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Functional *MIF* promoter haplotypes modulate Th17-related cytokine expression in peripheral blood mononuclear cells from control subjects and rheumatoid arthritis patients

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ABSTRACT

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease characterized by elevated levels of pro-inflammatory cytokines, such as interleukin (IL)-6, IL-17, and macrophage migration inhibitory factor (MIF). MIF induces IL-17 secretion and *MIF* promoter polymorphisms influence the expression of selected downstream mediators. The aim of this study was to investigate the relationship between known functional *MIF* haplotypes and Th17-related cytokine secretion profile in peripheral blood mononuclear cells (PBMC) from control subjects (CS) and RA patients stimulated with lipopolysaccharide (LPS) and recombinant human MIF (rhMIF). The −794 $CATT_{5\text{-}8}$ and −173G > C polymorphisms of the *MIF* gene were determined by conventional PCR and PCR-RFLP, respectively. The most frequent haplotypes of the *MIF* polymorphism and PBMC were identified from three subjects homozygous for each haplotype and in both study groups, the PBMC were obtained and stimulated with LPS or rhMIF. The secretion of cytokines related to the Th17 profile was determined by a multiplex immunoassay (MAGPIX). LPS stimulation induced the secretion of cytokines related to the Th17 profile in PBMC from CS and RA patients, whereas, rhMIF only stimulated this response in PBMC from RA patients. PBMC from CS carriers of the *MIF* 7C haplotype showed more IL-17A, IL-17F, IL-22, and IL-23 secretion than non-7C carriers after LPS stimulation. In the case of rhMIF stimulation, the PBMC from CS carriers of the 7C haplotype secreted more IL-17A and IL-23 than non-7C carriers. In conclusion, genetic variants of the *MIF* promoter modulate the secretion of cytokines related to the Th17 profile in PBMC from CS inducing a differential response in comparison to PBMC from RA patients.

1. Introduction

Rheumatoid arthritis (RA) is an autoimmune inflammatory disease characterized by diarthrodial joint damage [1]. The etiology of RA is not clear; however, the different genetic, hormonal, and environmental factors contribute to its development [2,3]. The synovial microenvironment in RA is regulated by a network of cytokines and chemokines produced by innate and adaptive immune cells, such as macrophages and T cells, respectively [1]. Particularly, T-helper 17 cells (Th-17) subset has been recognized as main orchestrator in the development and establishment of RA, since these cells produce interleukin (IL)-17, IL-21, and IL-22, which play a crucial role on synovial joint inflammation [4,5] as well as the presence of an increase in the percentage of Th17 cells in peripheral blood from RA patients [6].

Macrophage migration inhibitory factor (MIF) is a cytokine secreted mainly by macrophages and activated T cells, involved in the

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pathogenesis of RA, and it is a pleiotropic pro-inflammatory molecule highly expressed in the joints of RA patients [7,8]. It has been reported that MIF serum levels are increased in RA patients and associated with disease severity [9]. MIF owns several functions that make it an important cytokine involved in RA pathogenesis, such as the promotion of T lymphocyte proliferation and B lymphocyte activation, the enhancement of pro-inflammatory cytokine expression, as well as the induction of phospholipase A2 (PLA2) and cyclooxygenase-2 (COX-2) activation [10–17]. Moreover, some studies conducted in murine and human *in vitro* systems have reported that MIF can induce the expression of IL-17 [18,19].

The *MIF* gene is located on chromosome 22q11.23 and comprises three exons and two introns [20]. Two functional polymorphisms have been identified within the MIF promoter. The first is a short tandem repeat (STR) at position −794 with five to eight repetitions of a CATT tetranucleotide ($CATT_{5\text{-}8}$, *rs5844572*). The second is a single nucleotide polymorphism (SNP) at position -173, which consists of a transversion of G per C (−173 G > C, *rs755622*). The *MIF* $CATT_7$ allele and *MIF* -173C allele, as well as the *MIF* 7C haplotype, correlate with increased *MIF* mRNA expression and high levels of soluble MIF [17,21–23]. In addition, these polymorphisms have been associated with susceptibility to RA [9,17,24].

Few studies have examined *MIF* haplotypes associated with the production of pro-inflammatory cytokines. The aim of this study was to investigate the relationship between *MIF* haplotypes and Th17-related cytokine secretion profile in peripheral blood mononuclear cells (PBMC) stimulated with lipopolysaccharide (LPS) and recombinant human MIF (rhMIF) from control subjects (CS) and RA patients.

2. Materials and methods

2.1. Study site

This study was performed in the Instituto de Investigación en Ciencias Biomédicas, Centro Universitario de Ciencias de la Salud (CUCS) of the Universidad de Guadalajara in collaboration with the Departamento de Reumatología of the Hospital Civil de Guadalajara "Fray Antonio Alcalde" in Guadalajara, Jalisco, Mexico.

2.2. Subjects

The study was divided into two stages. The first stage included 281 CS and 210 RA patients from an unrelated Mexican-Mestizo population. The patients who fulfilled the classification criteria proposed by the 2010 American College of Rheumatology/European League Against Rheumatism (ACR/EULAR) [25] were included in the study. A group of healthy volunteers by self-report were recruited as the CS group. They were matched by the age and sex and none of them were taking any drugs at the time of inclusion in the study. A blood sample was collected from all participants in order to determine erythrocyte sedimentation rate (ESR) and perform genotyping of *MIF* promoter polymorphisms. In addition, the disease activity was assessed in the RA group by the Disease Activity Score 28 (DAS28) [26]. After the genotype identification in each group, the second stage consisted in selecting nine CS and nine RA patients, homozygous to the most frequent haplotypes of the *MIF* gene. The second blood sample was obtained to isolate PBMC in order to perform the cell cultures, stimulation, and cytokine quantification.

2.3. Ethical considerations

The present study was approved by the Ethics, Research and Biosafety Committee of the Universidad de Guadalajara (REI 103/UG-JAL/2008, CI-11908 and CI-3417/2017) and informed consent was obtained from all subjects prior to participation, according to ethical principles for medical research involving human subjects of the 2013 Declaration of Helsinki [27].

2.4. MIF polymorphism genotyping

Genomic DNA was extracted from peripheral blood leukocytes by the modified salting out method [28]. *MIF* -794 $CATT_{5\text{-}8}$ genotyping was performed by conventional polymerase chain reaction (PCR) and *MIF* −173 G > C by PCR-Restriction Fragment Length Polymorphism (RFLP) technique, using the primers reported by Radstake et al. and Makhija et al., respectively [17,29]. PCR conditions to identify both polymorphisms consisted of an initial denaturalization at 94 °C for 3 min followed by 30 cycles at 94 °C, 60 °C, and 72 °C, 30 s at each temperature, and a final extension at 72 °C for 1 min. The products obtained from the PCR to evaluate the −794 $CATT_{5\text{-}8}$ polymorphism were analyzed by polyacrylamide gel electrophoresis on a 19:1 (7%) polyacrylamide gel at 180 V during 4 h and stained with 0.02% $AgNO_3$. The genotyping of *MIF* −173 G > C polymorphism was performed as previously described in a study of the research group [30].

2.5. Isolation of PBMC and cell culture

A venous blood sample was used to isolate PBMC by density gradient using Lymphoprep™ NYCOMED; ρ 1.077 ± 0.001 g/mL. The cells were washed and resuspended in RPMI-1640 medium (GIBCO BRL, Rockville, MD, USA) supplemented with an antibiotic-antimycotic solution (50 U/mL penicillin and 50 μg/mL streptomycin, 1% (v/v), Sigma Aldrich, St. Louis, MO, USA). Later, the cell viability was measured using the Trypan blue exclusion test and the total number of cells per mL was quantified by direct counting using the Neubauer chamber. Subsequently, 1×10^5 cells/well were cultured in triplicate in 96-well polystyrene plates (Thermo Scientific Nunc Edge, USA) with 30 ng/mL *E. coli* LPS (CAT-L-2880, SIGMA®) or 200 ng/mL rhMIF (CAT-289 MF, R &D system™) and each well was left with a final volume of 300 μL of RPMI-1640 medium. Finally, the cells were incubated for 24 h at 37 °C in a humidified 5% CO_2 atmosphere. PBMC cultures without any agents at all were used as a negative control. The conditioned supernatants were collected and stored at −80 °C for further analysis.

2.6. Quantification of cytokines related to Th17 profile

The cytokines IL-6, IL-17A, IL-17F, IL-21, IL-22 and IL-23 were quantified from culture supernatants by means of a magnetic beads-based multiplex immunoassay method using the Bio-Plex Pro Human Th17 Cytokine Panel (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and the MAGPIX® System (Luminex, Austin, TX, USA). The standard curves of the cytokines were developed with the recombinant standards provided in the assay.

2.7. Statistical analysis

Continuous variables were expressed as the mean ± standard deviation (SD), nominal variables were expressed as frequencies and nonparametric variables as median and interquartile ranges 25th–75th. The heat maps were constructed for graphical representation and analysis of the data by means of R v3.4.1 software and to normalize the data, the Z score was used. The normal distribution of the data was evaluated by the Shapiro-Wilk test and the differences between the nonparametric quantitative determinations of two groups were determined by the Mann-Whitney *U* test. Data analysis was performed using SPSS v22 and GraphPad Prism v5.0 software and a p-value < 0.05 was considered statistically significant.

Table 1
Genotype and haplotype frequencies of the −794CATT$_{5-8}$ and −173G > C polymorphisms of the *MIF* gene in CS and RA patients.

	CS		RA	
−794CATT$_{5-8}$ genotype	n = 281	%	n = 210	%
*5,5	**8**	**2.8**	**11**	**5.2**
5,6	69	24.6	48	22.9
5,7	23	8.2	22	10.5
*6,6	**97**	**34.5**	**46**	**21.9**
6,7	66	23.5	70	33.3
*7,7	**18**	**6.4**	**13**	**6.2**
−173G > C genotype	**n = 123**	**%**	**n = 70**	**%**
GG	92	74.8	39	55.7
GC	17	13.8	23	32.9
CC	14	11.4	8	11.4
−794CATT$_{5-8}$/−173G > C haplotype	**n = 106**	**%**	**n = 47**	**%**
5G	6	5.7	5	10.6
5C	0	0	0	0
6G	84	79.2	33	70.2
6C	0	0	0	0
7G	2	1.9	1	2.1
7C	14	13.2	8	17.1

* Homozygotes subjects of the −794CATT$_{5-8}$ polymorphism are indicated in bold, followed by the frequencies of the −173G > C polymorphism that were determined. The homozygotes for both polymorphisms were used to calculate the haplotype frequencies. *Abbreviations:* CS, control subjects group; RA, rheumatoid arthritis group.

3. Results

3.1. Genotype and haplotype frequencies of MIF polymorphisms

A total of 281 CS and 210 RA patients were included in the study of *MIF* gene haplotypes (Table 1). First, the 123 CS and 70 RA patients homozygous for the −794 CATT$_{5-8}$ polymorphism were identified; these subjects were selected for further analysis of the −173 G > C polymorphism. Next, the homozygous frequencies of *MIF* gene haplotypes were obtained. In this study, a high frequency of the 6,6 genotype of −794 CATT$_{5-8}$ polymorphism in the CS group was observed but in RA patients, the 6,7 genotype, was the most frequent. On the other hand, in the homozygous subjects of the −794 CATT$_{5-8}$ polymorphism, it was detected that the GG genotype of the −173 G > C polymorphism was the most frequent in both CS and RA patients. In terms of haplotypes, the 6G haplotype was the most frequent in homozygosity, followed by the 7C haplotype in both groups, which is considered the high-expression haplotype [17,22,31,32]. In addition, the 5C and 6C haplotypes were not found in homozygosity in any groups of the studied population.

3.2. Characteristics of subjects included in cell culture experiments

Nine homozygous individuals for the three most frequent haplotypes identified as 5G, 6G and 7C, were selected to establish primary cell cultures in order to study the functional effect of the *MIF* gene haplotypes and their relationship with the secretion of cytokines related to the Th17 profile. Demographic and clinical characteristics of these subjects are represented in Table 2. All the subjects were female and the mean age in both groups was not statistically significant. Mean RA duration was 8.2 years, which is considered as long-standing RA. All the patients had severe disease activity, which is reflected by a mean DAS28 score of 5.6 [26]. As expected, RA patients exhibited an increased ESR.

3.3. Th17 profile-related cytokines secretion

Cytokine levels were compared in PBMC supernatants from CS and RA patients prior to and after stimulation with LPS and rhMIF. A statistically significant increase was observed in the secretion of IL-6 ($p < 0.001$), IL-17A ($p < 0.05$), IL-17F ($p < 0.001$), IL-21 ($p < 0.001$), IL-22 ($p < 0.01$), and IL-23($p < 0.001$) in PBMC from CS stimulated with LPS (Fig. 1A and B). In LPS stimulated PBMCs of RA patients, a significant increase in the expression of the Th17 cytokine profile (IL-6, $p < 0.01$; IL-17A, $p < 0.001$; IL-17F, $p < 0.001$; IL-21, $p < 0.05$; IL-22, $p < 0.01$), was observed, except for IL-23 (Fig. 1A and C).

LPS stimulation was used as a positive control and PBMC from CS secreted higher IL-6 ($p < 0.05$) and IL-23 ($p < 0.001$) levels than PBMC from RA patients after stimulation (Fig. 2).

A significant increase in the IL-17A ($p < 0.001$), IL-17F ($p < 0.01$), IL-21 ($p < 0.05$), IL-22 ($p < 0.05$) and IL-23 ($p < 0.05$) levels was found after stimulation with rhMIF in the RA group (Fig. 3A and C) while no significant differences were observed in the CS group (Fig. 3A y B).

Table 2
Demographic and clinical characteristics of CS and RA patients.

Variables	CS (n = 9)	RA (n = 9)	*p*
Age (years)*	41.7 ± 12.8	46.9 ± 10.2	0.352
ESR (mm/h)*	18.0 ± 10.9	38.3 ± 12.9	**0.002**
Disease duration (years)*	–	8.2 ± 6.5	–
DAS28+	–	5.6 (5.3–5.7)	–
Treatment°			
Chloroquine[a]	–	55.6 (5/9)	–
Methotrexate[b]	–	77.8 (7/9)	–
Sulfasalazine[c]	–	33.3 (3/9)	–

Student's *t*-test was used to evaluate the differences between groups. *Abbreviations:* CS, control subjects; DAS28, disease activity score 28; ESR, erythrocyte sedimentation rate; RA, rheumatoid arthritis.
* Data is shown as the mean ± standard deviation (SD).
+ Data is shown as median (interquartile ranges 25th–75th).
° Data shown as % (n).
[a] 150 mg per day.
[b] 10 mg per week.
[c] 500 mg per day.

3.4. Effect of MIF haplotypes on the secretion of cytokines related to Th17 profile in PBMC stimulated with LPS and rhMIF

The cytokine levels related to Th17 profile of PBMC cultures from CS and RA patients carriers of the high-expression 7C-haplotype *versus* non-carriers (non-7C) were compared after the stimulation with LPS and rhMIF for 24 h. Higher levels of IL-17A, IL-17F, IL-22, and IL-23 ($p < 0.01$ in all cases) were observed in PBMC from CS carriers of the 7C haplotype than non-carriers after LPS stimulation, nevertheless, this was not observed in the RA group (Fig. 4A–F).

After rhMIF stimulation, the PBMC from CS carriers of 7C haplotype exhibited higher levels of IL-17A ($p < 0.01$) and IL-23 ($p < 0.01$) than non-7C carriers. In addition, a non-significant increase in the levels of IL-6, IL-17F, IL-21, and IL-22 in the supernatants of the 7C-haplotype carriers, was observed in this group. Furthermore, in the RA group, no significant differences in the secretion of cytokines related to Th17 profile were found when comparing 7C-haplotype carriers *versus* non-7C carriers after stimulation (Fig. 5A–F).

4. Discussion

Several studies have reported that genetic factors play an important role in the development of RA, estimating that the RA heritability is approximately 60% [33,34] and about 45 non-*HLA* variants explain approximately 15% of RA heritability [35]. Nonetheless, a few genetic markers associated with RA have been studied in order to explain their

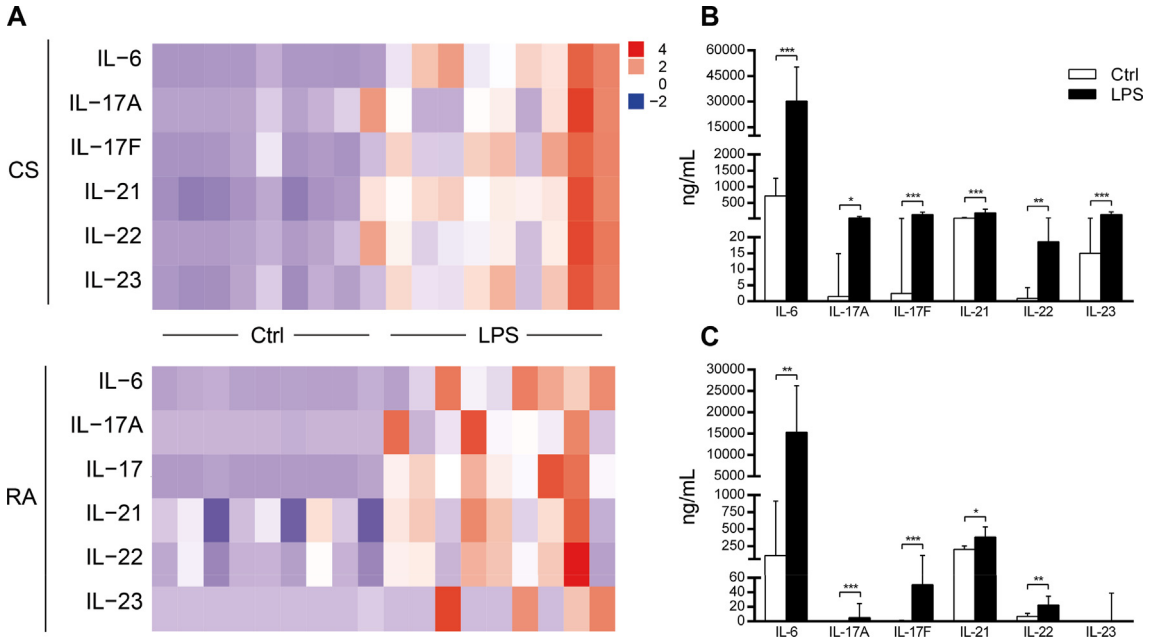

Fig. 1. Cytokine levels related to the Th17 profile in supernatants of PBMC from CS and RA patients before and after 24 h of stimulation with LPS (30 ng/mL). (A) The heat map of the cytokine secretion in non-stimulated (Ctrl) and LPS-stimulated PBMC in both study groups. The CS samples are shown in the upper part of the heat map and RA samples in the lower one. The left-side panels show the cytokine levels of non-stimulated PBMC and the right-side panels show the cytokine levels of LPS-stimulated PBMC from subjects of both groups. The heat map colors represent Z-score normalized values and a legend that represented these values is shown next to the heat maps. (B) The secretion of cytokines related to the Th17 profile was determined in supernatants of PBMC from CS without (Ctrl) or with 30 ng/mL of LPS. (C) The secretion of cytokines of the Th17 profile was determined in supernatants of PBMC from RA patients non-stimulated (Ctrl) or stimulated with 30 ng/mL of LPS. All the cultures were performed in triplicate (n = 9 per group). The results are presented as medians and interquartile ranges 25th–75th. The medians were compared using the Mann-Whitney *U* test. *p < 0.05, **p < 0.01, ***p < 0.001. Abbreviations: CS, control subjects; LPS, lipopolysaccharide; PBMC, peripheral blood mononuclear cells; RA, rheumatoid arthritis.

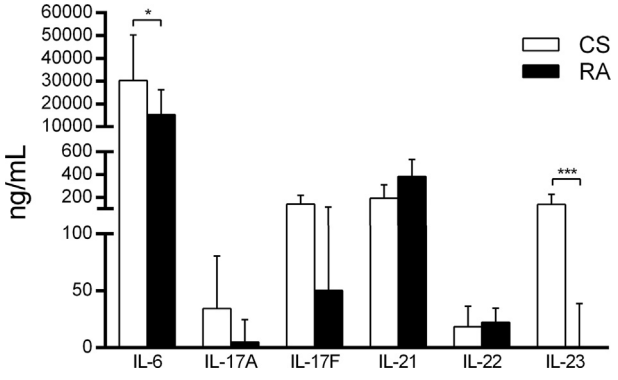

Fig. 2. Differential response in the secretion of cytokines related to the Th17 profile in PBMC from CS and RA patients stimulated with LPS (30 ng/mL) for 24 h. The results are presented as medians and interquartile ranges 25th–75th. The medians were compared using the Mann-Whitney *U* test. *p < 0.05, ***p < 0.001. *Abbreviations*: CS, control subjects; LPS, lipopolysaccharide; PBMC, peripheral blood mononuclear cells; RA, rheumatoid arthritis.

functional effect on the RA phenotype [36].

A non-*HLA locus* associated with RA susceptibility is the *MIF* gene, which encodes the upstream MIF cytokine [37]. In the previous study of this research group, an association of the heterozygous genotype 6,7 of −794 $CATT_{5-8}$ polymorphism and of the 7C haplotype of the *MIF* gene with RA susceptibility in a western Mexican population, was shown [30]. Genotype and allele frequencies reported in this study coincide with previous reports in a population of the United Kingdom and thus, strengthens the results found by Llamas-Covarrubias *et al* in 2013 [23,30]. Importantly, the -794 $CATT_8$ allele, which is considered an allele of very low frequency worldwide [23], was not identified in the studied population.

The most frequent homozygous haplotype in both groups was the 6G haplotype, however, the 5C and 6C haplotypes were not identified in homozygosity in the studied population. These results coincide with a previous report regarding a Mexican population, however, the low frequency of the C allele of −173 G > C polymorphism, as well as the strong linkage disequilibrium found in the population (D′ = 0.87), may explain the absence of 5C and 6C haplotypes in homozygosity [30].

The secretion of cytokines related to Th17 profile in LPS-stimulated PBMC from CS and RA patients was also explored. In both groups, IL-6, IL-17A, IL-17F, IL-21, and IL-22 levels were significantly higher after LPS stimulation, which is representative of a Th17-type immune response and IL-23 levels were only increased in PBMC from CS. Previous studies have reported that bacterial LPS might contribute to the pathogenesis of RA [38,39] and that LPS can induce IL-6, IL-17, and IL-21 secretion in CS and RA patients [40,41]. Furthermore, IL-6 enhances the Toll-like receptor (TLR) mediated cytokine production [42,43] and high levels of IL-6 may cause a Th17/Treg cell imbalance during RA, which is corrected with anti-IL-6 treatment [44]. However, this is the first study to show that LPS induces IL-22 secretion in PBMC, which is a cytokine with a recently recognized pathogenic role in RA [45]. Although these results and other reports show that LPS promotes the cytokine secretion of the Th17 profile, the mechanism is not totally understood.

In this study, the higher levels of IL-6 and IL-23 and similar levels of IL-17A, IL-17F, IL-21, and IL-22 were observed in LPS-stimulated PBMC from CS compared to RA patients. The data is in disagreement with Zivojinovic et al. [46], who found that IL-6 and IL-23 production in *ex vivo* LPS-stimulated whole blood from RA patients, was comparable with those of healthy subjects. Moreover, IL-17A and IL-21 levels were significantly higher in RA patients than in CS. Several studies have reported that treatment with chloroquine [47,48], methotrexate [49,50], and sulfasalazine [51] diminishes cytokine secretion in LPS-stimulated cells. In addition, methotrexate monotherapy and

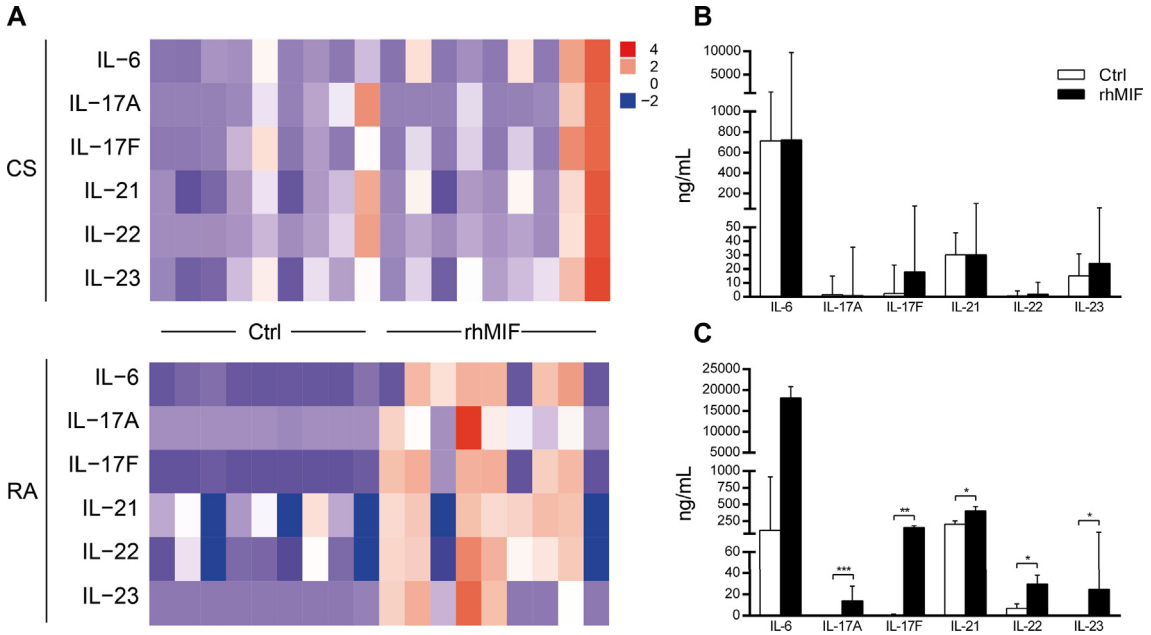

Fig. 3. Cytokine levels related to Th17 profile in supernatants of PBMC from CS and RA patients before and after 24 h of stimulation with rhMIF (200 ng/mL). (A) The heat map of the cytokine secretion in non-stimulated (Ctrl) and rhMIF-stimulated PBMC in both study groups. The CS samples are shown in the upper part of the heat map and RA samples in the lower one. The left-side panels show the cytokines levels of non-stimulated PBMC and the right-side panels show the cytokine levels of rhMIF-stimulated PBMC from subjects of both groups. The heat map colors represent Z-score normalized values and a legend that represented these values is shown next to the heat maps. (B) The secretion of cytokines related to Th17 profile was determined in supernatants of PBMC from CS without (Ctrl) or with 200 ng/mL of rhMIF. (C) Secretion of cytokines of the Th17 profile was determined in supernatants of PBMC from RA patients non-stimulated (Ctrl) or stimulated with 200 ng/mL of rhMIF. All the cultures were performed in triplicate (n = 9 per group). The results are presented as medians and interquartile ranges 25th–75th. The medians were compared using the Mann-Whitney *U* test. *p < 0.05, **p < 0.01, ***p < 0.001. *Abbreviations*: CS, control subjects; PBMC, peripheral blood mononuclear cells; RA, rheumatoid arthritis; rhMIF, recombinant-human Macrophage Migration Inhibitory Factor.

methotrexate/adalimumab treatment decrease IL-23 serum levels up to 13% in RA patients [52]. It should be noted that the RA study group was under treatment with chloroquine, methotrexate, and sulfasalazine, which could explain the differences observed between CS and RA patients.

It was demonstrated that MIF stimulates IL-17 production in murine lymph node cells [18] and recently, this research group has reported that MIF can induce secretion of IL-17A, IL17F, and IL-21 in rhMIF-stimulated PBMC from healthy subjects [19]. The IL-17A, IL-17F, IL-21, IL-22, and IL-23 levels were significantly increased in rhMIF-stimulated

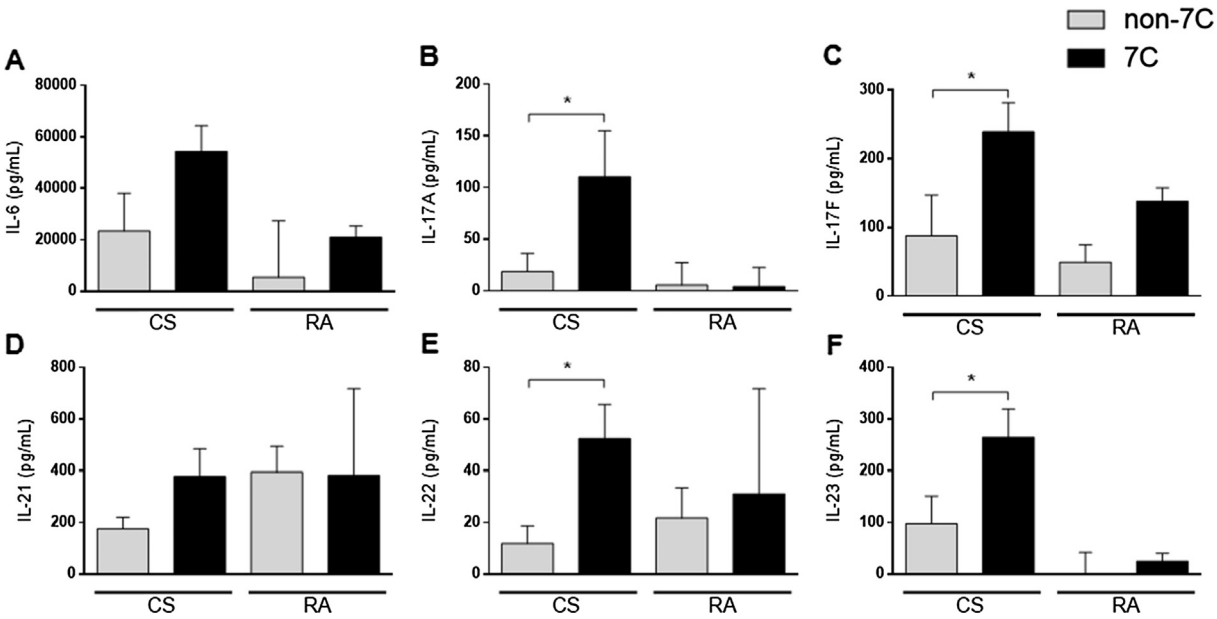

Fig. 4. Cytokine levels of the Th17 profile according to MIF haplotypes in PBMC after LPS-stimulation. The levels of IL-6, IL-17A, IL-17F, IL-21, IL-22, and IL-23 in supernatants of PBMC cultured with LPS (30 ng/mL, 24 h) were compared in CS and of RA patients groups according to the presence of the high-expression haplotype (7C, n = 3) or low-expression haplotypes (non-7C, n = 6) of the *MIF* gene. The cell cultures were performed in triplicate. The results are presented as medians and interquartile ranges 25th–75th. The medians were compared using the Mann-Whitney *U* test. *p < 0.05, *Abbreviations*: CS, control subjects; MIF, Macrophage Migration Inhibitory Factor; PBMC, peripheral blood mononuclear cells; RA, rheumatoid arthritis.

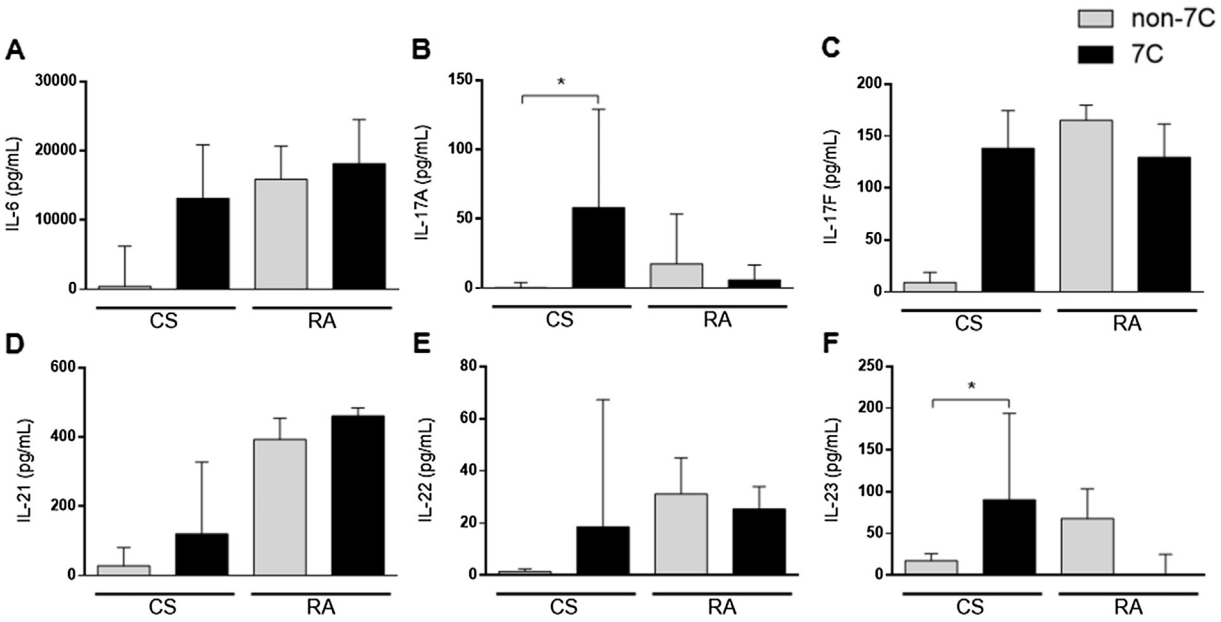

Fig. 5. Cytokine levels of the Th17 profile according to MIF haplotypes in PBMC after rhMIF-stimulation. The levels of IL-6, IL-17A, IL-17F, IL-21, IL-22, and IL-23 in supernatants of PBMC cultured with rhMIF (200 ng/mL, 24 h) were compared in CS and of RA patients groups according to the presence of the high-expression haplotype (7C, n = 3) or low-expression haplotypes (non-7C, n = 6) of the *MIF* gene. The cell cultures were performed in triplicate. The results are presented as medians and interquartile ranges 25th–75th. The medians were compared using the Mann-Whitney *U* test. *p < 0.05, *Abbreviations*: CS, control subjects; MIF, Macrophage Migration Inhibitory Factor; PBMC, peripheral blood mononuclear cells; RA, rheumatoid arthritis; rhMIF, recombinant-human Macrophage Migration Inhibitory Factor.

PBMC from RA patients but not from CS; this is an indication of the activation of the Th17- type immune response in RA patients after rhMIF stimulation. This Th17 profile polarization could occur by an indirect mechanism involving MIF dependent IL-6 and IL-21 induction, which can promote human Th17 differentiation. In addition, MIF promoted IL-23 production, which is also required for the full terminal differentiation of Th17 and ultimately, its activity in increasing IL-22 secretion but not IL-21 [53–55].

MIF can act through CD74 and CD44 [56,57], as well as CXC chemokine receptor (CXCR) 2, CXCR4, and CXCR7 [58–60]. The differential response in cytokine production after rhMIF stimulus observed in RA and CS, may be explained in terms of these receptors. It has been described that RA patients have high CXCR4 expression levels in synovial tissue CD4(+) memory T cells [61], and that CXCR7 inhibition reduced clinical arthritis scores and the numbers of vessels in the inflamed synovial tissue in mice with collagen-induced arthritis [62].

In this context, a limitation of the study design was that it did not allow to determine if the observed increased response of cytokines to stimulation with rhMIF reflects an upregulated secretion by a specific cell subset or by a differential expression of MIF receptors. However, it can be assumed that a substantial contribution of Th17 cells in the total measurable IL-17A, IL-17F, IL-21, and IL-22 levels in the experiment since previous evidence shows an increased proportion of IL-17-producing cells in RA [44,63].

The relationship between *MIF* haplotypes and the Th17-related cytokine levels in supernatants of PBMC cultures from CS and RA patients was analyzed. The supernatants of PBMC from CS carriers of the 7C haplotype had higher levels of IL-17A, IL-17F, IL-22, and IL-23 than CS carriers of the non-7C haplotype after LPS stimulation, and the 7C haplotype was associated with an increased secretion of IL-17A and IL-23 in the same group after rhMIF stimulation. Nevertheless, in the RA group, no association between *MIF* haplotypes and the Th17 profile related cytokines levels was observed, and as far as it is known these associations have not been explored before. Although *MIF* 7C haplotype is associated with juvenile idiopathic arthritis [21,22] and RA [24], minor evidence focuses on the functional effect of the *MIF* gene haplotypes. One study describes an association of both -794 $CATT_7$ allele and 7C haplotype with decreased total plasmatic IgE levels in a subset of Korean atopic dermatitis patients, where the authors suggest that *MIF* polymorphisms might be a marker for this disease [64]. A second study reports that in a synovial fibroblast line stratified by −794 *MIF* genotype, cells carrying the low-expression genotype exhibited a lower CD44 expression at the cell surface when compared with carrier cells of the high-expression genotype [57], which could explain the increased MIF response in those subjects.

It should be noted that the differential response observed in the results may be due to the fact that several transcription factors can regulate *MIF* expression by joining to the consensus sequences present in the gene promoter, which include the −794 $CATT_{5-8}$ and −173 G > C polymorphisms. In this regard, it has been reported that the transcription factor ICBP90, also known as UHRF1, is the major protein interacting with the *MIF* STR. This transcription factor is essential for *MIF* transcription in T and B lymphocytes, monocytes, macrophages and synovial fibroblasts. The affinity of ICBP90 to the *MIF* promoter increases in the presence of a higher number of CATT repetitions and this is associated with a higher *MIF* expression in TLR-induced *MIF* transcription [65]. Moreover, the transcription factor Pit-1 also interacts with the *MIF* promoter region at the microsatellite polymorphism and increases *MIF* transcription in human THP-1 monocytes [66]. Finally, it has been demonstrated that HBP1 can act as an inhibitor of *MIF* transcription by binding to a region that includes the STR *MIF* polymorphism [67]. Regarding the *MIF* −173*C allele, it has been reported that this polymorphism creates a potential binding site for the transcription factor AP-4 and this promotes *MIF* transcription [21]. For a complete understanding of this system, it is suggested to study if these transcription factors are differentially expressed in PBMC from CS and RA patients, as well as the possible interactions between ICBP90, Pit-1, HBP1, AP-4, and the *MIF* promoter haplotypes in different cell types.

Based on these results, MIF can play an important role in the modulation of the RA immune response by its ability to induce the secretion of Th17 cytokines. Furthermore, the genetic variants of the *MIF* promoter can influence the secretion of different cytokines related

with the Th17 profile in PBMC from CS, which is very important because they do not have the influence of any treatment, unlike RA patients, where we could not observe the same effect. However, further studies are needed to discern how *MIF* promoter haplotypes are implicated in the regulation of this process.

5. Disclosure statement

The authors report no conflicts of interest.

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