EXTENDED REPORT

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

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ABSTRACT

Objectives Interleukin (IL)-23 has been implicated in the pathogenesis of ankylosing spondylitis (AS). The aim of the study was to clarify the mechanisms underlying the increased IL-23 expression in the gut of AS patients.

Methods Consecutive gut biopsies from 30 HLA-B27$^+$ AS patients, 15 Crohn's disease (CD) patients and 10 normal subjects were obtained. Evidence for HLA-B27 misfolding was studied. Unfolded protein response (UPR) and autophagy were assessed by RT-PCR and immunohistochemistry. The contribution of UPR and autophagy in the regulation of IL-23 expression was evaluated in in vitro experiments on isolated lamina propria mononuclear cells (LPMCs).

Results Intracellular colocalisation of SYVN1 and FHCs but not a significant overexpression of UPR genes was observed in the gut of AS patients. Conversely, upregulation of the genes involved in the autophagy pathway was observed in the gut of AS and CD patients. Immunohistochemistry showed an increased expression of LC3II, ATG5 and ATG12 but not of SQSTM1 in the ileum of AS and CD patients. LC3II was expressed among infiltrating mononuclear cells and epithelial cells resembling Paneth cells (PC) and colocalised with ATG5 in AS and CD. Autophagy but not UPR was required to modulate the expression of IL-23 in isolated LPMCs of AS patients with chronic gut inflammation, CD patients and controls.

Conclusions Our data suggest that HLA-B27 misfolding occurs in the gut of AS patients and is accompanied by activation of autophagy rather than a UPR. Autophagy appears to be associated with intestinal modulation of IL-23 in AS.

INTRODUCTION

Subclinical ileal inflammation, resembling Crohn's disease (CD), has been demonstrated in up to 70% of ankylosing spondylitis (AS) patients apparently never resolving, suggesting the presence of chronic alterations in host–microbe interactions in the gut.[1–3] The terminal ileum of normal subjects basically produces interleukin (IL)-23 in the presence of commensal microbes[4] and its local excessive production has been found in the gut of AS patients with subclinical gut inflammation,[5] suggesting that intestinal mucosa may be a key site of IL-23 production in AS. IL-23 pathway has been hypothesised to be strongly involved in the pathogenesis of AS based on the cumulating genetic evidence from several genome-wide association study (GWAS) studies.[6–8]

Although increased concentrations of IL-23 have been demonstrated in the peripheral blood[9–11] and tissues of AS patients, the exact mechanism involved in IL-23 upregulation has not been definitively defined. In particular, it is not clear whether or not the overproduction of IL-23 could be linked to the misfolding of HLA-B27 through the induction of the unfolded protein response (UPR).[12] On the other hand, the demonstration that IL-23 is active at intestinal mucosal surfaces[4] and evidence that certain types of bacterial stimuli may influence its intestinal expression[13] could suggest a role for microbes in the IL-23 overexpression observed in AS.[14]

A key role in the intestinal innate immune response against bacterial infection is played by macroautophagy[15] (hereafter referred as autophagy), a basic cellular machinery responsible in eukaryotic cells for bulk degradation of cellular constituents[16] that also act as an effector of pattern recognition receptor response to pathogens,[17–19] directly eliminating intracellular microbes or their products. Another function of autophagy is connected to its ability to target improperly folded proteins for degradation in close connection with the endoplasmic reticulum stress response known as the UPR.[20–22]

In this study, we present data on autophagy as a probable regulator of IL-23 production in the gut of patients with subclinical gut inflammation. We also provide the first evidence that HLA-B27 misfolding occurs in the gut of HLA-B27+ AS patients.

METHODS

Patients

Multiple adjacent ileal mucosal biopsies from patients with AS (diagnosed according to the modified New York classification criteria),[23] CD and healthy subjects were consecutively obtained (baseline characteristics of patients and controls are specified in table 1). As a control group, 10 normal subjects undergoing to ileocolonscopy for diagnostic purposes but without evidence of underlying disease were also considered. Collection of ileal biopsies was approved by the ethical committee and

Table 1 Baseline characteristics of the patients and controls*

	AS patients no inflammation N=6	AS patients acute N=9	AS patients chronic N=15	CD patients N=15	Controls N=10
Age, mean (range) years	32 (22–45)	31 (20–50)	33 (20–53)	38 (18–51)	60 (35–70)
Sex, no. (%) male	5 (83)	7 (78)	13 (87)	9 (60)	7 (70)
HLA-B27, no. (%)	6 (100)	9 (100)	15 (100)	1 (6)	–
Disease duration, mean (range) months	26 (18–36)	28 (11–40)	20 (8–38)	15 (4–46)	NA
CRP (mg/l), mean (range)	1 (0.5–2)	1.2 (0.5–3)	1.9 (0.5–3)	6.5 (2–13)*	NA
Axial involvement, no. (%)	6 (100)	9 (100)	15 (100)	1	NA
Peripheral arthritis, no. (%)	–	3 (33)	4 (27)	–	NA
Enthesitis/dactylitis, no. (%)	1 (16)	2 (22)	2 (13)	–	NA
Uveitis, no. (%)	–		2 (13)	–	NA
Concomitant medical treatment, no. (%)					
NSAIDs	3 (50)	5 (55)	9 (60)	–	NA
Biological agents	–	–	–	–	NA
Immunosuppressants	–	–	–	–	NA
BASDAI score, mean (range)†	5.45 (4.2–8)	6 (4.4–9)	6.33 (5–9)	NA	NA
CDAI score, mean (range)‡	–	–	–	234 (170–334)	–

*p<0.0001.
†Scores for the Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) range from 0 to 10, with higher scores indicating more severe disease.
†Scores for the CDAI range from 150 to 450, with higher scores indicating more severe disease.
AS, ankylosing spondylitis; CD, Crohn's disease; CDAI, CD Activity Index; CRP, C reactive protein; NA, not applicable; NSAIDs, non-steroidal anti-inflammatory drugs.

the institutional review board of the University of Palermo and informed consent was obtained from each patient and control.

RNA extraction and quantitative TaqMan real-time polymerase chain reaction (RT-PCR) for human epithelial cell lines and ileal biopsies

Ileal biopsies, immediately after removal were stored in RNAlater solution (Applied Biosystems, Foster City, California, USA) and processed as previously described.[24] For quantitative TaqMan real-time PCR, sets of primers and probes were obtained from Applied Biosystems (see online supplementary table S2). Samples were run in triplicate using the Step-One Real-Time PCR system (Applied Biosystems). Relative changes in gene expression between controls and patients were determined using the $\Delta\Delta C_t$ method as previously described.[24] Final values were expressed as fold of induction.

Histomorphological grading and immunohistochemistry

Specimens from patients with AS were divided into three subgroups:[5] those with normal gut histology, those with acute inflammation and those with chronic inflammation. Immunohistochemistry was performed as previously described.[24] The primary and secondary antibodies used are listed in table 2. The number of immunoreactive cells was determined by counting the reactive cells on microphotographs obtained from three randomly selected high-power microscopic fields (original magnification ×400). To specifically address the presence of heavy chains (HCs)–HRD1 complexes, as a marker of misfolding,[25] a double staining was performed on paraffin-embedded sections of human ileum and the sections were treated with FITC- or Rhodamine Red-conjugated antimouse or antirabbit antibodies plus RNasi (200 ng/ml) and counterstained using Toto-3 iodide (642/660; Invitrogen). Confocal analysis was used to acquire fluorescence staining.

Isolation of LPMCs and flow cytometry analysis of surface and intracellular antigens

Lamina propria mononuclear cells (LPMCs) were isolated from gut biopsy specimens of 12 patients with AS and chronic gut inflammation, 10 CD patients and 10 healthy controls as previously described.[24 26] In order to evaluate the role of UPR and autophagy in regulating the production of IL-23p19 by lamina propria macrophages and dendritic cells, isolated cells were stimulated with thapsigargin (1 μM, to activate UPR), 3-methyl-adenine (100 μg/ml, to inhibit autophagy) and anisomycin (10 μg/ml, to inhibit CAM) with or without lipopolysaccharide (LPS). All cultures were set up in triplicate and cells were used for real-time polymerase chain reaction (RT-PCR) and flow-cytometric analyses. Before intracellular staining, cells were stimulated with phorbol myristate acetate (PMA) (1 μg/ml) plus ionomycin (0.5 μg/ml) for 4 h. After 2 h Brefeldin A (10 μg/ml; Sigma Aldrich, St Louis, Missouri, USA) was added. After simulation, the cells were stained with PerCP-labelled CD11c (Becton Dickinson, New Jersey, USA), fixed in 4% paraformaldehyde, permeabilised with 0.1% saponin (Sigma Aldrich) and then stained with PE-conjugated mouse anti-human IL-23p19 antibody (R&D systems, Minnesota, USA). Three-colour flow cytometric analysis was performed using a FACSCalibur (Becton Dickinson) and cell death was assessed by trypan blue exclusion. At least 50 000 cells (events) were acquired for each sample. LPMCs were expressed as percentage of cells within the lymphocytes gate. The acquired data were analysed using the CellQuest software program (Becton Dickinson).

Statistical analysis

Statistical analysis of quantitative variables was performed using the Mann–Whitney rank-sum test. Spearman's correlation analysis was used to quantify the expression associations between the genes of interest. p Values less than 0.05 were considered significant.

RESULTS

Patients

No significant differences among Bath Ankylosing Spondylitis Disease Activity Index score, clinical manifestations and non-steroidal anti-inflammatory drugs assumption were observed in the different subgroups of AS patients (table 1). Representative images of the intestinal biopsies of patients with AS enrolled are shown in online supplementary figure S1.

Table 2 List of primers and antibodies for IHC

Primers and sequences		Primary and secondary antibodies		
IL-23p19	Hs00372324_m1	Rabbit anti-human HSPA5	1:100	LSBio, Seattle, WA
HSPA5	Hs00607129_gH	Rabbit anti-human XBP-1	1:100	Novus Biological, Littleton, CO
PDIA4	Hs01115905_m1	Rabbit anti-human LC3II	1:100	Novus Biological, Littleton, CO
GADD34	Hs00169585_m1	Rabbit anti-human ATG5	1:500	Novus Biological, Littleton, CO
XBP-1	Hs00231936_m1	Rabbit anti-human ATG12	1:500	Novus Biological, Littleton, CO
ATF6	Hs00232586_m1	Rabbit anti-human SQSTM1	1:100	Novus Biological, Littleton, CO
PERK	Hs00984006_m1	rabbit IgG control antibody ab27472	1:100	AbCam, Cambridge, UK
ATG5	Hs00169468_m1	Mouse anti-human conformational HCs W6/32	1:100	Abcam, Cambridge, UK
ATG12	Hs01047860_g1	Mouse anti-human free HCs (HC10)	1:100	Reference 24
HSPA8	Hs03044880_gH	Rabbit-anti-human-SYVN-1	1:100	Novus Europe, Cambridge, UK
HSP90AA1	Hs00743767_sH	Biotinylated goat anti-rabbit Ig (ab64256)	1:100	AbCam, Cambridge, UK
ATG16L1	Hs00250530_m1			
IRGM	Hs01013699_s1			
MAP1LC3A	Hs01076567_g1			

Autophagy is differentially regulated in the gut of AS patients and CD patients

We first studied the gene expression of proteins involved in autophagy and assessed their correlation with the expression of IL-23p19 gene. As shown in figure 1 a strong and significant upregulation of ATG16L1 (figure 1A), immunity-related GTPase family (IRGM) (figure 1B) and MAP1LC3A (figure 1C) together with increased IL-23p19 levels (figure 1D) was observed in the ileum of CD patients and in the chronic inflamed ileum of AS patients compared with the other AS patients and controls. Expression levels of ATG16L1, IRGM and MAP1LC3A were correlated with the IL-23p19 levels only in AS patients with chronic gut inflammation (r^2=0.78, 0.67 and 0.83, respectively, p<0.0001; data not shown) and CD patients (r^2=0.66, 0.68, 0.77, respectively, p<0.0001; data not shown). Differently from the other autophagy genes evaluated, ATG5 and ATG12 transcripts levels were only modestly and not significantly increased in the ileum of patients with CD and AS with chronic gut inflammation (figure 1E,, respectively). Interestingly, HSPA8 (figure 1G) and HSP90AA1 (figure 1H) that are markers of chaperone-mediated autophagy (CMA) were significantly downregulated in the gut of all AS and CD patients compared with controls (p<0.0001), and were inversely correlated with the IL-23p19 levels in both chronically inflamed AS

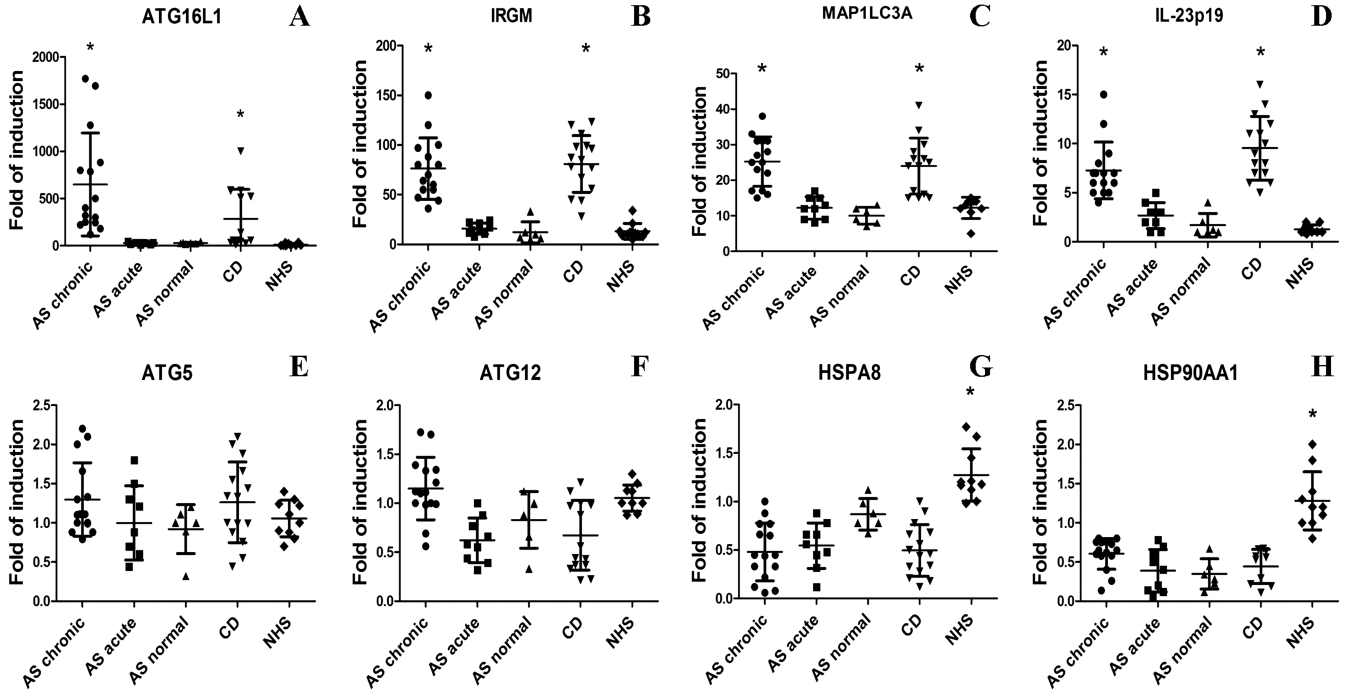

Figure 1 Macroautophagy and chaperone-mediated autophagy in the gut of ankylosing spondylitis (AS) patients. (A–H) Relative m-RNA quantification of ATG16L1 (A), immunity-related GTPase family (B), MAP1LC3A (C), IL-23p19 (D), ATG5 (E), ATG12 (F), HSPA8 (G) and HSP90AA1 (H) was assessed by quantitative RT-PCR in ileal biopsy specimens obtained from 30 AS, 15 Crohn's disease patients and 10 controls. Patients with AS were further divided into three groups: those with normal histological findings, those with acute inflammation and those with chronic inflammation. Data are shown as mean (SD). *p<0.0001.

patients (r^2=–0.63 and –0.58, respectively, p<0.05, data not shown) and CD patients (r^2=–0.71 and –0.64, respectively, p<0.005, data not shown) suggesting differential level of regulation of intestinal autophagy machinery.

In light of the differential expression of autophagy genes, we evaluated the protein expression of ATG5, ATG12 and LC3 by immunohistochemistry. A strong diffuse expression of LC3 was found only in the paraffin-embedded AS ileal samples with chronic gut inflammation (figure 2A–D) and CD patients (data not shown). The classic punctate LC3 staining, characteristic of autophagosome buildup, was clearly observed only in the frozen ileal samples (figure 2C) among infiltrating mononuclear cells and among some epithelial cells that we hypothesised to be Paneth cells (PC) because of their pyramidal shape (figure 2A–C, E). Despite the absence of a clear upregulation at transcript levels, immunohistochemical analysis showed that ATG5 (figure 2F–H,L) and ATG12 proteins (figure 2M–O,Q) were strongly upregulated only in the gut of CD and AS patients with chronic gut inflammation. Accordingly, to the increased activation of autophagy pathway, expression of SQSTM1 was observed only in few epithelial cells in normal controls (see online supplementary figure S2A) whereas no SQSTM1 immunoreactivity was detectable in the gut of AS patients with chronic gut inflammation and CD (supplementary figure 2B and C, respectively). In order to confirm that the immunoreactivity for LC3-II observed was associated with autophagosomes, we also examined the colocalisation of LC3 with ATG5. As shown in online supplementary figure S2, a significant colocalisation of LC3II and ATG5 was observed in the gut of AS patients with chronic gut inflammation (supplementary figure 2E–G) and CD patients (supplementary figure 2H–L) compared with the other AS patients (data not shown) and healthy controls (supplementary figure 2M–O).

UPR is not upregulated in the inflamed ileum of AS

Since there is a close relationship between autophagy and UPR, we also analysed the UPR response in the gut of AS patients and controls. The expression of the heat shock 70 kDa protein 5 (HSPA5) was assessed as a marker of global UPR.[27] The three main UPR pathways were also evaluated through the measure of the expression of PDIA4, GADD34, ATF6, protein kinase-like endoplasmic reticulum kinase (PERK) and XBP1. The expression levels of HSPA5 (figure 3A), PDIA4 (figure 3B), GADD34 (figure 3C) and ATF6 (figure 3D) were similar in the ileal samples from patients and controls, whereas PERK expression was significantly less in the gut of all AS and CD patients (figure 3E). Different regulation of XBP-1 was observed. Unspliced XBP-1 (figure 3F) was in fact upregulated in the gut of AS

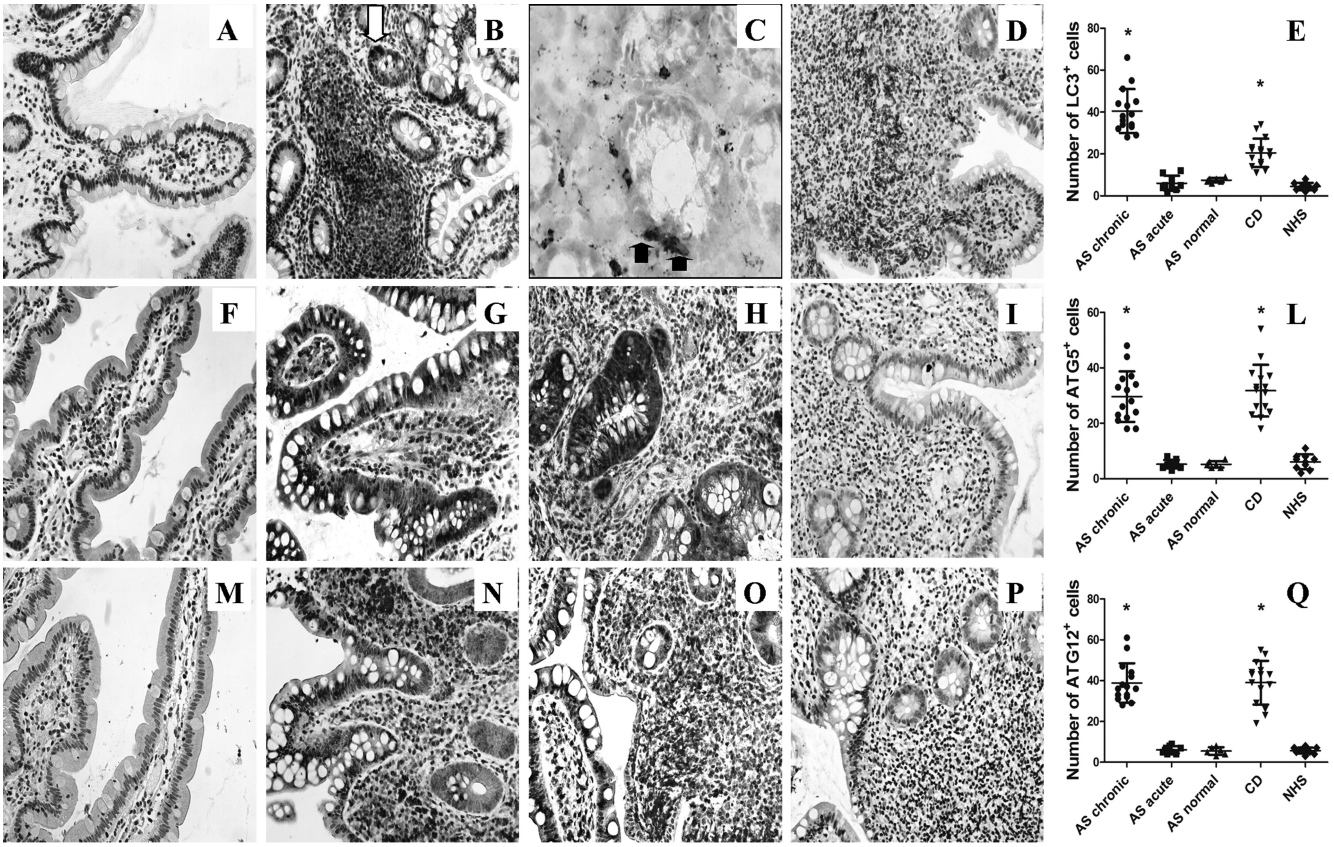

Figure 2 Autophagy is upregulated in the gut of ankylosing spondylitis (AS) patients. A 5 μm-thick paraffin-embedded sections of ileal biopsies obtained from AS, Crohn's disease (CD) patients and controls were stained with anti-LC3II, anti-ATG5 and anti-ATG12 antibodies. (A–C) Representative microphotographs showing LC3II immunostainings in heavy chain (HC) (A), and AS patients with chronic inflammation (B, C). (C) Representative immunostaining of LC3II staining on frozen samples obtained from AS patients. (E–G) Representative microphotographs showing ATG5 immunostainings in HC (E), AS patients with chronic inflammation (F) and CD patients (G). (I–M) Representative microphotographs showing ATG12 immunostainings in HC (I), AS patients with chronic inflammation (L) and CD patients (M). Diffuse expression of LC3II, ATG5 and ATG12 was observed in epithelial cells and infiltrating mononuclear cells of AS patients with chronic gut inflammation and CD patients compared with controls. Intense LC3 expression was observed in some epithelial cells of AS patients highly resembling Paneth cells (PC) for their pyramidal shape (black arrow) (C). The classic punctate staining was highly evident among infiltrating mononuclear cells and PC (arrows). (A–B, D, F–I, M–P) Original magnification ×250; (C) original magnification ×630. (D, H, N) Number of LC3+, ATG5+, ATG12+ cells in the ileal mucosa; *p<0.0001.

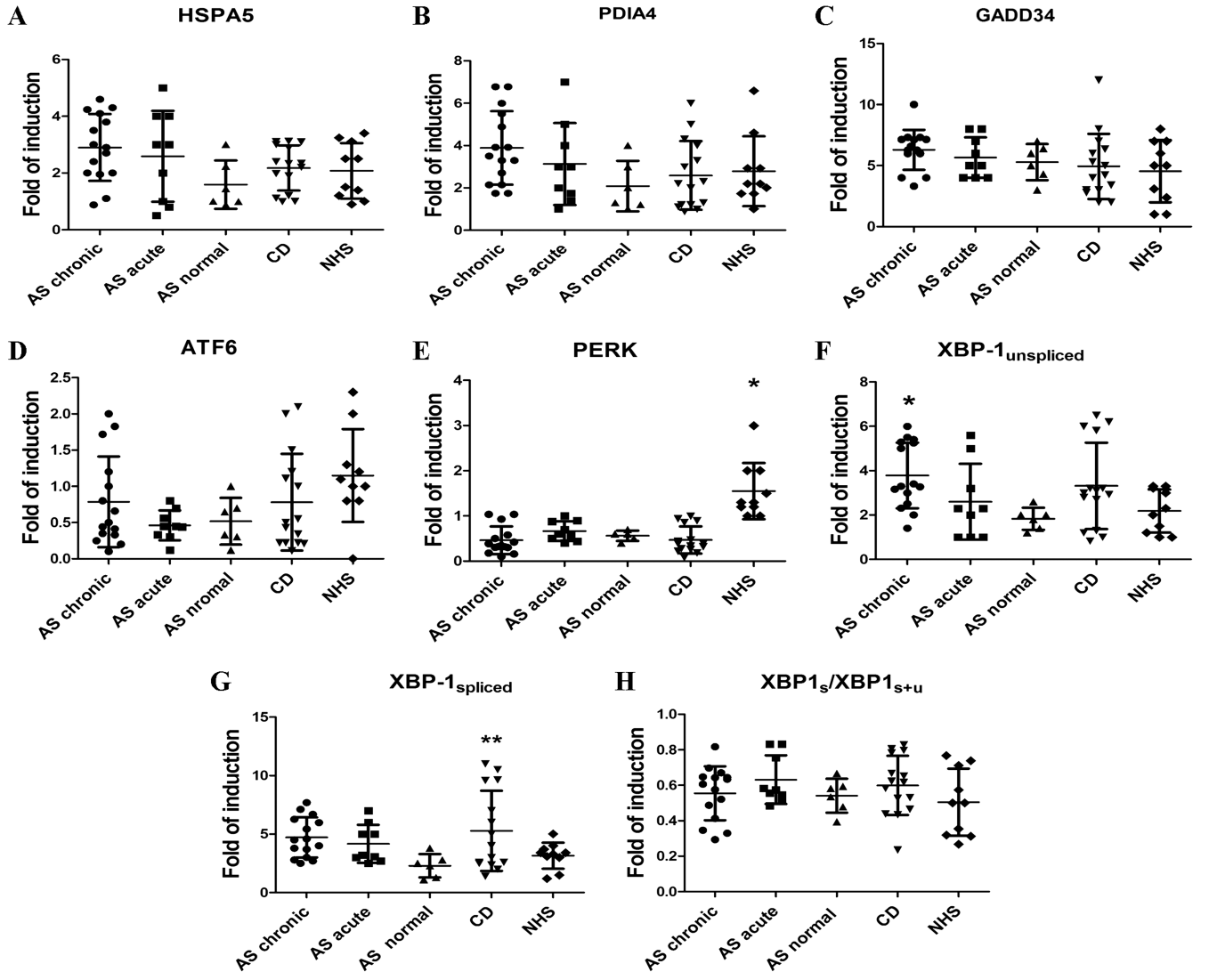

Figure 3 Unfolded protein response in the gut of ankylosing spondylitis (AS) and Crohn's disease (CD) patients and healthy subjects (HS). (A–H) Relative m-RNA quantification of HSPA5 (A), PDIA4 (B), GADD34 (C), protein kinase-like endoplasmic reticulum kinase (D), ATF6 (E), XBP1-unspliced (F), XBP1-spliced (G) and XBP1 ratio (H) was assessed by quantitative RT-PCR in ileal biopsy specimens from AS patients, CD patients and controls. Data are shown as mean (SD). *p<0.0001; **p<0.05.

patients with chronic intestinal inflammation and CD patients compared with controls. Conversely, XBP-1 spliced (figure 3G) levels were significantly upregulated only in the gut of CD patients, whereas XBP-1 splicing, evaluated as the ratio of XBP1s(XBP-1s+XBP-1u),[28] did not significantly differ between patients and controls (figure 3H). The protein levels of HSPA5 and XBP-1 were also evaluated by immunohistochemistry. As shown in online supplementary figure S3, no significant differential expression of HSPA5 (supplementary figure 3A–C,E) and XBP-1 (supplementary figure F–H,L) was observed in AS and CD patients compared with controls.

HC misfolding occurs in the gut of AS patients

The absence of a clear UPR activation prompted us to investigate the occurrence of HC accumulation and possible misfolding. Since SYVN1 is involved in the degradation of non-β2m bound misfolded major histocompatibility complex (MHC) class I HCs but not properly conformed MHC I-β2m-peptide heterotrimers,[29] double immunofluorescent confocal microscopy analysis was performed by using two monoclonal antibodies for HCs (the W6/32, specific for fully assembled MHC class I molecules and the mAb HC10, specific for β2m-free HLA-B and C HCs (FHCs), including misfolded forms of HLA-B27) and the anti-SYVN1 antibody. Properly folded (data not shown) and SYVN1 were both overexpressed in the gut of AS (figure 4A,D,E,H) and CD (figure 4I,N) patients compared with controls (figure 4O,R). Conversely, a significantly higher amount of free HCs was detected intracellularly only in the gut of AS patients independently by the presence/absence of intestinal inflammation (figure 4B,D,F,H) and in the HLA-B27+ CD patient (data not shown); rare colocalisation of properly-folded HCs and SYVN1 was observed in either patients and controls (data not shown). Interestingly, in both groups of AS patients (independent of gut inflammation) (figure 4C,D,G–H) and HLA-B27+ CD patients (data not shown) a strong colocalisation of free HCs and SYVN1 was observed, providing the first evidence of HLA-B27 misfolding in AS patients.

Autophagy but not UPR regulates IL-23p19 production in LPMC

We next examined the contribution of UPR and autophagy to the regulation of IL-23 expression. In this regard, isolated mononuclear cells from 12 AS, 10 CD patients and 10 controls were

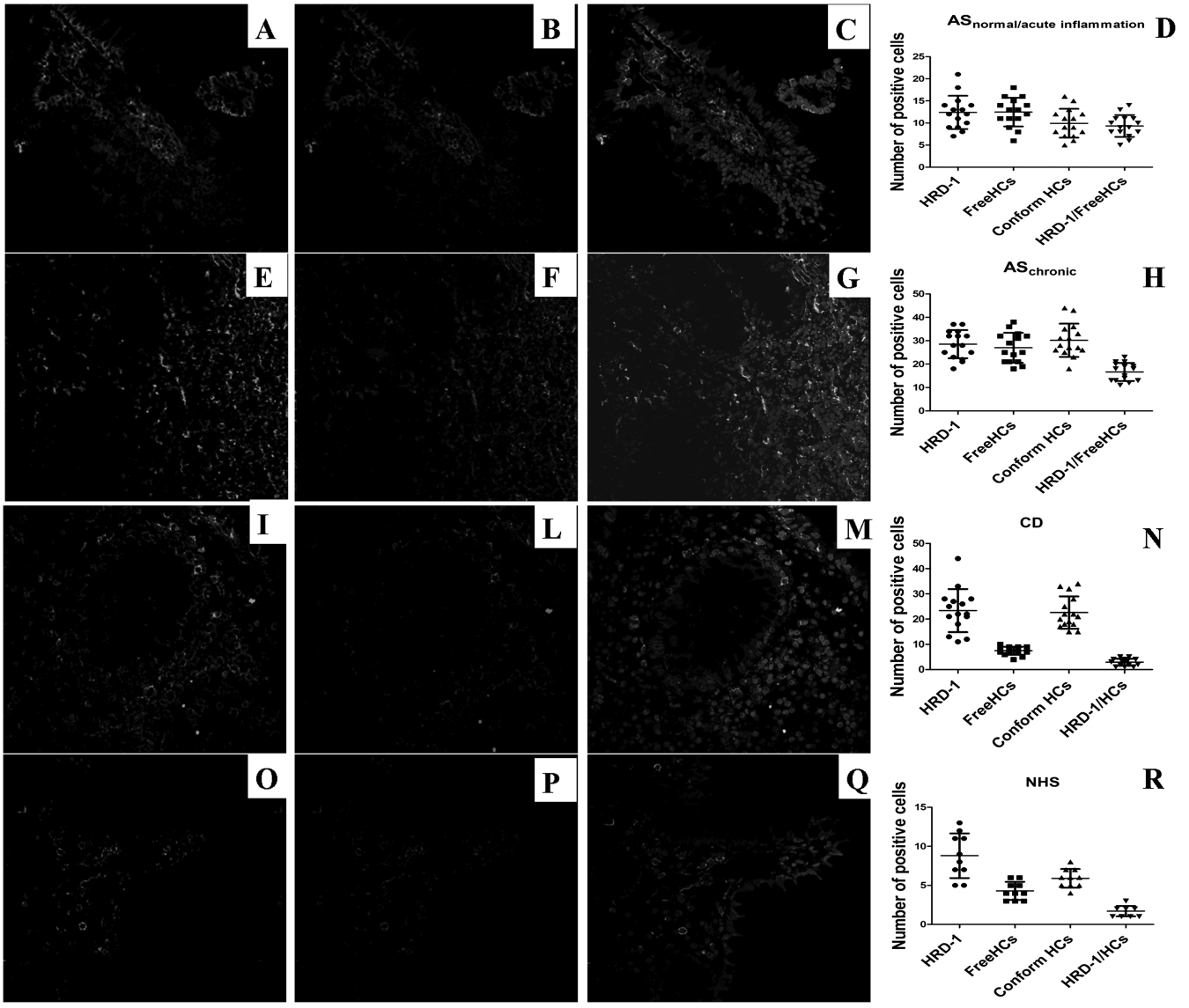

Figure 4 Free heavy chains (HCs) and SYVN1 co-colocalise in the gut of ankylosing spondylitis (AS) patients. Immunolocalisation by confocal microscopy of SYVN1 (green staining) and HCs (red staining) in AS, CD and control ileal biopsies. Paraffin-embedded section from patients and controls were stained with rabbit antihuman SYVN-1 and mouse antihuman free HCs (HC10) antibodies and treated with FITC-conjugated antirabbit antibody and Rhodamine Red-conjugated antimouse antibody. SYVN1 and free HCs expression was significantly increased in the gut of AS patients independently by the degree of intestinal inflammation (A, B, D and E, F, H) compared with controls (O–P, R). Significant colocalisation of SYVN1 and free HCs was detected in the gut of AS patients independently by the degree of intestinal inflammation (C, D, G, H) compared with CD (M, N) and controls (Q, R). (A–C, E–G, I–M, O–Q: original magnification ×250).

analysed for the IL-23p19 expression before and after stimulation with LPS, in the presence or absence of thapsigargin (TG; to induce UPR) and 3-methyladenine (MA) (to block autophagy). Unstimulated LPMC from AS patients with chronic gut inflammation (figure 5A) and CD patients (figure 5C) produced significantly higher amounts of IL-23p19 compared with controls (figure 5E). Incubation with LPS significantly increased the number of IL-23p19 producing LPMC from control subjects (figure 5F) but not from AS and CD patients (figure 5B,D respectively), suggesting a maximal preactivation of LPMC to produce IL-23p19. Treatment and/or pretreatment of LPMC with thapsigargin did not result in greater LPS-induced upregulation of IL-23p19-producing cells in AS, CD or normal controls (figure 5A,B), arguing against a role for UPR in the modulation of IL-23 production in LPMC from patients and controls. Finally, only pretreatment with the autophagy inhibitor 3-MA and CMA-inhibitor anisomycin similarly reduced the number of IL-23p19-expressing cells in patients and controls. Modulation of IL-23 m-RNA levels by UPR and autophagy was also evaluated by RT-PCR. IL-23p-19 mRNA levels in AS and CD patients were significantly increased only by the combination of 3-MA+LPS and anisomycin+LPS (figure 5B,D). In healthy controls LPMCs, LPS increased IL-23p19 expression and the preincubation with 3-MA/anisomycin further enhances this effect. Altogether these experiments suggest that autophagy process but not the UPR activation may be involved in regulating IL-23 in the gut of AS patients.

DISCUSSION

In this study, we confirm our previous results that showed that IL-23 is overexpressed in the gut of AS patients[5] and demonstrate for the first time that autophagy but not the UPR is

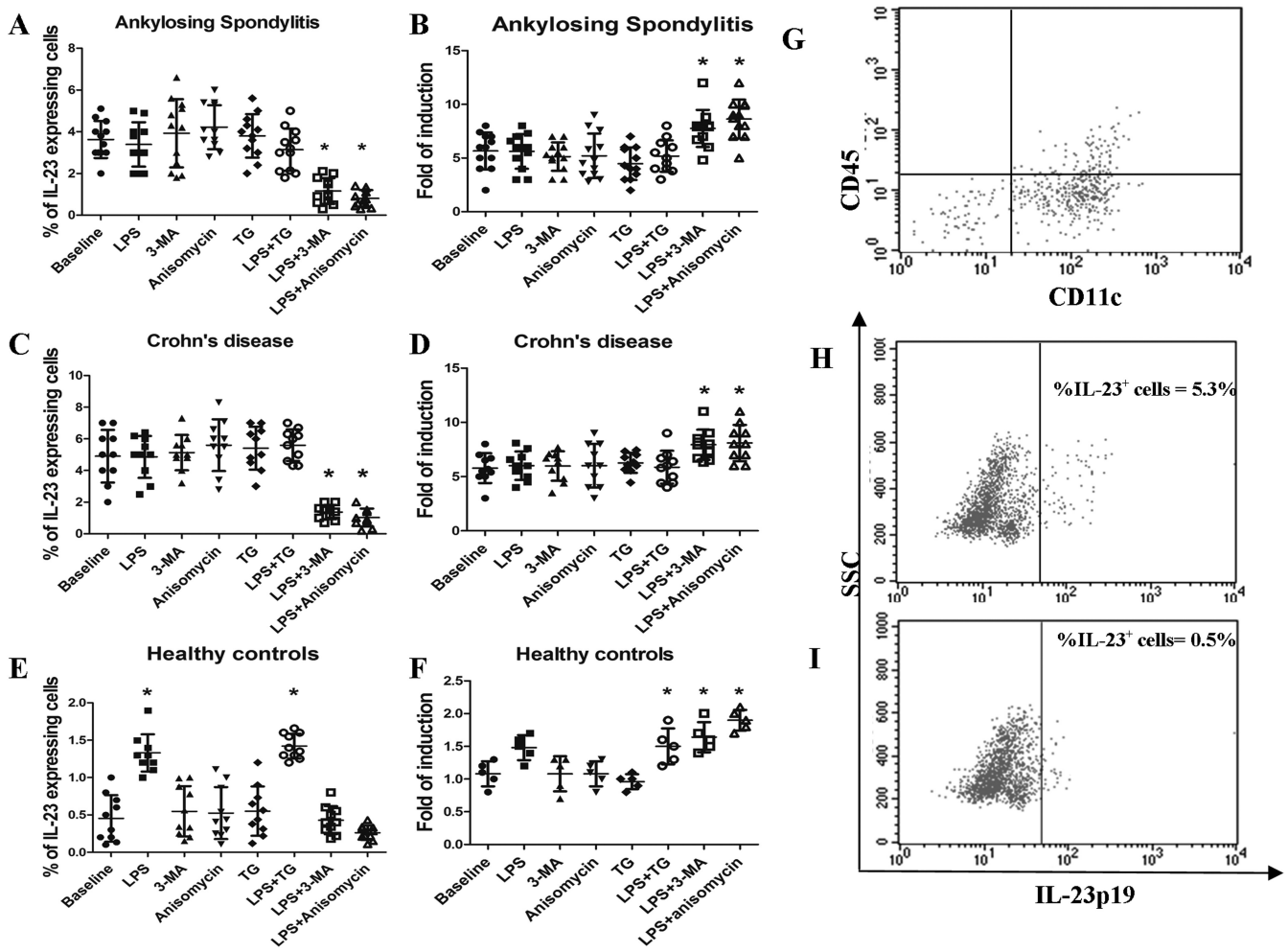

Figure 5 Autophagy but not unfolded protein response (UPR) regulates the production of IL-23 in lamina propria mononuclear cells (LPMCs). LPMCs isolated from patients with ankylosing spondylitis (AS), Crohn's disease (CD) and heavy chains (HCs) were cultured in the presence of lipopolysaccharide (LPS), 3-MA/anisomycin (to block autophagy) and TG (to induce UPR) alone and with LPS+TG and LPS+3-MA. The percentage of IL-23-producing cells and the m-RNA levels of IL-23p19 were evaluated by flow cytometry and RT-PCR, respectively. The percentage of IL-23 producing cells and the m-RNA levels were modified in AS (A) and CD (C) only by the combination of 3MA/anisomycin and LPS. In normal controls (E), LPS alone increased the percentage of IL-23-producing cells and the levels of IL-23 that were not further increased by the co-incubation with TG. Blocking of autophagy and chaperone-mediated autophagy significantly reduced the number of IL-23-producing cells in AS and CD and restrained the LPS-dependent IL-23 expression in LPMC from controls, also increasing the IL-23p19 m-RNA levels (B, D, F). *p<0.05. (G) Representative dot plot of CD45 versus CD11c+ cells among LPMC from AS patients. (H, I) Representative dot plot showing the percentage of IL-23 expressing cells before (H) and after (I) 3-MA/LPS incubation.

differentially regulated and may be modulating production of IL-23p19 in the LPMCs. Finally, we provide the first evidence that HLA-B27 misfolding is occurring in the gut of AS patients.

A considerable amount of genetic, immunological and therapeutic studies have demonstrated that IL-23 may be an important driver in AS aetiopathogenesis. The identification of the IL-23 pathway as a key regulator of entheseal pro-inflammatory responses[30] has added new arguments in favour of a pathophysiological role of this cytokine in AS.[5 9–11] Why IL-23 is overexpressed in the gut of AS is not still known, however. The discovery that the HLA-B27 HC is prone to misfold-inducing endothelial reticulum (ER) stress and activating the so-called UPR and the demonstration in a murine model that UPR activation in macrophages can promote IL-23p19 induction in response to LPS provide a possible explanation.[12 31] However, UPR activation was not observed in human macrophages from AS patients where IL-23 production was substantially greater than controls [9] rendering the exact role of misfolding unclear.

MHC I HCs are retained intracellularly becoming associated with the endoplasmic reticulum-associated protein degradation (ERAD)-associated E3 ubiquitin-protein ligase (SYVN1) and targeted for ERAD.[29] In the absence of SYVN1, misfolded HLA-B27 accumulates in the cells.[29] Thus, the co-colocalisation of SYVN1 together with non-β2m bound (HC-10-reactive) MHC class I HCs discriminates misfolded MHC class I from properly folded MHC I-β2m-peptide heterotrimers.[29] In our study, in the chronically inflamed gut of AS, both conformational and free HCs were overexpressed but only the unbound HCs significantly colocalised with SYVN1, strongly suggesting the occurrence of HLA-B27 misfolding in the intestine of HLA-B27+ AS patients.

IL-23 is constitutively produced in the terminal ileum in the presence of commensal microbes and plays an important role in the host defence against bacterial infection.[4] Subclinical gut inflammation has been demonstrated in a considerable percentage of AS patients and it seems to be immunologically

characterised by the increased expression of IL-23[5] perhaps as the result of chronic alterations in host–microbe interactions. In the gut of AS patients, the main source of IL-23 is represented by infiltrating inflammatory mononuclear cells and PC,[5] a subset of specialised epithelial cells that is considered to have a role in innate immune function through the secretion of antimicrobial peptides.[23] This secretory function seems to be closely related to the autophagy pathway, as suggested by the PC granule abnormalities observed in CD patients who have polymorphisms in the autophagy gene Atg16L1.[32]

Autophagy is a mechanism of controlled digestion of damaged organelles within a cell that may be differentiated in macroautophagy, in which entire regions of cytosol are sequestered in vesicular compartments and CMA that is exclusively dedicated to degradation of soluble proteins.[33] During infection, autophagy may be also induced as an innate defence mechanism to eliminate bacteria or toxins[20 21] and activation of autophagy has been demonstrated in PC of patients with CD independently of disease-associated variants of ATG16L1 or IRGM.[34]

In our study in the inflamed gut of AS and CD patients, a different regulation of autophagy and CMA was observed. The expression of macroautophagy genes was in fact significantly upregulated in comparison with healthy subjects whereas CMA appears to be importantly downregulated. The macroautophagy activation was confirmed by the immunohistochemistry experiments as recommended by the recently published guidelines for the use and interpretation of assays for monitoring autophagy.[35] LC3II, a marker of global autophagy, was demonstrated to be in fact upregulated in the gut of AS and CD patients and associated with autophagosome formation, as suggested by the strong colocalisation of with ATG5. Two additional markers of autophagy, ATG5 and ATG12, were also found to be increased at protein level in the gut of AS and CD patients. Finally, SQSTM1, a ubiquitin binding protein that accumulates when autophagy is inhibited, and is reduced when autophagy is induced, was not detectable in the gut of CD patients and AS patients with chronic gut inflammation.

Interestingly, increased autophagy gene expression was correlated with augmented IL-23p19 levels. In this regard, numerous cytokines have been shown to regulate autophagy in macrophages and dendritic cells and, obviously, we cannot exclude that the increased autophagy expression in the AS gut may be the consequence rather than the cause of IL-23 overexpression. Since the important role of PC in the defence against microorganisms and their activation is to release antimicrobial peptides in both AS and CD ileal samples,[36] we can also speculate that specific microbiota alterations could drive activation of autophagy in CD and AS.

However, our data suggest that autophagy may be one of the immunological factors regulating the production and secretion of IL-23 in AS. Specifically, inhibition of autophagy and CMA (with 3-MA and anisomycin) was sufficient, in the presence of LPS, to reduce the percentage of IL-23 expressing cells at the same time increasing the mRNA levels for IL-23p19 in the LPMCs isolated from the gut of patients and controls. The increased expression of IL-23p19 mRNA after LPS/3-MA and LPS/anisomycin stimuli could suggest that the reduced percentage of IL-23-producing cells observed in flow-cytometry may be attributable to the active cellular release of IL-23. However, the significant downregulation of CMA observed in inflamed gut and its negative correlation with IL-23 levels could suggest a role of specific branch of autophagy pathway in modulating IL-23 response. Nevertheless, the in vitro activation of LPMCs with PMA/ionomycin before the incubation with 3-MA is an experimental artifice that could limit the strength of our results. In agreement with this result is however the recent demonstration that autophagy regulates IL-23 secretion in dendritic cells[37] and our immunohistochemical observation that autophagy is localised to the two main cellular sources of IL-23 in the gut of AS, the infiltrating inflammatory mononuclear cells and in those that we consider to be PC.

Our study aimed also to investigate the UPR activation in the gut of AS patients. Similar to a previous study in macrophages,[9] other than increased total (unspliced) XBP1 mRNA, we found no evidence for UPR activation playing a role in IL-23 production. UPR signalling is initiated by the activation of at least three distinct stress sensors located at the ER membrane known as the ER-resident transmembrane protein kinase-endoribonucleases (RNase) (ERN1/IRE1α), the protein kinase PERK EIF2AK3/PERK and a family of type II transmembrane transcription factors, whose most prominent member is ATF6α. When unfolded proteins accumulate in the ER, eukaryotic cells can upregulate the expressions of the chaperone protein HSPA5 (which is used as a marker of global UPR).[38] Activation of the IRE1 kinase activity was assayed indirectly in our study by measuring the levels of unspliced and spliced XBP1 mRNA (XBP1u and XBPs, respectively), the latter of which is generated by removal of a 26-nucleotides and results in the generation of a functional protein (sXBP1). Activation of the PERK and ATF6 branches were evaluated by assessing the expression levels of transcriptionally activated target genes, GADD34 and PDIA4, respectively. No increased expression levels of UPR genes was observed in the ileal mucosa of AS and CD patients compared with healthy controls. Since our results support the presence of HLA-B27 misfolding, we cannot obviously exclude that low levels of ER stress caused by HLA-B27 in the gut of AS patients contribute to the intense activation of autophagy, which in turn may further limit the full UPR activation by enhancing the removal of misfolded protein. Consistent with this concept, ER stress has been shown to activate autophagy through PERK[39] and/or XBP1.[40] Interestingly, misfolded monomeric proteins have been demonstrated to be degraded by CMA[41 42] and the downregulation of CMA could account, at least in part, for the accumulation of misfolded proteins observed in AS gut.

In conclusion, we provide evidence that autophagy, rather than robust UPR activation, may be an important regulator of IL-23 production in the gut of AS patients. Since autophagy plays an important role in the intestinal immune response against microorganisms, our results could suggest a fundamental role of AS intestinal microbiota in regulating intestinal IL-23 expression. We also provide the first evidence that HLA-B27 misfolding occurs in the gut of AS. Further studies are required to better study the specific role of autophagy and CMA in regulating the immunology of the gut of AS patients.

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