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The peroxisome-proliferator activated receptor- γ agonist pioglitazone modulates aberrant T cell responses in systemic lupus erythematosus

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Abstract

PPAR- γ agonists can suppress autoimmune responses and renal inflammation in murine lupus but the mechanisms implicated in this process remain unclear. We tested the effect of the PPAR- γ agonist pioglitazone in human lupus and control PBMCs with regards to gene regulation and various functional assays. By Affymetrix microarray analysis, several T cell-related pathways were significantly highlighted in pathway analysis in lupus PBMCs. Transcriptional network analysis showed IFN- γ as an important regulatory node, with pioglitazone treatment inducing transcriptional repression of various genes implicated in T cell responses. Confirmation of these suppressive effects was observed specifically in purified CD4⁺ T cells. Pioglitazone downregulated lupus CD4⁺ T cell effector proliferation and activation, while it significantly increased proliferation and function of lupus T regulatory cells. We conclude that PPAR- γ agonists selectively modulate CD4⁺ T cell function in SLE, supporting the concept that pioglitazone and related-agents should be explored as potential therapies in this disease.

Keywords

systemic lupus erythematosus; T cells; gene expression

Many abnormalities in the phenotype and function of T cells have been described and are considered important in disease pathogenesis. Indeed, aberrant T cell activation, disrupted T cell cytotoxic responses, and decreased numbers and function of T regulatory cells (Tregs)

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occur in human and murine lupus systems[1-4]. Furthermore, various therapies known to be effective in SLE act, at least in part, by modulating T cell responses[5-7]. However, many of these therapies are associated with significantly deleterious global immunosuppressive effects. Therefore, there is a need for less immunosuppressive T cell modulatory approaches that may specifically target pathogenic immune responses characteristic of SLE.

The peroxisome proliferator activated receptor (PPAR)- γ is a type II nuclear receptor that regulates fatty acid storage and glucose metabolism[8]. PPARs form heterodimers with retinoid X receptor (RXR) and regulate gene transcription. PPAR- γ also plays essential roles in the regulation of cellular differentiation and development[9]. Ligands for PPARs have anti-inflammatory and vasculoprotective activities in numerous disease models and various exogenous agonists have been developed[10, 11]. Among these, the thiazolidinediones (TZDs) pioglitazone and rosiglitazone are approved for the treatment of diabetes mellitus[9]. These drugs have been found to be anti-inflammatory in various disease settings in rodent and human systems, and to have vasculoprotective roles in various disease conditions beyond diabetes[11-13].

Recently, our group reported that pioglitazone improves cardiometabolic risk and renal inflammation in lupus-prone New Zealand Black/New Zealand White F1 mice. Indeed, these mice developed significantly decreased renal immune complex deposition and T cell inflammatory infiltrates and lower expression of renal inflammatory markers[14]. Similar findings were reported by Aprahamian et al. when rosiglitazone was studied in another lupus model, where downregulation of autoimmune responses, T and B cell activation and renal inflammation was prominent[15]. Further supporting that TZDs may effectively modulate T cell responses, newly appreciated regulatory roles have been reported for the PPAR- γ pathway in Th cell skewing, T cell activation, and in the induction of Tregs. Indeed, TZDs induce Treg synthesis and improve their function in non-lupus models[16, 17] and PPAR- γ agonists inhibit allogeneic human memory T cell responses[18]. Further, suppression of Th17 differentiation has been reported by these compounds, through inhibition of the transcription factor ROR- γ T [19], while PPAR- γ levels, in combination with specific hormonal exposures, can determine T cell fate with regards to Th1 versus Th17 differentiation[20]. In adipose tissue, PPAR- γ -mediated anti-inflammation occurs primarily through a T-cell related effect[13, 20].

Higher levels of PPAR- γ were recently reported in SLE PBMCs and proposed to regulate T cell activation [21]. However, it remains unclear if PPAR- γ agonists have immunomodulatory roles in human SLE that may provide an important therapeutic tool to mitigate autoimmune responses in these patients. We now report the results of a study that assessed if PPAR- γ agonists modulate phenotype and function of human Lupus PBMCs.

Patients and Methods

Patient recruitment, PBMC and T cell isolation and treatment with pioglitazone

Research was conducted according to the principles expressed in the Declaration of Helsinki and informed consent was obtained. Study was approved by the University of Michigan's IRB. Participants provided written informed consent to participate according to IRB

policies. Lupus patients were recruited from the comprehensive lupus clinic, general rheumatology clinics and inpatient services at the University of Michigan. Age- and gender-matched healthy controls were enrolled by advertisement. Lupus patients fulfilled ACR criteria for the disease[22]. Lupus disease activity was quantified by SLEDAI as previously described[23]. Patients were excluded if they had symptoms of recent or active infection or were pregnant. Immunosuppressive medications, antimalarials and steroid use were carefully recorded. No patient or control recruited was taking pioglitazone or other PPAR- γ agonists. Demographic and clinic characteristics of patients and controls are included in Supplementary Table 1. Peripheral blood was obtained by venipuncture in EDTA-coated tubes. PBMCs were isolated as previously described by us, using Ficoll Hypaque gradient[24]. Freshly isolated PBMCs were cultured in RPMI/10% FBS in the presence or absence of 1 μ M pioglitazone (Sigma-Aldrich, St. Louis, MO, USA) for various timepoints. For microarray studies, PBMCs were cultured with pioglitazone for 6 h, harvested and RNA isolated as mentioned below.

In additional experiments, CD4⁺, CD8⁺ and pan-T cells were isolated from PBMCs by negative selection using a CD4⁺ T cell isolation Kit II, CD8⁺ T cell isolation Kit and pan-T cell isolation Kit II, respectively (Miltenyi Biotec, Auburn, CA), and experiments performed as stated below. Purity was >95%

RNA isolation

Total RNA from PBMCs and T cells was isolated with Tripure (Roche, Indianapolis, IN), following manufacturer's recommendations. For microarray analysis of PBMCs, RNA was further purified using an RNeasy micro kit (Qiagen, Valencia, CA). RNA samples were processed on an Agilent 2100 Bio-Analyzer (Agilent Technologies, Santa Clara, CA) to assess integrity.

Microarray data processing, analysis and pathway mapping

Affymetrix Human U133 Plus 2.0 Genechips (Affymetrix, Inc, Santa Clara, CA) were processed in a single batch and normalized exactly as described previously[25]. Of the 17527 gene IDs (corresponding to the 54675 Affymetrix probesets), 16148 were expressed above the Poly-A Affymetrix control expression baseline (negative controls) and used for further analyses. Statistical paired analyses were performed using Significance Analysis of Microarrays (SAM) method implemented in MultiExperiment Viewer application (MeV), comparing untreated and pioglitazone-treated lupus PBMCs (n=4 in each group) to healthy control pioglitazone-treated or untreated PBMCs (n=5 in each group) [26, 27]. Unpaired analyses were done in the comparisons of lupus PBMCs to healthy control PBMCs and lupus pioglitazone-treated PBMCs to healthy control pioglitazone-treated PBMCs. The significantly regulated genes between the groups (q-value <0.05, depicting the False Discovery Rate) were analyzed by building biological literature-based networks using Genomatix Pathway System (GePS) (www.genomatix.de). Canonical pathways were analyzed using the Ingenuity Pathway Analysis (IPA) Software (www.ingenuity.com) as well as GePS; a q-value < 0.05 was considered statistically significant.

Quantification of gene expression in T cells by real-time PCR

Real-time PCR reactions were run on an ABI Prism 7900HT in duplicate using 23SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). Oligonucleotide primers (IDT, Coralville, IA) used in the reactions were:

IFN- γ : 5'-TCT TGG CTT TTC AGC TCT GCA TCG-3'(forward), 5'-GCT GGC GAC AGT TCA GCC ATC A-3'(reverse);

IL-10: 5'-ATG CCC CAA GCT GAG AAC CAA GAC CC-3'(forward), 5'-TCT CAA GGG GCT GGG TCA GCT ATC CC-3'(reverse);

IL-17: 5'-ACT CCT GGG AAG ACC TCA TTG G-3'(forward), 5'-GGC CAC ATG GTG GAC AAT CG-3'(reverse)

CD40L: 5'-CACAGCATGATCGAAACATACAACC-3'(forward), 5'-ATCCTTCACAAAGCCTTCAAACCTG-3'(reverse);

CD27: 5'-CGGCACTGTAACCTCTGGTCTT-3'(forward), 5'-TTGCCCGTCTTGTAGCATGT-3'(reverse);

PPAR γ 1:5'-AAA GAA GCC GAC ACT AAA CC-3'(forward);

PPAR γ 2: 5'-GCG ATT CCT TCA CTG ATA C-3'(forward);

PPAR γ 1/2: 5'-CTT CCA TTA CGG AGA GAT CC-3'(reverse);

PPAR α : 5'-TGCAGATCTCAAATCTCTGG-3'(forward), 5'-ATC ACA GAA GAC AGC ATG GC-3'(reverse);

RXR:5'-AGGCCTACTGCAAGCACAAGTAC-3'(forward), 5'-GGCAGGCGGAGCAAGAG-3'(reverse);

GAPDH:5'-TTGCCATCAATGACCCCTTCA-3'(forward), 5'-CGCCCCACTTGATTTTGGA-3' (reverse).

Assessment of T cell activation, apoptosis and proliferation

To examine whether pioglitazone has an effect on T cell activation and proliferation, CD4⁺ T cells were stimulated with phytohemagglutinin (PHA, 5 μ g/ ml, Sigma-Aldrich), or with anti-human-CD3 plus anti-human-CD28 (5 μ g/mL each, Ancell, Bayport, MN), for 48 h at 37°C, in the presence or absence of 1 μ M pioglitazone. Cells were then incubated with PE-anti-human CD4, FITC-anti-human-CD25 or FITC-anti-human-CD154 (BD Bioscience, San Jose, CA, USA). The expression of activation markers was analyzed with FACS Calibur III flow cytometer. Both % cells expressing the markers and mean fluorescent intensity were calculated. To assess proliferation, DNA synthesis was quantified with propidium iodide (PI, BioLegend, San Diego, CA, USA). Pioglitazone-treated or untreated CD4⁺ T cells were washed with PBS, incubated for 1h at 4°C in the dark with a solution containing Triton X-100(0.1%), sodium citrate(0.1%), and PI (50 μ g/ ml). Cells were analyzed with a flow

cytometer equipped with a 488-nm argon laser on the FL2 channel. T cell apoptosis was quantified by Annexin-V staining by flow using a kit from BD Biosciences, as previously described by us[28].

Isolation of Treg and T effector (Teff) cells

This was performed as previously described[29]. In brief, CD4⁺ T cells were isolated as above. CD4⁺CD25⁺CD127^{-/low} (Tregs) and CD4⁺CD25⁻CD127⁺ (Teff) T cells were further purified by cell sorting on a FACS Calibur, by labeling cells with FITC-anti-human-CD25 and APC-anti-human-CD127 mAbs (BD Biosciences). Cell purity was >98%.

In vitro Treg proliferation assay

To analyze Treg proliferative capacity, 2×10^4 CD4⁺CD25⁺CD127^{low} Tregs labeled with CFSE were plated in round-bottomed 96-well plates and cultured in the presence of anti-human-CD3/CD28 (5µg/mL each) and IL-2 (100u/mL) in the presence or absence of pioglitazone (1µM) in RPMI1640 (10%FBS). After 7 days in culture, cells were stained with anti-human mAbs to CD4, CD25 and CD127. CD4⁺CD25⁺CD127^{low} cells were gated and the dilution of CFSE was quantified by flow cytometry.

In vitro suppression assay

An *in vitro* suppression assay of Teff proliferation by Tregs was performed as previously described [30, 31]. In brief, Tregs were cultured with or without pioglitazone (1uM) in RPMI1640 (10%FBS) for 16 h. Teffs were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE; Invitrogen Molecular Probe, Eugene, OR), according to the manufacturer's instructions. Tregs were co-cultured with autologous CFSE-labeled Teffs at 1:1 ratios in a 96-well round-bottom plate pre-coated with anti-human-CD3 and anti-human-CD28 mAb (5ug/mL each) for 3 days, in the presence or absence of APCs, as described[31, 32]. The reaction was stopped with cold PBS. Dilution of CFSE at 72 h post-stimulation was determined by FACS to calculate proliferation of Teffs, as previously described by us and others[24, 29].

IL-10 quantification

Tregs were cultured in the presence or absence of 1uM pioglitazone for 72 h, followed by harvesting of supernatants to quantify IL-10 levels by ELISA (eBioscience), per manufacturer's recommendations. A Biotek ELISA plate (Biotek, Winooski, VT) reader was used to quantify absorbance.

Assessment of autoantibody synthesis in vitro

CD4 T cells were isolated as above, and Tregs were depleted using a Treg purification kit (Miltenyi). B cells were purified with the CD19⁺ B cell Kit (Miltenyi) and T and B lymphocytes were cocultured at a 1:1 ratio (1×10^5 T cells and B cells/well) in the presence of anti-human-CD3/CD28 (5µg/mL each), recombinant IL-2(100 U/mL) and BAFF (25 ng/mL, Millipore) for 7 days with or without pioglitazone (1uM) in RPMI1640 (10%FBS). At 7 days, culture supernatants were harvested and anti-dsDNA IgG was quantified using the anti-dsDNA IgG ELISA kit (GenWay Biotech) and manufacturer's instructions.

Statistical analysis

The difference between means was analyzed using paired *t* test or one-way ANOVA with *post hoc* analysis and Bonferroni correction. Spearman and Pearson's correlation were used to assess correlation between different variables. A value of $p < 0.05$ was considered to be statistically significant.

Results

Pioglitazone differentially regulates PBMC gene signatures in SLE

Gene expression profiles were compared between untreated lupus and control PBMCs and between cells exposed to pioglitazone *in vitro* for 6 h. In the case of lupus PBMCs, a total of 1362 genes were significantly modified with pioglitazone ($q\text{-value} < 0.05$, Supplementary Table 2), including 850 that were transcriptionally repressed in the presence of the drug, and 512 genes that were upregulated (Figure 1A). In contrast, only 215 mRNAs were modified by pioglitazone treatment on control PBMCs, when compared to non-treated control PBMCs ($q\text{-value} < 0.05$, supplementary table 3).

Several T-cell related pathways were significantly highlighted in the pathway analysis of the 1362 transcripts modified by pioglitazone treatment in SLE PBMCs (Table 1 and supplementary figure 1); these included the T-cell receptor signaling pathway in which most of the molecules were transcriptionally repressed (Figure 1B). Transcriptional network analysis using Genomatix Pathway System (GePS) confirmed these findings. Indeed, from the 272 genes having the most differential expression based on a stringent filter criteria (fold-change 1.5 for the up-regulated genes and 0.7 for the down-regulated genes) and using the shortest path network algorithm (which calculates the optimal set of interactions for a network without losing relevant information), GePS built a transcriptional network of 128 genes where connections are based on the PubMed literature co-citations. The resulting network identified *Interferon- γ* (IFN- γ) as an important regulatory node (Figure 1C). The transcripts belonging to this node were mainly repressed in SLE PBMCs exposed to pioglitazone and included several T-cell related genes (*LCK*, *CD40LG*, *CD27*, *ITK*, *TXK*, among others).

In addition, the 1362 genes modified by pioglitazone in SLE PBMCs were enriched with 147 transcription factors. Among them, 62 had a binding site in the promoter of at least one of the 1362 regulated genes, most of them being transcriptionally repressed by pioglitazone (Supplementary Table 2). Several of these transcription factors, including *TP53* or *FOXQ1*, have been reported to be implicated in T-cell regulation[33].

Overall, these results indicate that pioglitazone preferentially modulates gene expression of lupus PBMCs, when compared to controls, and that the modulated genes are primarily related to T-cell related pathways. Given these findings, confirmation of the microarray data was performed by real-time PCR in purified CD4⁺ and CD8⁺ T cells. Several of the genes found to be preferentially regulated by pioglitazone in lupus PBMCs, including IFN- γ , CD40L and CD27 were similarly found to be regulated in purified lupus CD4⁺ T cells, but not in control CD4⁺ or CD8⁺ T cells or in lupus CD8⁺ T cells (Figure 2 and not shown). Decreased synthesis of IFN- γ upon exposure to pioglitazone was also confirmed at the

protein level (Figure 2). Furthermore, there were no consistent differences in the expression of PPAR receptors and RXR between SLE and control T cells (not shown). These results suggest that the effects of pioglitazone on T cell gene expression occur primarily in CD4+ lupus T cells.

Pioglitazone modulates activation status of CD4+ lupus T cells

Overall, there were no significant correlations between lymphocyte counts, the use of specific immunosuppressive drugs by SLE patients or SLEDAI, and the responses elicited by pioglitazone in the various T cell assays presented below (data not shown).

SLE is characterized by abnormal T cell activation and potential targeted therapies currently being explored for this disease focus on inhibiting this phenomenon[34]. There were no significant differences in mean fluorescent intensity or in the percentage of CD4+ T cells expressing the activation markers CD25 and CD154, between control and SLE cells. Upon stimulation with anti-CD3/CD28 or with PHA, lupus CD4+ T cells show significantly enhanced upregulation of CD154 by MFI, when compared to controls (not shown). Exposure of CD4+ lupus T cells to pioglitazone led to significant downregulation of activation markers CD25 and CD154, following activation stimuli PHA or anti-CD3/CD28, when compared to untreated lupus CD4+ T cells. In contrast, these inhibitory trends following pioglitazone treatment were not significant in control CD4+ T cells, with the exception of CD154 expression in response to anti-CD3/CD28 stimulation (Figure 3). Similar results were observed when mean fluorescent intensity was quantified by FACS. Indeed, pioglitazone significantly downregulated CD25 expression on activated lupus CD4+ T cells, following stimulation with PHA ($p=0.016$) or anti-CD3 and anti-CD28 ($p=0.043$), and CD154 expression following stimulation with anti-CD3 and anti-CD28 ($p=0.042$), with a non-significant trend for PHA stimulation ($p=0.1$). In contrast, MFI of activation markers was not significantly altered in CD4+ control T cells following activation, when comparing pioglitazone treated and untreated cells.

Pioglitazone suppresses DNA synthesis and proliferation in CD4+ lupus T cells but does not alter autoantibody synthesis in vitro

Pioglitazone did not modify viability of control and lupus T cells, as assessed by Annexin V expression (not shown). In untreated CD4+ T cells, proliferation in response to stimulation was not significantly different between lupus and control cells, as assessed by PI staining. Proliferation was significantly decreased in stimulated CD4+ lupus T cells after exposure to pioglitazone for 3 days. A similar, but less significant effect was observed when stimulated control CD4+ T cells were exposed to the drug (Figure 3). No changes were observed in proliferation of CD8+ lupus or control T cells in response to pioglitazone. These results indicate that pioglitazone modulates proliferation of CD4+ T cells without affecting their viability, and that this effect is more pronounced in lupus when compared to control cells.

Previous reports indicate that PPAR agonists can modulate Th17 responses in non-lupus models [20]. While exposure to pioglitazone led to a trend in downregulation of Th17 mRNA in control T cells, no significant changes were observed in the level of mRNA of this cytokine in lupus T cells (Figure 4A).

When effector T cells were cocultured with B cells in the presence or absence of pioglitazone, no modulation of anti-dsDNA synthesis was detected by this compound in the lupus cocultures (Figure 4B).

Overall, these results indicate that pioglitazone selectively decreases the percentage and degree of activation of CD4⁺ lupus T cells that undergo activation in response to stimulation while it does not modify autoantibody synthesis in vitro.

Pioglitazone improves Treg function in SLE

Tregs have a fundamental role in the establishment and maintenance of peripheral tolerance, and there is extensive compelling evidence that deficits in the numbers and/or function of different types of Tregs can lead to various autoimmune processes. Indeed, Tregs are dysfunctional in human and murine SLE and this phenomenon may exacerbate autoimmune responses in this disease[2, 4, 35, 36]. The transcription factor Foxp3, a member of the forkhead-winged helix family, appears to be critical in the suppressive abilities of Tregs and its levels have been found to be decreased in lupus Tregs[4]. One of the cytokines synthesized by Treg subsets is IL-10, a pleiotropic antiinflammatory cytokine that regulates a variety of functions of hemopoietic cells[37].

Recent evidence has implicated PPAR- γ as an important pathway in the regulation of phenotype and function of Tregs[16, 17]. Confirming these observations, pioglitazone treatment led to significant increases in mRNA levels of *Foxp3* and *IL-10* in lupus, but not in control, Tregs. IL-10 synthesis was confirmed at the protein level, where significantly higher levels of this molecule were detected by ELISA in Treg supernatants after exposure to pioglitazone, when compared to untreated cells (Figure 5A). In contrast, synthesis of IL-10 was not significantly modified by pioglitazone in lupus T effector cells exposed to pioglitazone (not shown). Furthermore, pioglitazone improved proliferation of lupus Tregs in vitro (Figure 5B). These results indicate that pioglitazone specifically modulates the expression of molecules implicated in Treg function and enhances Treg proliferation.

As previously reported[4], lupus Tregs were dysfunctional in a Teff suppression assay, when compared to control Tregs, and failed to suppress their proliferation. This effect was abrogated when the lupus Tregs were pre-treated with pioglitazone (Figure 5C). Results did not differ depending on whether APCs were added or not to the coculture. These results indicate that pioglitazone improves the phenotype and function of lupus Tregs and enhances their immunosuppressive capabilities.

Discussion

While aberrant T cell phenotype and function have been clearly implicated as important drivers in autoimmune responses in SLE, the pharmacologic strategies to suppress these abnormal adaptive responses have been accompanied by significant complications secondary to immunosuppression. As such, it is important to identify novel targets that act as T cell immunomodulators without causing broad immunosuppression.

The thiazolidinedione (TZDs) class of drugs that include pioglitazone and rosiglitazone are synthetic ligands that demonstrate high affinity binding to PPAR- γ , and these compounds function as full agonists of the receptor[9]. In addition to well-described roles for these drugs in repressing synthesis of various proinflammatory molecules in APCs, recent studies have indicated that TZDs can suppress the differentiation of T_H17 and T_H1 cells [19, 38] and increase numbers and function of Tregs, while inhibiting memory T cell responses in various chronic inflammatory states[16-18]. Furthermore, murine lupus models have shown beneficial responses in inflammatory pathways and disease activity when exposed to chronic pioglitazone or rosiglitazone[14, 39]. However, it has been unclear if TZDs may have beneficial immunomodulatory roles in human SLE, and this hypothesis is attractive because these drugs do not appear to have prominent immunosuppressive effects. In this study, we identified pioglitazone as a selective modulator of lupus human T cell responses. These included pleiotropic effects on T cell-related genes and pathways, suppression of IFN- γ synthesis, decreases in T_H17 activation and proliferation and upregulation of Treg function.

Characterizing which changes in phenotype and function of lupus T cells may prove beneficial for disease pathogenesis is complex. As described by many groups, lupus T cells display various biochemical abnormalities that lead to hyper-excitable phenotypes but also to defective gene transcription programs[1]. These complex aberrant processes lead to a distinct phenotype in SLE, where T cells display properties of activated/effector cells but also evidence of an anergic state[1]. Several of the abnormalities previously described in lupus T cells were modulated by pioglitazone. For example, IFN- γ has been reported to play prominent pathogenic roles in SLE and associated nephritis[40], and transcription of this cytokine was significantly repressed by pioglitazone in lupus cells. Similarly, pioglitazone decreased markers of T cell activation and suppressed proliferation in SLE CD4⁺ T cells, a phenomenon that could suppress autoimmune networks in this disease[34]. Importantly, this drug had prominent effects in improving lupus Treg function, an effect that could lead to abrogation of deleterious immune responses.

The mechanisms by which pioglitazone preferentially modulates lupus T cells responses, compared to healthy control T cells, remain unclear. A previous report suggested that levels of PPAR- γ were increased in lupus PBMCs and played a role in suppressing costimulatory pathways[21]; therefore, we could speculate that enhanced levels of this receptor may promote increased responses to the agonist. However, we could not find consistent differences in the levels of expression of PPAR receptors or RXR between lupus and control CD4⁺ T cells. It is possible that *in vivo* levels of activation, Th skewing or other factors related to the multiple abnormalities in T cell phenotype and function described in SLE play an important role in the differential response, given that these cells have a hyperactivated state that may render them more susceptible to the effects of this drug. Hormonal influences have a significant impact on immune cell homeostasis and function and may play key roles in the development of autoimmune responses in SLE. PPAR molecules have been implicated as important players related to sex differences in the development of T cell-mediated autoimmune diseases[41]. Further, there are recently described complex interactions between PPARs, sexual hormone receptors and hormonal exposure with regards to immunomodulation[20]. As such, it is possible that exposure to a dysregulated hormonal

milieu characteristic of SLE could be implicated in enhanced response to pioglitazone observed in lupus T cells. This hypothesis requires additional experimental testing.

It will be important to assess in the future if the modulatory role of pioglitazone in lupus T cell function is through a TZD class effect or can be observed with other non-TZD PPAR- γ ligands. In addition, it will be important to assess if the pioglitazone immunomodulatory effects are partially independent of PPAR signaling, as recently demonstrated for Tregs [17]. Given evidence that Th17 and Th1 responses are potentially important in SLE, the role that PPAR agonists play in downmodulation of these responses *in vivo* in animal models of this disease should be investigated [19, 34]. Whether these compounds modulate other T cell subsets potentially important in lupus pathogenesis, such as T follicular cells, may be an interesting future line of research. Finally, other pathways were differentially regulated by pioglitazone in lupus PBMCs, including mitochondrial biogenesis-related pathways. Future studies should examine additional pathways by which these compounds may modify the phenotype and function of various lupus PBMC subsets; this was beyond the scope of this work which focused primarily on T cells.

Extensive literature supports a role for TZDs and related compounds in cardiovascular protection in human and murine systems in various diseases [12, 14], which appear independent of the role that these drugs play in mitigating insulin resistance. Given the significantly increased cardiovascular risk that has been well described in SLE [42, 43], identifying a therapy with potential dual roles in the control of aberrant adaptive immune responses and cardiovascular damage is very attractive in this disease. However, moving forward with the possibility of exploring the role of TZDs in lupus activity, it will be important to be cautious, given the potential risk that these agents may pose with regards to heart failure, bladder cancer and disruptions in bone biology, as described in other patient groups [44-46]. Indeed, newer compounds are being explored and may prove particularly effective and safer in the future. Specifically, the ongoing development of a newer class of “modulator” compounds, non-agonist PPAR γ ligands, may prove particularly useful, given their apparent lack of at least some of the side effects associated with TZDs [47]. In addition, exploring how “pan” PPAR ligands, so-called *glitazars*, which bind two or more PPAR isoforms, modulate T cell function in SLE may be the focus of future investigations [48]. Certainly, the cardiovascular risk profile and the differential effects of pioglitazone and rosiglitazone on lipids would favor exploring the former as a therapy in SLE in proof of concept studies [49, 50].

Many of the current and novel promising therapies in SLE and other autoimmune diseases are associated with significant immunosuppression [51-52]. As such, exploring alternative pathways that are immunomodulatory and anti-inflammatory without obvious induction of global immunosuppression, is an important priority in SLE. Given their reported beneficial effect in murine lupus and their lack of association with complication related to immunosuppression, these results further support the concept that PPAR- γ agonists should be explored for potential therapeutic effects in human SLE.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Pioglitazone selectively modulates CD4+ T cell function in SLE.
- Pioglitazone induces transcriptional regulation of T cell pathways in lupus PBMCs.
- Pioglitazone modulates phenotype and function of lupus regulatory T cells.
- PPAR agonists should be further explored as potential therapeutic targets in SLE.

Figure 1. Pioglitazone differentially modulates gene transcription in lupus PBMCs

A. Venn diagram illustrates the number of genes (in brackets) regulated by pioglitazone in control PBMCs (black left circle) and in lupus PBMCs (red right circle), as well as their directionality (bar graphs). In the overlap of both, the Venn diagram shows in the middle the number of genes modified by pioglitazone in control as well as in lupus PBMCs, and on each side the number of genes modified by pioglitazone only in control PBMCs (left) or only in lupus PBMCs (right). In the genes modified by pioglitazone in control and lupus PBMCs, 9 genes were not regulated in the same direction (down-regulated in control PBMCs, but up-regulated in lupus PBMCs or vice-versa). **B.** T-cell receptor signaling pathway in SLE PBMCs is modulated by pioglitazone, as assessed by Ingenuity Pathway Systems. Genes highlighted in green are significantly transcriptionally repressed in the pioglitazone-treated PBMCs, while genes highlighted in red are upregulated by the drug. **C.** Resulting transcriptional network analysis displays IFN- γ as an important regulatory node in pioglitazone-treated lupus PBMCs. T-cell related genes are in red characters. The figure displays the transcriptional network (Genomatix Pathway System) obtained from the genes that were co-cited in PubMed abstracts in the same sentence linked to a function word (128 of 372 genes regulated with a q-value<0.05 and fold-change 1.5 for the up-regulated genes and 0.7 for the down-regulated genes).

Figure 2. Transcriptional regulation of CD4+ T cells by pioglitazone

A-C. Some of the genes that were transcriptionally regulated by pioglitazone in lupus PBMCs were confirmed in CD4+ T cells by real-time PCR. Results are adjusted to housekeeping gene GAPDH and expressed relative to unstimulated healthy control CD4+ T cells. Results are mean±SEM of controls and SLE CD4+ T cells (n=5/group). **D.** Confirmation of downregulation of IFN-γ synthesis in SLE CD4+ T cells was performed at the protein level by ELISA. Results represent mean±SEM of controls and SLE CD4+ T cells (n=5/group).

Figure 3. Pioglitazone selectively modulates SLE CD4+ T cell activation and proliferation

A. Pioglitazone decreases upregulation of activation markers in CD4+ lupus T cells.

Results represent mean+SEM % expression of activation markers following stimulation with PHA or with anti-CD3/anti-CD28 Abs for 3 days, in the presence or absence of pioglitazone (controls n=4; SLE n=5 samples).

B. Pioglitazone decreases CD4+ T cell proliferation.

Bar graph represents mean+SEM %proliferating cells, as assessed by PI expression in the presence of pioglitazone, and results are relative to proliferation in the absence of pioglitazone (assessed as 100%); Y axis indicates % decrease in proliferation*=p<0.05 when comparing with untreated control and lupus T cells, and also when comparing downregulation of proliferation between pioglitazone-treated control and lupus T cells (n=5/group). Representative density plots from one control and one SLE patient represent % cycling cells. X axis represents PI and Y axis is side scatter.

Figure 4.

A. Pioglitazone does not repress IL-17 mRNA in lupus T cells Results are adjusted to housekeeping gene GAPDH and expressed relative to unstimulated healthy controls CD4+ T cells. Results are mean±SEM of controls and SLE CD4+ T cells (n=5/group). **B.**

Pioglitazone does not modulate autoantibody synthesis in T-B cell cocultures. Results are mean±SEM pg/mL levels of anti-dsDNA antibodies in controls and SLE CD4+ T cells (n=5/group);p=Non significant.

Figure 5. Pioglitazone selectively modulates phenotype and function of lupus Tregs

A. Pioglitazone modulates levels of Foxp3 and IL-10 in SLE Tregs. Results represent mRNA levels of *Foxp3* (top left) and *IL-10* (top right) in CD4+CD25+CD127^{low} control and lupus Tregs isolated by cell sorting, in the presence or absence of pioglitazone treatment for 24 h. Bottom left panel displays quantification of IL-10 protein by ELISA performed in supernatants of Tregs after 24 h treatment with pioglitazone (n=5/group). **B. Pioglitazone increases lupus Treg proliferation in vitro.** Results represent mean±SEM % proliferation of control and SLE Tregs, as assessed by CFSE dilution (n=5/group). **C. Pioglitazone increases suppressor function of Tregs.** CD4+CD25+CD127^{low} Tregs were stimulated with pioglitazone for 24h. CFSE-labeled CD4+CD25⁺ Tregs and pioglitazone-treated Tregs were cocultured with anti-human anti-CD3+anti-CD28 Abs at a ratio of 1:1 for 72 h. Teff proliferation was then quantified by FACS. The suppression of Teff proliferation by pioglitazone-treated Tregs was expressed relative to that of Teffs alone (quantified as 100%). Results represent mean±SEM % Teff proliferation; Control n=3, lupus n=5. Representative histograms of one SLE and one control samples demonstrating improvement in Treg suppressive function in SLE upon treatment with pioglitazone after 3 days in culture. Percentages demonstrate Teff proliferating cells.

Table 1

Top 20 canonical pathways from the 1362 genes regulated in Pioglitazone-treated SLE PBMCs compared to non-treated (the total number of genes in each pathway is indicated in brackets). T-cell related pathways are highlighted in bold.

<i>As assessed by Ingenuity Pathway Analysis (IPA)</i>		<i>p-value</i>	<i>Number of regulated genes</i>	
1	CCR5 Signaling in Macrophages (94)	0.000	16	
2	Atherosclerosis Signaling (107)	0.000	18	
3	Colorectal Cancer Metastasis Signaling (257)	0.000	34	
4	Altered T Cell and B Cell Signaling in Rheumatoid Arthritis (92)	0.000	16	
5	Inhibition of Angiogenesis by TSP1 (39)	0.001	9	
6	IL-12 Signaling and Production in Macrophages (132)	0.001	18	
7	Communication between Innate and Adaptive Immune Cells (109)	0.001	14	
8	Glucocorticoid Receptor Signaling (295)	0.002	33	
9	T Cell Receptor Signaling (109)	0.002	17	
10	Hepatic Fibrosis/Hepatic Stellate Cell Activation (147)	0.003	20	
11	Primary Immunodeficiency Signaling (63)	0.004	9	
12	Nicotinate and Nicotinamide Metabolism (135)	0.005	15	
13	Caveolar-mediated Endocytosis Signaling (85)	0.005	12	
14	IL-17A Signaling in Gastric Cells (25)	0.009	6	
15	Crosstalk between Dendritic Cells and Natural Killer Cells (97)	0.010	13	
16	Regulation of IL-2 Expression in Activated and Anergic T Lymphocytes (89)	0.010	13	
17	RAR Activation (183)	0.012	21	
18	TNFR2 Signaling (34)	0.016	6	
19	Airway Pathology in Chronic Obstructive Pulmonary Disease (9)	0.018	3	
20	NF- κ B Activation by Viruses (82)	0.018	11	
<i>As assessed by Genomatrix Pathway System (GePS)</i>		<i>p-value</i>	<i>Number of regulated genes</i>	<i>Number of expected genes</i>
1	Calcineurin-regulated NFAT-dependent transcription in lymphocytes (49)	0.000	16	4.37
2	The co-stimulatory signal during T-cell activation (20)	0.000	8	1.78
3	IL12 signaling mediated by STAT4 (36)	0.000	11	3.21
4	IL12-mediated signaling events (63)	0.000	15	5.62
5	Downstream signaling in naive CD8⁺ T cells (69)	0.001	15	6.16
6	Aurora A signaling (31)	0.001	9	2.77
7	IL12 and STAT4 dependent signaling pathway in Th1 development (15)	0.001	6	1.34
8	TCR signaling in naive CD8⁺ T cells (56)	0.003	12	5.00
9	Calcium signaling in the CD4⁺ TCR pathway (30)	0.004	8	2.68
10	LCK and FYN tyrosine kinases in initiation of TCR activation (13)	0.004	5	1.16
11	ATM signaling pathway (19)	0.005	6	1.70
12	Pertussis toxin-insensitive CCR5 signaling in macrophage (9)	0.005	4	0.80
13	Activation of CSK by cAMP-dependent protein kinase inhibits signaling through the T-cell receptor (41)	0.009	9	3.66

<i>As assessed by Ingenuity Pathway Analysis (IPA)</i>		<i>p-value</i>	<i>Number of regulated genes</i>	
14	T-cell receptor signaling pathway (56)	0.009	11	5.00
15	HIF-2-alpha transcription factor network (35)	0.010	8	3.12
16	Role of MEF2D in T-cell apoptosis (30)	0.014	7	2.68
17	HIF-1-alpha transcription factor network (68)	0.015	12	6.07
18	Endothelins (61)	0.017	11	5.44
19	TCR signaling in naive CD4⁺ T cells (69)	0.017	12	6.16
20	TSP-1 induced apoptosis in microvascular endothelial cell (7)	0.019	3	0.62

Table II

List of the 62 regulated transcription factors with a binding site in the promoter of at least one of the 1362 genes regulated by pioglitazone in SLE PBMCs.

Gene ID	Transcription factor	Description	# of genes in the 1362 gene list with a binding site for the TF	Fold-change	q-value
7157	TP53 (V\$P53F)	Tumor protein p53	276	0.78	0.0137
3725	JUN (V\$APIF)	Jun oncogene	255	1.35	0.0252
2353	FOS (V\$APIF)	FBJ murine osteosarcoma viral oncogene homolog	146	1.49	0.0172
5468	PPARG (V\$PERO)	Peroxisome proliferator-activated receptor gamma	139	0.72	0.0054
2113	ETS1 (V\$SETSF)	V-ets erythroblastosis virus E26 oncogene homolog 1 (avian)	86	0.68	0.0028
51176	LEF1 (V\$LEFF)	Lymphoid enhancer-binding factor 1	84	0.73	0.0030
4602	MYB (V\$MYBL)	V-myb myeloblastosis viral oncogene homolog (avian)	78	0.70	0.0052
196	AHR (V\$AHRR)	Aryl hydrocarbon receptor	68	0.70	0.0062
4088	SMAD3 (V\$SMAD)	SMAD family member 3	58	0.74	0.0059
4306	NR3C2 (V\$GREF)	Nuclear receptor subfamily 3, group C, member 2	43	0.79	0.0264
3280	HES1 (V\$HESF)	Hairy and enhancer of split 1, (Drosophila)	42	1.58	0.0000
1959	EGR2 (V\$EGRF)	Early growth response 2	40	0.67	0.0044
7494	XBPI (V\$CREB)	X-box binding protein 1	39	0.82	0.0219
25	ABL1 (V\$CABL)	C-abl oncogene 1, receptor tyrosine kinase	37	1.24	0.0408
10320	IKZF1 (V\$IKRS)	IKAROS family zinc finger 1 (Ikaros)	35	0.72	0.0046
7291	Twist1 (V\$HAND)	Twist homolog 1 (Drosophila)	35	1.38	0.0492
3394	IRF8 (V\$IRFF)	interferon regulatory factor 8	34	0.84	0.0492
7067	THRA (V\$RXRF)	Thyroid hormone receptor, alpha (erythroblastic leukemia viral (v-erb-a) oncogene homolog, avian)	33	0.72	0.0043
3087	HHEX (V\$HOMF)	Hematopoietical ly expressed homeobox	32	1.51	0.0083
10365	KLF2 (V\$KLFS)	Kruppel-like factor 2 (lung)	31	0.78	0.0296
4286	MITF (V\$MITF)	Microphthalmia-associated transcription factor	31	0.59	0.0000
2354	FOSB (V\$APIF)	FBJ murine osteosarcoma viral oncogene homolog B	31	1.33	0.0306
2627	GATA6 (V\$GATA)	GATA binding protein 6	31	0.77	0.0296
639	PRDM1 (V\$PRDF)	PR domain containing 1, with ZNF domain	29	0.70	0.0063
3660	IRF2 (V\$IRFF)	Interferon regulatory factor 2	27	0.76	0.0205

Gene ID	Transcription factor	Description	# of genes in the 1362 gene list with a binding site for the TF	Fold-change	q-value
5916	RARG (V\$RXXRF)	Retinoic acid receptor, gamma	26	0.73	0.0053
1870	E2F2 (V\$E2FF)	E2F transcription factor 2	26	0.73	0.0065
1879	EBF1 (V\$NOLF)	Early B-cell factor 1	25	1.89	0.000
4603	MYBL1 (V\$MYBL)	V-myb myeloblastosis viral oncogene homolog (avian) -like1	23	0.74	0.0055
2119	ETV5 (V\$ETSF)	Ets variant 5	21	1.25	0.0428
6932	TCF7 (V\$LEFF)	Transcription factor 7(T-cell specific,HMG-box)	20	0.65	0.0000
4610	MYCL1 (V\$EBOX)	V-myc myelocytomato sis viral oncogene homolog 1,lung carcinoma derived (avian)	19	0.72	0.0084
4929	NR4A2 (V\$NBRE)	Nuclear receptor subfamily 4,group A,membre r2	18	1.28	0.0352
3202	HOXA5 (V\$HOXC)	Homeobox A5	18	1.36	0.0492
4150	MAZ (V\$MAZF)	MYC-associated zinc finger protein (purine-binding transcription factor)	17	0.83	0.0352
94234	FOXQ1 (V\$FKHD)	Forkhead box Q1	12	2.19	0.0027
8521	GCM1 (V\$GCMF)	Glial cells missing homolog 1(Drosophila)	12	1.59	0.0221
23764	MAFF (V\$APIR)	V-maf musculo aponeurotic fibrosarcoma oncogene homolog F (avian)	11	0.82	0.0428
64919	BCL11B (V\$EV11)	B-cell CLL/lymphoma 11B (zinc finger protein)	11	0.70	0.0000
55502	HES6 (V\$HESF)	Hairy and enhancer of split 6 (Drosophila)	10	1.32	0.0264
6615	SNAI1 (V\$MYOD)	Snail homolog1 (Drosophila)	10	1.65	0.0060
5090	PBX3 (V\$HOXC)	Pre-B-cell leukemia homeobox 3	9	1.39	0.0252
22806	IKZF3 (V\$IKRS)	IKAROS family zinc finger 3 (Aiolos)	8	0.51	0.0029
1628	DBP (V\$SPARF)	D site of albumin promoter (albumin D-box) binding protein	8	0.72	0.0176
1749	DLX5 (V\$DLXF)	Distal-less homeobox 5	8	1.32	0.0428
57801	HES4 (V\$HESF)	Hairy and enhancer of split 4 (Drosophila)	6	1.41	0.0153
5324	PLAG1 (V\$PLAG)	Pleiomorphic adenoma gene 1	6	0.61	0.0000
9572	NR1D1 (V\$RORA)	Nuclear receptor subfamily 1,group D,member 1	6	0.60	0.0000
401	PHOX2A (V\$CART)	Paired-like homeobox 2a	5	1.25	0.0428
4520	MTF1 (V\$MTF1)	Metal-regulatory transcription factor 1	5	0.80	0.0358
56033	BARX1 (V\$HOMF) GRHL1	BARX homeobox 1	5	1.35	0.0306
29841	(V\$GRHL)	Grainy headlike 1 (Drosophila)	5	1.24	0.0428

Gene ID	Transcription factor	Description	# of genes in the 1362 gene list with a binding site for the TF	Fold-change	q-value
4862	NPAS2 (V\$SHIFF)	Neuronal PAS domain protein 2	4	0.80	0.0249
9603	NFE2L3 (V\$APIR)	Nuclear factor (erythroid-derived 2)-like 3	4	0.70	0.0133
84159	ARID5B (V\$ARID)	AT rich interactive domain 5B (MRF1-like)	3	0.76	0.0252
9095	TBX19 (V\$BRAC)	T-box 19	3	1.28	0.0358
2004	ELK3 (V\$ETSF)	ELK3 ETS-domain protein (SRF accessory protein 2)	3	1.43	0.0409
55509	BATF3 (V\$APIF)	Basic leucine zipper transcription factor, ATF-like 3	2	0.75	0.0227
1389	CREB12 (V\$CREB)	cAMP responsive element binding protein-like 2	2	0.81	0.0188
116113	FOXP4 (V\$FKHD)	Forkhead box P4	2	1.39	0.0113
7629	ZNF76 (V\$STAF)	Zinc finger protein 76 (expressed intestis)	2	0.84	0.0378
2306	FOXD2 (V\$FKHD)	Forkhead box D2	1	1.28	0.0492