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Ankylosing spondylitis macrophages produce greater interleukin-23 in response to lipopolysaccharide without significant Unfolded Protein Response induction

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Abstract

OBJECTIVE—Previous work from the HLA-B27 transgenic rat model of ankylosing spondylitis (AS) suggests that macrophages develop an intracellular stress response called the Unfolded Protein Response (UPR) and as a result secrete increased cytokines in response to Toll like receptor agonists such as lipopolysaccharide (LPS). Our objective was to determine if macrophages from AS patients also undergo an UPR and secrete increased cytokines/chemokines in response to LPS.

METHODS—Peripheral blood monocytes isolated from 10 AS patients and healthy controls were differentiated *in vitro* with M-CSF. Select samples were treated with IFN-γ to up-regulate MHC class I (HLA-B) expression prior to stimulation with LPS for 3h (for RNA collection), or 8–24h (for supernatant collection). UPR induction was assessed by expression of ERdj4, BiP and CHOP mRNA.

RESULTS—Although IFN-γ treatment up-regulated HLA-B expression 2-fold(p=0.0094), neither IFN-γ nor LPS enhanced BiP or CHOP expression substantially (<1.3 fold). ERdj4 expression increased weakly but insignificantly in IFN-γ+LPS AS samples (2.2 fold, p=0.31). In response to LPS, AS macrophages secreted more CXCL9, IL-10, IL-12p70, IL-23, and TNF-α than controls($p \leq 0.025$)The most striking difference was observed for IL-23 (median AS patient 265 pg/mL vs. control 9 pg/mL, p=0.0007). We did not detect significant differences in IL-6, IL-8, or IFN-β production.

CONCLUSIONS—Greater IL-23 production by AS patient macrophages in response to LPS provides further support for the development of Th17/IL23-directed therapy. Since significant UPR induction was not detected in AS patient macrophages, the relationship between UPR and inflammatory cytokine production remains unclear.

Ankylosing spondylitis (AS) is an insidious spinal inflammatory disease that affects young adults and incurs a high rate of disability (1, 2). Inflammation can also involve the eyes, skin, gut, peripheral joints and tendinous insertions (entheses). AS is a complex genetic disease, with the presence of the MHC class I allele HLA-B27 conferring up to 40% of genetic risk; this allele is found in 90–95% of AS patients but only 8–9% of the Caucasian population (3–6). HLA-B27/human β_2 microglobulin-expressing rats (B27-Tg) develop a spondyloarthritis-like disease involving gut inflammation and joint swelling that mimics human disease and highlights the importance of HLA-B27 as a causative factor (7). However, almost 40 years after the strong linkage to HLA-B27 was discovered, it is still not clear how this allele predisposes to disease (8). Although the physiologic role of MHC class

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I is to present intracellular peptides to CD8+ T cells, depletion of CD8+ T cells in the B27-Tg rat does not impact disease (9).

One hypothesis for HLA-B27-related pathogenesis stems from the observation that HLA-B27 displays an unusual propensity to fold slowly and misfold during its biosynthesis (10, 11). HLA-B27 forms aberrant dimers that can be recognized by leukocytes on the cell surface and that accumulate in the endoplasmic reticulum (ER) (12–14). Accumulation of misfolded protein in the ER triggers a conserved intracellular stress response, the Unfolded Protein Response (UPR), that decreases protein production, enhances size and folding capacity of the ER, and degrades excess ER protein. The UPR profoundly alters cellular metabolism predominantly through the induction of gene expression (15). Evidence for HLA-B27 induction of the UPR, including up-regulation of binding immunoglobulin protein (BiP/GRP78), CCAAT/-enhancer-binding protein homologous protein (CHOP), and UPR-dependent splicing of the X-box binding protein-1 (XBP1) transcription factor mRNA, has been detected in cells transfected with HLA-B27 as well as in bone marrow macrophages from diseased B27-Tg animals (16). In the animal model, the magnitude of UPR induction correlated strongly with HLA-B27 expression; in pre-morbid animals, visualization of the UPR required acute up-regulation of MHC class I by inflammatory cytokines such as TNF-α and IFN (16, 17). The clinical relevance of these observations is supported by BiP over-expression in knee synovial fluid mononuclear cells from HLA-B27 positive spondyloarthropathy patients (18). However, the relationship between HLA-B27 triggered UPR and disease remains unclear: in B27-Tg rats with intermediate HLA-B27 expression, additional human β2 microglobulin induces arthritis and spondylitis in the absence of intestinal inflammation, while simultaneously reducing B27 misfolding and BiP expression evident in conconavalin A treated spleen cells.(19).

Our previous studies suggested a link between HLA-B27, the UPR, and innate immune responses: macrophages stimulated with pharmacologic UPR inducers(e.g. tunicamycin)respond to Toll-like receptor (TLR) agonists such as lipopolysaccharide (LPS/endotoxin) with increased production of inflammatory cytokines, in particular IFN-β and IL-23 (20, 21). Bone marrow macrophages from diseased animals expressing UPR target genes also expressed type I IFN regulated genes (16). B27+ macrophages from transgenic rats stimulated *in vitro* with IFN-γ (to induce the UPR) produce increased IFN-β and IL-23 in response to LPS. IL-23 drives IL-17 production from pathogenic Th17 T cells and is implicated in multiple autoimmune and inflammatory diseases (22). Involvement of the IL-23/Th-17 axis in AS pathogenesis is further supported by studies in the B27-Tg rat colon, colonic biopsies from AS patients, and AS peripheral blood mononuclear cells (PBMC) (21, 23, 24).

Macrophages pre-dominate in biopsies from early spondyloarthritis inflammatory lesions (25). Also, macrophages appear to be more susceptible to UPR induction than splenocytes in the B27-Tg rat model (17). For this study, we examined peripheral blood derived macrophages from AS patients to determine if they undergo an UPR and whether UPR induction correlates with increased cytokine/chemokine responses to LPS. Despite a 2-fold up-regulation of HLA-B by IFN-γ treatment, we did not observe significant UPR induction as measured by BiP, CHOP, and ERdj4 (an XBP1 gene target) expression. Except for IL-12 secretion, IFN-γ treatment minimized differences between patients and controls. In response to LPS alone, even in the absence of obvious UPR induction, AS patient macrophages produced strikingly greater levels of IL-23 ($p=0.0007$) than controls. These results have implications for AS pathogenesis and further support the development and application of anti-IL23 therapeutics to AS.

PATIENTS AND METHODS

Study Subjects

Nine patients meeting modified New York criteria for AS were included in the study along with one HLA-B27+ patient with thoracic syndesmophytes and sacroiliac pain (26). None of the patients were related to each other. In one AS patient sample, we were unable to collect culture supernatants. In another AS patient sample, we only had enough cells to collect RNA for no stimulation and IFN-γ conditions only (final numbers per group are indicated in figures and tables). Control subjects were unrelated, and had no personal or family history of AS. For subject characteristics see Table 1. All subjects were recruited from within the University of Wisconsin Hospitals and Clinics. Informed consent was obtained prior to participation and the study protocol was approved by the UW Health Sciences Institutional Review Board.

Macrophage derivation and *in vitro* treatments

Whole blood was obtained by venipuncture and collected in 3 × 10 mL ACD tubes (acid citrate dextrose, BD Biosciences, San Jose, CA). Samples were processed within 18h of collection. Samples were diluted with PBS, underlaid with lymphocyte separation medium (Mediatech, Menassas, VA) and centrifuged to obtain PBMC buffy coats. Cells were washed twice at 900 rpm to decrease platelets. Monocytes were purified by negative selection using MACS columns from Miltenyi Biotech (Auburn, CA) according to the manufacturer's instructions. Purity was 87–92% CD 14 positive by flow cytometry. Monocytes were plated in serum free RPMI in 6-well dishes for RNA analysis (350,000–500,000/well) and 24-well dishes (150,000–250,000)/well) for cytokine analysis. RNA and cytokine results were normalized (described below) to account for cell number differences. For further purification, non-adherent cells were removed after 2h. The adherent cells were then cultured with RPMI 1640 containing 10% Fetal Bovine Serum, penicillin-streptomycin, and L-glutamine (HyClone, Logan, UT). Cultures were supplemented with 20 ng/mL recombinant human M-CSF (R&D Systems, Minneapolis, MN), replaced on days 2 and 4. On day 5, 1000U/mL IFN-γ (PeproTech, Rocky Hill, NJ) was added to select cultures for 24h prior to stimulation on day 6 with either 10 ng/mL or 100 ng/mL LPS (S. enteriditus, Sigma, St. Louis, MO). IFN-γ and M-CSF contained less than 0.01 EU endotoxin detected by limulus assay (GenScript, Piscataway, NJ). For the tunicamycin (Sigma, St. Louis, MO) assay, macrophages were stimulated with 0.1–1 μg/mL for 4–16h starting on day 5. Cells obtained after 5 days of culture were relatively homogeneous, large, and had a "fried egg" appearance. By flow cytometry, cells were >99% CD11b+, 94–99% HLA-DR+, and expressed macrophage markers CD163 and CD206 (80–87% and 86–88% respectively). IFN-γ and LPS treatments affected viability (assessed by propidium iodide and trypan blue exclusion) by the end of day 7 compared to no stimulation as follows: LPS 10 ng/mL 73+/−7%, LPS 100 ng/mL 87+/−7%, IFN-γ 92+/−11%, IFN-γ + LPS 10 ng/mL 60+/−12%, IFN-γ + LPS 100 ng/mL 55+/−5%.

HLA-B, HLA-B27, and UPR gene expression

RNA was obtained following 24h of IFN-γ treatment plus 3h LPS stimulation (in select samples)on day 6 by resuspending the cells in TriZol (Invitrogen, Carlsbad, CA), and processing according to the manufacturer's instructions. RNA was treated with DNase (Invitrogen), and reverse transcribed to cDNA using reverse transcriptase (Promega, Fitchburg, WI) with random primers (Promega) according to standard protocols. Relative expression of HLA-B, and the UPR-regulated genes BiP, CHOP, and ERdj4 were determined by quantitative real time polymerase chain reaction (PCR) using a MyiQ or iCycler (Bio-Rad, Hercules, CA) with normalization to 18S rRNA expression. HLA-B27 specific primers have been previously described by Bon et al.(27). Other primers were

designed using Beacon Design 7.0 software (Premier Biosoft, Palo Alto, CA); primer sequences are available upon request from the corresponding author.

Cytokine/Chemokine secretion

Cell culture supernatants were collected at 8h (0.5 mL from the 1.5 mL culture) and at 24h: at 24h, all media was removed. Two time points were selected because of production kinetics for early and late peaking inflammatory mediators (e.g. IFN-β and CXCL9). Cells were lysed in 100 μL SDS lysis buffer and total protein quantified by BCA assay (Pierce, Rockford, IL) to account for differences in cell numbers/well. Supernatants were frozen at −80°C until analysis by Luminex assay (Millipore, Billerica, MA) measured on a Luminex 100 (Austin, TX). Multiplex analytes included IFN-γ, IFN-α2, IL-1β, IL-10, IL-12 (p70), IL-15, IL-1α, IL-6, IL-8, IP-10, MCP-1, TNF-α, and VEGF. CXCL9 and IL-23 Luminex assays were run individually. IFN-β production was quantified by colorimetric ELISA (PBL, Piscataway, NJ) read on a 354 Multiskan Ascent plate reader (Thermo Labsystems, Beverly, MA). Initially we screened 6 patient and 4 control samples for the 15 cytokines and chemokines listed above. For analytes where we observed a $p<0.15$ by student's T-test comparing controls and patients within a treatment group (e.g. LPS 10 ng/mL, IFN-γ+LPS 10 ng/mLetc.) we completed evaluation of the other 3 patient and 6 control samples. Duplicate samples were used to normalize between Luminex runs. Thus we measured CXCL-9, IFN-β, IL-6, IL-8, IL-10, IL-12 (p70), IL-23, and TNF-α in 9 AS patient and 10 control subjects. Only data from the complete analysis of 8 chemokines and cytokines in 19 subjects is presented below.

Statistical Analysis

UPR-regulated gene expression and up-regulation of HLA-B and HLA-B27 were analyzed using Linear Mixed Effects (LME) models. These models included a random effect for subject and fixed effects for group (patient vs. control), IFN-γ, LPS 10 ng/mL, and LPS 100 ng/mL plus two-way interactions between group and all other terms and between IFN-γ and the two LPS doses. An LME model (also called a repeated measures ANOVA) is similar to ANOVA except that at least one additional error term is included. Subject was included as a random effect in the LME model, to model within-subject correlation among all the measurements taken on a particular subject. The UPR responses were transformed to the log scale before analysis to obtain approximately normally distributed residuals. For BiP and HLA-B, the significant main effect of IFN-γ in the complete model was further investigated by examining individual treatment comparisons. Correlations were performed using log-transformed data. Response to tunicamycin was also analyzed using an LME model with fixed effects for dose and time and a random effect for blood sample. Responses were transformed to the log scale before analysis. Wilcoxon Rank Sum tests were used to assess the differences in cytokine and chemokine production between patient and control groups for all the responses under each of the treatment conditions at both 8 and 24 hours.

RESULTS

Up-regulation of HLA-B

Previous examination of GM-CSF-derived macrophages from AS patients by microarray did not show evidence of an UPR (28). However, HLA-B up-regulation was also not detected in that study. In the B27-Tg rat model, up-regulation of HLA-B27 appears to play a critical role in visualizing the UPR; in pre-morbid animals, acute up-regulation of HLA-B27 expression by inflammatory cytokines was essential, and the magnitude of UPR induction strongly correlated with the fold-increase in HLA-B27 expression (16, 17). To further examine the role of the UPR in AS, we derived macrophages from PBMC with either GM-CSF or M-CSF and stimulated with 100–1000U/mL TNF-α, IFN-α or IFN-γ for 24–48h

prior to analyzing surface expression of HLA-B by flow cytometry (data not shown). Derivation with M-CSF and 24h stimulation with 1000U/mL IFN-γ yielded the greatest increase in HLA-B (1.82 ± 0.12 fold) with the least impact on viability as determined by propidium iodide exclusion (+11% ± 30%). These conditions were used for the following studies.

HLA-B expression was analyzed in macrophages from AS patients and controls by quantitative real time PCR (Table 2). AS patient and control macrophages responded similarly to treatment and only IFN-γ had a significant impact on mean expression ($p<0.0001$). In pair-wise tests combining patients and controls, IFN-γ, IFN-γ+LPS 10 ng/mL and IFN-γ+LPS 100 ng/mL were all significantly increased over no stimulation (NS), ($p 0.02$). In the AS patients, HLA-B expression increased from NS to IFN-γ an average of 2.21(range 1.09–5.95, $p=0.0063$);from NS to IFN-γ + LPS 10 ng/mL the increase was 1.83-fold (0.34–4.98, $p=0.02$);for NS to IFN-γ + LPS 100 ng/mL an increase of 2.44(1.3–6.75, $p=0.0027$)was observed. In this last group, all AS patients up-regulated HLA-B expression (Figure 1A).

Others have demonstrated increased HLA-B27 expression on patient PBMC compared to healthy HLA-B27+ controls, in the absence of overall increased HLA class I expression(29). Thus we specifically examined HLA-B27 expression in patient samples. Using a linear effects model, HLA-B27 behaved similarly to HLA-B, in that only IFN-γ treatment was significant ($p=0.0005$). IFN-γ induced a mean of 2.24-fold increase (range 0.86–7.8, $p=0.016$), IFN-γ + LPS 10 ng/mL 3.74-fold (0.61–10.4, $p=0.063$) and IFN-γ+LPS 100 ng/mL 1.96-fold (1.32–11.23, $p=0.016$). These ranges are slightly larger compared to HLA-B, consistent with previously published data(29).

UPR gene expression

Part of our hypothesis predicted that IFN-γ would induce the UPR by acutely up-regulating HLA-B expression in AS patient-derived macrophages. We chose to study the expression of CHOP, ERdj4 and BiP, as these gene targets are robustly up-regulated by the UPR, and relatively specific for the three major identified signaling pathways of the UPR initiated by activation of the ER-resident proteins protein kinase receptor-like ER kinase (PERK), inositol requiring 1 (IRE-1), and activating transcription factor 6 (ATF6) respectively (15, 30, 31). In previous studies in the transgenic rat model, 20–24h IFN-γ had been sufficient to observe UPR induction and a further 3h of LPS for additional XBP1 splicing(16, 20). As seen in Figure 1A, these gene products are sensitive to change following treatment with very low doses of tunicamycin. All tunicamycin doses showed significant elevation over no stimulation (p-values ranged from 0.048 to <0.001) with the exception of ERdj4 at 0.1 μg/mL ($p=0.094$). The increase from 4h to 16h was significant for BiP ($p=0.0044$) All other effects of increased time were not significant (p-values of 0.057 to 0.88).

Contrary to our prediction, *in vitro* stimulation with IFN-γ and LPS had minimal impact on mean UPR-regulated gene expression, even under conditions of the greatest HLA-B up-regulation (IFN-γ + 100 ng/mL LPS, Figure 1). AS patient mean gene expression was not significantly elevated compared to controls in any treatment groups. There was no statistical difference between the responses of patients and controls to the various treatments (Table 3). Combining patients and controls, the only significant difference compared to no treatment was seen in response to IFN+10 ng/mL LPS, where BiP and CHOP decreased (fold changes of 0.68 and 0.7 respectively, $p<0.02$). ERdj4 showed a trend towards differential regulation in AS patient and control macrophages: In the IFN + LPS treatment groups, control macrophages had unchanged or slightly decreased mean ERdj4 expression, whereas the AS patient macrophages tended to up-regulate ERdj4 (a significant patient-IFN interaction, $p=0.03$). However, ERdj4 mean expression in AS patient-derived macrophages remained

below control mean expression. In AS patient macrophages there was no significant correlation between fold up-regulation of HLA-B and UPR-regulated gene expression.

We further analyzed the AS patients according to whether they were treated with DMARDs (6 DMARD+ and 4 DMARD−). Few cases were significant: in response to IFN-γ, BiP expression changed by 0.64-fold in the DMARD+ and 1.16-fold in DMARD− (p=0.038). In response to IFN-γ+100 ng/mL LPS, CHOP changed 0.71-fold in DMARD+ vs. 0.93-fold in DMARD− (p=0.016) and ERdj4 changed 1.31-fold in the DMARD+ vs. 4.83-fold in the DMARD− (p=0.032). Thus, in select cases, DMARD− patients tended to show greater increase in gene induction or less of a decrease as compared to the DMARD+. Our study was most likely underpowered to detect other differences between DMARD groups.

Cytokine/Chemokine production

The evaluation of inflammatory mediator production was based upon previously described differences between AS patients and controls as well as studies examining the regulation of inflammation by the UPR. We had previously described a marked synergy between the UPR and LPS induction of both IFN-β and IL-23, in the context of both pharmacologic UPR induction and HLA-B27+ Tg rat macrophages undergoing an UPR (20, 21). We hypothesized that IFN-γ-treated AS patient macrophages would produce excess IFN-β and IL-23 in response to LPS stimulation. In addition to IFN-β and IL-23, we also examined macrophage production of CXCL-9, IL-6, IL-8, IL-10, IL-12 (p70), and TNF-α in 9 AS patients and 10 controls (Table 4).

We observed statistically significant increases in CXCL9, IL-10, IL-12 (p70), IL-23, and TNF-α production by AS patient macrophages compared to controls (Table 4 and Figure 2). We did not detect significant differences in production of IL-6, IL-8 or IFN-β. Apart from IL-12 (p70), these differences were primarily found in the LPS only treatment groups, where there was no HLA-B up-regulation or UPR gene induction. Contrary to our prediction, IFN-γ pre-treatment minimized differences for all inflammatory mediators tested except IL-12 (p70) (Table 4 and data not shown). Median differences between AS patients and controls for IL-10, IL-12 (p70), and CXCL9 ranged from 1.9 to 4.6 fold. Statistical differences in IL-12 production were only detected at 8h, but not 24h (data not shown). Several *in vitro* treatments revealed differences between AS patients and control macrophages in TNF-α production, although p-values reflected relatively less variability rather than profound differences in median production (< 2 fold). The most striking AS patient-control differences were observed for median IL-23 production following 24h LPS stimulation(see Table 4 and Figure 2): AS patient macrophages produced greater than a log-fold more IL-23 compared to controls (median AS production of 265–357 pg/mL vs. control 9–15 pg/mL, p=0.0022–0.0007). In our initial screen of 4 controls and 6 patients, no differences in IL-23 production were observed for IFN-γ pre-treated cells, and 8h LPS stimulation was comparable to 24h (median AS production of 213–341 pg/mL vs. control 24–64 pg/mL).

DISCUSSION

Based on the B27-Tg rat spondyloarthritis model we hypothesized that increased HLA-B27 expression and misfolding during inflammation induces a UPR that renders innate immune cells (macrophages and dendritic cells) more pro-inflammatory and hyper-responsive to bacterial TLR agonists. UPR-driven cells secrete excessive IL-23 (Th17 activation) and type I IFN (20, 21). However, the transgenic rat highly over-expresses HLA-B27 and thus HLA-B27 misfolding effects would most likely be exaggerated. It is important to assess the UPR-TLR inflammatory model in human disease, where there are at most 2 copies of the HLA-B27 gene.

In this study we did not detect significantly increased UPR-regulated gene expression in AS patient macrophages relative to controls at baseline, nor did we observe significant up-regulation of classic UPR target genes following *in vitro* IFN-γ and LPS stimulation. Recently, LPS was shown to activate IRE-1 dependent XBP1 splicing via the induction of an NADPH oxidase while suppressing activation of the PERK and ATF6 arms of the UPR (32). In this report, 10 ng/mL LPS also tended to decrease mean UPR gene expression in AS patients. However, we did not detect increased UPR gene expression following IFN-γ stimulation even in the absence of the additional LPS treatment. The significance of the increase in ERdj4 mRNA following IFN-γ+LPS treatment in AS patients is unclear, since mean expression remained below that observed in control subject macrophages.

The simplest explanation for the difference in our findings and the B27-Tg animal studies lies in the magnitude of HLA-B up-regulation. In the report describing the correlation of UPR magnitude and HLA-B27 expression in transgenic rat macrophages, IFN-γ treatment up-regulated HLA-B27 four to ten fold; although even a two fold up-regulation of HLA-B27 by TNF-α treatment yielded a two-fold induction of BiP expression (17). The mean two to three-fold HLA-B27 induction we observed may not have been sufficient to detect UPR induction in patient samples, particularly given patient to patient variability. Our small sample sizes may have been underpowered to detect subtle upregulation of gene expression. In addition, the representative UPR target genes we chose (BiP, CHOP, ERdj4) may not be the most sensitive indicators of an HLA-B27 UPR, although they are widely utilized to detect the UPR (15, 32). Alternatively, the UPR observed in the B27-Tg rats may depend upon dramatic HLA-B27 over-expression related to multiple transgene copy numbers, and thus be less relevant to human disease. However, the increased BiP expression found in the synovial cells from spondyloarthritis patients would argue against this possibility (18). Another possibility is that factors in addition to IFN-γ activation and HLA-B27 up-regulation are required to induce a UPR in AS macrophages. Our *in vitro* system may have been too simplistic compared to the *in vivo* inflammatory milieu. Future examination of inflamed patient tissues may be more revealing. Related to all these factors, our results do not rule out a role for the UPR in pathogenesis.

The study by Tran et al. showing worse arthropathy in B27-Tg rats where the UPR has been modulated by β2 microglobulin throws into question the relationship between B27-related UPR and joint disease(19). Multiple non-UPR hypotheses have sought to explain the striking contribution of HLA-B27 to genetic risk (8). For instance, expanded numbers of natural killer and CD4 T cells that recognize cell surface HLA-B27 dimers have been identified in the circulation of AS patients (14). Other efforts have focused on specific antigen presentation by HLA-B27 (33). With some variability across studies, HLA-B27 has also been shown to alter survival and persistence of intracellular organisms through unclear mechanisms (8, 34). Ultimately, our data suggest that non-UPR, cytokine modulatory mechanisms may contribute towards a pro-inflammatory diathesis and that non-UPR hypotheses bear greater exploration.

The tendency of IFN-γ to minimize differences in inflammatory mediator production is consistent with our previous data showing a global decrease in IFN-γ regulated gene expression in AS patient macrophages that could be recovered with exogenous IFN-γ treatment (28). This study and others have highlighted a relative deficit in the production of Th1 cytokines such as IFN-γ by AS patient cells (28, 35, 36). However, the IL-12 (p70) data (Table 4) suggests that under certain infectious or inflammatory conditions where IFN-γ is produced, AS patients could potentially mount an even more robust Th1 response than controls. Our findings of increased CXCL9, TNF-α and IL-10 in AS patient macrophages corroborate previous studies of patient samples (37, 38). Increased CXCL9, CXCL10/IP-10 and IL-12/23p40 are present in synovial fluid from patients with spondyloarthropathy (37,

39, 40). Reports describing serum and PBMC from AS patients have demonstrated variable increases in TNF-α, IL-1β, IL-6, IL-8, VEGF, and IL-10(38). What is unique to this study is removal of monocytes from the context of patient inflammation and medication. The *in vitro* derivation and stimulation conditions are controlled; thus the increased cytokine/chemokine production most likely reflects an intrinsic, genetically determined property of AS macrophages.

The most striking finding from this study is that even in the absence of an obvious UPR, AS macrophages produced significantly more IL-23 in response to LPS alone. The one known HLA-B27 negative patient produced IL-23 close to the median AS patient level (335 pg/mL in response to 10 ng/mL LPS and 405 with 100 ng/mL LPS). The increased IL-23 was much more robust than the differences observed for TNF-α. ER stress induced CHOP has recently been shown to directly regulate IL-23 expression(41). Although UPR and IL-23 production have also been closely associated in the B27-Tg rat model, our results suggest the potential for excess IL-23 production by patient macrophages in the absence of an overt UPR; in this study mean CHOP expression actually decreased following IFN-γ treatment and early during the LPS stimulation period (21). In the recent report by Martinon et al., LPS stimulation decreased pharmacologically induced UPR target gene expression (BiP, CHOP, ERdj4) out to 9h(32). In the present study we did not collect RNA after 24h of LPS, and thus cannot absolutely rule out late occurrence of an LPS-induced UPR.

Although this study is underpowered to assess the impact of HLA-B27 positivity in patients, our results support the hypothesis that AS macrophages produce an overabundance of IL-23 in response to TLR4 agonists in the infectious environment, thus predisposing to the development of inflammatory lesions. The IL-23/Th17 axis has become increasing recognized as a key component of anti-bacterial immunity, thus explaining genetic pressures to maintain risk alleles for a debilitating arthritic condition over the centuries (42). Given the responses we observed to LPS, it will be important to determine whether macrophages from AS patients produce excess IL-23 in response to colonic and infectious organisms. The finding of robust IL-23 expression in subclinical gut inflammation from AS patients suggests this may be the case, however more defined studies will be helpful in elucidating pathogenesis(24).

Our IL-23 results extend and support other recent studies examining the IL-23-Th17 axis in AS. Polymorphisms in the IL-23R were identified in genome wide association studies as susceptibility alleles for AS (43). Increased IL-23 and IL-17 levels have been observed in the serum and cultured PBMC supernatants from AS patients with active disease (23, 44). AS patients also have a higher proportion of IL-17 producing CD4+ T cells in circulation (23).

Anti-IL12p40 agents (e.g. ustekinumab), that block both IL-12-and IL-23-mediated effects, are highly efficacious in psoriasis, more than etanercept (a TNF-blocker) (45). In this study, the overproduction of IL-23 by AS patient macrophages was much more impressive than that observed for TNF-α, even though TNF-blockers are currently the therapy of choice for AS (46). The evidence from this study and others, implicating IL-23 expression in AS pathogenesis provides strong rationale for development of IL-23 blocking agents as treatment for AS.

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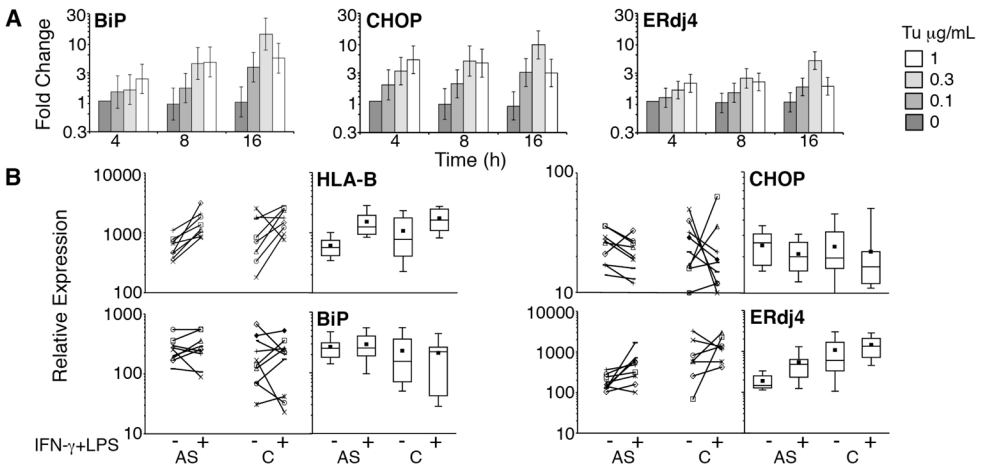

Figure 1. HLA-B and UPR-regulated gene expression

A) Human macrophages were treated with various doses of tunicamycin (Tu μg/mL) for the times indicated. Gene expression was normalized to 4h untreated controls to yield fold change. Data was combined from 4 independent experiments with bars representing geometric means and vertical bars showing +/− standard errors of the mean. The geometric mean best represents the typical fold change but downweights large fold changes. B) Analysis of AS patients (AS) and controls (C): individual data points with lines connecting unstimulated and stimulated samples are on the left, and box plots showing median, 25th percentile, 75th percentile (boxes), means (black squares) and 10th and 90th percentiles (bars), are on the right. Subject macrophages were unstimulated (−), or treated with IFN-γ for 24h followed by 100 ng/mL LPS (+) for 3h. Relative gene expression was determined by quantitative PCR with normalization to 18S rRNA. HLA-B expression represents data from 8 patients and 8 controls. P=0.0006 for upregulation of HLA-B in combined patient and control samples. CHOP and BiP expression represent data from 9 patients and 10 controls. ERdj4 expression shows data from 9 patients and 7 controls.

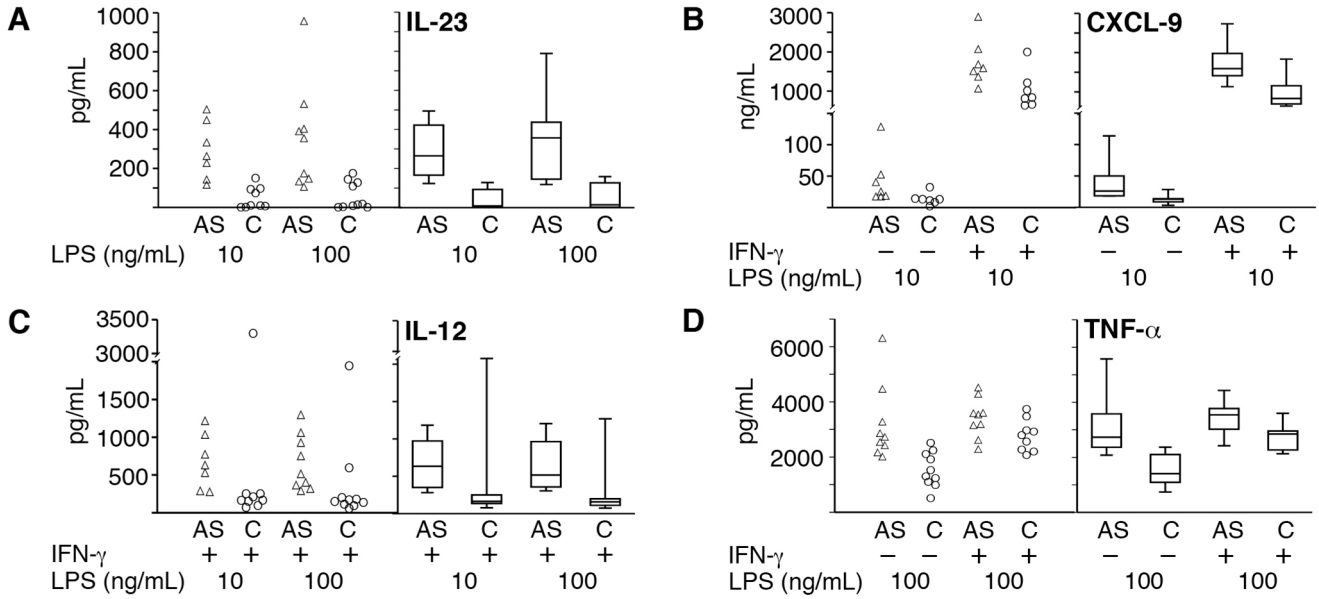

Figure 2. Increased IL-23, CXCL9, IL-12(p70) and TNF-α production in AS patient macrophages

Individual sample data points are on the left and box plots showing median, 25th and 75th percentiles (boxes) and 10th and 90th percentiles (error bars) are on the right. A) IL-23: AS patient (AS, open triangles) or control samples (C, open circles) were treated with 10 or 100 ng/mL LPS for 24h. See Table 3 for associated p-values and numbers per group. B) CXCL-9: AS patient and control samples were left untreated (−) or treated with IFN-γ (+) for 24h, and then stimulated with 10 ng/mL LPS for another 24h. C) IL-12 (p70): All samples were pre-treated with IFN-γ for 24h and then stimulated with either 10 or 100 ng/mL LPS for another 8h. The outlier points for IL-12 production in response to both doses of LPS were from the same control subject. D) TNF-α: Macrophages were left untreated (−) or treated with IFN-γ (+) for 24h and then stimulated with 100 ng/mL LPS for 8h.

Table 1

Ankylosing Spondylitis patient and control characteristics

	AS patient(n=10)	Control (n=10)
Age median (range) years:	47 (18–58)	45 (28–57)
Sex, %male:	90	90
Disease duration median (range) years:	15 (2–32)	NA
HLA-B27, number positive:	9	0
BASDAI, median (range):	3.2 (0–5.4)	N.A.
Arthritis meds:		
NSAIDs:	6	1*
Methotrexate:	2	0
TNF-blockers:	5	0
Other:	sulfasalazine (1)	allopurinol (1)
	glucosamine (1)	glucosamine (1)
	fosamax (1)	

* 1 Control was taking 325 mg aspirin for history of myocardial infarction. Not included above were 3 other controls taking anti-platelet doses of aspirin (81 mg).

NA: not applicable, ND: not determined, BASDAI: Bath ankylosing spondylitis disease activity score, NSAIDS: non-steroidal anti-inflammatories, TNF: Tumor necrosis factor. 6 patients were on disease modifying anti-rheumatic drugs (DMARDS: Methotrexate and/or TNF blockers).

Table 2

HLA-B expression following *in vitro* treatment with IFN-γ or LPS*

Treatment	Patient (mean, 95% CI)	Control (mean, 95% CI)
No stimulation	559 (356, 875)	770 (492, 1204)
LPS 10 ng/mL	664 (413, 1065)	1551 (969, 2479)
LPS 100 ng/mL	596 (380, 933)	855 (546, 1336)
IFN-γ	1249 (799, 1949)	1239 (793, 1933)
IFN-γ+LPS 10 ng/mL	1071 (668, 1713)	1911 (1195, 3052)
IFN-γ+LPS 100 ng/mL	1375 (881, 2145)	1577 (1010, 2459)

*Means were derived from log transformed expression values (see Methods). Patients did not respond differently than controls to treatment ($p=0.119$). The only significant effect was by IFN-γ treatment, which increased the response for all treatments (IFN-γ only or IFN-γ+LPS) for both patients and controls ($p<0.0001$). N=7–8 AS patients and controls per group.

Table 3

UPR-regulated genes following *in vitro* treatment with IFN-γ or LPS

Gene	Treatment	Patient (mean, 95% CI)	Control (mean, 95% CI)
BiP	No stimulation	262 (136, 499)	150 (76, 299)
	LPS 10 ng/mL	197 (95, 400)	205 (99, 414)
	LPS 100 ng/mL	259 (131, 501)	185 (93, 360)
	IFN-γ	206 (106, 399)	115 (57, 223)
	IFN-γ+LPS 10	204 (98, 414)	69 (30, 147)
	IFN-γ+LPS 100	261 (133, 505)	133 (67, 258)
CHOP	No stimulation	15.4 (9, 23.9)	11.1 (5.8, 18.2)
	LPS 10 ng/mL	8.1 (3, 15.1)	10.5 (4.7, 18.4)
	LPS 100 ng/mL	10.9 (5.5, 18.2)	7 (2.6, 12.9)
	IFN-γ	11.7 (6.3, 19)	7.3 (3, 13.1)
	IFN-γ+LPS 10	7.9 (2.8, 14.9)	4.5 (0.4, 10.1)
	IFN-γ+LPS 100	10.9 (5.5, 18.2)	9.2 (4.4, 15.7)
ERdj4	No stimulation	185 (83, 397)	593 (268, 1295)
	LPS 10 ng/mL	110 (40, 275)	644 (248, 1651)
	LPS 100 ng/mL	214 (94, 476)	651 (282, 1486)
	IFN-γ	151 (67, 327)	284 (131, 604)
	IFN-γ+LPS 10	213 (83, 520)	237 (94, 579)
	IFN-γ+LPS 100	391 (175, 860)	486 (219, 1064)

N = 10 patients and controls for no stimulation and IFN-γ, 7 patients and controls for LPS 10 ng/mL and IFN-γ + LPS 10, and 9 patients and 9–10 controls for LPS 100 ng/mL and IFN-γ +LPS 100 ng/mL. Mean expression derives from log-transformed data (see Methods).

Table 4

Cytokine and chemokine production by AS patients and controls

Mediator Pg/mL	Time	Stimulation	Patient median(25%,75%)	Control median(25%,75%)	p-value
CXCL9 **	24h	no stimulation	0.06 (0,0.14)	0.47(0.29,0.64)	0.1832
		LPS 10 ng/mL	40.6 (22.3,154.5)	12.6 (9.4,13.4)	**0.0041** *
		LPS 100 ng/mL	27.7 (23.4,71.3)	31.9 (16.5,35.1)	0.5457
		IFN-γ	1796 (1661,2026)	1091 (1.4,2269)	0.885
		IFN-γ+LPS 10	1593 (1447,1884)	832 (731,1104)	**0.0175** *
		IFN-γ+LPS 100	1650 (1263,2031)	833 (603,1677)	0.1615
IFN-β	8h	no stimulation	0	0	NA
		LPS 10 ng/mL	76 (0,132)	0 (0,65)	0.3507
		LPS 100 ng/mL	51 (0,148)	0 (0,78)	0.4530
		IFN-γ	0	0	NA
		IFN-γ+LPS 10	522 (302,1087)	362 (274,460)	0.7577
		IFN-γ+LPS 100	627 (225,1181)	301 (260,377)	0.7802
IL-6	8h	no stimulation	6 (3,8)	7 (6,22)	0.4568
		LPS 10 ng/mL	2413 (1211,3569)	3012 (2811,3771)	0.6065
		LPS 100 ng/mL	2305 (1616,3440)	2972 (2192,3713)	0.6965
		IFN-γ	19 (14,26)	7 (3,12)	0.2000
		IFN-γ+LPS 10	8904 (6456,12302)	5600 (4698,7336)	0.1142
		IFN-γ+LPS 100	8037 (5606,13858)	5161 (3228,8601)	0.0831
IL-10	24h	no stimulation	34 (20,47)	8 (8,37)	0.2000
		LPS 10 ng/mL	2240 (1523,4869)	486 (383,1153)	**0.0229** *
		LPS 100 ng/mL	2126 (1438,2749)	682 (471,1725)	0.0653
IL-12p70	8h	no stimulation	0	0	NA
		LPS 10 ng/mL	3 (1,4)	0 (0,0)	0.0203 §
		LPS 100 ng/mL	2 (0,5)	0 (0,1)	0.0929
		IFN-γ	0	0	NA
		IFN-γ+LPS 10	634 (411,908)	165 (150,250)	**0.0110** *

Mediator Pg/mL	Time	Stimulation	Patient median(25%,75%)	Control median(25%,75%)	p-value
		IFN-γ+LPS 100	516 (369, 931)	158 (118,194)	**0.0101** *
IL-23	24h	no stimulation	0	0	NA
		LPS 10 ng/mL	265 (188,393)	9 (6,92)	**0.0007** *
		LPS 100 ng/mL	357 (149,405)	15 (3,122)	**0.0022** *
TNF-α	8h	no stimulation	3 (2,4)	3 (2,13)	0.8571
		LPS 10 ng/mL	2667 (2260,2719)	1393 (1094,2397)	0.0562
		LPS 100 ng/mL	2732 (2431,3284)	1410 (1128,2053)	**0.0016** *
		IFN-γ	10(7,16)	4 (2,13)	0.8571
		IFN-γ+LPS10	3077 (2952,3663)	2806 (2625,3193)	0.0556
		IFN-γ+LPS100	3545 (3156,3604)	2671 (2212,2949)	**0.0408** *
	24h	no stimulation	7 (5,7)	3 (2,6)	0.2938
		LPS 10 ng/mL	2430 (2352,2550)	1617 (1224,1739)	**0.0068** *
		LPS 100 ng/mL	2620 (1964,2957)	1536 (1157,2213)	**0.0177** *

*Significant p-values;

**CXCL9 is ng/mL and all other cytokines are pg/mL. The number of subjects for chemokine/cytokine experiments were 4–5 for unstimulated and IFN-γ only, 7 patients and 7–9 controls for LPS 10 ng/mL and IFN-γ+LPS 10 ng/mL, and 9 patients and 9–10 controls for LPS 100 ng/mL and IFN-γ+LPS 100 ng/mL. NA: not applicable.

§Values at detection threshold for assay. No significant differences were detected for IL-8 production (data not shown).