# Upmodulation of Unfolded Protein Response and ER Stress-Related IL-23 Production in M1 Macrophages From Ankylosing Spondylitis Patients

**Alireza Rezaiemanesh**
  Kermanshah University of Medical Sciences

**Mahdi Mahmoudi**
  Tehran University of Medical Sciences

**Ali Akbar Amirzargar**
  Tehran University of Medical Sciences

**Mahdi Vojdanian**
  Tehran University of Medical Sciences

**Farhad Babaie**
  Urmia University of Medical Sciences

**Jila Mahdavi**
  Payame Noor University

**Ahmad Reza Jamshidi**
  Tehran University of Medical Sciences

**Mohammad Hossein Nicknam** ( ✉ mhnicknam@sina.tums.ac.ir )
  Tehran University of Medical Sciences   https://orcid.org/0000-0001-9132-1675

---

---

# Abstract

## Background

The inflammatory interleukin (IL)-23/IL-17 axis plays an important role in the pathogenesis of ankylosing spondylitis (AS), but with an unknown regulatory mechanism. The aim of this study was to investigate the role of endoplasmic reticulum (ER) stress and autophagy pathway in the expression of IL-23 in peripheral blood-derived macrophages in AS patients.

## Methods

Peripheral blood samples were obtained from 15 AS and 15 healthy control subjects. MACS was used to isolate monocytes from PBMCs. Then, M-CSF was used to differentiate monocytes to M2 macrophages. IFN-γ and/or LPS was used to activate macrophages and M2 polarization toward M1 macrophages. Thapsigargin was used to induce ER stress and 3-MA to inhibit autophagy. The purity of extracted monocytes and macrophage markers was evaluated by flow cytometry. mRNA expression of HLA-B and-B27, ER stress-related genes, autophagy-related genes, and IL-23p19 was performed using RT-qPCR. Soluble levels of IL-23p19 were measured using ELISA.

## Results

Significant increase in mRNA expression of HLA-B, HLA-B27, BiP, XBP1, CHOP, and PERK mRNAs was observed in macrophages of AS patients before and after stimulation with IFN-γ and LPS. No significant change in autophagy gene expression was detected. mRNA and soluble levels of IL-23p19 demonstrated a significant increase in macrophages of AS patients compared to healthy subjects. ER stress induction led to a significant increase in IL-23p19 in macrophages. Inhibition of autophagy had no effect on IL-23 expression.

## Conclusion

ER stress, unlike autophagy, in macrophages of AS patients is associated with increased IL-23 levels.

## Key Messages

- ER stress in macrophages from AS patients plays a role in increased production of IL-23.
- HLA-B27 is not involved in ER-stress related over-production of IL-23 in AS macrophages.
- Autophagy pathway is not involved in modulation of IL-23 production by AS macrophages.

## 1. Introduction

Ankylosing spondylitis (AS), as the most common type of spondyloarthritis (SpA), is a chronic inflammatory arthritis that predominantly involves the axial skeleton. Despite the significant contribution of the human leukocyte antigen (HLA)-B27 in conferring genetic risk of AS, several other genes have also been identified in association with the disease [1, 2]. Alteration in the sequence of HLA-B27 is involved in the modulation of peptide-binding ability and stability of the antigen presentation [3, 4]. That notwithstanding, no certain 'arthritogenic peptide' has been recognized in induced abnormal immune response in AS, and no differences have been found among peptides that are loaded onto AS-associated and non-associated HLA-B27 subtypes [5].

In the synovial tissues and gut of SpA patients as well as HLA-B27-transgenic rats, an accumulation of unconventional HLA-B27 forms, like the free heavy chain (FHC), has been observed [6]. The uncommon characteristic of HLA-B27 molecules in the slow rate of folding and misfolding during biosynthesis in the ER. Such unconventional HLA-B27 forms are able to accumulate in the endoplasmic reticulum (ER), resulting in ER stress, which stimulates the unfolded protein response (UPR). On the other side, UPR has been associated with the promoted production of inflammatory IL-23 cytokine in AS patients [7].

Unconventional HLA-B27 molecules, like FHC dimers expressed on the surface of antigen-presenting cells (APCs), are able to induce IL-23 receptor (IL-23R) expressing T cells to generate IL-17 [8]. Killer cell immunoglobulin-like receptor (KIR) 3DL2 expressed on these T cells is bound to FHC of unconventional HLA-B27 molecules, leading to the upregulation of T helper (Th) 17 specific transcription factor, namely RAR-related orphan receptor-gamma t (RORγt). This, in turn, results in Th17 polarization and increased production of pro-inflammatory IL-17 in AS [9−11].

Autophagy is considered as a protective cellular process that is involved in clearing the dying cellular organelles as well as misfolded proteins to enhance cell survival [12]. Autophagy has been suggested to be related to the pathogenesis of AS through digesting misfolded and unconventional HLA-B27 molecules and, hence, modulation of UPR. Modifications in the autophagy pathways have also been reported to be tissue-specific since dysregulated autophagy was no seen in the PBMCs or synovium of AS patients [13].

Our previous investigations demonstrated that UPR is activated in the macrophage-colony stimulating factor (M-CSF)-derived macrophages of AS patients, along with the upregulation of HLA-B27 expression in these cells, which was linked to the overproduction of IL-23 in macrophages from AS [14]. Seeking for a broader insight, in the current study, the behavior of macrophages derived from peripheral blood monocytes of AS patients and healthy controls in establishing ER stress, induced either by treatment with IFN-γ (which triggers HLA-B27) or by a chemical ER stress inducer and, ultimately, production of IL-23 were assessed. In addition, the behavior of AS macrophages in response to the inhibition of autophagy was studied.

## 2. Methods And Study Subjects

## 2.1. AS patients and controls

This was a case-control study, 15 AS patients and 15 healthy controls were included. AS patients were recruited from the rheumatology outpatient clinic of Rheumatology Research Center, Shariati Hospital affiliated by Tehran University of Medical Sciences, Tehran, Iran. Patients fulfilled the classification index of the modified New York criteria for the diagnosis of AS. Patients were evaluated for functional capacity through examining the Bath Ankylosing Spondylitis Functional Index (BASFI), for spinal mobility by Bath Ankylosing Spondylitis Metrology Index (BASMI), and for disease activity via Bath Ankylosing Spondylitis Disease Activity Index (BASDAI). AS the inclusion criteria, AS patients having a BASDAI score further than 4 were chosen. Healthy controls were age- and sex-matched with the patient group, had no familial relation with AS cases, and had no family history of AS as well as other autoimmune disorders (supplementary Table 1). From all study subjects, fresh whole blood samples were obtained by venipuncture and collected into a 9-ml vacuum blood collection tube containing EDTA anticoagulant.

## 2.2. Monocyte isolation and M1 macrophage polarization

In order to isolate peripheral blood mononuclear cells (PBMCs), blood samples were first diluted in with Phosphate-buffered saline (PBS; GIBCO Invitrogen, Carlsbad, CA), and then were separated using Ficoll/Hypaque 1.077 g/ml (Lymphodex, inno-Train, Kronberg, Germany) and gradient density centrifugation. The cells were then washed twice at 300 and 200×g to reduce impurity. In order to isolate monocytes, magnetic-activated cell sorter columns (Miltenyi Biotec, San Diego, CA) was used for positive selection of CD14 + cells from PBMCs according to the manufacturer's guidelines. The monocytes were assessed for purity using flow cytometry that was approximately 90−94% (Supplementary Fig. 1). To further purify the samples, monocytes were cultured in serum-free RPMI 1640 media (GIBCO Invitrogen, Carlsbad, CA) in 12-well media at a density of $55 \times 10^5$ cells/well and then were kept in the humidified atmosphere under 5% $CO_2$ at 37° C for 2 hrs. Afterward, non-adherent cells were removed, and differentiation of the adherent monocyte cells to M2 macrophages was implemented using 50 ng/ml of recombinant human M-CSF (eBioscience, San Diego, CA) in R10 media (containing RPMI-1640, 10% FBS, 4 mM L-glutamine, 1% penicillin/streptomycin antibiotic) for seven days in the humidified atmosphere under 5% $CO_2$ at 37° C. Thereafter, interferon (IFN)-γ cytokine alone or in combination with LPS was used to polarize the M2 to M1 macrophage cells [15]. On the seventh day, the supernatant was removed from each well of culture media, and RPMI-1640 and 50 ng/ml IFN-γ (R&D Systems) alone or with 100 ng/ml Lipopolysaccharides (LPS; Sigma-Aldrich) obtained from *Salmonella enterica* serotype Enteritidis were added to each well.

To confirm the polarization of M2 to M1 macrophages, expressions of specific mRNAs of *CD36* and Mannose receptor C-type 1 (*MRC1*) genes for M1 macrophages and CC-chemokine receptor 7 (*CCR7*) and Indoleamine 2,3-dioxygenase 1 (*IDO1*) genes for M2 macrophages were evaluated (Supplementary Fig. 2) [15−17].

## 2.3. Flow cytometry

For phenotypic comparison of macrophages and monocytes, the flow cytometry approach was used. First, monocytes and macrophages collected from culture plates were washed twice with PBS. Cells were then incubated for 1 h in PBS containing 5% FBS (GIBCO Invitrogen, Carlsbad, CA) at 4° C to interrupt the non-specific monoclonal antibody binding. Afterward, cells were stained with APC-conjugated anti-human CD163, PE-Cy5-conjugated anti-human HLA-DR (Biolegend, San Diego, CA), and FITC-conjugated anti-human CD206 (Miltenyi Biotec, San Diego, CA) in the dark for 30 min at 4° C. In parallel, irrelevant isotype-matched controls labeling was also carried out. Upon after, the cells were rinsed and resuspended in 500 μl PBS, then chilled on ice, and evaluation of fluorescence was conducted using FACSCalibur (Becton Dickinson, Franklin Lakes, NJ) and analyzed using FlowJo software (Tree Star, Ashland, OR). At least 10,000 events were counted for each sample. Trypan blue staining was used to determine cell death.

## 2.4. Induction of ER stress in M1 macrophages

Thapsigargin (TPG) was used to induce ER stress in macrophages derived from blood monocytes of AS patients and then evaluate the effect of ER stress inhibition on IL-23 production. To this end, macrophages on the seventh day of culture were treated with 500 nM TPG (TOCRIS) along with or without IFN-γ and LPS for 12 hrs.

## 2.5. Inhibition of Autophagy pathway in M1 macrophages

To investigate the inhibition of autophagy pathway in macrophages derived from blood monocytes of AS patients and determine the inhibition effect on IL-23 production, 3-Methyladenine (3MA) was used. For this purpose, macrophages on the seventh day of culture were treated with 5 mM 3MA (TOCRIS) along with or without IFN-γ and LPS for 24 hrs.

## 2.6. RNA extraction, cDNA synthesis, and quantitative Real-time PCR

In order to extract the total RNA from M1 macrophages on day 7, the High Pure RNA Isolation Kit (Roche, Basel, Switzerland) was used, according to the company's instructions. Synthesis of complementary DNA (cDNA) was conducted exerting the Transcriptor First Strand cDNA Synthesis Kit (Roche, Basel, Switzerland) using random hexamer primers, based on the manufacturer's protocol. The quantitative mRNA expression levels of differentiation markers of macrophages, including CD36, MRC1, CCR7, and IDO1, HLA genes, ER stress-associated genes, autophagy-related genes, and IL-23p19 were determined to employ the Real-time PCR performed by the StepOnePlus™ Real-Time PCR system (Applied Biosystems, Foster City, CA), SYBR Green master mix (Ampliqon, Odense, Denmark), and specific primers (Supplementary Table 2). The primers were designed through the Primer Express 3.0 tool (Applied Biosystems, Foster City, CA, USA) and check for the specificity by the Primer-BLAST Tool of the National Center for Biotechnology Information (NCBI) website (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Finally, the primers were generated by the custom oligonucleotide synthesis service (Metabion, Martinsried, Germany). Moreover, the specification of the HLA-B27 specific primers was obtained from the study be Zeng *et al.* [15]. The comparative $C_T$ approach, defined by Livak and Schmittgen [18], was used to determine the relative transcription levels. Normalization of target gene mRNAs was done in

corresponding to the expression level of 18S ribosomal RNA as the housekeeping gene to calculate the relative amounts of each gene transcription using "Relative Expression = $2^{-\Delta Ct}$" equation.

## 2.7. ELISA

To measure the IL-23p19 cytokine concentration, the supernatant of each well was collected after different treatments of macrophages and then stored in -70°C for conducting the enzyme-linked immunosorbent assay (ELISA). The optical density in the samples was determined using the Human IL-23 ELISA development kit (HRP, Mabtech, Stockholm, Sweden).

## 2.8. Statistical analysis

Data analysis and illustrations by graphs were both conducted using GraphPad Prism v.8 (GraphPad Software, San Diego, CA). Friedman test was used to compare three different macrophages (M2 macrophages, IFN-γ-induced M1 macrophages, and IFN-γ + LPS-induced M1 macrophages) from patients or controls. Additionally, before-after treatment analyses of comparing means were performed by Wilcoxon signed-rank test. The nonparametric spearman's rho test was performed to assess the correlation between mRNA expression of different genes with mRNA and soluble levels of IL-23p19. Data were expressed as means ± standard error of the mean (SEM) and a $P$ value less than 0.05 was considered as statistically significant level.

## 3. Results

## 3.1. Expression of HLA-B and HLA-B27 molecules

There was a statistically significant difference in mRNA expression of HLA-B among IFN-γ-induced M1 macrophages, IFN-γ + LPS-induced M1 macrophages, and M2 macrophages in AS-B27[+], AS-B27[−], Con-B27[+], and Con-B27[−] (Fig. 1A). There was a statistically significant difference in mRNA expression of HLA-B27 among IFN-γ-induced M1 macrophages, IFN-γ + LPS-induced M1 macrophages, and M2 macrophages in both AS patients and control group (Fig. 1B). Refer to supplementary table 3 for fold change values and $P$ values with more details.

## 3.2. mRNA expression of ER stress-associated genes

A statistically significant difference was observed in mRNA expression of BiP (Fig. 2.A), XBP1 (Fig. 2.B), and PERK (Fig. 2.C) between different groups of AS patients and controls as well as between IFN-γ-induced M1 macrophages, IFN-γ + LPS-induced M1 macrophages, and M2 macrophages. However, the mRNA expression of ATF6 (Fig. 2.D) and CHOP (Fig. 2.E) did not show significant difference between different cells. Refer to supplementary table 3 for fold change values and $P$ values with more details.

## 3.3. mRNA expression of autophagy-related genes

In the IFN-γ + LPS-induced M1 macrophages, there were upregulated levels of ATG5 mRNA in the AS-B27[+] patients compared with the AS-B27[−] patients and Con-B27[+] group (Fig. 3.A). Significantly altered

expression of ATG12 mRNA was detected in the M2 macrophages between patients and controls. AS-B27$^+$ patients expressed increased levels of ATG12 mRNA in the M2 macrophages in comparison to the Con-B27$^-$ group. Moreover, mRNA expression of ATG12 was significantly upregulated in the Con-B27$^+$ in relation to the Con-B27$^-$ group (Fig. 3.B). Analysis indicated statistically significant difference in mRNA expression of ATG16L among IFN-γ-induced M1 macrophages, IFN-γ + LPS-induced M1 macrophages, and M2 macrophages in AS-B27$^+$, Con-B27$^+$, and Con-B27$^-$ groups (Fig. 3.C). Refer to supplementary table 3 for fold change values and $P$ values with more details.

## 3.4. mRNA expression of IL-23

There was a statistically significant difference in the mRNA expression of IL-23p19 among IFN-γ-induced M1 macrophages, IFN-γ + LPS-induced M1 macrophages, and M2 macrophages in AS-B27$^+$, AS-B27$^-$, Con-B27$^+$, and Con-B27$^-$. In comparison to M2 macrophages, the mRNA expression of IL-23p19 was overexpressed in the IFN-γ + LPS-induced M1 macrophages in AS-B27$^+$, AS-B27$^-$, Con-B27$^+$, Con-B27. Furthermore, the mRNA expression of IL-23p19 was upregulated in the IFN-γ + LPS-induced M1 macrophages in comparison to the IFN-γ-induced M1 macrophages in AS-B27$^+$, AS-B27$^-$, Con-B27$^+$, and Con-B27$^-$ individuals (Fig. 2.F). Refer to supplementary table 3 for fold change values and $P$ values with more details.

## 3.5. Effect of ER stress induction on mRNA expression of IL-23

After induction of ER stress in the IFN-γ-induced M1 macrophages, the mRNA expression of IL-23p19 was upregulated in AS-B27$^+$, AS-B27$^-$, Con-B27$^+$, and Con-B27$^-$ in comparison to non-ER stress-induced IFN-γ-induced M1 macrophages (Fig. 4.A). Similar results were observed in the IFN-γ + LPS-induced M1 macrophages.

Furthermore, there was an overexpression of the mRNA expression of IL-23p19 in the ER stress-induced IFN-γ + LPS-induced M1 macrophages from AS-B27$^+$ patients in comparison to Con-B27$^+$ group, as well as in the AS-B27$^-$ patients compared with the Con-B27$^+$ group and Con-B27$^-$ group (Fig. 4.B). Refer to supplementary table 4 for fold change values and $P$ values with more details.

## 3.6. Effect of ER stress induction on soluble level of IL-23

The measurement range of kit used (Mabtech, Stockholm, Sweden) in the detection of soluble levels of IL-23p19 was between 7 and 700 pg/ml. Levels of IL-23p19 were not detectable in the M2 macrophages and IFN-γ-induced M1 macrophages due to very low levels. However, the cytokine levels were measured in the IFN-γ + LPS-induced M1 macrophages before and after induction of ER stress.

Analysis indicated that in comparison to the non-ER stress-induced IFN-γ + LPS-induced M1 macrophages, the soluble levels of IL-23p19 was higher in the supernatant of ER stress-induced IFN-γ + LPS-induced M1 macrophages in AS-B27$^+$, AS-B27$^-$, Con-B27$^+$, and Con-B27$^-$. In the supernatant of ER

stress-induced IFN-γ + LPS-induced M1 macrophages, soluble levels of IL-23p19 was higher in the AS-B27$^+$ patients in comparison to the Con-B27$^+$ group, as well as in the AS-B27$^-$ patients compared to the Con-B27$^+$ and Con-B27$^-$ groups (Fig. 4.C).

## 3.7. Effect of autophagy pathway inhibition on mRNA expression of IL-23

Inhibition of the autophagy pathway did not alter the mRNA expression of IL-23 in none of the macrophages between patients and controls (Fig. 5A and B).

## 3.8. Effect of autophagy pathway inhibition on soluble levels of IL-23

After inhibition of the autophagy pathway in the IFN-γ + LPS-induced M1 macrophages, no altered levels of soluble IL-23p19 were detected between patients and controls (Fig. 5.C).

## 3.9. Correlation analysis

In order to evaluate the relation between the ER stress-related genes and autophagy genes with IL-23p19, the correlation analysis was conducted. It was observed that mRNA expression of several ER stress-related and autophagy genes was correlated significantly with mRNA expression as well as soluble level of IL-23p19 in three types of macrophages (Supplementary Tables 5 and 6).

## 4. Discussion

In the current study, we tried to evaluate the role of UPR in the modulation of AS macrophages to establish an inflammatory setting by the production of IL-23. The behavior of macrophages derived from peripheral blood monocytes isolated from AS patients and healthy controls in stimulating the ER stress-induced either by treatment with IFN-γ (HLA-B27 enhancer stimulant) or by ER stress inducer (TPG) and IL-23 production was assessed. Moreover, the macrophage response against the inhibition of autophagy (by 3MA agent) was evaluated.

A bulk of investigations have shown increased IL-23 in tissue, serum, and peripheral blood cells in AS patients [19, 20]. However, the specific pathway that increases IL-23 in this disease has not yet been clearly identified. Among the different hypotheses presented for AS pathogenesis, the theory of misfolding and accumulation of HLA-B27 in the ER and induction of ER has been suggested. According to this hypothesis, stress-induced in the ER by misfolding of the HLA-B27 molecule can induce the UPR cellular response, leading to increased inflammatory cytokines like IL-23 [21, 22].

Here in this study, IFN-γ was used to differentiate M2 to M1 macrophages and increase HLA-B27 expression in these cells. It was observed that M2 macrophages, IFN-γ-induced M1 macrophages, and IFN-γ + LPS-induced M1 macrophages obtained from AS-B27$^+$ patients had upregulated mRNA expression of HLA-B in comparison to the AS-B27$^-$ patients and control group. All these macrophage cells

also overexpressed mRNA of HLA-B in AS-B27[+] patients compared with Con-B27[+] individuals. Previous studies using immunofluorescence, flow cytometry [23], and quantitative PCR [24], showed increased HLA-B27 expression in the PBMCs from HLA-B27[+] AS patients compared to healthy HLA-B27[+] subjects.

Considering increased expression of the HLA-B27 in the IFN-γ-induced M1 macrophages and IFN-γ + LPS-induced M1 macrophages, the behavior of macrophages with respect to ER stress and mRNA expression of related genes were investigated. The results showed that M2 macrophages, as well as IFN-γ-induced M1 macrophages and IFN-γ + LPS-induced M1 macrophages of AS-B27[+] patients compared to AS-B27[−] patients and control group overexpressed ER stress-associated genes of BiP, XBP1, PERK, and CHOP, implying to ER stress in these cells. It should be noted, however, that the differences in the mRNA expression of BiP and CHOP were not different between the three macrophages. PERK mRNA expression in the IFN-γ + LPS-induces M1 macrophages was higher than IFN-γ-induced M1 macrophages in AS-B27[+] patients. Only mRNA expression of XBP1 showed a significant increase under IFN-γ treatment alone or with LPS. Feng *et al.* also indicated increased expression of UPR genes, such as BiP, CHOP, and XBP1 in the peripheral blood as well as the synovial fluid of SpA patients [25].

Similarly, using microarray and gene expression profile analysis, Gu *et al.* reported the increase of BiP in the synovial fluid mononuclear cells (SFMC) in SpA patients [26]. However, Lamina propria mononuclear cells (LPMC) isolated from the gut of AS patients did not show significant changes in the expression of BiP, XBP1, PERK, and ATF6 genes [27]. Zeng *et al.*, also could not show a significant increase in UPR associated genes in macrophages of AS patients after treatment with IFN-γ and LPS [15].

Our experiments also indicated that macrophages from AS-B27[+] patients produced higher levels of IL-23p19 in comparison to AS-B27[−] patients and healthy individuals. It should be noted that after treatment with IFN-γ, there was no significant difference in the level of IL-23p19 expression between M2 macrophages and IFN-γ-induced M1 macrophages. On the other hand, increased expression of HLA-B27 could not result in a difference in IL-23p19 production. However, data on IFN-γ + LPS-induced M1 macrophages treated with both IFN-γ and LPS indicated that LPS could increase IL-23 in both patients and controls that had a significant difference with M2 macrophages and IFN-γ- induced M1 macrophages. IFN-γ + LPS-induced M1 macrophages from AS-B27[+] patients expressed further amounts of IL-23p19 in comparison to other subjects.

Experiments by induction of ER stress using TPG implied to the different behavior of macrophages from patients compared to the control group. TPG treatment of macrophages significantly increased both mRNA expression and protein levels of IL-23p19 in both patients and controls. This increase was significant for macrophages that were not treated with TPG. Additionally, TPG treated macrophages of patients showed higher IL-23p19 cytokine levels than healthy controls. The results of the present study were in line with increased IL-23 levels in macrophages of AS patients and demonstrated the previously reported involvement of IL-23/IL17 inflammatory pathway in the pathogenesis of AS. Many genome-wide association studies (GWASs) have reported the association of *IL23 receptor* gene polymorphisms with AS susceptibility [28, 29]. In a study by Goodall *et al.*, it was shown that the ER stress-related gene CHOP

directly regulated IL-23. In fact, CHOP is an ER stress-induced transcription factor and possesses a specific binding site to the *IL23* gene regulatory domain [30]. Animal studies have also shown that TPG-induced ER stress can increase IL-23 production in rat bone marrow-derived macrophages [22, 31]. This increase by ER stress has also been detected in human dendritic cells (DCs) [30].

Experiments on the autophagy genes indicated no significant difference in expression of ATG5 and ATG12 in the three macrophage groups between patients and healthy individuals. Only IFN-γ + LPS-induced M1 macrophages from AS-B27+ patients expressed higher levels of ATG16L than M2 macrophages and IFN-γ-induced M1 macrophages. In addition, mRNA expression of ATG12 in M2 macrophages from patients was upregulated compared to controls. In a 2013 study by Ciccia and colleagues on LPMCs isolated from the gut of AS patients, it was found that the autophagy pathway in these cells was more active than the control group. They showed that the expression levels of ATG5, ATG12 and LC3II were higher in the gut of AS patients than in healthy individuals and that the autophagy pathway was involved in regulating IL-23 production in the gut of these patients [27]. Data from autophagy inhibition in M1 macrophages by 3MA and IL-23p19 cytokine analysis showed that inhibition of the autophagy pathway could not have a significant effect on IL-23p19 expression and production. Contrary to the results of the present study, a study by Castro *et al*. on the autophagy pathway showed that both mouse bone marrow-derived macrophages and human macrophages and DCs were more susceptible to autophagy inhibition either by chemical inhibitors or by interfering RNA. Autophagy pathway inhibition increased the production of IL-23 in the cells mentioned [32].

## 5. Conclusion

Considering all the facts, this study revealed that AS patients express higher levels of HLA-B27 than healthy controls. Macrophages in these patients can also produce higher levels of IL-23, but increased HLA-B27 was not associated with increased IL-23 levels. The increased expression of a number of genes involved in the UPR response indicated an ER stress state in macrophages of AS patients. Evaluation of the ER stress pathway revealed that AS macrophages behave differently towards ER stress compared to healthy subjects. Hence, ER stress can be proposed as a regulatory pathway for IL-23 production in macrophages of AS patients. Inhibition of autophagy pathway did not alter IL-23 production and probably could not be considered as the regulatory pathway of this cytokine in macrophages of AS patients. As a result, it is suggested that IL-23 inhibitors may serve as a promising tool for AS treatment. The precise understanding of the pathways involved in increased IL-23 production can be further contributing to the treatment of AS disease.

## 6. Abbreviations

3MA, 3-Methyladenine; APC, Antigen-presenting cell; AS, Ankylosing spondylitis; BASDAI, Bath Ankylosing Spondylitis Disease Activity Index; BASFI, Bath Ankylosing Spondylitis Functional Index; BASMI, Bath Ankylosing Spondylitis Metrology Index; CCR7, CC-chemokine receptor 7; DC, Dendritic cells; ER, Endoplasmic reticulum; FHC, Free heavy chain; GWAS, Genome-wide association study; HLA-B27, Human

leukocyte antigen B27; IDO1, Indoleamine 2,3-dioxygenase 1; IFN-γ, Interferon Gamma; IL-23, Interleukin-23; IL-23R, IL-23 receptor; KIR, Killer cell immunoglobulin-like receptor; LPMC, Lamina propria mononuclear cells; LPS, Lipopolysaccharide; M-CSF, Macrophage-colony stimulating factor; MRC1, Mannose receptor C-type 1; PBMC, Peripheral blood mononuclear cell; PBS, Phosphate-buffered saline; RORγt, RAR-related orphan receptor-gamma t; SFMC, Synovial fluid mononuclear cells; SpA, Spondyloarthritis; Th17, T helper 17; TPG, Thapsigargin; UPR, Unfolded protein response.

# 7. Declarations

## 7.1. Ethical statement

The protocol of the study was endorsed by the ethical committee of Tehran University of Medical Sciences (Ethical code: IR.TUMS.REC.1394.671) for use of human participants. All study subjects signed informed consent forms before sampling. All research was performed in accordance with relevant guidelines and regulations of Tehran University of Medical Sciences.

## 7.2. Consent for publication

Written informed consent was obtained from all participating individuals.

## 7.3. Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## 7.4. Competing interests

The authors have no competing interests to disclose.

## 7.5. Funding

## 7.6. Authors' contributions

A.R. conceived the study and wrote the manuscript. M.V. and A.R.J. contributed to sample preparation. F.B. and J.M. took the lead in writing the manuscript. M.M. and A.A.A. contributed to the interpretation of the results. M.H.N. critically revised the manuscript and provided the final approval. All authors reviewed the manuscript.

## 7.7. Acknowledgements

**7.8. Authors' information:**

1. Alireza Rezaiemanesh, PhD, Department of Immunology, School of Medicine, Kermanshah University of Medical Sciences, Kermanshah, Iran. alireza.rezaiemanesh@kums.ac.ir

2. Mahdi Mahmoudi, PhD, Rheumatology Research Center, Tehran University of Medical Sciences, Tehran, Iran. mahmoudim@tums.ac.ir

3. Ali Akbar Amirzargar, PhD, Department of Immunology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran. Molecular Immunology Research Center, Tehran University of Medical Sciences, Tehran, Iran. amiralizar@yahoo.com

4. Mahdi Vojdanian, MD, Rheumatology Research Center, Tehran University of Medical Sciences, Tehran, Iran. vojdanian@gmail.com

5. Farhad Babaie, PhD, Department of Immunology and Genetic, School of Medicine, Urmia University of Medical Sciences, Urmia, Iran. farhad.babai@gmail.com

6. Jila Mahdavi, MSc, Department of Biology, Payame Noor University, Tehran, Iran. mscjilamahdavi@gmail.com

7. Ahmad Reza Jamshidi, MD, Rheumatology Research Center, Tehran University of Medical Sciences, Tehran, Iran. jamshida@tums.ac.ir

8. Mohammad Hossein Nicknam, PhD, Department of Immunology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran. Molecular Immunology Research Center, Tehran University of Medical Sciences, Tehran, Iran. mhnicknam@sina.tums.ac.ir

### Corresponding author

Correspondence to Mohammad Hossein Nicknam

# References

1. Ranganathan V, Gracey E, Brown MA, Inman RD, Haroon N. Pathogenesis of ankylosing spondylitis—recent advances and future directions. Nat Rev Rheumatol. 2017;13:359.

2. Mahmoudi M, Aslani S, Nicknam MH, Karami J, Jamshidi AR. New insights toward the pathogenesis of ankylosing spondylitis; genetic variations and epigenetic modifications. Modern rheumatology. 2017;27:198–209.

3. García-Medel N, Sanz-Bravo A, Alvarez-Navarro C, Gómez-Molina P, Barnea E, Marcilla M, et al. Peptide handling by HLA-B27 subtypes influences their biological behavior, association with ankylosing spondylitis and susceptibility to endoplasmic reticulum aminopeptidase 1 (ERAP1). Molecular Cellular Proteomics. 2014;13:3367–80.

4. Dashti N, Mahmoudi M, Aslani S, Jamshidi A. HLA-B* 27 subtypes and their implications in the pathogenesis of ankylosing spondylitis. Gene. 2018;670:15–21.

5. Schittenhelm RB, Sian TCLK, Wilmann PG, Dudek NL, Purcell AW. Revisiting the arthritogenic peptide theory: quantitative not qualitative changes in the peptide repertoire of HLA–B27 allotypes. Arthritis rheumatology. 2015;67:702–13.

6. Rysnik O, McHugh K, van Duivenvoorde L, van Tok M, Guggino G, Taurog J, et al. Non-conventional forms of HLA-B27 are expressed in spondyloarthritis joints and gut tissue. J Autoimmun. 2016;70:12–21.

7. Colbert RA, DeLay ML, Klenk EI, Layh-Schmitt G. From HLA-B27 to spondyloarthritis: a journey through the ER. Immunological reviews. 2010;233:181–202.

8. Bowness P, Ridley A, Shaw J, Chan AT, Wong-Baeza I, Fleming M, et al. Th17 cells expressing KIR3DL2 + and responsive to HLA-B27 homodimers are increased in ankylosing spondylitis. J Immunol. 2011;186:2672–80.

9. Babaie F, Hasankhani M, Mohammadi H, Safarzadeh E, Rezaiemanesh A, Salimi R, et al. The role of gut microbiota and IL-23/IL-17 pathway in ankylosing spondylitis immunopathogenesis: New insights and updates. Immunology letters. 2018;196:52–62.

10. Rezaiemanesh A, Abdolmaleki M, Abdolmohammadi K, Aghaei H, Pakdel FD, Fatahi Y, et al. Immune cells involved in the pathogenesis of ankylosing spondylitis. Biomed Pharmacother. 2018;100:198–204.

11. Ridley A, Hatano H, Wong-Baeza I, Shaw J, Matthews KK, Al-Mossawi H, et al. Activation-Induced Killer Cell Immunoglobulin-like Receptor 3DL2 Binding to HLA–B27 Licenses Pathogenic T Cell Differentiation in Spondyloarthritis. Arthritis rheumatology. 2016;68:901–14.

12. Ciccia F, Haroon N. Autophagy in the pathogenesis of ankylosing spondylitis. Clin Rheumatol. 2016;35:1433–6.

13. Neerinckx B, Carter S, Lories R. IL-23 Expression and Activation of Autophagy in Synovium and PBMCs of HLA-B27 Positive Patients with Ankylosing Spondylitis. Arthritis Rheumatology. 2014;66:844-S.

14. Rezaiemanesh A, Mahmoudi M, Amirzargar AA, Vojdanian M, Jamshidi AR, Nicknam MH. Ankylosing spondylitis M-CSF-derived macrophages are undergoing unfolded protein response (UPR) and express higher levels of interleukin-23. Modern rheumatology. 2017;27:862–7.

15. Zeng L, Lindstrom MJ, Smith JA. Ankylosing spondylitis macrophage production of higher levels of interleukin-23 in response to lipopolysaccharide without induction of a significant unfolded protein response. Arthr Rhuem. 2011;63:3807–17.

16. Svensson J, Jenmalm MC, Matussek A, Geffers R, Berg G, Ernerudh J. Macrophages at the fetal–maternal interface express markers of alternative activation and are induced by M-CSF and IL-10. J Immunol. 2011;187:3671–82.

17. Brown MB, von Chamier M, Allam AB, Reyes L. M1/M2 macrophage polarity in normal and complicated pregnancy. Frontiers in immunology. 2014;5:606.

18. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative CT method. Nature protocols. 2008;3:1101–8.

19. Ciccia F, Bombardieri M, Principato A, Giardina A, Tripodo C, Porcasi R, et al. Overexpression of interleukin-23, but not interleukin-17, as an immunologic signature of subclinical intestinal inflammation in ankylosing spondylitis. Arthritis Rheumatism: Official Journal of the American College of Rheumatology. 2009;60:955–65.

20. Mei Y, Pan F, Gao J, Ge R, Duan Z, Zeng Z, et al. Increased serum IL-17 and IL-23 in the patient with ankylosing spondylitis. Clin Rheumatol. 2011;30:269–73.

21. Appel H, Maier R, Bleil J, Hempfing A, Loddenkemper C, Schlichting U, et al. In Situ Analysis of Interleukin–23–and Interleukin-12–Positive Cells in the Spine of Patients With Ankylosing Spondylitis. Arthr Rhuem. 2013;65:1522–9.

22. DeLay ML, Turner MJ, Klenk EI, Smith JA, Sowders DP, Colbert RA. HLA–B27 misfolding and the unfolded protein response augment interleukin-23 production and are associated with Th17 activation in transgenic rats. Arthr Rhuem. 2009;60:2633–43.

23. Cauli A, Dessole G, Fiorillo M, Vacca A, Mameli A, Bitti P, et al. Increased level of HLA-B27 expression in ankylosing spondylitis patients compared with healthy HLA-B27-positive subjects: a possible further susceptibility factor for the development of disease. Rheumatology. 2002;41:1375–9.

24. Liu S-Q, Yu H-C, Gong Y-Z, Lai N-S. Quantitative measurement of HLA-B27 mRNA in patients with ankylosing spondylitis–correlation with clinical activity. J Rhuematol. 2006;33:1128–32.

25. Feng Y, Ding J, Fan CM, Zhu P. Interferon-γ contributes to HLA-B27-associated unfolded protein response in spondyloarthropathies. J Rhuematol. 2012;39:574–82.

26. Gu J, Rihl M, Märker-Hermann E, Baeten D, Kuipers JG, Song YW, et al. Clues to pathogenesis of spondyloarthropathy derived from synovial fluid mononuclear cell gene expression profiles. The Journal of Rheumatology. 2002;29:2159–64.

27. Ciccia F, Accardo-Palumbo A, Rizzo A, Guggino G, Raimondo S, Giardina A, et al. Evidence that autophagy, but not the unfolded protein response, regulates the expression of IL-23 in the gut of patients with ankylosing spondylitis and subclinical gut inflammation. Ann Rheum Dis. 2014;73:1566–74.

28. Rahman P, Inman RD, Gladman DD, Reeve JP, Peddle L, Maksymowych WP. Association of interleukin-23 receptor variants with ankylosing spondylitis. Arthr Rhuem. 2008;58:1020–5.

29. Reveille JD, Sims A-M, Danoy P, Evans DM, Leo P, Pointon JJ, et al. Genome-wide association study of ankylosing spondylitis identifies non-MHC susceptibility loci. Nat Genet. 2010;42:123.

30. Goodall JC, Wu C, Zhang Y, McNeill L, Ellis L, Saudek V, et al. Endoplasmic reticulum stress-induced transcription factor, CHOP, is crucial for dendritic cell IL-23 expression. Proceedings of the national academy of Sciences. 2010;107:17698-703.

31. Turner MJ, Sowders DP, DeLay ML, Mohapatra R, Bai S, Smith JA, et al. HLA-B27 misfolding in transgenic rats is associated with activation of the unfolded protein response. J Immunol. 2005;175:2438–48.

32. de Castro CP, Jones SA, Cheallaigh CN, Hearnden CA, Williams L, Winter J, et al. Autophagy regulates IL-23 secretion and innate T cell responses through effects on IL-1 secretion. J Immunol. 2012;189:4144–53.
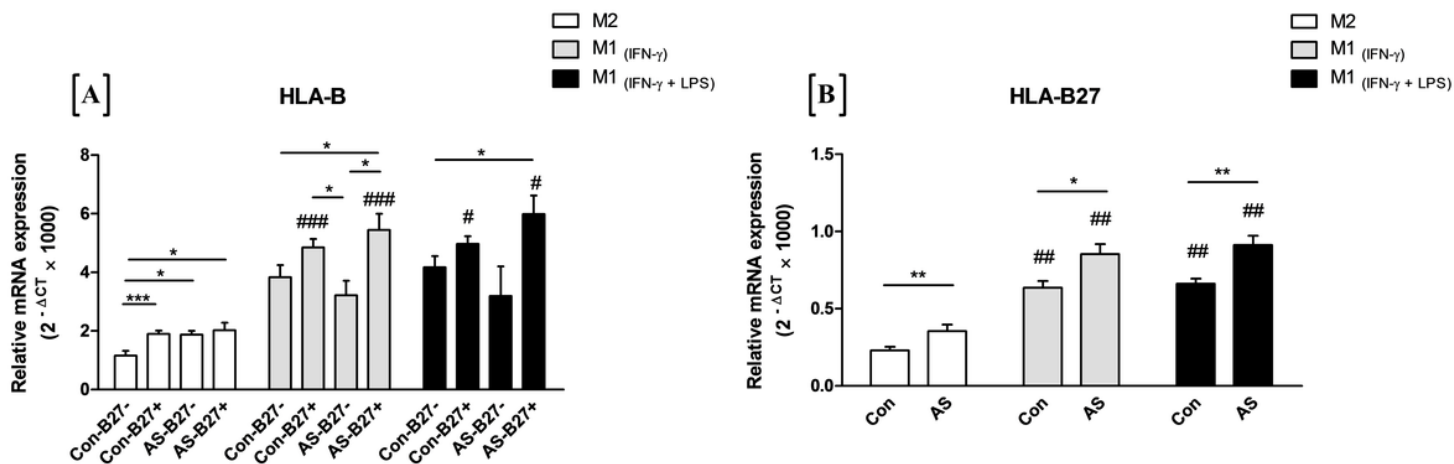
# Figures



**Figure 1**

mRNA expression levels of HLA-B (A) and HLA-B27 (B) in AS patients and healthy subjects. Monocytes were isolated from 10 HLA-B27 positive AS patients (AS-B27+), 5 HLA-B27 negative AS patients (AS-B27-), 10 HLA-B27 positive healthy controls (Con-B27+) and 5 HLA-B27 negative healthy controls (Con-B27-) and were differentiated into M2 macrophages upon treating with M-CSF in 7 days. M2 macrophages were then polarized into M1 macrophages after treating with IFN-γ alone or in combination with LPS for 24 hrs. The expression levels of HLA-B mRNA (A) and HLA-B27 mRNA (B) in these three cell groups were compared between patients and controls. Results are presented as mean ± standard error of the mean (SEM). Significance within each cell group was indicated with "*" sign, between M1 (IFN-γ) and M1 (IFN-γ + LPS) group compared to M2 with "#" sign, and between M1 (IFN-γ) and M1 (IFN-γ + LPS) group by "$" sign.
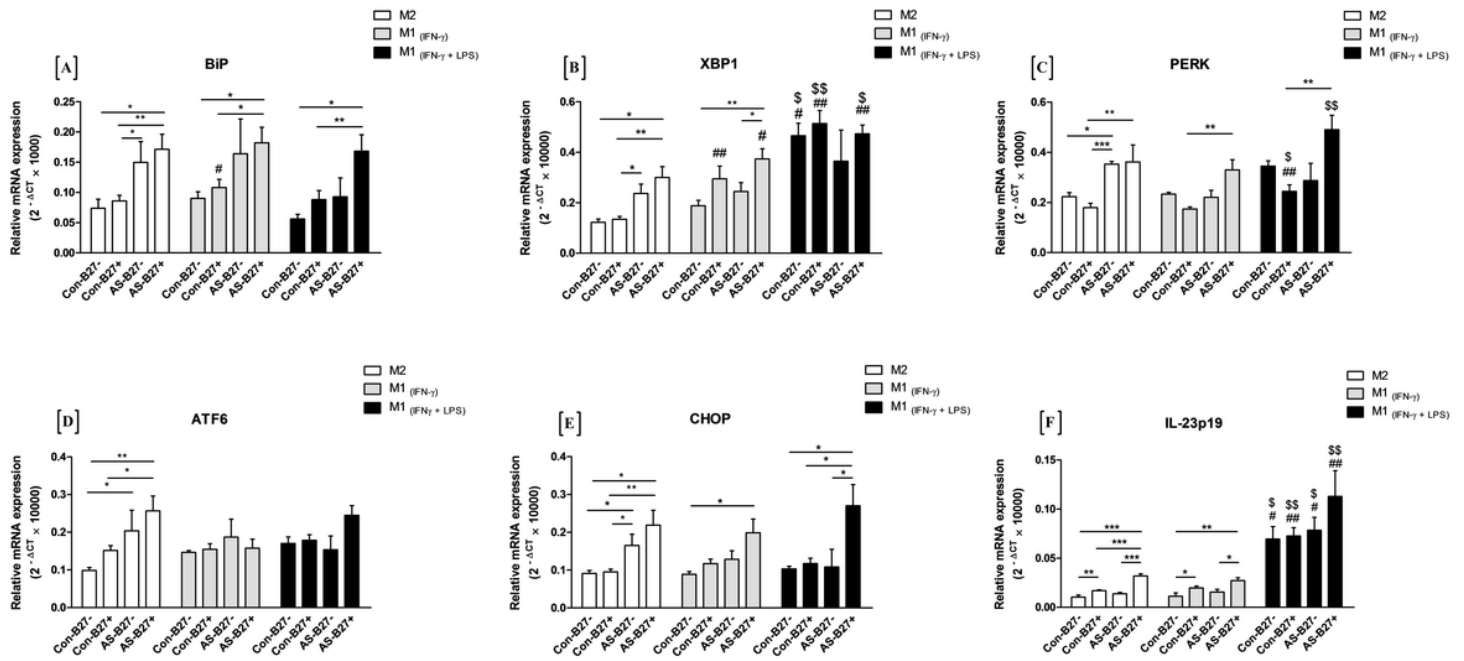
## Figure 2

mRNA expression levels of ER-stress related genes, including BiP (A), XBP1 (B), PERK (C), ATF6 (D), CHOP (E), and IL-23p19 (F) in AS patients and healthy subjects. Monocytes were isolated from 10 HLA-B27 positive AS patients (AS-B27+), 5 HLA-B27 negative AS patients (AS-B27-), 10 HLA-B27 positive healthy controls (Con-B27+) and 5 HLA-B27 negative healthy controls (Con-B27-) and were differentiated into M2 macrophages upon treating with M-CSF in 7 days. M2 macrophages were then polarized into M1 macrophages after treating with IFN-γ alone or in combination with LPS for 24 hrs. The mRNA expression levels of the mentioned genes in these three cell groups were compared between patients and controls. Results are presented as mean ± standard error of the mean (SEM). Significance within each cell group was indicated with "*" sign, between M1 (IFN-γ) and M1 (IFN-γ + LPS) group compared to M2 with "#" sign, and between M1 (IFN-γ) and M1 (IFN-γ + LPS) group by "$" sign.
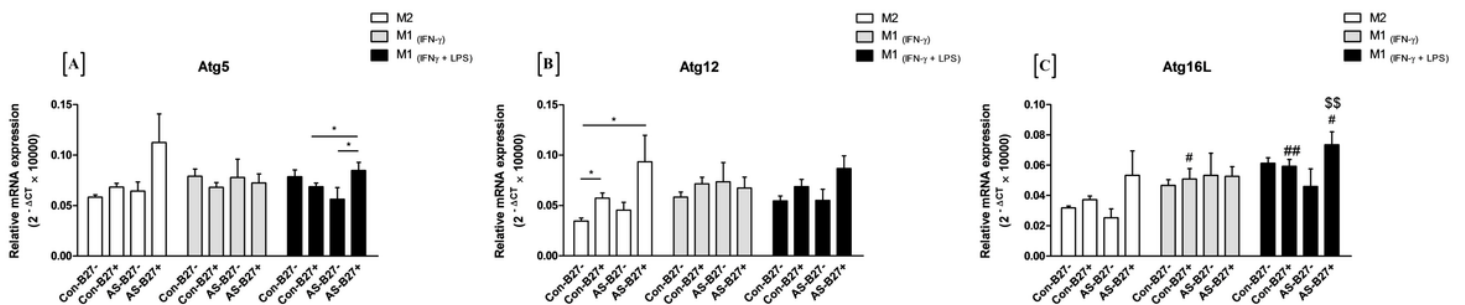


## Figure 3

mRNA expression levels of autophagy-related genes, including Atg5 (A), Atg12 (B), and Atg16L (C) in AS patients and healthy subjects. Monocytes were isolated from 10 HLA-B27 positive AS patients (AS-B27+), 5 HLA-B27 negative AS patients (AS-B27-), 10 HLA-B27 positive healthy controls (Con-B27+) and 5 HLA-

B27 negative healthy controls (Con-B27-) and were differentiated into M2 macrophages upon treating with M-CSF in 7 days. M2 macrophages were then polarized into M1 macrophages after treating with IFN-γ alone or in combination with LPS for 24 hrs. The mRNA expression levels of the mentioned genes in these three cell groups were compared between patients and controls. Results are presented as mean ± standard error of the mean (SEM). Significance within each cell group was indicated with "*" sign, between M1 (IFN-γ) and M1 (IFN-γ + LPS) group compared to M2 with "#" sign, and between M1 (IFN-γ) and M1 (IFN-γ + LPS) group by "$" sign.
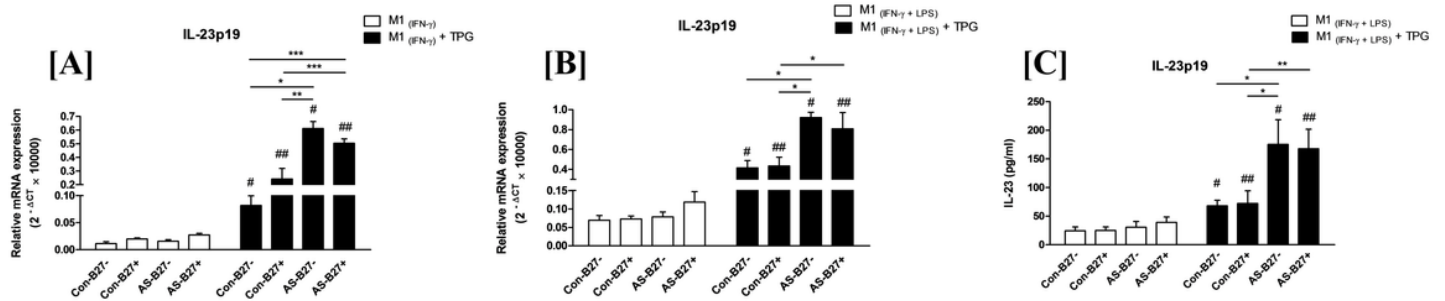


**Figure 4**

Bar graphs indicate the mRNA expression and soluble protein levels of IL-23p19 in the patients and healthy controls before and after TPG treatment (to induce ER stress). Monocytes were isolated from 10 HLA-B27 positive AS patients (AS-B27+), 5 HLA-B27 negative AS patients (AS-B27-), 10 HLA-B27 positive healthy controls (Con-B27+) and 5 HLA-B27 negative healthy controls (Con-B27-) and were differentiated into M2 macrophages upon treating with M-CSF in 7 days. M2 macrophages were then polarized into M1 macrophages after treating with IFN-γ alone or in combination with LPS for 24 hrs. To induce ER stress, TPG at a concentration of 500 nM was added into M1 macrophages and incubated for 12 hrs. The mRNA expression and serum levels of IL-23p19 were compared between patients and controls. Soluble levels of IL-23p19 cytokine in the supernatant of the IFN-γ+LPS-induced M1 macrophages were measured by ELISA and then were compared between patients and controls. A; mRNA expression of IL-23p19 in the IFN-γ-induced M1 macrophages. B; mRNA expression of IL-23p19 in the IFN-γ+LPS-induced M1 macrophages. C; Soluble leves of IL-23p19 in the supernatant of the IFN-γ+LPS-induced M1 macrophages. Results are presented as mean ±standard error of the mean (SEM). Significance within each cell group is marked with "*" sign, and between different cell groups with # sign.
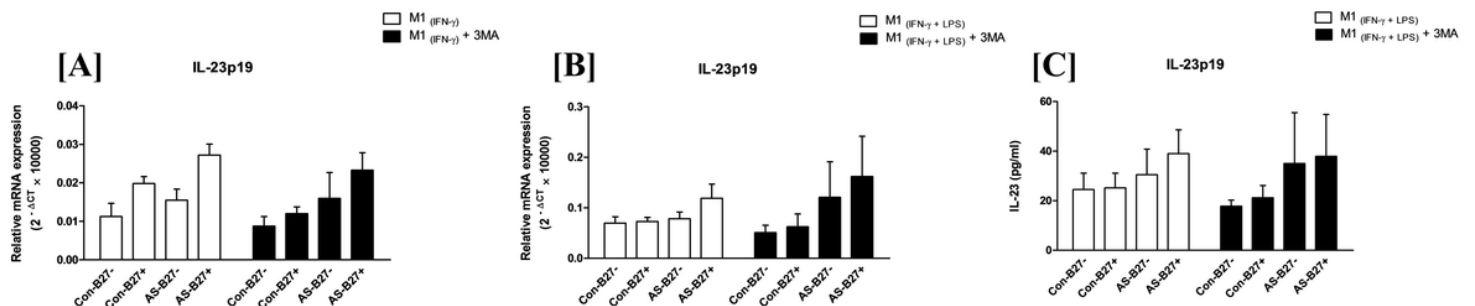


**Figure 5**

Bar graphs indicate the mRNA expression and soluble protein levels of IL-23p19 in the patients and healthy controls before and after 3MA treatment (to inhibit autophagy pathway). Monocytes were isolated from 10 HLA-B27 positive AS patients (AS-B27+), 5 HLA-B27 negative AS patients (AS-B27-), 10 HLA-B27 positive healthy controls (Con-B27+) and 5 HLA-B27 negative healthy controls (Con-B27-) and were differentiated into M2 macrophages upon treating with M-CSF in 7 days. M2 macrophages were then polarized into M1 macrophages after treating with IFN-γ alone or in combination with LPS for 24 hrs. For inhibiting the autophagy pathway, 3MA at a concentration of 5 mM was added into M1 macrophages and incubated for 24 hrs. The expression levels of IL-23p19 mRNA in these cell groups were compared between patients and controls. Soluble levels of IL-23p19 cytokine in the supernatant of the IFN-γ+LPS-induced M1 macrophages were measured by ELISA and then were compared between patients and controls. A; mRNA expression of IL-23p19 in the IFN-γ-induced M1 macrophages. B; mRNA expression of IL-23p19 in the IFN-γ+LPS-induced M1 macrophages. C; Soluble leves of IL-23p19 in the supernatant of the IFN-γ+LPS-induced M1 macrophages. Results are presented as mean ±standard error of the mean (SEM).

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementary.docx