Supplemental methods

Patients and controls

Fifty consecutive HLA-B27 AS patients, fulfilling the ASAS classification criteria (28 of them also fulfilling the modified New York criteria), [1-2] 33 coming from the University of Palermo and 17 from the University of Gent were enrolled in this study. All the patients had active disease defined as an Ankylosing Spondylitis disease activity score (ASDAS) [3] of 2 or more. Independent of the presence of gastrointestinal symptoms, all the patients underwent ileocolonscopy and multiple adjacent ileal mucosal biopsies were obtained from each patient for a total of 165 biopsies. Twenty consecutive healthy subjects, enrolled at the University of Palermo, unmatched for gender, undergoing ileocolonoscopy for diagnostic purposes but without evidence of intestinal disease, were considered as controls (baseline characteristics of patients and controls are specified in supplemental Table 1). Peripheral blood mononuclear cells (PBMCs) were also obtained from all the patients and controls. The ratio of urinary excretion of lactulose to mannitol (LA/MA) was used to measure intestinal mucosal permeability in 20 AS patients and 20 healthy controls who underwent colonoscopy, with higher ratios indicative of increased intestinal permeability as previously described.[4]

Characterization of bacteria and immunohistochemistry

In order to detect bacteria, we first examined the presence of bacterial bodies in the small intestinal biopsies using the highly sensitive Warthin-Starry silver/nitrate-based staining method as described.[5] Bacteria were further characterized by using the Gram staining and an antibody directed against bacterial lipopolysaccharide (LPS). Bacterial quantification was performed in 10 representative fields at a final observed magnification of 600x. The ten fields included bacteria observed within two well-defined mucosal compartments: (1) bacteria contained beyond the epithelial layer (invasive bacteria) and (2) bacteria attached to the surface epithelium (adherent bacteria). The degree of bacterial

infiltration was blinded scored by AR and FC as 0 (no infiltration), 1 (mild focal infiltration), 2 (moderate focal infiltration), or 3 (severe infiltration). Immunohistochemistry for occludin and claudin 4 was performed on paraffin-embedded sections as previously described [6] on AS and controls ileal samples. Immunohistochemistry for zonulin was performed on snap frozen ileal samples of AS patients and controls as previously described. [7] Briefly, frozen tissue block were cut at -20°C with a thickness of 5 µm using a cryotome. Tissue sections were dried overnight at room temperature and fixed in pre-cooled acetone (-20°C) for 10 min. Slides were rinsed in 300 ml of 10 mM phosphate buffered saline (PBS) at a neutral pH for 2 changes, 5 min each and incubated in 0.3% H_2O_2 solution in PBS at room temperature for 10 min to block endogenous peroxidase activity. 100 µl of anti-zonulin antibody, diluted 1:100 in 0.5% bovine serum albumin in PBS were then added to the sections on the slides and incubated in a humidified chamber for 1 h at room temperature. 100 µl an of diluted biotinylated secondary antibody (using the antibody dilution buffer) were then applied to the sections on the slides and incubated in a humidified chamber at room temperature for 30 min. 100 µl of pre-diluted Sav-HRP conjugates were then applied to the sections on the slides and incubated in a humidified chamber at room temperature for 30 min (keep protected from light). 100 µl DAB substrate solution were finally applied to the sections on the slides to reveal the color of the antibody staining for 3 minutes. A list of primary and secondary antibodies used is provided in supplementary table 2. The number of positive cells was determined as previously described by counting epithelial and infiltrating positively stained cells on photomicrographs obtained from 3 random high-power microscopic fields (400X magnification).[6] In order to study the integrity of gut vascular-barrier, RT-PCR and triple immunostainings were performed by using anti-CD31, glial fibrillary acidic protein (GFAP) and plasmalemma associated protein-1 (PV1) and the images evaluated by confocal microscopy analysis.

Isolation and culture of aerobic and facultative anaerobic bacteria

Briefly, three biopsy samples (15 mg each) were first washed in 500 ml of physiological saline with 0.016% dithioerythritol to remove the mucus and then washed three times in 500 ml of physiological saline by shaking for 30 second each time. Then the biopsies were hypotonically lysed by vortexing for 30 min in 500 ml distilled water to analyse mucosal aerobic and facultative-anaerobic bacteria. After hypotonic lysis the cell debris (100 ml) was plated in tenfold dilution steps onto elective and differential media (Oxoid, Wiesel, Germany). For the bacteria isolation all the culture media used in the study from Conte et al were used [8]. Columbia blood agar for total microorganisms, selective Columbia blood agar with colistin-nalidix acid supplement for Gram-positive microorganisms, MacConkey agar without supplements for Enterobacteriaceae, Enterococcus-selective bile–aesculin azide agar for Enterococcus spp, Schiemann Cefsulodin, Irgasan, Novobiocin medium for Yersinia spp, Skirrow selective medium for Campylobacter spp, Oxford agar for Listeria spp and Sabouraud medium for yeasts were used. Further investigations were performed on single colonies. Biochemical identification was determined by api 32 ID, api NE, api Strep, api Staph (bio-Merieux-Italia, Rome, Italy) following the manufacture's instructions.

RNA extraction and quantitative TaqMan real-time PCR (RT-PCR) for ileal biopsies

A total of 1 µg of RNA was reverse-transcribed to complementary DNA (cDNA) using a ThermoScript First-Strand cDNA Synthesis kit (Invitrogen, Waltham, MA). For quantitative TaqMan real-time PCR, sets of primers and probes were obtained from Applied Biosystems (Foster City, CA) (see supplemental table 2). Both 18S and GADPH were used as housekeeping genes, giving comparable results. GADPH was used for the final results shown. Relative changes in gene expression between controls and patients were determined using the ΔΔCt method as previously described.[7] Final values were expressed as fold of induction.

ELISA for circulating LPS, iFABP and zonulin

Blood was collected via EDTA vacutainer tubes. All samples were centrifuged at 1200 rpm for 10 minutes at 4°C immediately after collection, flash freezing in liquid nitrogen in order to avoid protein degradation and then stocked at -80°C until the analysis. The range of storage for patients was 12±6 months for the patients and 9±5 months for the controls. Human zonulin was analyzed in sera employing a sandwich-ELISA kit (Elabscience, China) following the manufacturer's instructions (measurable concentration range of 0.781 to 50ng/mL). Analysis of I-FABP was carried out from sera diluted 1:6 using a commercial ELISA kit (Hycult Biotech Inc., USA) following the manufacturer's instructions (measurable concentration range of 47 to 3000 pg/ml). LPS was determined in sera, diluted 1:100, by the Hycult Biotech LAL assay (Hycult Biotech Inc, USA) that detects and measure bacterial endotoxin, employing an enzymatic reaction (measurable concentration range of 0.04 to 10 EU/ml). All results were analyzed using a five parameter-logistic (5PL) function for fitting standard curves obtained from recombinant protein standards.

Cell cultures

The effect of zonulin on human umbilical vein endothelial cells (HUVECs) and peripheral blood mononuclear cells (PBMC) isolated from 5 AS patients enrolled at the University of Palermo was evaluated by incubating human endothelial cells (100000/well) and PBMC (500000 cells/well) with 50 μg/ml of zonulin (Novoprotein, Summit, US) for 24 hours at 37 °C in CO_2 5% as previously described.[9] The modulation of vascular tight and adherens junctions was then evaluated by RT-PCR and immunofluorescence and the effect of zonulin on PBMC by flow cytometry. The effects of LPS and soluble CD14 (sCD14) stimulation on IL-23 production by monocytes was evaluated by incubating PBMC with LPS (100 ng/ml) and sCD14 (0.25 μg/ml) and examined by flow cytometry. All cultures were set up in triplicate.

Flow cytometry analysis of surface and intracellular antigens

PBMCs were isolated from the peripheral blood of 20 patients with AS and 10 healthy controls as previously described.[7] A list of the antibodies used is provided in supplemental table 2. Flow cytometric analysis was performed using a FACSCanto and FACScalibur (Becton Dickinson, Franklin Lakes, New Jersey), and cell death was assessed by trypan blue exclusion. At least 50 000 cells (events) were acquired for each sample. Lamina propria mononuclear cells (LPMCs) were expressed as percentage of cells within the lymphocyte gate. The acquired data were analysed using CellQuest and FlowJo software programs.

HLA-B27 TG rats

Wild type PVG rats (PVG/OlaHsd) were purchased from Harlan and bred in-house. Animals were screened for expression of HLA-B27 by flow cytometry. Age-matched non-transgenic littermates were used as controls. All procedures were approved by the University of Glasgow Ethical Review Panel and performed under licenses from the UK Home Office, see online methods. Adult (8-10 wk) animals were treated with 50mg/kg/day vancomycin and meropenem, daily (Monday - Friday) by gavage, for three weeks. All animals were at least 12 weeks old when tissue was harvested, under terminal anaesthesia. Tissues were immediately fixed in neutral buffered formalin. They were dissected, paraffin embedded, and sectioned (5micrometers), before staining with anti-rat occludin antibody, anti-Rat IL-23 and Warthin-Starry silver/nitrate-based staining method. The effects of antibiotic treatment on intestinal inflammation was assessed by histological assessment of H&E-stained section of jejunum, ileum, and colon.

Supplemental references

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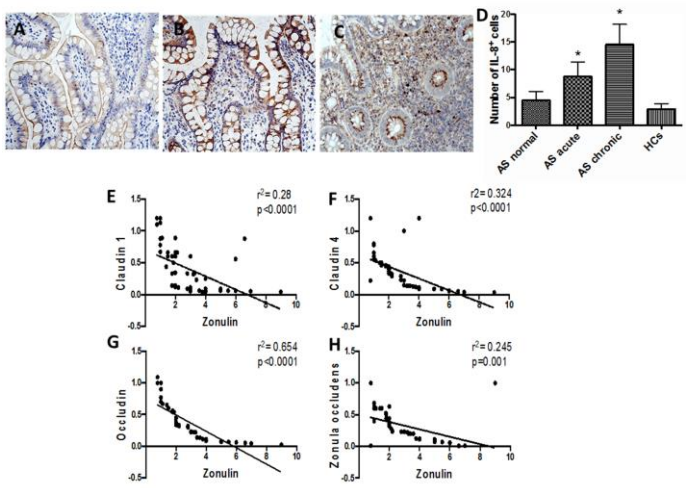
Supplemental Figure 1. A-C: representative images showing IL-8 immunohistochemistry for IL-8 in HCs (A), in AS patients with acute (B) and chronic inflammation (C). D: semiquantitative evaluation of IL-8+ cells in HC, AS patients without intestinal inflammation, AS patients with acute and chronic inflammation and healthy controls. E-H: correlations between zonulin and tight junction proteins. E: correlation between claudin1 and zonulin mRNA levels. F: correlation between claudin 4 and zonulin mRNA levels. G: correlation between occluding and zonulin mRNA expression. H: correlation between zonula occludens and zonulin mRNA expression. A-C: original magnification x250.



Supplemental figure 2. Genetic factors, such as HLA-B27, may shape the composition of intestinal microbiome in AS patients resulting in gut dysbiosis. Dysbiosis induces the production of high amount of zonulin that acts, in turn, deeply altering the integrity of epithelial and vascular barriers. The resulting translocation into the systemic circulation of bacterial products may result in monocyte anergy (indicated by the down regulation of CD14 and HLA-DR). Zonulin is also released in the systemic circulation and may induce the expansion of M2 macrophages.