Immunomodulation in systemic lupus erythematosus: induction of M2 population in monocyte-derived macrophages by pioglitazone

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Macrophages have recently gained attention in systemic lupus erythematosus (SLE) pathogenesis for their role in the anti-inflammatory clearance of apoptotic cells. The M1/M2 polarization of macrophages improves efferocytic capability. Peroxisome proliferator-activated receptor γ is proposed to function in the expansion of the M2 subpopulation. Pioglitazone is a peroxisome proliferator-activated receptor γ agonist with a variety of anti-inflammatory effects. In this paper, we investigated the ex vivo alterations of monocyte-derived macrophages of 15 newly diagnosed SLE patients and 10 normal subjects triggered by apoptotic cells among SLE patients following pioglitazone treatment. The phagocytosis capacity of macrophages and M1/M2 polarization (CD86/CD163) was evaluated. The supernatants were also analyzed for the expression of interleukin (IL)-10, IL-12, transforming growth factor β and TNF-α. The mRNA expression of IL-1β and mannose receptor C-type 1 were also quantified among treated and non-treated monocyte-derived macrophages. We found that efferocytosis is defective among monocyte-derived macrophages of SLE patients and might be a major underlying mechanism involved in the sustained inflammation. Pioglitazone could enhance alternative activation of monocyte-derived macrophages and consequently immunomodulation in these patients. Lupus (2017) 26, 1318–1327.

Key words: Efferocytosis; immunomodulation; macrophage polarization; peroxisome proliferator-activated receptor γ; pioglitazone; systemic lupus erythematosus

Introduction

Systemic lupus erythematosus (SLE) is a heterogeneous and chronic inflammatory autoimmune disorder with a wide range of clinical manifestations.¹,² Different molecular mechanisms have been suggested for the pathogenesis of SLE including genetic susceptibility, environmental triggers and infections which give rise to the immune complex formation. In the absence of suppressive networks, the abnormal immune response occurs which results in persistent inflammation and consequently nephritis, rash, arthritis and other organ damage.³ Regarding the fact that lupus autoantigens are expressed on the surface of apoptotic blebs, the accumulation of apoptotic cells may be relevant in initiating and propagating SLE.⁴,⁵ Accordingly, an efficient apoptotic cell removal system is crucial for immune regulation. Macrophages can be polarized from a classically activated M1 state to alternatively activated M2 subset, and vice versa under different conditions.⁶ Pathogen-clearing M1 produce pro-inflammatory cytokines in response to lipopolysaccharide and other inflammatory triggers.⁷ On the other hand, resolving M2 are the mononuclear phagocytes which could engulf apoptotic cells in the early stages of apoptosis in an anti-inflammatory manner called ‘efferocytosis’.⁸,⁹ This is associated with an increase in the production of anti-inflammatory and decreasing pro-inflammatory cytokines.¹⁰,¹¹ Therefore, M2 macrophages regulate immune responses by increasing anti-inflammatory cytokines including interleukin (IL)-10 and transforming growth factor (TGF)-β.¹²,¹³ Thus, failure in the alternative activation of macrophages and removal of apoptotic cells may be a crucial underlying mechanism in SLE pathogenesis.
Peroxisome proliferator-activated receptor γ (PPARγ) is a ligand-activated nuclear receptor which is abundantly expressed in macrophages especially upon differentiation of monocytes.12,13 The activation of PPARγ elicits the alternative activation of macrophages and modulates the inflammatory immune response.13 The antidiabetic pioglitazone is a specific ligand which activates PPARγ.14,15 PPARγ activation among macrophages of SLE patients may expand the M2 subpopulation and result in enhancing the removal of apoptotic cells and improving effecytosis.

In the present study, we evaluated the ex vivo M2/M1 polarization of monocyte-derived macrophages (MDMs) among SLE patients following pioglitazone treatment to assess its immunomodulatory effects on these cells.

Patients and methods

Patients and controls

Fifteen newly diagnosed SLE patients, fulfilling four out of 11 items of the revised American College of Rheumatology criteria for SLE,16 were recruited from Sayyad Shirazi educational Hospital, Rheumatology department, Gorgan, Iran. Clinical and laboratory characteristics of all patients are listed in Table 1. The disease activity was calculated by systemic lupus erythematosus disease activity index (SLEDAI). Ten age-matched healthy controls were recruited. All cases and controls were female subjects. Data collection sheets including laboratory tests and clinical data were filled by a specialist at each visit. A volume of 15 mL whole blood was collected from each participant in a sterile conical tube and transferred immediately to the cell culture laboratory. All patients with active infection or inflammation history within the past 6 months, pregnant patients, receiving anti-TNF drugs within the past 3 months or taking glucocorticoids in the past month were excluded from the study. All individuals with a history of any autoimmune disorder were also not included in the study. All subjects signed an informed consent, which was approved by the ethical committee of Golestan University of Medical Sciences.

Preparation of MDMs and pioglitazone treatments

Monocytes were isolated using the attachment method, as previously described.17,18 Briefly, each sample was diluted (1:4 ratio) in sterile phosphate buffered saline (PBS) (37°C). Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque (Baharafshan, Tehran, Iran) density-gradient centrifugation, as described.19 PBMCs were resuspended in high glucose RPMI 1640 (Gibco, Life Technologies, USA) supplemented with 0.5% human AB serum (Gibco, Life Technologies, USA) and incubated in tissue culture-treated T25 flasks for 3 hours at 37°C with 5% carbon dioxide. The floating cells were removed

Table 1 Clinical characteristics and laboratory parameters of newly diagnosed systemic lupus erythematosus patients

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<th>SLEDAI</th>
<th>Anti-dsDNA titre (μM)</th>
<th>WBC count (per μL)</th>
<th>ESR</th>
<th>RF-IgG (μM)</th>
<th>Lupus nephritis</th>
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<td>43.87 ± 5.59</td>
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*Data are presented as means ± SE (standard error) for continuous measures and number of positive patients (%) for nominal variables. P: patient; SLEDAI: systemic lupus erythematosus disease activity index; WBC: white blood cells; ESR: erythrocyte sedimentation rate; RF: rheumatoid factor.
and the attached cells (monocytes) were washed vigorously by PBS. The cells were detached using ethylene diamine tetraacetic acid (EDTA) 5 mM and 1 × 10^6 cells/mL were plated in a six-well culture plate. Monocytes were then cultured in RPMI 1640, supplemented with 10% fetal bovine serum (FBS; Gibco, Life Technologies, USA), stimulated with human macrophage colony-stimulating factor (M-CSF; Biolegend, San Diego, CA, USA) (100 U/ml) and human granulocyte-macrophage colony-stimulating factor (GM-CSF; Biolegend, San Diego, CA, USA) (10 ng/ml) and cultured for 6 days to differentiate into MDMs. At the beginning of the differentiation process (day 4), MDMs were treated with 100 nM pioglitazone, which was provided by Dr Reddy’s Laboratories Ltd. (Miyapur, Hyderabad, India).

Preparation of apoptotic Jurkat cells and phagocytosis assay

Human Jurkat cells (National Cell Bank of Iran, Pasteur Institute, Iran) were cultured in RPMI 1640 supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin (Gibco, Life Technologies, USA) and 2 mM Glutamax (Gibco, Life Technologies, USA) in a fully humidified atmosphere at 37°C with 5% carbon dioxide. Apoptotic Jurkat cells were generated by UVB irradiation at the beginning of day 6 with minor modifications, as described: 3–4 × 10^6 apoptotic cells were added to each well excluding the controls. The efficiency of apoptosis was evaluated using FITC-conjugated annexin V and PI double staining detection kit (eBioscience, San Diego, USA) (data not shown). Jurkat apoptotic cells were also labeled with calcein-AM (Sigma–Aldrich, St Louis, USA) to evaluate the phagocytosis capacity of MDMs by flow cytometry. MDMs were incubated with apoptotic cells for 16–18 hours.

ELISA cytokine assay

Cell culture supernatants of treated and non-treated macrophages were collected, centrifuged at 1000g for 5 minutes to remove any cellular remnants. The protein expression levels of anti-inflammatory (IL-10 and TGF-β1) and inflammatory cytokines (TNF-α, IL-12) were determined using commercially available ELISA kits (eBioscience, San Diego, USA) according to the manufacturer’s instructions. A Biotek ELISA reader ELX800 (Biotek, USA) was used to obtain the optical density of each sample at the wavelength of 450 nm. All samples were assayed in triplicate and the results were reported as picograms of cytokines per mL. IL-10/IL-12 and TGF-β1/TNF-α ratios demonstrate the percentage of M2 polarized macrophages in response to each treatment.

Flowcytometric analysis of intracellular and cell surface markers

MDMs were detached from culture plates using EDTA 5 mM. They were then washed and resuspended in PBS. Flow cytometric characterization of MDMs was performed by staining cells with intracellular FITC-conjugated anti-human CD68 antibody (333806; Biolegend, San Diego, CA, USA) cell marker, as described. The expression level of PE-conjugated anti-human CD86 (305406; Biolegend, San Diego, CA, USA) and PerCP/Cy5.5-conjugated anti-human CD163 (333608; Biolegend, San Diego, CA, USA) antibodies were analyzed by gating on the CD68^+ population of macrophages. The ratio of CD163^+ /CD86^+ cell surface markers demonstrates the percentage of M2 polarized macrophages in response to each treatment. Samples were run using a BD Accuri C6 flow cytometer (BD PharMingen, San Diego, CA, USA) and analyzed with BD Accuri C6 Flow analysis software.

RNA extraction and real time RT–PCR

Total RNA was also extracted from detached MDMs using Biozol (Bioron, Germany) according to the manufacturer’s protocols. One microgram of total RNA was reverse transcribed to cDNA with random hexamer primers. Polymerase chain reaction (PCR) amplifications were performed using Bioron master mix (Bioron, Germany). Real-time reverse transcriptase–polymer chain reaction (RT–PCR) was conducted using the Bioer real-time PCR detection system (Bioron Technology, Hangzhou High Tech, China); 18S ribosomal RNA (18S rRNA) was used as a suitable internal control for gene expression normalization using the following primers: forward: 5'-CAGCCACCCCGAGA TTGAGCA-3'; reverse: 5'-TAGTACGACCCGG CCGGTGTG-3'. Gene-specific primers for mannose receptor C-type 1 (MRC1; CD206) (18S) as a M2 marker were as follows: forward: 5'-ATGAGGTACCCCTGCTC-3'; reverse: 5'-TAGAACGGGAATGCA CAGGTT-3'. The mRNA expression of IL-1β was also quantified as a major cytokine among M1 macrophages with the following primers: forward: 5'-GGCTTATACAGTGCCAAATG-3'; reverse: 5'-TAGTGGTGGTCCGAGATT-3'.

Immunomodulation in SLE by PGZ treated macrophages

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All primers were designed and evaluated to span exon–exon junctions.

Statistical analysis

All of the experiments were repeated in triplicate and data were demonstrated as means ± SE (standard error). Statistical software SPSS22.0 and Graphpad Prism 5.0 were used for data analysis. Two-way analysis of variance (ANOVA) with Bonferroni posttest was used for comparing means of multiple samples. The independent samples $t$-test or Mann–Whitney U test were used to compare differences between two independent groups. $P$ values lower than 0.05 were considered statistically significant.

Results

The phagocytic capacity of macrophages among SLE patients compared to healthy subjects

MDMs of both groups engulfed labeled apoptotic cells efficiently (Figure 1). The engulfment capacity of differentiated macrophages was quantified and compared by detecting the green fluorescent emitted beam of engulfed apoptotic cells using

Figure 1 Phagocytosis assay: macrophages engulfed calcein-AM labeled apoptotic cell (ACs) (marked by arrows) successfully (a). (b) Blue-stained nucleus of the same section with DAPI. Merged (a) and (b) indicates cytoplasmic position (c). The calcein-AM labeled ACs imaged by fluorescent microscopy as a control (d) (magnification 100×). (e) Engulfment capacity of macrophages; the engulfment capacity of systemic lupus erythematosus (SLE) macrophages is not different from normal subjects. Data obtained from experiments in triplicates ($N = 3$). Data of each bar demonstrates means ± SE. ns: not significant.
flow cytometry. The apoptotic cell engulfment capacity among macrophages of SLE patients was not significantly different from that of healthy subjects (Figure 1(e)).

The expression of CD163 and CD86 upon pioglitazone treatment among macrophages

The expression of CD86 (M1 marker) and CD163 (M2 marker) among CD68⁺ macrophages was evaluated using flow cytometry (Figure 2(a)). The expression of CD86 is higher among macrophages of SLE patients treated with apoptotic cells and pioglitazone compared to that of healthy subjects (Figure 2(b)). CD163 is overexpressed among macrophages of healthy subjects in all groups (Figure 2(c)). Pioglitazone-treated macrophages of SLE patients express significantly higher levels of CD163 compared to the macrophages treated with pioglitazone.

**Figure 2** The expression of CD86 (M1) and CD163 (M2) markers upon pioglitazone treatment; flow cytometric histogram overlay for the intracellular expression of CD68 (patients in red and normal subjects in green lines as indicated) were overlayed and compared with respective isotype controls (blue lines) (a–f1). The expression of CD86 (V7-R) and CD163 (V8-R) among CD68⁺ macrophages (M1 gate in P1) were evaluated using flow cytometry (a). Flowcytometric histogram overlay for the surface expression of CD86 and CD163 (patients in red and normal subjects in green lines as indicated) were overlayed and compared with respective isotype controls (blue lines) (a–f2). CD163 is overexpressed (b) upon pioglitazone treatment while CD86 is down-regulated in macrophages of systemic lupus erythematosus (SLE) patients(c). AC: apoptotic cells; PGZ: pioglitazone; Mock: non-treated monocyte-derived macrophages. Data of each bar demonstrates means ± SE. P values lower than 0.05 were considered statistically significant. Ns: not significant; *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.
apoptotic cells ($P < 0.01$) (Figure 2(c)). There was also a significant decline in the expression of CD86 among pioglitazone-treated macrophages of SLE patients in comparison with the macrophages treated with apoptotic cells ($P < 0.01$) (Figure 2(b)).

**Pioglitazone treatment alters the expression of cytokines**

ELISA cytokine assay was performed on the cell culture supernatant of all treated and non-treated macrophages of SLE patients and healthy subjects to investigate the expression of anti-inflammatory and pro-inflammatory cytokines in each combination. The expression of IL-10 as an anti-inflammatory cytokine was higher among macrophages of healthy subjects prior to any treatment ($P < 0.001$). The expression of IL-10 was not significantly different upon apoptotic cell treatment between patients and healthy subjects. However, pioglitazone treatment increased the expression of IL-10 among macrophages of SLE patients significantly ($P < 0.01$) (Figure 3(a)). The secretion levels of IL-12 and TGF-β1 were not changed significantly in response to any treatment (Figure 3(b) and (c)). However, TNF-α as a major pro-inflammatory cytokine, which was overexpressed among macrophages of SLE patients in response to apoptotic cells, endured a significant decrease upon pioglitazone treatment (Figure 3(d)).

**The mRNA expression of MRC1 and IL-1β**

In order to investigate further the effect of pioglitazone treatment, the mRNA expression of MRC1 (as a main M2 marker) and IL-1β (as a major pro-inflammatory cytokine and M1 marker) was examined. The basal expression of MRC1 is lower among non-treated MDMs of SLE patients ($P < 0.01$). Although the challenge of apoptotic cells decreased the expression of MRC1 ($P < 0.001$), pioglitazone treatment enhanced the expression of MRC1 among macrophages of SLE patients ($P < 0.0001$) (Figure 4(a)). The expression level of IL-1β was higher among non-treated macrophages of SLE patients ($P < 0.001$). Pioglitazone treatment downregulated the expression of IL-1β drastically ($P < 0.0001$) (Figure 4(b)).

**Pioglitazone enhances IL-10/12, TGF-β1/TNF-α and CD163/CD86 ratios among macrophages of patients**

The overexpression of anti-inflammatory cytokines (IL-10 and TGF-β1) and downregulation of pro-inflammatory cytokines (IL-12 and TNF-α) alters...
the ratios of IL-10/IL-12, TGF-β1/TNF-α and CD163/CD86. Moreover, the changes in the expression of CD163 and CD86 cell surface markers changes the ratio and determines the percentage of M2/M1 polarized macrophages. Here, we showed that the ratios of IL-10/IL-12, TGF-β1/TNF-α and CD163/CD86 are significantly increased among macrophages of SLE patients in response to pioglitazone treatment (Figure 5).

**Discussion**

As a fundamental function of macrophages within the innate immune system, efferocytosis is the action of removing apoptotic cells in the early stages of apoptosis resulting in the resolution of inflammation. This function is probably defective among SLE patients. Therefore, the accumulation of self-antigens (especially on the surface of apoptotic cells blebs) is proposed as a possible mechanism in initiating and propagating this autoimmune disorder. Although the nomenclature of M2 macrophages (alternatively activated) was termed to mimic T helper (Th)2 cell responses, macrophage polarization and relevant biological functions such as efferocytosis became increasingly extended to a wider range. Macrophages are plastic cells with several subtypes including the resolving M2 which is responsible for efferocytosis. Although the nomenclature of M2 macrophages (alternatively activated) was termed to mimic T helper (Th)2 cell responses, macrophage polarization and relevant biological functions such as efferocytosis became increasingly extended to a wider range. Macrophages are plastic cells with several subtypes including the resolving M2 which is responsible for efferocytosis. Normal immunosuppressive therapies such as the administration of glucocorticoids has been shown to exert the M2 subtype of macrophages. However, long-term use of glucocorticoid-based therapies could be associated with harmful side-effects. Moreover, some recent data have shown that glucocorticoids may imbalance the Th17/Th1 response which is in favour of persistent inflammation and SLE propagation. Therefore, finding suitable pathways in order to design harmless therapies on the basis of macrophage polarization could be considered. It has been suggested that the activation of PPARγ by different agonists such as docosahexaenoic acid (DHA) may elicit the expansion of the M2 subset of macrophages and consequently modulating the inflammatory immune response. DHA may also exert its anti-inflammatory effects over macrophages through other mechanisms while pioglitazone is a specific PPARγ agonist with anti-inflammatory effects. Pioglitazone has also been shown to regulate innate immunity among antigen presenting cells (APCs) such as dendritic cells by PPARγ activation and inhibiting mitogen-activated protein kinase (MAPK) and nuclear factor (NF)-κB pathways. Recent studies have also suggested that PPARγ agonists such as pioglitazone and rosiglitazone could be beneficial as a treatment merely in the early stages of murine lupus. Pioglitazone could also ameliorate atherosclerosis, a major cause of death in SLE patients, even in established or severe disease. However, precise molecular mechanisms were not addressed. Pioglitazone has been shown to modulate selectively the phenotype and function of lupus regulatory T-cells by inducing transcriptional regulation of T-cell pathways in lupus PBMCs. Here, we studied the ex vivo anti-inflammatory effect of pioglitazone and its modulatory action on the expanding M2 subset of macrophages among SLE patients.

![Figure 4](image-url)

**Figure 4** The mRNA expression of mannose receptor C-type 1 (MRC1) and interleukin (IL)-1β; quantitative real-time reverse transcriptase–polymerase chain reaction (RT–PCR) on MRC1 (a) and IL-1β (b) was conducted to investigate further the changes in the expression of M1 and M2 markers among monocyte-derived macrophages (MDMs) of systemic lupus erythematosus (SLE) patients upon pioglitazone treatment. Data are expressed relatively to mRNA levels in the non-treated group (Mock) of healthy subjects, arbitrarily set at the value of 1. Data of each bar represents the mean ± SE. $P$ values lower than 0.05 are considered statistically significant. Ns: not significant; **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$. 

Lupus
In order to assess the possible differences in the engulfment capacity of macrophages, we evaluated the parameter among each testing group using phagocytosis assay. We found that the phagocytic capacity of macrophages among SLE patients was not different from that of healthy subjects (Figure 1), which was in accordance with previous reports.\textsuperscript{32} It has been shown that autoantibodies from SLE patients are able to opsonize apoptotic cells and inhibit their uptake by macrophages via an Fc\textsubscript{\gamma}R-dependent mechanism.\textsuperscript{33} PPAR\textsubscript{\gamma} agonists are capable of regulating B-cell differentiation, antibody production including IgG and also enhancing opsonization.\textsuperscript{34} Despite the rise in the apoptotic cells in the inflammatory milieu, we suggest that there might be an intrinsic defect among macrophages of SLE patients regarding the fact that they are capable of efficient phagocytosis. Thus, we examined the cell surface markers representing the polarization of macrophages to the major subsets.\textsuperscript{12,35} It was revealed that the expression of CD86 as a M1 marker is higher among macrophages of SLE patients while the M2 marker (CD163) is overexpressed among macrophages of healthy subjects (Figure 2). Interestingly, pioglitazone showed promising effects as expected.\textsuperscript{13,36} Pioglitazone-treated macrophages of SLE patients expressed higher levels of CD163 and lower levels of CD86 (Figure 2), which represents the effectiveness of this treatment on the enhancement of alternative activation. We also quantified the mRNA expression of MRC1 (CD206) and IL-1\textbeta among MDMs of SLE patients. MRC1 has recently been introduced as a novel marker to delineate the M2 subtype of macrophages.\textsuperscript{37} On the other hand, IL-1\textbeta is reported to be upregulated among M1 macrophages and is known as a M1 marker.\textsuperscript{38} The MRC1 gene was downregulated in comparison with normal subjects. Pioglitazone treatment exerted a significant upexpression of MRC1 among macrophages of SLE patients. Moreover, IL-1\textbeta was downregulated upon pioglitazone treatment, which denotes that pioglitazone may enhance alternative activation in an anti-inflammatory manner (Figure 4).

To address the anti-inflammatory effects of pioglitazone and also bring evidence in favour of alternative activation we tested the secretion level of different cytokines. IL-10 was secreted at lower levels among macrophages of SLE patients while TNF-\textalpha was more abundant (Figure 3). Pioglitazone treatment turned the secretion profile of tested cytokines anti-inflammatory by giving rise to IL-10 and reducing TNF-\textalpha. IL-12 and TGF-\textbeta\textsuperscript{1} were not different in any group of treated or non-treated macrophages. It has also been suggested that the IL-10/IL-12,\textsuperscript{23} TGF-\beta1/TNF-\textalpha and...
CD163/CD86$^{11}$ ratios (Figure 5) represent the quantity of alternative activation. The obtained results revealed that pioglitazone successfully enhances the alternative activation among macrophages of patients.

In response to pioglitazone treatment as a PPARγ agonist, with potential anti-inflammatory properties, we observed an obvious decrease in the expression of pro-inflammatory cytokines and also an increase in the secretion of anti-inflammatory cytokines in the supernatant of treated macrophages of SLE patients. Moreover, the expression of M2 markers was significantly higher among pioglitazone-treated macrophages while the same treatment decreased the expression of M1 markers in the macrophages of SLE patients. The activation of PPARγ by pioglitazone has anti-inflammatory effects on the macrophages of SLE patients and consequently the immunomodulation. Thus, after further research pioglitazone could be introduced as a choice of anti-inflammatory therapy for SLE patients.

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