Immunoregulatory effects of indole-3-carbinol on monocyte-derived macrophages in systemic lupus erythematosus: A crucial role for aryl hydrocarbon receptor

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To cite this article: Saeed Mohammadi, Ali Memarian, Sima Sedighi, Nasser Behnampour & Yaghoub Yazdani (2018): Immunoregulatory effects of indole-3-carbinol on monocyte-derived macrophages in systemic lupus erythematosus: A crucial role for aryl hydrocarbon receptor, Autoimmunity, DOI: 10.1080/08916934.2018.1494161

To link to this article: https://doi.org/10.1080/08916934.2018.1494161

Published online: 05 Oct 2018.

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ABSTRACT

Macrophages are versatile phagocytic cells in immune system with immunoregulatory functions. However, the removal of apoptotic cells by macrophages is disturbed in systemic lupus erythematosus (SLE). Aryl hydrocarbon receptor (AhR) is a ligand-activated cytoplasmic receptor and transcription factor with diverse effects on immune response. Indole-3-carbinol (I3C) is an AhR agonist which has been implicated as a beneficial factor in regulating inflammation and cytokine expression in murine models of SLE. However, the molecular mechanisms are not thoroughly studied. Here, we aimed to investigate the ex vivo effects of I3C on polarization of monocyte-derived macrophages (MDMs) in SLE patients and the expression of regulatory cytokines upon AhR activation. MDMs from 15 newly diagnosed SLE patients and 10 normal subjects were induced by Jurkat apoptotic bodies (JABs) and treated with I3C. I3C enhanced the nuclear accumulation of AhR among MDMs of SLE patients and altered the expression of AhR target genes including CYP1A1, IL1-β, IDO-1 and MRC-1. The imbalanced expression of pro- and anti-inflammatory cytokines (IL-10, IL-12, TGFβ1, TNFα, IL-23, IL-6 and IFN-γ) was compensated in response to I3C. AhR activation was also associated with the overexpression of M2 markers (CD163) and downregulation of M1 markers (CD86). Thus, macrophages are activated alternatively in response to I3C. The obtained data indicate that I3C-mediated AhR activation possess immunoregulatory effects on macrophages of SLE patients by exerting an obvious downregulation in the expression of pro-inflammatory and overexpression of anti-inflammatory cytokines. Therefore, AhR could be targeted and further investigated as a choice of anti-inflammatory therapies for autoimmune disorders such as SLE.

Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disorder in which the immune tolerance is broken [1]. In the absence of an efficient immunoregulatory response, the immune system targets self-antigens which results in the production of autoantibodies against multiple organs, an insuppressible chronic inflammation and irreversible damages [2,3]. The underlying molecular mechanism for SLE aetiology is not completely understood. However, several factors including genetic susceptibility [4,5], environmental triggers [6] and specific viral infections [7] have been proposed.

Macrophages are specialized cells of innate immune system involved in regulation of inflammation and immune response modulation [8]. Moreover, macrophages are committed phagocytic cells in fighting against pathogens and clearance of senescent, apoptotic or damaged cells [9]. The clearance of apoptotic cells is normally a non-inflammatory process which is called “efferocytosis” where macrophages give rise to anti-inflammatory and decrease pro-inflammatory cytokines [10]. Macrophage abnormalities such as efferocytosis defects may contribute to the accumulation of damaged cells and consequently the persistent inflammation in SLE. Macrophages are also a source of immunomodulatory cytokines [11]. Deregulated production of cytokines by defective macrophages may be involved in the onset and propagation of SLE and aberrant activation of the adaptive immune system [12]. Macrophages are versatile cells which possess plasticity and could be divided into different subsets from a classically activated M1 subset to alternatively activated M2 state with specialized functions [13]. Under inflammatory conditions such as active phase of infection and in response to LPS, M1 subset is favoured which give rise to pro-inflammatory cytokines [14]. However, M2 macrophages are the subpopulation with interest in the clearance of apoptotic cells in an anti-inflammatory manner...
We previously reported that MDMs of SLE patients are aberrantly polarized in response to apoptotic cells which is accompanied by overproduction of pro-inflammatory cytokines [16]. It has been also expressed that in vitro phagocytosis of apoptotic cells is defected in MDMs of SLE patients that may deteriorate the burden of inflammation [16,17]. Moreover, sera from SLE patients could enhance the capacity of apoptosis induction [18]. However, the phagocytic defect in macrophages of SLE patients could be compensated by sera from normal subjects [19]. Therefore, defects in efferocytosis and alternative activation among macrophages could be attributed as a major underlying mechanism in the pathogenesis of SLE.

Several pathways and molecules have been proposed to be involved in controlling macrophage polarization including peroxisome proliferator-activated receptor gamma (PPAR-γ) activation [15,16] and histone deacetylase inhibition (HDACi) [20]. The Aryl hydrocarbon Receptor (AhR) is a ligand-activated cytoplasmic receptor and transcription factor which is best known for its role in detoxification of environmental pollutants [21]. Furthermore, several studies have demonstrated the role of AhR in immune regulation through different mechanisms [22–24] as AhR plays a fundamental role in differentiation of regulatory and IL-17-producing helper T cells [25]. Macrophages and lymphocytes in AhR-null mice are more susceptible to produce higher levels of IFN-γ, TNF-α and IL-12 pro-inflammatory cytokines [26,27]. It has been proposed that production of type I IFN and expression of IFN-stimulated genes in AhR knockout mice is higher than in wild-type mice and AhR negatively regulates type I IFN signalling and production and the development of murine lupus [28]. In a recent study, it has been reported that disruption of AhR gene alters the macrophage polarization [29] which may affect the development of several infectious and autoimmune disorders including SLE. Moreover, Shinde et al. indicated that the phagocytosis of apoptotic cells can directly induce AhR activity in macrophages which highlights AhR as a key molecular regulator in anti-inflammatory disposal of apoptotic cells. They also detected an enhanced AhR-related transcriptional signature in patients with SLE, which indicated that this anti-inflammatory pathway might be upregulated during autoimmunity to restrict pathology [30]. Therefore, AhR activation by means of a specific ligand with anti-inflammatory properties may influence the alternative activation of macrophages among SLE patients and enhance efferocytosis. Several chemical compounds including exogenous environmental pollutants, nutritional metabolites and endogenous molecules could be recognized by the ligand binding domain of AhR with various affinities and may exert diverse effects on immune response on the basis of their interaction with the AhR molecule. Indole-3-carbinol (I3C) is a specific AhR agonist with potential anti-inflammatory properties which is normally found in cruciferous vegetables [31]. Moreover, I3C could facilitate shifting oestrogen metabolism towards less estrogenic metabolites [32]. According to the significance of steroid hormones in SLE pathogenesis, the SLE patients could benefit from anti-estrogenic effects of I3C. Moreover, I3C has been implicated as a beneficial factor in regulating inflammation and cytokine expression in murine models of SLE by inducing tandem B- and T-cell differentiation blockades [33–35]. However, the underlying mechanisms of I3C as an AhR agonist should be further investigated to target the apoptotic cell-induced AhR activity and modulate immune response in macrophages of SLE patients.

In the present study, we examined the ex vivo effects of I3C on polarization of monocyte-derived macrophages (MDMs) among SLE patients in comparison to normal subjects and investigated the expression of anti-inflammatory cytokines upon AhR activation and its immunoregulatory properties.

**Materials and methods**

**Patients and controls**

Fifteen newly diagnosed SLE patients, fulfilling the revised American College of Rheumatology (ACR) classification criteria for SLE [36] and 10 age matched normal subjects were enrolled in this study. All of the subjects were recruited from outpatient clinics and inpatient wards of Rheumatology department, Sayyad Shirazi educational hospital, Gorgan, Iran. All of the cases and controls were female subjects. Individuals with the history of other autoimmune diseases, active or persistent inflammation within last six months, pregnancy and malignancies or receiving anti-TNF therapies within last 3 months and taking glucocorticoids in the previous month were not included. A trained specialist filled data collection sheets including clinical data and laboratory tests at each visit (Data not shown). A total volume of 15 mL whole blood was taken from all participants in a sterile conical tube and transferred immediately to the cell culture laboratory. A written informed consent following the declaration of Helsinki [37] was taken from all participants.

**Culture and differentiation of monocyte-derived macrophages and treatments**

A modified attachment method was used to isolate monocytes, as previously described [38]. Briefly, peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque (Baharafshan, Tehran, Iran) density-gradient centrifugation. 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% CO2. The floating cells were removed and the attached cells (monocytes) were washed repeatedly by PBS. EDTA 5 mM was used to detach monocytes and 1 × 10⁶ cells/mL was plated in a 6 well culture plate. Monocytes were then cultured in a differentiation media containing RPMI 1640, 10% fetal bovine serum (FBS) (Gibco, Life Technologies, USA), human macrophage colony-stimulating factor (hM-CSF) (Biolegend, San Diego,
CA, USA) (100 U/mL) and human Granulocyte-macrophage colony-stimulating factor (hGM-CSF) (Biolegend, San Diego, CA, USA) (10 ng/mL) and cultured for 6 days to differentiate into MDMs. MDMs were treated with 100 nM 13C (Abcam, Cambridge, UK) at the beginning of the differentiation process (Day 4) which can efficiently activate AhR with minimal cytotoxic effects (Data not shown). UVB-irradiated human Jurkat cells (National Cell Bank of Iran, Pasteur Institute, Iran) were used to prepare apoptotic cells, as previously described [19]. 3-4 x 10⁸ apoptotic cells were added to each well excluding the non-treated mock wells. MDMs were then treated with apoptotic cells for 16-18 hours.

**Immunofluorescence staining of MDMs**

In order to evaluate the expression and cellular localization of AhR among MDMs of SLE patients in all combinations, the immunofluorescence staining method was applied. The endogenous expression of AhR was detected in macrophages using anti-AhR monoclonal antibody (MA1-513; Thermo Fisher Scientific, MA, USA) (1/100) and visualized using DyLight 488 conjugated goat anti-rabbit IgG (H+L) secondary antibody (35552; Thermo Fisher Scientific, Massachusetts, USA) (1/200). In order to address possible nonspecific antibody bindings, cells were incubated with secondary antibody alone as a negative control. Nucleus was visualized by 4', 6-diamidino-2-phenylindole (DAPI) (422801; Biolegend, San Diego, CA, USA) staining. Cells were washed and immediately visualized by fluorescence microscopy (Nikon, Japan). To identify the AhR expression within nuclei, Photoshop CS 5 extended (Adobe Systems, München, Germany) software was used. All nuclei containing the green fluorescent associated with AhR expression were highlighted and counted. Results are demonstrated as percentages of positive AhR expression in each nucleus in comparison to all nuclei.

**Flowcytometric analysis of intracellular and cell surface markers**

Flowcytometric immunophenotyping was conducted to address the phenotype alterations and polarization status of MDMs in each combination using selected M1 and M2 markers. Macrophages were detached using EDTA 5 mM followed by vigorous aspiration. Cells were then washed repeatedly and resuspended in 37°C PBS. Flowcytometric 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) cell surface markers were analysed by gating on CD68⁺ population of macrophages. In all experiments, CD163⁺/CD86⁺ ratio demonstrates the percentage of M2 polarized macrophages. BD Accuri flow cytometer (BD PharMingen, San Diego, CA, USA) was used to run the samples and data were analysed using BD Accuri C6 Flow analysis software. CD163/CD86 ratios were quantified for all experiments to address the efficiency of alternative activation among macrophages.

**ELISA cytokine assay**

Cell culture supernatants were collected from 13C- and JAB-treated or non-treated MDMs and centrifuged at 1000 g for 5 minutes to remove any cellular debris and stored at -80°C for the measurement of cytokines. The expression levels of pro-inflammatory (IL-12, TNF-α, IL-23, IL-6 and IFN-γ) and anti-inflammatory (IL-10 and TGF-β1) cytokines were assessed by commercially available ELISA kits (eBioscience, San Diego, CA, USA) according to the manufacturer's protocols. Biotek ELISA reader ELX800 (Biotek, USA) was used to obtain the optical density of each sample at the wavelength of 450 nm. All of the experiments for each sample were assayed in triplicates and the results were reported as picograms of cytokines per mL. IL-10/IL-12 and TGF-β1/TNF-α ratios were calculated for each combination which represent the percentages of M2 polarized macrophages.

**RNA extraction and real-time RT-PCR**

Total RNA was extracted from detached MDMs using Biozol (Boer, China) according to the manufacturer's instructions. One microgram of total RNA was reverse transcribed to cDNA with random hexamer primers (Bioron, Germany) followed by DNaseI treatment (CinnaGen, Iran). PCR amplifications were also performed using Bioron SYBR green master mix (Bioron, Germany). Real-time RT-PCR was conducted using Bior Real-time PCR detection system (Boer Technology, Hangzhou high tech, China). 18s ribosomal RNA (18s rRNA) was used as a suitable internal control for gene expression normalization [39]. In order to further investigate the efficient AhR activation, the mRNA expression of IL1-β (as a major pro-inflammatory cytokine and M1 marker) [40], CYP1A1 (as a major AhR target gene) [41], IDO-1 (as a putative SLE biomarker and M1 marker) [42] and MRC-1 (as a main M2 marker) [16] were quantified. Gene specific primers are summarized in Table 1. All primers were designed and evaluated to span exon-exon junctions. All experiments were tested in triplicates and 2-ΔΔCt method was used to calculate the mRNA expression level for each combination.

<table>
<thead>
<tr>
<th>Table 1. Gene specific primers used for real time RT-PCR.</th>
<th>Primer (accession)</th>
<th>Sequence (5'&gt;3')</th>
<th>Tm</th>
<th>Amplicon size</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A1 (NM_000499)</td>
<td>F: TCTGTGAGGCTCACTCTGATT</td>
<td>61</td>
<td>200 bp</td>
<td></td>
</tr>
<tr>
<td>R: TCTTTGTTGTTCTGTGTTGG</td>
<td></td>
<td></td>
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<tr>
<td>IL1β (NM_000576)</td>
<td>F: GGGTTTTAATCATGGGAAATG</td>
<td>60</td>
<td>135 bp</td>
<td></td>
</tr>
<tr>
<td>R: TAGTTGGTGTGGAGATT</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IDO-1 (NM_002164)</td>
<td>F: TACCATGTCAAAATCTGGTAAGAATG</td>
<td>60</td>
<td>84 bp</td>
<td></td>
</tr>
<tr>
<td>R: AAGGGCTTTCAGAGCTTATCTC</td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>MRC-1 (NM_0002438)</td>
<td>F: ATGAGCCTACCCTGCTGCTC</td>
<td>61</td>
<td>217 bp</td>
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<tr>
<td>R: TGAACGGGATGCCAACAGGTT</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>18s rRNA (M10098)</td>
<td>F: CAGCACCACCGGAGTGGAACCA</td>
<td>61</td>
<td>252 bp</td>
<td></td>
</tr>
<tr>
<td>R: TAGTACGAGGCGGGCGGTG</td>
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</table>
Statistical analysis

All of the experiments were repeated in triplicates. Data were demonstrated as means ± SE (standard error) for normally distributed values and medians ± IQR (interquartile range) for skewed variables. Statistical software SPSS 22.0 and Graphpad Prism 5.04 were used for data analysis and preparation of graphs. One-Way ANOVA with Tukey’s post hoc test or non-parametric Kruskal–Wallis with Dunn–Bonferroni post hoc test was used to compare the means of multiple samples. Independent Samples t-test or nonparametric Mann–Whitney U test was used to compare the means of two groups. p-values lower than .05 were considered as statistically significant.

Results

AhR translocation among MDMs of SLE patients

In order to determine whether I3C treatment is capable to activate AhR in macrophages, we evaluated the nuclear accumulation of AhR in comparison to the threshold intensity of immunofluorescence. Figure 1(A) represents the nuclear accumulation of AhR among macrophages of SLE patients and normal subject. The per cent of positive nuclei are shown in Figure 1(B). The percentages of positive nuclei have significantly increased upon I3C treatment in both groups. However, the nuclear accumulation of AhR among macrophages of SLE patients was drastically higher in comparison to normal subjects.

The expression of M1 and M2 cell surface markers

In order to investigate M2 polarization, the expression of CD86 (M1 cell surface marker) and CD163 (M2 cell surface marker) were evaluated among CD68⁺ MDMs using flow cytometry (Figure 2A). Macrophages of SLE patients expressed higher levels of CD86 in comparison to normal subjects (p < .0001). The expression of CD86 was significantly raised upon JAB treatment in both groups. I3C-treated macrophages of SLE patients expressed lower levels of CD86 in comparison to JAB-treated counterparts (p < .0001) (Figure 2B). CD163 expression was higher among macrophages of normal subjects in all combinations in comparison to SLE patients. The expression of CD163 was markedly increased upon I3C treatment among macrophages of SLE patients (p < .0001) and normal subjects (p = .021) in comparison to JAB-treated MDMs (Figure 2C).

I3C and alterations in the expression of cytokines

ELISA cytokine assay was conducted on the cell culture supernatant of macrophages among SLE patients and normal subjects to address the secretion level of pro- and anti-inflammatory cytokines upon each treatment. Although JAB induction downregulated IL-10 expression in both groups, I3C treatment markedly increased IL-10 level among macrophages of SLE patients (p = .005) and normal subjects (p = .037) (Figure 3A). IL-12 was merely downregulated in response to I3C among macrophages of SLE patients (p = .038) (Figure 3B). The secretion levels TGF-β1 were not significantly changed in any of the combinations (Figure 3C). TNF-α as a major pro-inflammatory cytokine showed a significant decrease upon I3C treatment among SLE patients.
Figure 2. The expression of CD86 (M1) and CD163 (M2) cell surface markers. Flowcytometric 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 and CD163 among CD68⁺ macrophages (M1 Gate in P1) were evaluated using flowcytometry (A). The intensities of total positive cells for SLE patients and a normal subject are represented in quadrants (Q-LL: CD86⁻, CD163⁻; Q-LR: CD86⁺, CD163⁻; Q-UL: CD86⁻, CD163⁺; Q-UR: CD86⁺, CD163⁺) (A-f2). CD86 is merely downregulated in macrophages of SLE patients (B) while CD163 is overexpressed among MDMs of both groups upon I3C treatment compared to JAB-treated cells (C). Flowcytometry data are presented as frequency distribution histograms showing signal intensities on the x-axis and counts on the y-axis. One-way ANOVA with Tukey’s post hoc test were used to compare the means of multiple samples. All of the experiments were repeated in triplicates for each sample (number of patients: 15, number of normal subjects: 10). Data of each bar demonstrate means ± SE. p < .05 were considered as statistically significant. *p < .05, **p < .01, ***p < .001, and ****p < .0001. NS, not statistically significant; Mock, non-treated monocyte-derived macrophages; JAB, Jurkat apoptotic bodies; I3C, indole-3-carbinol; SE, standard error.
Figure 3. ELISA cytokine assay. IL-10 is overexpressed in response to I3C in both groups (A). IL-12 is downregulated in response to I3C among macrophages of SLE patients in comparison to JAB-treated cells (B). TGF-β1 expression levels are not altered in response to I3C (C). I3C-treated MDMs express lower levels of TNF-α in SLE patients (D). IL-23 and IL-6 levels decreased upon I3C treatment among MDMs of SLE patients without any significant change in normal subjects (E and F). IFN-γ merely downregulated among MDMs of SLE patients upon I3C treatment (G). One-way ANOVA with Tukey's post hoc test or Kruskal–Wallis with Dunn–Bonferroni post hoc test were used to compare the means of multiple samples. All of the experiments were repeated in triplicates for each sample (number of patients: 15, number of normal subjects: 10). Data of each bar demonstrate means ± SE for normally distributed values (TNF-α, IL-23 and IL-6) and medians ± IQR for skewed (IL-10, IL-12, TGF-β1 and IFN-γ) variables. *p < .05, **p < .01, ***p < .001, ****p < .0001. NS, not statistically significant; Mock, non-treated monocyte-derived macrophages; JAB, Jurkat apoptotic bodies; I3C, indole-3-carbinol; SE, standard error; IQR, interquartile range.
IL-23, IL-6 and IFN-γ proinflammatory cytokines were altered among macrophages of SLE patients and not normal subjects. IL-23 was overexpressed upon JAB induction \( (p < .0001) \) and downregulated in response to I3C treatment \( (p < .0001) \) (Figure 3E). The expression level of IL-6 was also increased among JAB-treated macrophages \( (p < .0001) \), while I3C-treated MDMs expressed lower levels of IL-6 \( (p < .0001) \) (Figure 3F). Although the overexpression of IFN-γ among JAB-induced macrophages of SLE patients was not statistically significant \( (p = .217) \), I3C-treated MDMs showed lower levels of IFN-γ significantly \( (p = .012) \) (Figure 3G).

**The mRNA expression of CYP1A1, IL1-β, IDO-1, and MRC-1**

The mRNA expression of CYP1A1 as a major AhR target gene was quantified in macrophages of SLE patients to further investigate the efficient AhR activation. JAB-treated macrophages of normal subjects expressed higher levels of CYP1A1 \( (p = .003) \). CYP1A1 was significantly overexpressed upon I3C treatment in macrophages of SLE patients as an approval marker for proper AhR activation \( (p < .0001) \) (Figure 4A). Moreover, the expression level of IL1-β (as a major proinflammatory cytokine and M1 marker), IDO-1 (as a putative SLE biomarker and M1 marker) and MRC-1 (as a main M2 marker) were examined. IL1-β was higher among macrophages of SLE patients in comparison to normal subjects \( (p < .001) \). I3C-treated macrophages of SLE patients expressed lower levels of IL1-β in comparison to non-treated \( (p = .001) \) and JAB-induced cells \( (p = .001) \) (Figure 4B). IDO-1 as a putative M1 marker was overexpressed among MDMs of SLE patients \( (p = .027) \). I3C treatment decreased the mRNA level of IDO-1 in comparison to JAB-treated macrophages in SLE patients \( (p < .0001) \) (Figure 4C). The basal expression of MRC-1 among JAB-treated macrophages of SLE patients was significantly higher from MDMs of normal subjects \( (p = .038) \). I3C-treated MDMs of SLE patients expressed higher levels of MRC-1 (Figure 4D) which is a clue to alternative activation.

**I3C enhances alternative activation among macrophages of SLE patients**

In order to investigate the alternative activation in macrophages of SLE patients and normal subjects, IL-10/IL-12,
TGF-β1/TNF-α and CD163/CD86 ratios were quantified [43]. All of the possible alterations in the expression of cytokines and cell surface markers determine the inflammatory origin and polarization status of the cells in ratios. Here, we demonstrated that I3C treatment successfully increased IL-10/IL-12, TGF-β1/TNF-α and CD163/CD86 ratios in MDMs of SLE patients (Figure 5). We also showed that CD163/CD86 ratio was not significantly different between I3C-treated and non-treated MDMs in normal subjects (p = .505) and SLE patients (p = .097).

**Discussion**

The immune tolerance is breached and consequently the immune response is imbalanced in autoimmune disorders such as SLE [1,3,39]. Despite several proposed mechanisms in initiation and propagation of SLE, the molecular mechanism is not completely understood. However, the accumulation of apoptotic and necrotic cells is known to be associated with the persistent inflammation in SLE [17,19,44]. *Efferocytosis* is a triggered process in which apoptotic bodies and dying cells are removed in an anti-inflammatory manner [10]. Macrophages are the most specialized mediators of efferocytosis within innate immune system [17]. Ineffective efferocytosis may result in releasing the content of dying cells and subsequently initiation of an inflammatory immune response [10,19]. Abnormal clearance of apoptotic cells by monocyte-derived macrophages in SLE patients was demonstrated previously [17]. However, sera from healthy subjects are capable of removing the efferocytosis defects in macrophages of SLE patients [18].

The cytokine profile is also imbalanced in autoimmune disorders including SLE in which the pro-inflammatory cytokines are aberrantly overexpressed and immune response is deregulated consequently [45]. Macrophages are involved in the regulation of immune response through production of immunomodulatory cytokines [14]. Imbalanced cytokine production by macrophages is associated with the aberrant activation of adaptive immune response and chronic inflammation in SLE [12]. Moreover, macrophages are plastic cells which gain specialized functions through polarization to each subset from pathogen-clearing M1 subtype to resolving M2 subpopulation [13,43]. Although administration of glucocorticoids (GCs) as regular immunosuppressive therapies are semi-efficient compounds in M2 expansion [46] and expression of immunomodulatory molecules [47], long-term use of GC-based treatments are associated with irreversible side-effects [48]. Furthermore, some GC-based therapies are in favour of inducing Th17/Th1 imbalance which leads to insuppressible inflammation [49]. Thus, therapeutic methods should be designed on the basis of anti-inflammatory compounds which may target pathways involved in macrophage polarization.
Among various proposed pathways in controlling macrophage polarization [15,20], the AhR signalling pathway is underestimated. AhR is a ligand-activated cytoplasmic receptor and transcription factor which is mainly involved in processing environmental toxicants such as 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD) [22]. In addition to the well-known functions of AhR in regulating the expression of xenobiotic-metabolizing enzymes such as CYP1A1, CYP1A2 and CYP1B1, most recent studies have brought growing evidence to the role of AhR as a negative regulator of immune response [21,50]. According to the role of AhR in cell cycle regulation [24], it is also involved in differentiation of regulatory and IL-17-producing helper T cells [25]. It has been suggested that macrophages and lymphocytes of AhR-null mice express higher levels of IFN-γ, TNF-α and IL-12 pro-inflammatory cytokines in comparison to wild type (WT) mice [26]. The AhR-null mice are more susceptible to LPS-induced endotoxin shocks and exhibit exacerbated immune response against certain pathogens such as Leishmania major [23]. Recently, the polarization of murine macrophages was assessed by targeting AhR signalling pathway in which the disruption of AhR gene affected the balance between inflammatory M1 subtype and the anti-inflammatory M2 phenotype [29]. Interestingly, Lee et al. proposed that production of type I IFN and expression of IFN-stimulated genes in AhR knockout pristine-induced lupus mice is higher than in wild-type mice and AhR negatively regulates type I IFN signalling and production and the development of murine lupus [28]. In a most recent study by Shinde et al. the AhR activity induced by apoptotic cell phagocytosis was proposed to maintain peripheral tolerance and the disease progression in SLE mice correlated with strength of the AhR signal, and the disease course could be altered by modulation of AhR activity [30]. Thus, AhR activation by an exogenous ligand could be associated with improved efferocytosis effects in macrophages of SLE patients. Therefore, we investigated the ex vivo regulatory effects of I3C as a specific AhR agonist on immune response and cytokine balance through alternative activation of MDMs in SLE.

As previously stated [16], there is no significant difference between the uptakes of apoptotic cells in macrophages of SLE patients in comparison to normal subjects. Thus, the accumulation of dying cells in the inflammatory milieu might be due to an endogenous deficiency among macrophages of SLE patients. In order to address the efficient activation of AhR by means of I3C, the nuclear accumulation of AhR (Figure 1) and the mRNA expression of CYP1A1 (Figure 4A) were quantified. The increased percentages of positive nuclei and CYP1A1 mRNA (which is strongly induced by AhR and is known as a marker of the transcriptional activity of AhR) [51] overexpression in response to I3C denotes an efficient AhR activation among macrophages of SLE patients which is consistent with previous reports [30,52].

The immuno-phenotyping results of major M1 and M2 cell surface markers showed that CD86 is overexpressed among macrophages of SLE patients while CD163 levels were higher among normal subjects. CD86 was overexpressed upon JAB induction in both groups, while CD163 was decreased among macrophages of SLE patients. The overexpression of CD163 and downregulation of CD86 unveiled the anti-inflammatory effects of I3C on macrophages through enhancing M2 expansion (Figure 2).

In addition to the basal increased level of pro-inflammatory and decreased expression of anti-inflammatory cytokines, JAB induction deteriorated inflammatory status among macrophages of SLE patients. However, the production of cytokines remained partially constant among macrophages of normal subjects in response to JAB induction or I3C treatment which may reflect the presence of sufficient ligands and proper AhR activation in this group. To be more precise, JAB-treated macrophages expressed lower levels of IL-10 in both groups and higher levels of IL-6 and IL-23 among SLE patients. It has been suggested that activation of AhR by apoptotic cells in normal macrophages drives IL-10 production [30]. Although the anti-inflammatory IL-10 level was increased upon I3C treatment in either SLE patients or normal subjects, the protein expressions of pro-inflammatory cytokines (IL-12, TNF-α, IL-23, IFN-γ and IL-6) were markedly decreased (Figure 3). Previously, IL-1β mRNA level was reported to be altered upon AhR activation [53]. Real time RT-PCR findings showed that macrophages of SLE patients express higher levels of IL1-β in comparison to normal subjects. However, a significant downregulation of IL1-β was demonstrated among I3C-treated macrophages of SLE patients (Figure 4B) which is in favour of anti-inflammatory effects of AhR. We also measured the mRNA expression of MRC-1 (CD206; M2 marker) [54] and IDO-1 (M1 marker) [55] among MDMs of SLE patients. To bring more compelling data in favour of alternative activation, I3C-treated macrophages of SLE patients expressed higher levels of MRC-1 and lower levels of IDO-1 (Figure 5). Previous findings reported that AhR activation may lead to significant induction of IDO-1[56]. However, controversial studies showed that The IDO inhibitors may activate AhR in immune cells [57]. The systemic overexpression of IDO-1 in SLE [39] might be due to the raised population of classically activated macrophages during inflammation. Although AhR activation has been reported to be capable of controlling monocyte differentiation into dendritic cells versus macrophages [58], the unchanged ratio of CD163/CD86 and stable expression of CD68 among non-treated MDMs compared with I3C-treated macrophages shows that the addition of I3C after 4 days of ex vivo culture may not alter the fate of monocytes differentiation. Finally, the overexpressed IL-10/IL-12, TGF-β1/TNF-α and CD163/CD86 ratios revealed that I3C could enhance alternative activation successfully.

I3C has been introduced as a natural AhR ligand with potential therapeutic modalities against inflammatory disorders such as SLE. However, the mechanism by which it exerts such modulations is not thoroughly investigated [59]. Apoptotic cell-induced AhR activity is proposed to be necessary for immunological tolerance and suppression of autoimmunity in human macrophages, while aberrant AhR activation in lupus macrophages is responsible for ineffective efferocytosis and persistent inflammation [30]. Thus, drug targeting AhR by a specific ligand with anti-inflammatory properties could alleviate the effects of excessive accumulation of apoptotic cells. Moreover, I3C is a nutritional compound that can shift
oestrogen metabolism towards less estrogenic metabolites and women with SLE can manifest a metabolic response to I3C and benefit from its anti-estrogenic effects (60). According to the fact that activated AhR could inhibit oestrogen receptor (ER) through a number of different mechanisms and the role of ERs in SLE pathogenesis(61), the ER-AhR crosstalk in addition to the direct role of I3C-dependent AhR activation could be proposed as a potential mechanisms.

**Conclusions**

AhR activation by means of I3C (which was confirmed by the nuclear translocation of AhR and expression of AhR target gens) exerts an obvious downregulation in the protein expression of pro-inflammatory and overexpression of anti-inflammatory cytokines in SLE patients. Macrophages are activated alternatively in response to I3C, while an obvious increase in the expression of M2 markers and a significant decrease of M1 markers is reported. The obtained data indicate that I3C-mediated AhR activation possess immunoregulatory effects on macrophages of SLE patients. Therefore, AhR could be targeted and further investigated as a choice of anti-inflammatory therapies for autoimmune disorders which give raise to apoptotic cells such as SLE.

**Acknowledgments**

The authors thank Dr Marie Saghaeian-Jazi, Dr Ayyoob Khosravi, Dr Sareh Zhand, Mr Mohammad Shariati and Mrs Haydari for their scientific and technical support.

**Disclosure statement**

The authors declare no financial or non-financial conflicts of interest related to the subject matter or materials discussed in the article.

**Funding**

This work was supported by Gorgan School of Advanced Technologies in Medicine, Golestan University of Medical Sciences [grant number 931128255], [grant number 31078693122410].

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