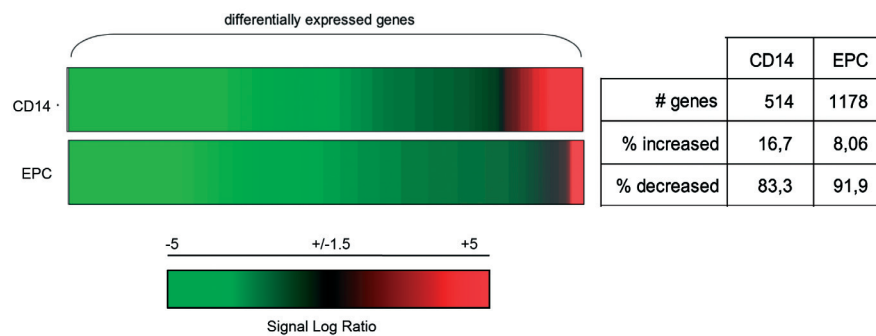


Figure 3: Transrepression of differentially expressed genes occurred in both CD14⁺ cells and EPC in patients treated with Pioglitazone.

Genetic profiles of both pioglitazone and placebo treatments (background values) are compared of CD14⁺ cells and EPC and only the differentially expressed genes are depicted.

Signature of inflammatory responsive genes by pioglitazone treatment.

In order to evaluate the effect of pioglitazone treatment on inflammatory responsive genes in both cell types we looked for the genes present on the HG-U133A that represent the gene ontology class “Immune response” (GO:00069). We extracted about 50 different probe sets from this GO-class and we calculated the ratios of the probe signals (placebo=background). By plotting these ratios, the fingerprint of this specific GO-class of genes became visible (figure 4).

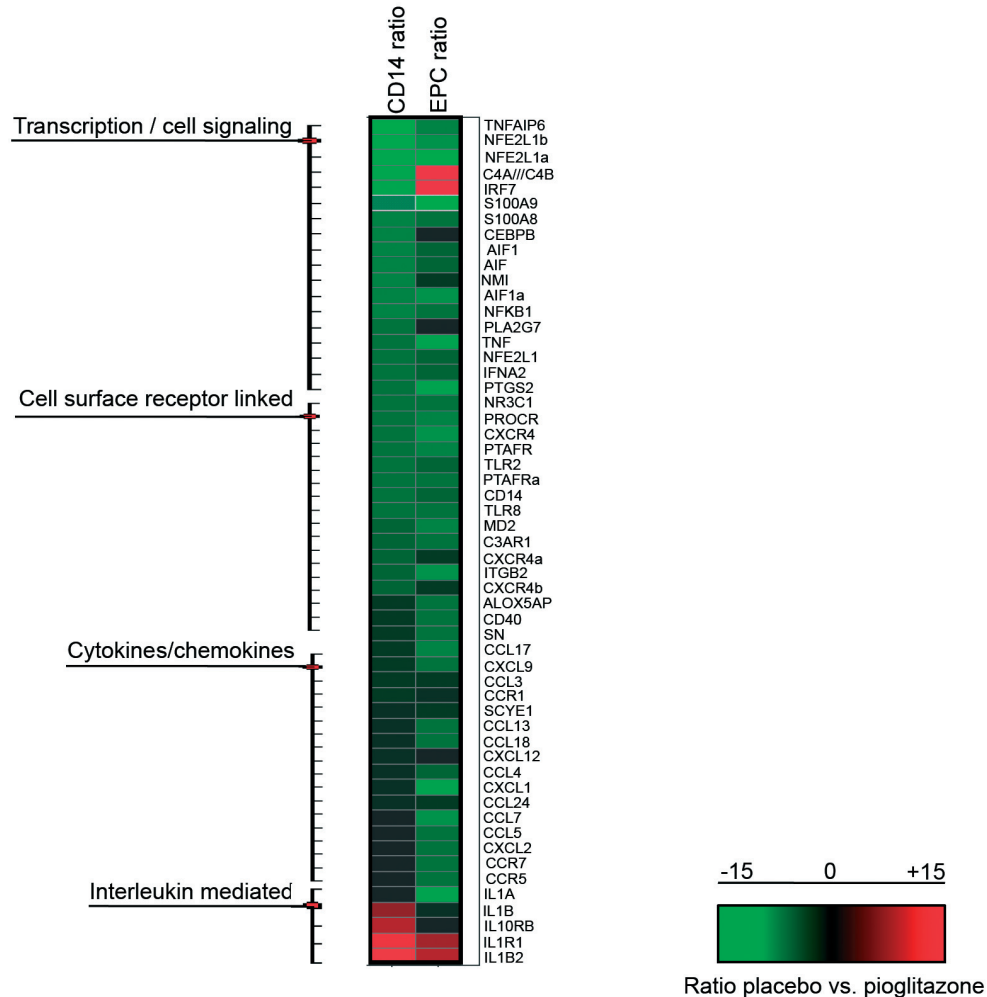


Figure 4: The effect of pioglitazone treatment on inflammatory responsive genes in CD14⁺ cells and EPC.

Some of the genes are represented several times in the figure but different probe sets were analysed for these.

Validation of microarrays by quantitative real time PCR analyses

To validate if the gene arrays were representing the mRNA levels in the different cells, four genes of interest were analyzed by quantitative rt-PCR. Expression ratio's of the different genes were normalized to two different normalization genes (GAPDH/Actin) and results are shown in figure 5A. Raw data signals from the various genes (figure 5B) were assembled from the array analysis and compared to the expression data derived from the

quantitative rt-PCR. Complement factor C1q was analyzed in the patient samples (figure 5A) as well as in the various differentiation stages of endothelial cells (figure 5C). C1q, a major component of the classical complement pathway, was not significantly differentially regulated by treatment of pioglitazone in both cell types (figure 5A); however it showed a marked upregulation in the EPC differentiation stage when compared to monocytes or mature EC (figure 5C). Quantitative analyses of factor P mRNA levels, also known as Properdin, a positive regulator of complement activation showed marked higher levels in CD14⁺ cells when compared to EPC. No significant differential expression after pioglitazone treatment was observed in EPC, however in CD14⁺ cells we found a marked reduction in Factor P ($P = 0.05$). P21 mRNA levels showed the same pattern as mRNA levels of C1q in the patient samples, revealing no significant differential expression in both

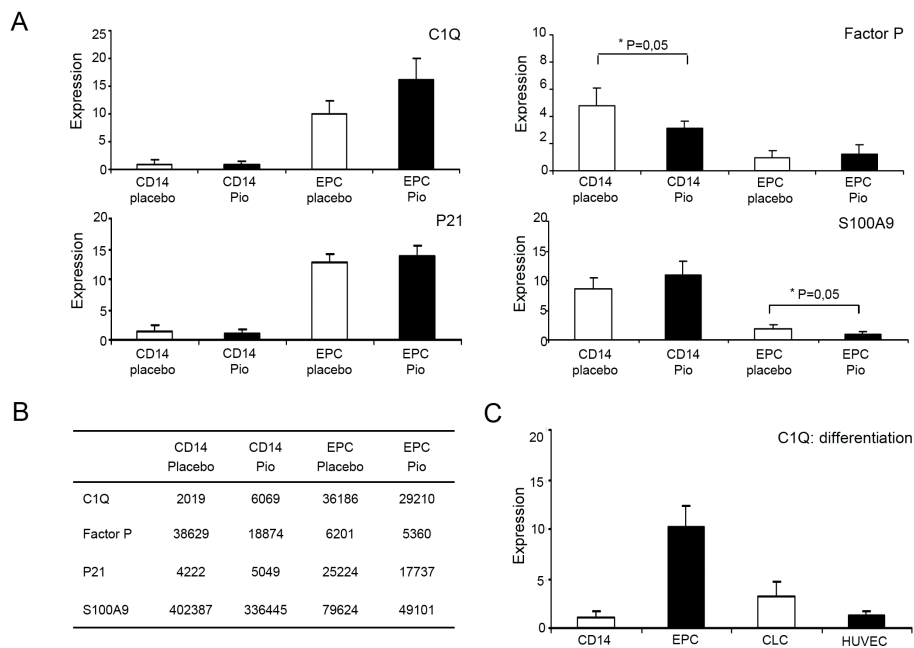


Figure 5: Validation of microarrays by quantitative real time PCR analyses.

Gene expression profiles of CD14 cells and EPC derived from patients with (Pio) or without (placebo) pioglitazone treatment were validated. Normalized relative expression ratio's of mRNA levels of C1q, Factor P, P21 and S100A9 were analyzed using quantitative real time PCR techniques (A). Raw data signals of the microarrays were plotted to be able to compare these expression levels with the expression ratio's generated by quantitative PCR (B). In addition to the patient samples C1q profiles of various differentiation stages of endothelial cells were further explored using quantitative PCR. Table 2 shows the raw data signals of the microarray experiments and here these data are verified (C).

cell types when patients were treated with pioglitazone but a marked increased expression in EPC when compared to CD14 cells. When we quantified S100A9, a Ca^{2+} -binding protein known to be involved in inflammatory responses of monocytes and macrophages¹⁸, we noticed that CD14⁺ cells had a higher expression of the protein when compared to EPC but EPC showed a significant reduction in the level of S100A9 after pioglitazone treatment ($P = 0.05$).

In summary, for all genes tested, observations obtained by quantitative rt-PCR were in line with the microarray analyses, showing that the gene expression levels from the microarray analyses did represent mRNA levels in the cell isolations.

Discussion

Recently attention has been drawn to the anti-inflammatory and anti-atherosclerotic effects of synthetic PPAR- γ ligands. The current study provides a rationale for these effects by demonstrating generalised trans-repression in myeloid cells that are involved in inflammation and repair processes in patients with type II diabetes by the PPAR- γ agonist pioglitazone.

The classical paradigm is that thiazolidinediones, synthetic ligands for PPAR γ , act through transcriptional activation in adipocytes resulting in improved free fatty acids metabolism and glucose handling^{10,11}. However, in recent years data have emerged that also show direct vascular effects of these drugs. For example inhibition of atherosclerosis progression was observed in non-diabetic patients¹⁹. It was suggested that this effect was independent of changes in metabolism and could possibly be attributed to anti-inflammatory effects. Indeed, thiazolidinediones have been shown to reduce tissue inflammation in conditions such as psoriasis and kidney disease²⁰. The exact nature of these effects is not exactly known yet.

The current study shows that patients with type 2 diabetes that have been treated with pioglitazone have a generalised transcriptional repression in circulating monocytes (figure 3). This trans-repression includes important genes involved in cell activation such as cell surface receptor linked mechanisms, the cytokine and chemokine production and genes involved in control of transcription (figure 4). It has been proposed that such trans-repression may be secondary to a physical interaction between PPAR- γ and the co-repressor complexes that keep inflammatory transcription suppressed¹⁵. Binding of PPAR- γ to this

repressive complex prevents recruitment of the ubiquitination machinery normally required to clear the co-repressor complex from the activation site. The current study extrapolates these *in vitro* concepts to the *in vivo* situation in patients with type 2 diabetes. As monocyte activation and transformation into foam cells is fundamental to atherogenesis and plaque disruption, one may postulate that these molecular effects of thiazolidinediones contribute to the beneficial effects on atherosclerotic disease of these drugs. Indeed, reduced progression of intima media thickness, as an intermediate marker of atherosclerosis, has been observed both in diabetic patients²¹ as well as in non-diabetic patients¹⁹. Moreover in a recent large endpoint driven study, the PRO-active study, it was shown that pioglitazone could produce cardiovascular events and associated mortality²².

The present short-term study only shows very limited effects on metabolic indices. Only small changes in free fatty acid fluxes could be observed, while glucose and lipid levels did not change significantly. For metabolic changes the treatment period could be too short as they are generally only seen from 8 weeks of treatment²⁰. This makes a direct transrepressor effect by pioglitazone more likely than an indirect effect through improvement of insulin resistance.

We recently could demonstrate that myeloid cells may not only give rise to inflammatory cells but are also implicated in repair processes of the vessel wall. For example they have been shown to be able to reendothelialize² and to enhance angiogenesis^{23,24} and lymphangiogenesis^{4,25}. The current study shows that despite the generalized transrepressive effects of pioglitazone, differentiation into an endothelial cell phenotype is still possible. The number of endothelial like cells (general referred to as endothelial progenitor cells, EPC) that can be cultured from the mononuclear cells is the same whether the patients are treated with placebo or with pioglitazone (figure 2B). Nevertheless when these cells are in the process of transdifferentiation towards an endothelial phenotype they show a similar profound reduction in expression of genes that are involved in cell activation. Previously, using a mice model it was observed that thiazolidinediones might even increase the capacity to form endothelial cells from bone marrow. It should, however, be noticed that these were healthy mice that did not have diabetes-associated recruitment and differentiation defects²⁶.

We also characterised the expression profiles of myeloid cells while they are in transition of myeloid to endothelial cells. When cells are cultured for 4 days under conditions that promote the formation of endothelial cells, expression of endothelial specific genes such as

endoglin, VEGF-B become upregulated (table 2). However these cells still bear more characteristics of myeloid cells. For example they express HLA-DR and CD14. Only after prolonged culture a full endothelial phenotype (CLC) with a similar expression signature to that of e.g. HUVEC appears. This would imply that the early re-endothelializing cells still could be considered as cells that are part of the innate immune system. In this respect they demonstrated some interesting features that do distinguish them from monocytes. For example the complement factor C1q is strongly upregulated. (figure 5C, table 2). Recent data from our laboratory have shown that C1q produced by myeloid cells acts as an immune-modulatory factor that allows for phagocytosis and clearance of apoptotic cells but blocks T-cell activation^{27,28}. This suggests that these EPC also participate in immune surveillance. It is of interest to notice that treatment with pioglitazone upregulates C1q in the monocytes while the monocyte complement propagation factor properdin, which allows for full complement activation, is downregulated. In this respect pioglitazone treatment shifts the expression in monocytes of diabetes patients towards that of immature immune cells.

In sum, our study provides first *in vivo* evidence of a novel mode of action of thiazolidinediones that may contribute to organ protection in diabetes.

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