Macrophage polarization in spondyloarthritis
Ambru, C.A.

Citation for published version (APA):

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The effects of endoplasmatic reticulum stress on macrophage polarization and cytokine production: relevance for HLA-B27+ spondyloarthritis

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The effects of endoplasmatic reticulum stress on macrophage polarization and cytokine production: relevance for HLA-B27+ spondyloarthritis

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ABSTRACT

Objective: Spondyloarthritis (SpA) synovitis is characterized by altered macrophage phenotype and cytokine production. In experimental settings, misfolding of HLA-B27 in the endoplasmic reticulum (ER) can induce an unfolded protein response (UPR) and increase pro-inflammatory cytokine production. Therefore, we investigated the role of ER stress in macrophage polarization and cytokine production in HLA-B27+ human SpA.

Methods: Peripheral blood-derived macrophages (PBDMs) from healthy donors (HD) were stimulated with thapsigargin (TG), polarized by IFN-γ (MΦ_{IFN-γ}) or IL-10 (MΦ_{IL-10}), and phenotyped by flow cytometry. Polarized PBDMs from HD, HLA-B27- and HLA-B27+ SpA patients, and rheumatoid arthritis (RA) patients were stimulated with TG and/or LPS. ER stress markers (BiP, CHOP, ERdj4) and cytokines (IL-23, IL-12, TNF, IL-10) were measured by qRT-PCR and/or Luminex.

Results: ER stress induced by TG in vitro impaired macrophage polarization towards a MΦ_{IL-10} phenotype. TG also impaired the LPS-induced production of TNF (p < 0.05) and to a lesser extent IL-23, IL-12 and IL-10 by MΦ_{IFN-γ} but strongly increased the TNF production by MΦ_{IL-10} (p < 0.01). Polarized PBDMs of HLA-B27+ SpA did not show elevated expression of ER stress markers. Ex vivo LPS stimulated MΦ_{IFN-γ} from HLA-B27+ SpA patients tended to express more IL-23p19 versus IL-10 than MΦ_{IFN-γ} from HLA-B27- SpA (p = 0.09) and RA patients (p = 0.07). MΦ_{IL-10} expressed less IL-10 in HLA-B27+ versus HLA-B27- SpA patients upon LPS stimulation (p < 0.05).

Conclusion: These data question the relevance of HLA-B27-induced ER stress for the cytokine production of polarized macrophages in SpA, since the in vitro TG data do not correspond with ER stress marker and cytokine profiles of HLA-B27+ SpA PBDMs. The latter are mainly characterized by decreased IL-10 expression.
ER stress in macrophages is not driving the altered cytokine production in HLA-B27+ spondyloarthritis

INTRODUCTION

Spondyloarthritis (SpA) displays a strong familial aggregation, which is related to the genetic background of the disease [1]. Among these genes, HLA-B27 is responsible for approximately 40% of the inherited risk of developing ankylosing spondylitis (AS), which is the prototype of SpA, and plays also a dominant role in the other SpA subtypes [2,3]. Originally, HLA-B27 has been proposed to contribute to the disease pathogenesis by presenting bacterial antigens and/or cross-reactive autoantigens to CD8+ T lymphocytes [4]. However, depletion of CD8+ cells did not prevent disease in the HLA-B27/human β2m (B27/hβ2m) transgenic rat model of SpA [5,6]. More generally, the absence of common autoimmune genetic risk factors, female predominance, autoantibodies, and response to T or B cell targeted therapies questioned the role of autoimmune lymphocytes in the pathogenesis of the disease [7]. Several lines of evidence indicate a prominent role of innate immune cells rather than lymphocytes in SpA [1,7–9]. Among these, immunopathological analysis indicated an altered macrophage polarization and cytokine production in SpA synovitis [10–13]. This concept was supported by the shifted TNF/IL-10 balance in innate immune cells from HLA-B27 transgenic rats [14], as well as by the reverse IFN-γ signature [15] and increased LPS-induced IL-23 production by human AS monocyte-derived macrophages [16]. Several mechanisms, including changes in TLR expression [14,17], may contribute to the altered macrophage function in SpA. Of particular interest is the role of HLA-B27 in this process, since this molecule has a tendency to misfold in the endoplasmic reticulum (ER) and thereby generate ER stress [18,19]. ER stress can inhibit protein translation, upregulate ER chaperone molecules such as BiP and ERdj4, and activate transcription factors like CHOP. In combination with TLR stimuli, chemical induction of this so-called unfolded protein response (UPR) leads to a specific increase in IL-23 production by both rat bone marrow-derived macrophages (BMDMs) [20] and human monocyte-derived dendritic cells (DCs) [21]. Furthermore, an UPR as a result of HLA-B27 overexpression was shown in the B27/hβ2m rats [22,23] and was characterized by a predominant increase in IL-23 production and Th17 activation [20]. Taken together, these data suggest that HLA-B27-induced UPR may contribute to the altered macrophage polarization and function in SpA.

In contrast with these experiments using either strong chemical induction of ER stress or non-physiological HLA-B27 overexpression, however, the recent study by Zeng at al could not correlate the altered LPS-induced cytokine production of AS macrophages with significant induction of UPR [16]. Therefore, this study aimed to investigate in more detail whether the link between UPR and altered macrophage function, which has been well established in experimental setting, could also explain the aberrant macrophage polarization and cytokine production in HLA-B27+ SpA. Since our previous work suggested an IL-10 polarized...
macrophage (MΦ_{IL-10}) signature (alternative macrophage activation) in SpA versus an IFN-γ polarized (MΦ_{IFN-γ}) signature (classical macrophage activation) in rheumatoid arthritis (RA) [10–13], we studied the effects of ER stress in these two macrophage subsets.

MATERIALS AND METHODS

Patients

Peripheral blood samples were obtained from 6 healthy controls (HCs), 17 SpA and 9 RA patients. SpA and RA patients fulfilled the criteria of the European Spondyloarthropathy Study Group (ESSG) [24] and the ACR classification criteria for RA [25], respectively. The demographic and clinical data of the patients are shown in Table 1. All patients had active disease as defined by at least one swollen joint and/or inflammatory back pain and none of the patients was treated with biologicals. All patients gave written informed consent to participate to the study, as approved by the Medical Ethics Committee of the Academic Medical Centre/University of Amsterdam.

<table>
<thead>
<tr>
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<th>HD (n=6)</th>
<th>SpA (n=17)</th>
<th>RA (n=9)</th>
</tr>
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<tbody>
<tr>
<td>Number of men/women</td>
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<td>11/6</td>
<td>3/6</td>
</tr>
<tr>
<td>Age (years)</td>
<td>31 (2)</td>
<td>47 (15)</td>
<td>52 (15)</td>
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<tr>
<td>Disease duration (years)</td>
<td>NA</td>
<td>8 (11)</td>
<td>7 (6)</td>
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<tr>
<td>Swollen joint count</td>
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<td>1,5 (2,6)</td>
<td>4,3 (2,8)</td>
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<tr>
<td>CRP, mg/L</td>
<td>-</td>
<td>11 (18)</td>
<td>7 (9)</td>
</tr>
<tr>
<td>ESR, mm/hour</td>
<td>-</td>
<td>12 (13)</td>
<td>19 (16)</td>
</tr>
<tr>
<td>HLA-B27 positivity, %</td>
<td>0</td>
<td>53</td>
<td>0</td>
</tr>
<tr>
<td>BASDAI</td>
<td>NA</td>
<td>4,8 (1,9)</td>
<td>NA</td>
</tr>
<tr>
<td>DMARDs, %</td>
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<td>29</td>
<td>100</td>
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</table>

Table 1. Demographic and clinical features of healthy donors, spondyloarthritis (SpA) and rheumatoid arthritis (RA) patients. Unless indicated otherwise, data are represented as mean (standard deviation). NA = not applicable. CRP = C-reactive protein. ESR = erythrocyte sedimentation rate. BASDAI = Bath Ankylosing Spondylitis Disease Activity Index. DMARD = Disease Modifying Anti-Rheumatic Drugs.

Monocyte isolation and in vitro polarization and stimulation

Monocytes were isolated from peripheral blood by gradient centrifugation as previously described [26]. For the analysis of cell phenotype, we stimulated freshly isolated monocytes with TG 0,01 uM for 1 h, followed by washing of the medium and 4 days polarization in the presence of human recombinant IFN-γ (50 ng/ml; R&D Systems, Abingdon, UK) or IL-10 (50 ng/ml; R&D Systems). In the experiments investigating the effect of TG on the ER stress marker and cytokine expression, we first generated MΦ_{IFN-γ} and MΦ_{IL-10} and subsequently stimulated them with TG 0,01 uM for 2 h, in the presence or absence of LPS 500 ng/ml or 10 ng/ml for an additional 4 h.
ER stress in macrophages is not driving the altered cytokine production in HLA-B27+ spondyloarthritis

**Flow cytometry**

Cell viability after TG/LPS stimulation was measured by flow cytometry (BD FACS Calibur Flow Cytometer, Erembodegem, Belgium) using TO-PRO®-3 stain (Invitrogen, Bleiswijk, The Netherlands) and was circa 90%. Phenotypic marker expression on in vitro polarized macrophages was measured using fluorochrome-labeled monoclonal antibodies against CD32 (clone AT10, abcam, Cambridge, UK), CD64 (clone 10.1, BioLegend, Uithoorn, The Netherlands), CD80 (clone L307.4, BD Pharmingen, Breda, The Netherlands) and CD163 (clone GHI/61, BD Pharmingen), as previously described [26]. Values were expressed as the ratio of the geometric mean fluorescence intensity (gMFI) of the marker of interest over the gMFI of the isotype control.

**Quantitative real-time PCR**

Total RNA isolation from in vitro polarized macrophages and RNA concentration measurement was done as previously described [27]. The primer sequences are shown in Table 2. The mRNA expression levels were normalized to those of the human housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Oligonucleotide primers were designed using the online tool for Real-time PCR (TaqMan) Primer Design (Genscript) (IL-10, IL-12, IL-23 and TNF) and the online tool qPrimerDepot of the National Institutes of Health (NIH) (BiP, CHOP and ERdj4) and obtained from Invitrogen.

**Multiplex immunoassay**

Supernatants from the TG and/or LPS stimulated MΦ<sub>IFN-γ</sub> and MΦ<sub>IL-10</sub> were collected and analyzed using a multiplex immunoassay as previously described [28].

<table>
<thead>
<tr>
<th></th>
<th>Forward</th>
<th>Reverse</th>
</tr>
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<tbody>
<tr>
<td>BiP</td>
<td>CATCACGCGGTCTATGTCG</td>
<td>CGTCAAAGACCGTGTTCTTCG</td>
</tr>
<tr>
<td>CHOP</td>
<td>AGCCAAAATCAGAGCTGGAA</td>
<td>TGGATCAGTCTGGAAAGCA</td>
</tr>
<tr>
<td>ERdj4</td>
<td>AATGCAGATTGCCAGTGAAAGATGAAA</td>
<td>CAGCTCTGTGGAGGACG</td>
</tr>
<tr>
<td>IL-10</td>
<td>GATGCTTCAGCACAGTGAA</td>
<td>CCCAGGTAACCCTAAAGTC</td>
</tr>
<tr>
<td>IL-12p35</td>
<td>ACCAGGTGAGTCCAAGACC</td>
<td>TGGCACAGTCTCAGTGGTA</td>
</tr>
<tr>
<td>IL-12/-23p40</td>
<td>AAGGAGGCCGAGTTCTAAGC</td>
<td>TGGGTTCTTTCTGGTCTTT</td>
</tr>
<tr>
<td>IL-23p19</td>
<td>TTCTCTGCTCCCTGATAGCC</td>
<td>CCTCAGGCCTGAGGAGTT</td>
</tr>
<tr>
<td>TNF</td>
<td>CCCATGTTGTAGCAAAACCT</td>
<td>TGAGGTACAGGCCCTCTGAT</td>
</tr>
</tbody>
</table>

Table 2. qPCR primer sequences for BiP, CHOP, ERdj4, IL-10, IL-12p35, IL-12/-23p40, IL-23p19 and TNF.
Statistics
Statistical analysis was performed using Prism software (GraphPad, La Jolla, CA). Data were expressed as mean ± SEM, or median ± interquartile range. Paired t test (for the TG experiments) and Mann-Whitney test (for the experiments with PBDMs from HD and patients) were used for comparisons between 2 groups. One-way Anova followed by Bonferroni posttest (for the TG experiments) and Kruskal-Wallis followed by Dunn’s posttest (for the experiments with PBDMs from HD and patients) were used for comparisons between more than 2 groups. A P value of less than 0.05 was considered to be statistically significant.

RESULTS
TG-induced ER stress does not selectively promote polarization towards $\text{MΦ}_{\text{IL-10}}$
Previous immunopathological studies suggested that SpA is characterized by a $\text{MΦ}_{\text{IL-10}}$ signature compared to the $\text{MΦ}_{\text{IFN-γ}}$ signature in RA [10,11,13,15]. To assess whether ER stress promotes $\text{MΦ}_{\text{IL-10}}$ polarization, we stimulated HD peripheral blood monocytes with TG before polarizing them with IFN-γ or IL-10 and subsequently measured the expression of polarization markers CD80 and CD64 for $\text{MΦ}_{\text{IFN-γ}}$ and CD163 and CD32 for $\text{MΦ}_{\text{IL-10}}$ [26]. TG alone did not upregulate any of the polarization markers (data not shown). IFN-γ induced up-regulation of CD80 independently of exposure to TG (Figure 1A) but failed to upregulate CD64 after exposure to TG ($p < 0.05$ in comparison with cells without TG) (Figure 1B). As to IL-10 polarization, pre-incubation with TG prevented the up-regulation of both $\text{MΦ}_{\text{IL-10}}$ markers CD163 and CD32 ($p < 0.05$ in comparison with cells without TG) (Figure 1C and D). These results indicate that TG-induced ER stress does not selectively promote but, on the contrary, impairs $\text{MΦ}_{\text{IL-10}}$ polarization as assessed by phenotypical markers.

TG impairs LPS-induced cytokine production by $\text{MΦ}_{\text{IFN-γ}}$
TG-induced ER stress was reported to strongly induce IL-23 transcription and induction in M-CSF differentiated bone marrow-derived rat macrophages and human monocyte-derived DCs [20,21]. As $\text{MΦ}_{\text{IFN-γ}}$ are the major producers of pro-inflammatory cytokines upon TLR ligation in comparison with other macrophage subsets in humans [27], we first assessed whether TG also augmented IL-23 production in this human macrophage subset. Analysis of cytokines secreted in the supernatant indicated that TG alone did not affect IL-23, IL-12, TNF and IL-10 secretion (Figure 2A-D). As expected, LPS stimulation significantly upregulated all investigated cytokines ($p < 0.001$ for TNF, $p < 0.01$ for IL-23 and IL-12, $p < 0.05$ for IL-10) (Figure 2A-D). The combination of TG and LPS, however, decreased the LPS-induced production of TNF ($p < 0.05$) and to a lesser extent IL-23, IL-12 and IL-10 in comparison to LPS alone (Figure 2A-D). We performed 3 additional sets of experiments to confirm these data. Firstly, we
ER stress in macrophages is not driving the altered cytokine production in HLA-B27+ spondyloarthritis

Figure 1. Expression of MΦ_{IFN-γ} and MΦ_{IL-10} phenotypic markers after TG stimulation. Monocytes from peripheral blood of healthy donors were stimulated with TG for 1h, followed by polarization in the presence of IFN-γ or IL-10. The membrane expression of MΦ_{IFN-γ} markers CD80 (A) and CD64 (B) and MΦ_{IL-10} markers CD163 (C) and CD32 (D) in the presence (black bars) or absence of TG (white bars) was measured by flow cytometry. Bars represent the mean (SEM) of 4 HDs. *p < 0.05

Figure 2. Cytokine protein and mRNA expression by MΦ_{IFN-γ} after stimulation with TG and/or LPS. Graphs represent IL-23, IL-12, TNF and IL-10 production after stimulation with TG and/or LPS 500 ng/ml, measured by Luminex (A-D) and IL-23p19, IL-12p35, TNF, IL-10 and IL-12/-23p40 mRNA expression after stimulation with LPS ± TG, measured by qRT-PCR and normalized to the expression of the human housekeeping gene GAPDH (E-I). mRNA levels are expressed as fold increase induced by LPS ± TG compared to no stimulus. Bars represent the mean (SEM) of 6 HDs. *p < 0.05, **p < 0.01, ***p < 0.001
confirmed that TG did genuinely induce ER stress in our experiments by demonstrating an upregulation of BiP (2-fold), CHOP (7-fold) and ERdj4 (7-fold) in TG-exposed versus resting MΦ \(_{\text{IFN-γ}}\). Secondly, we repeated these experiments with lower doses of LPS (10 ng/ml instead of 500 ng/ml) and obtained similar findings (data not shown). Thirdly, we assessed the cytokine expression by qPCR in order to exclude that a toxic effect of TG on macrophages may cause the lower cytokine secretion in LPS+TG versus LPS stimulated MΦ \(_{\text{IFN-γ}}\). Also at mRNA level, TG alone failed to upregulate any of the investigated cytokines (data not shown). In contrast, LPS strongly upregulated the transcription of all cytokines but this was again tempered by addition of TG, with a trend towards statistical significance for IL-23p19 (p = 0.07) and TNF (p = 0.08) (Figure 2E-I). Taken together, these data indicate that TG-induced ER stress does not augment but, on the contrary, tends to impair the production of TNF, IL-23 and other cytokines by human MΦ \(_{\text{IFN-γ}}\).

**TG potentiates LPS-induced TNF production by MΦ \(_{\text{IL-10}}\)**

We next performed similar assays using MΦ \(_{\text{IL-10}}\) as, despite being poor cytokine producers in vitro [27], the presence of MΦ \(_{\text{IL-10}}\)-like cells strongly correlates with disease activity in vivo in SpA [29,30]. In contrast to MΦ \(_{\text{IFN-γ}}\), MΦ \(_{\text{IL-10}}\) secreted very low amounts of IL-23, IL-12, TNF and IL-10 in resting conditions as well as upon LPS stimulation (both 500 and 10 ng/ml) (data not shown). TG alone had no influence on the cytokine secretion (data not shown). Combination of TG with LPS, however, strongly upregulated the production of TNF (p < 0.01) but not of other studied cytokines in comparison to LPS stimulation alone (Figure 3A). The TNF levels in TG+LPS stimulated MΦ \(_{\text{IL-10}}\) (9.7 ± 2.2 ng/ml) were even comparable to those produced by TG+LPS stimulated MΦ \(_{\text{IFN-γ}}\) (10.7 ± 2.8 ng/ml). In the same manner as in the experiments with MΦ \(_{\text{IFN-γ}}\), we confirmed these data by a) demonstrating that TG induced the UPR-related gene BiP in MΦ \(_{\text{IL-10}}\) (data not shown), b) obtaining similar results with lower doses of LPS (10 ng/ml) (Figure 3B) and c) showing a similar albeit not statistically significant trend at the transcript level (Figure 3C). These data indicate that TG-induced ER stress has a differential effect on cytokine production by MΦ \(_{\text{IFN-γ}}\) and MΦ \(_{\text{IL-10}}\) and, in particular, strongly potentiates TNF secretion by MΦ \(_{\text{IL-10}}\).

**ER stress markers are not increased in peripheral blood-derived macrophages from HLA-B27+ SpA patients**

All previous experiments were performed with TG-induced ER stress in macrophages from HD. We next investigated if a similar mechanism may be operative in SpA macrophages under influence of HLA-B27 misfolding. We first assessed whether we could detect an increased expression of ER stress-induced genes in PBDMs from HLA-B27+ SpA patients in
ER stress in macrophages is not driving the altered cytokine production in HLA-B27+ spondyloarthritis.

Figure 3. TNF protein and mRNA expression by MΦ after stimulation LPS ± TG. Graphs represent TNF production after stimulation with LPS 500 ng/ml (A) or 10 ng/ml (B), measured by Luminex and TNF mRNA expression measured by qRT-PCR and normalized to the expression of the human housekeeping gene GAPDH (C). mRNA levels are expressed as fold increase induced by LPS ± TG compared to no stimulus. Bars represent the mean (SEM) of 6 HDs. **p < 0.01

Figure 4. Expression of IL-23p19 and IL-10 by LPS-stimulated MΦ derived from peripheral blood of HD, SpA and RA patients. Graphs represent mRNA expression of IL-23p19 (A) and IL-10 (B) by MΦ from HD, SpA and RA patients, mRNA expression of IL-23p19 (C) and IL-10 (D) by MΦ from HLA-B27- versus HLA-B27+ SpA patients and IL-23p19/IL-10 ratio in HD, HLA-B27- and HLA-B27+ SpA and RA patients (E). mRNA levels were measured by qRT-PCR, normalized to the expression of the human housekeeping gene GAPDH and expressed as fold increase induced by LPS compared to no stimulus. Bars represent the median (interquartile range) of the donors depicted in Table 1.
comparison with HD, HLA-B27- SpA and RA patients. MΦ_{IFN-γ} and MΦ_{IL-10} from HD, SpA and RA patients expressed similar BiP mRNA levels, both in the absence and in the presence of LPS stimulation (data not shown). There was also no difference in BiP expression between HLA-B27+ and HLA-B27- SpA (data not shown). Since IFN-γ is known to up-regulate the expression of HLA-B27, thus increasing the risk for ER stress, we additionally measured the mRNA levels of CHOP and ERdj4 in LPS-stimulated MΦ_{IFN-γ} and again found no differences between SpA and RA samples or between HLA-B27+ and HLA-B27- SpA (data not shown). In conclusion, ER stress markers were not increased in polarized macrophages from HLA-B27+ SpA patients compared to controls, even after LPS stimulation.

**Altered IL-23/IL-10 expression by MΦ_{IFN-γ} from HLA-B27+ SpA patients**

As the expression of ER stress-induced genes may not be sensitive enough to detect low grade UPR, we next measured the expression of cytokines by PBDMs of HLA-B27+ SpA patients versus controls to assess whether these cells display the same cytokine patterns as observed with TG in vitro. MΦ_{IFN-γ} from HD, SpA and RA patients expressed similar levels of IL-12p35, IL-12p40, and TNF mRNA upon LPS stimulation (data not shown). Also the levels of IL-23p19 and IL-10 were not statistically different (Figure 4A and B) but upon stratification of SpA according to HLA-B27 status, HLA-B27+ SpA tended to express more IL-23p19 (Figure 4C) and less IL-10 (Figure 4D) than HLA-B27- SpA (p = 0.09). Accordingly, the ratio of IL-23p19 to IL-10 was shifted in HLA-B27+ SpA versus HLA-B27- SpA (8-fold, p = 0.09), RA (9-fold, p = 0.07) and HC (11-fold, p = 0.43) (Figure 4E).

**Impaired IL-10 expression by MΦ_{IL-10} from HLA-B27+ SpA patients**

We performed a similar analysis in LPS-stimulated MΦ_{IL-10} from HD, SpA and RA patients. In contrast to MΦ_{IFN-γ}, LPS failed to induce IL-23p19 expression in MΦ_{IL-10} (Figure 5A). IL-10 expression was similar in all diseases (Figure 5B). Stratification of SpA according to HLA-B27 status showed no differences for IL-23p19 (Figure 5C) but a significant decrease in IL-10 expression in HLA-B27+ versus HLA-B27- SpA (p < 0.05) (Figure 5D). In contrast with the in vitro experiments with TG indicating that ER stress potentiates LPS-induced TNF production by MΦ_{IL-10}, TNF production by LPS-stimulated MΦ_{IL-10} in HLA-B27+ SpA was similar or even slightly lower than in HLA-B27- SpA, RA or HD (data not shown).

**DISCUSSION**

In this study, we aimed to assess the potential impact of ER stress on pro-inflammatory cytokine production by macrophages in SpA. Experimental studies have indicated that HLA-B27 misfolding can lead to ER stress and thereby lead to altered TLR-induced pro-
ER stress in macrophages is not driving the altered cytokine production in HLA-B27+ spondyloarthritis. Of particular interest is the specific upregulation of IL-23 as the genetic association of AS with IL-23R polymorphisms [31–33], the development of enthesitis in IL-23 overexpressing mice [34], and the beneficial effects of IL-12/-23p40 blockade in PsA and Crohn’s disease [35,36] suggest that aberrant IL-23 signalling plays a key role in the pathogenesis of SpA [37]. A recent study, however, questioned the relevance of ER stress-induced IL-23 production in human AS macrophages as a) not only IL-23 but also other pro-inflammatory and anti-inflammatory cytokines (including IL-10) were upregulated in comparison with healthy controls and b) there was no correlation between this altered cytokine production and ER stress [16]. This observation urged us to investigate in more detail the impact of ER stress on human macrophages and the relevance of this process to human SpA.

A first key finding of the present study is that the effect of ER stress on myeloid cells is highly dependent on the exact cell type. Using two different types of macrophages, MΦ_{IFN-γ} and MΦ_{IL-10}, derived from the same healthy controls and processed in parallel we observed...
marked differential effects of TG-induced ER stress on macrophage cytokine production after TLR stimulation. In particular, TG suppressed TNF production by MΦ<sub>IFN-γ</sub> but selectively potentiated TNF production by MΦ<sub>IL-10</sub>. These effects in MΦ<sub>IFN-γ</sub> and MΦ<sub>IL-10</sub> are also clearly different from the selective upregulation of IL-23 in human DCs [21]. Taken together, these data strengthen the concept that macrophage polarization has an important impact on their subsequent functional behaviour, not only as a response to TLR ligation [27], but also to ER stress induction and thus plead for careful analysis and description of the exact myeloid cell subtype used in different in vitro studies.

The most striking observation of the in vitro assays with TG was the marked enhancement of TNF production by MΦ<sub>IL-10</sub>. MΦ<sub>IL-10</sub> were originally classified as alternatively activated macrophages with immunoregulatory properties, as reflected by their steady state production of IL-10 [27,38]. MΦ<sub>IL-10</sub> are also known as deactivated macrophages since, in contrast to MΦ<sub>IFN-γ</sub> and MΦ<sub>IL-4</sub>, they fail to produce significant amounts of pro- and anti-inflammatory cytokines upon TLR stimulation [27,39]. Our data indicate that this concept is probably not correct as, after ER stress induction, they produce similar levels of TNF as MΦ<sub>IFN-γ</sub> upon LPS stimulation. Intriguingly, this effect seems to be specific for TNF and is not observed for IL-23 and IL-12, raising the question of the underlying molecular mechanisms. One hypothesis which deserves further research is that ER stress counteracts the immunomodulatory effects of IL-10, as suggested by the fact that TG-exposed macrophages failed to upregulate phenotypic MΦ<sub>IL-10</sub> markers. A cross-regulation between ER stress and IL-10 has been described previously in intestinal inflammation [40] and would fit with the observation that alleviation of HLA-B27-induced ER stress by beta2-microglobulin overexpression allowed to dissociate arthritis from colitis (which is very sensitive to IL-10 immunoregulation) in HLA-B27 transgenic rats [41].

The key question of the present study was whether these in vitro effects of TG-induced ER stress are relevant to the altered cytokine production by macrophages in HLA-B27+ SpA. Our ex vivo data with MΦ<sub>IFN-γ</sub> and MΦ<sub>IL-10</sub> could not completely reproduce the findings of Zeng et al in MΦ<sub>M-CSF</sub> [16] as our data indicate a modest but selective increase in IL-23 expression in HLA-B27+ SpA MΦ<sub>IFN-γ</sub> rather than a global increase of a broad panel of pro- and anti-inflammatory cytokines, including IL-23 and IL-10. This discrepancy, however, may relate to the previously highlighted functional difference between different polarized macrophage subsets used in these studies. More striking than the upregulation of IL-23, however, was the selective decrease in IL-10 expression in HLA-B27+ SpA macrophages. This decreased IL-10 production was found in both MΦ<sub>IFN-γ</sub> and MΦ<sub>IL-10</sub> and may thus reflect a more robust and general biological phenomenon in SpA.

Interestingly, the ex vivo cytokine profile alterations of HLA-B27+ SpA macrophages (increase
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in IL-23 in MΦ_{IFN-γ} and decrease in IL-10 in both subsets) did not correspond with the in vitro effects of TG-induced ER stress on the same polarized macrophage subsets. In agreement with the findings by Zeng et al [16], these data suggest that the altered cytokine production by HLA-B27+ SpA macrophages is not primarily related to the presence of ER stress. Again in agreement with the findings of Zeng et al, we could not find clear evidence of enhanced ER stress in HLA-B27+ SpA macrophages as measured by the expression of BiP, CHOP, and ERdj4. These data should be interpreted with caution, however, as we cannot exclude that this approach is not sensitive enough to detect discrete levels of HLA-B27-induced ER stress and/or that other signals besides HLA-B27 are required to lead to significant ER stress. It can also be envisaged that these additional signals are present in specific target tissues but not in peripheral blood macrophages of SpA. We previously proposed that several stress-signals (including but not restricted to HLA-B27 misfolding) might integrate at the level of CHOP to regulate pro-inflammatory cytokine production [37]. Further research to test this hypothesis should thus include functional analysis of macrophages recovered from inflamed joints and/or gut mucosa.

In conclusion, we demonstrate here that ER stress strongly influences the production of pro-inflammatory cytokines by specific human polarized macrophage subtypes in vitro and, in particular, potentiates the LPS-induced production of TNF by MΦ_{IL-10}. We hypothesize that ER stress may impair the responsiveness of macrophages to the immunomodulatory effects of IL-10. LPS stimulated PBDMs of HLA-B27+ SpA patients, however, do not display transcriptional or functional signs of increased ER stress compared to the controls, but consistently show impaired IL-10 expression. A combination of ER stress and impaired IL-10 expression in specific target tissues such as the gut or the joint may thus contribute to macrophage-mediated inflammation in SpA.

Acknowledgements
D. Baeten is supported by a VIDI grant from The Netherlands Organization for Scientific Research (NWO) and by a grant from the Dutch Arthritis Foundation (Reumafonds).

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