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Dysbiosis and Zonulin up-regulation alter gut epithelial and vascular barriers in patients with Ankylosing Spondylitis

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Abstract

Background: Dysbiosis has been recently demonstrated in AS patients but its implications in the modulation of intestinal immune responses have been never studied. The aim of this study was to investigate the role of ileal bacteria in modulating local and systemic immune responses in Ankylosing Spondylitis (AS).

Methods: Ileal biopsies were obtained from 50 HLA-B27$^+$ AS patients and 20 normal subjects. Silver stain was used to visualize bacteria. Ileal expression of tight and adherens junction proteins was investigated by TaqMan real-time (RT-PCR) and immunohistochemistry. Serum levels of lipopolysaccharide (LPS), LPS-binding protein (LPS-BP), intestinal fatty acid-binding protein (iFABP) and zonulin were assayed by ELISA. Monocyte immunological functions were studied in *in vitro* experiments. In addition the effects of antibiotics on tight junctions in HLA-B27 transgenic (TG) rats were assessed.

Results: Adherent and invasive bacteria were observed in the gut of AS patients with the bacterial scores significantly correlated with gut inflammation. Impairment of the gut-vascular barrier was also present in AS, accompanied by significant up-regulation of zonulin, and associated with high serum levels of LPS, LPS-BP, iFABP and zonulin. In *in vitro* studies zonulin altered endothelial tight junctions while its epithelial release was modulated by isolated AS ileal bacteria. AS circulating monocytes displayed an anergic phenotype partially restored by *ex vivo* stimulation with LPS+sCD14 and their stimulation with recombinant zonulin induced a clear M2 phenotype. Antibiotics restored tight junction function in HLA-B27 TG rats.

Conclusions: Bacterial ileitis, increased zonulin expression and damaged intestinal mucosal- and gut-vascular barriers, characterizes the gut of AS patients and are associated with increased blood levels of zonulin, and bacterial products. Bacterial products and zonulin influence monocyte behavior.

Keywords: dysbiosis; zonulin; epithelial barrier; vascular barrier; Ankylosing Spondylitis

Introduction

In healthy subjects, the gastrointestinal tract is colonized by a broad range of microbes, termed the gut microbiota.[1] In heathy individuals a gut-epithelial [2] and a gut-vascular barrier [3] control the translocation of bacteria and bacterial antigens into the bloodstream. The homeostasis of normal microbial flora in the gut is essential for intestinal health and its altered balance, termed dysbiosis, may influence intestinal permeability through the release of zonulin,[4] a protein that modulates the permeability of epithelial tight junctions of the digestive tract.

Dysbiosis has been recently demonstrated in the terminal ileum of AS patients togheter with the presence of subclinical gut inflammation.[5] It is unclear, however, whether this dysbiosis is a cause or consequence of the inflammation and whether dysbiosis modulates immune responses in AS. The aim of the present study was to study the tissue localization of bacteria in the gut of AS patients and the eventual changes in gut-epithelial and gut-vascular barriers integrity. We also assessed the role of zonulin in modulating intestinal permeability and monocyte activation. Finally, we analyzed whether alterations in gut permeability and microbiota composition are associated with systemic immune responses.

Methods

Consent was obtained from all enrolled subjects and the study was approved from the institute review board at the Universities of Palermo and Ghent. For more details about patients and controls see supplemental methods and supplemental table I.

Histomorphological grading and immunohistochemistry

One hundred and sixty-five biopsies were obtained from the 50 AS patients enrolled. Gut specimens from patients with AS were histologically divided as previously described in: normal gut histology, acute and chronic inflammation.[6-7] The degree of gut inflammation was also evaluated by using IL-8 as a general marker of inflammation.[8] For more details about bacteria characterization and immunohistochemistry see supplemental methods.

Isolation of bacteria

Ileal biopsy specimens from patients and controls enrolled at the University of Palermo, were immediately processed for bacteriological study in the Microbiology Laboratory, Azienda Ospedaliera Villa Sofia Cervello, Palermo, Italy according to Conte et al. [9] For more information, see supplemental methods.

Cultures for aerobic and facultative anaerobic bacteria

For bacterial culteres only ileal biopsies obtained from AS patients and controls enrolled at the University of Palermo were used. For more information, see supplemental methods.

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

Total RNA was extracted using the Qiagen RNeasy Mini kit, with on-column DNase I digestion as previously described.[7] For more information, see supplemental methods.

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.

ELISA for circulating LPS, iFABP and zonulin

Levels of PGE2 were analyzed in sera of all AS patients and controls. For more information, see supplemental methods.

Cell cultures

In order to evaluate the role of intestinal bacteria isolated from AS patients in modulating epithelial zonulin levels, bacteria were isolated from ileal AS samples obtained from patients enrolled at the University of Palermo as described by Conte MP et al [9] and incubated with Caco-2 epithelial cells. The modulation of zonulin mRNA was then assessed by RT-PCR. The effect of zonulin on human umbilical vein endothelial cells (HUVECs) and peripheral blood mononuclear cells (PBMC) was evaluated as previously described.[9] For more information, see supplemental methods.

HLA-B27 TG rats

HLA-B*2705 transgenic rats of line 33-3 (B27-TG) on a Fischer background (F344/NTac-Tg (HLA-B*2705, β2M) (Taconic, Hudson, NY) were backcrossed with PVG rats (PVG/OlaHsd) (Harlan, UK) for a minimum of ten generations before their use in experiments as previously described.[10] For more information, see supplemental methods.

Statistical analysis

The non-parametric Mann–Whitney test was used to calculate the statistical significance between groups. Spearman's rank correlation was used to calculate the correlation between different variables in AS. p values <0.05 were considered significant.

Results

Assessment of intestinal gut inflammation in AS

IL-8 was over-expressed in AS patients with chronic inflammation (Supplemental figure 1C-D) compared to those with acute inflammation (Supplemental figure 1B-D) and without inflammation (Supplemental figure 1D) and controls (Supplemental figure 1A and D). In AS patients, the number of IL-8 positive cells was correlated with the degree of intestinal inflammation (Supplemental figure 1D).

Adherent and invasive bacteria are present in the gut of AS patients

Adherent and invading rod-shaped bacteria were observed in 35 out of 50 AS patients (25/33 of the Palermo cohort and 10/17 of the Gent cohort) independent of the presence of acute or chronic inflammation. Among these patients, only 4 showing acute inflammation and 4 showing chronic inflammation were taking sulfasalazine. Of these, 2 out of 4 patients with acute inflammation and 1 out of 4 with chronic inflammation did not show cultivable bacteria. Bacteria were mainly detected within the epithelium and rarely in the context of lamina propria (Figure 1A-C). Absence of adherent and/or invasive bacteria was observed in normal ileum (Figure 1D). In particular, invasive bacteria, sometimes aggregated in clusters, were observed in 12 AS patients of the Palermo's cohort and in 7 AS patients of the Gent's cohort. The bacterial scores significantly correlated with the percentages of infiltrating inflammatory cells (r^2=0.57, p<0.0001) (Figure 1E). $Gram^+$ (F-G) and $Gram^-$ (H-I) bacteria were confirmed to be both adherent and invasive in AS patients. The presence of invasive bacteria in AS was invariably associated with histologic changes characterized by the detachment of basal membranes from the lamina propria, forming vacuoles inside the villi, and edematous lamina propria with extravasated red blood cells (Figure 1K-M and Supplemental Table 3). Isolated edematous lamina propria, without detachment of basal membranes and/or vasculitis, was observed in the intestine of all patients displaying adherent bacteria (Supplemental Table 3). Identification of the bacteria

from culture of ileal samples showed that all of AS patients of the Palermo's cohort had cultivable bacteria essentially the Gram-negative bacteria *Escherechia coli and Prevotella spp* (Figure 1N). Conversely, only 5 out of 20 control samples displayed cultivable bacteria (25%) being *Escherichia coli* the only Gram-negative species found (Figure 1N). No culture of ileal samples was performed in ileal samples from the Gent's cohort. Cultures of Prevotella and Escherichia coli were confirmed by PCR.

We next studied the expression of intestinal tight junction proteins. A significant down-regulation of claudin 1 (Figure 1O), claudin 4 (Figure 1P), occludin (Figure 1Q) and zonula occludens 1 (Figure 1R) was observed in the gut of AS patients (expecially in those with chronic gut inflammation) compared to controls. The significant down-regulation of the tight junction proteins in AS was confirmed by IHC demonstrating the reduced expression in AS of occludin (Figure 2A, C) and claudin 4 (Figure 2D, F) compared to controls (Figure 2B, C and Figure 2E, F).

Zonulin is up-regulated in the gut of AS patients and modulated by ileal bacteria

We next evaluated zonulin expression in the biopsies of all AS patients and controls. Significant up-regulation of zonulin mRNA was observed in the ileal samples of AS patients, expecially in those with chronic gut inflammation, (Figure 2G), inversely correlated with the expression levels of claudin 1 (r^2=0.28,p<0.0001) (Supplemental Figure 1E), claudin 4 (r^2=0.324, p<0.0001) (Supplemental Figure 1F), occludin (r^2=0.654, p>0.0001) (Supplemental Figure 1G) and zonula occludens 1 (r^2=0.245; p<0.001) (Supplemental Figure 1H). Zonulin has been identified as pre-haptoglobin 2, one of the two genetic variants (together with haptoglobin-1) of human haptoglobins.[3] Since that by RT-PCR we can not completely discriminate between pre-HP2 and HP2,[11] over-expression of zonulin was also confirmed by immunohistochemistry in frozen ileal samples obtained from AS patients by using a specific anti-zonulin antibody (Figure 2 H-J). Analysis of tissue distribution of zonulin demonstrated its expression among epithelial cells and

infiltrating mononuclear cells (Figure 2 H-I). Interestingly, the number of zonulin^{+} cells correlated with the number of IL-8^{+} cells (Figure 2K). We next evaluated *in vitro* the role of isolated ileal bacteria from AS patients in modulating zonulin expression. As shown in Figure 2L, co-culture of Caco-2 cells with bacteria isolated from ileal biopsies of 5 AS patients of the Palermo's cohort induced significant up-regulation of zonulin.

Impairment of gut-vascular barrier occurs in AS patients

In order to evaluate whether increased intestinal permeability was paralleled by impairment of the gut vascular barrier (GVB),[12] RT-PCR for junctional adhesion molecule-A (JAM-A), a vascular tight junctions protein, VE-cadherin, a vascular adherens junctions protein, and PV1, a marker of endothelial cells permeability, was performed. VE-cadherin and JAM-A (Figure 3A-B), were significantly down-regulated in the the inflamed ileum of AS patients togheter with a significant up-regulation of PV1 expecially in those patients with chronic gut inflammation compared to controls (Figure 3C). To confirm the alteration of GVB, confocal microscopy analysis of occludin and CD31 (a specific endothelial cell marker) expression and of CD31/GFAP/PV1 was next performed in ileal samples from AS patients and controls. As shown in Figure 3 D-F, endothelial occludin expression in HC showed a continuous staining of the junctional protein that surrounded cell borders. In comparison, endothelial cells from AS patients exhibited the disappearance of the classic Occludin continuous staining, showing a jagged and broken vascular distribution (Figure 3G-I). Analysis of GVB showed in AS a higher expression of PV1 (Figure 3N) compared to HC (Figure 3J) and confirmed the disorganized staining for CD31 (Figure 3O) and GFAP (Figure 3P).

Zonulin alters the expression of endothelial tight junctions

We next evaluated *in vitro* whether zonulin may influence the expression of endothelial tight junction proteins. As shown in figure 4, zonulin induced a significant down-regulation of occludin (Figure 4A) and VE-cadherin (Figure 4B). Corresponding with the alteration of

the GVB, increased serum zonulin levels (Figure 4C) were observed in AS. To establish whether serum zonulin levels are correlated with intestinal permeability, LA/MA urine ratio was determined in 20 AS patients and 20 controls, all enrolled at the University of Palermo. An increased intestinal permeability, significantly correlated with serum zonulin, was present in AS patients (LA/MA 0.052 ± 0.002, r^2=0.7236, *p*=0.01777) (Figure 4D) but not in healthy control (LA/MA 0.021 ± 0.0011; r^2=0.1858; p>0.05) (data not shown). Zonulin has a CD163 binding motif identical to that present in mature haptoglobin 2 [9,13] In order to assess the potential functional relevance of zonulin interaction with CD163, isolated PBMC from AS patients and controls were incubated with recombinant zonulin. As shown in Figure 4E-F and G-H, incubation with zonulin induced a significant expansion in of c-MAF$^+$CD163$^+$ cells identified as M2 polarized macrophages [14] in AS (Figure 4 E-F) but not in controls (Figure 4G-H).

Increased serum levels of iFABP, LPS and LPS-BP are found in AS patients

Since the alterations of epithelial and endothelial permeability, we next evaluated the serum levels of LPS, LPS-BP and iFABP in all the AS patients and controls. As shown in figure 5, significantly increased levels of LPS (Figure 5A), LPS-BP (figure 5B) and iFABP (Figure 5C) were observed in AS patients. Since it has been demonstrated that the presence of high LPS concentration down-regulates the expression of CD14,[15] we examined by flow cytometry, the expression of CD14 in circulating monocytes and the effects of LPS and soluble CD14 stimulation on IL-23 production. A significant reduction of CD14$^+$ monocytes (Figure 5D-F) but also of HLADR$^+$ monocytes (Figure 5G-H) was observed only in AS monocytes. Since the soluble form of CD14 (sCD14) has been demonstrated to enable CD14$^-$ cells to respond to LPS [16], we next evaluated whether sCD14 might rescue AS CD14$^-$ cells from their anergic state. Among AS CD14$^+$ cells, stimulation with LPS, but not with sCD14, modified the expression of IL-23 that was not further modified by the combination of LPS and sCD14 (Figure 5I-K). Conversely, only the

combination of sCD14 and LPS strongly up-regulated the production of IL-23 in AS CD14 negative monocytes (Figure 5I-K).

Alteration of epithelial tight junctions occurs in HLA-B27 rats and is restored by antibiotic treatment

In human HLA–B27 and β2-microglobulin transgenic rats (B27-TG), ileitis develops spontaneously.[17] In order to study whether alteration of tight junctions is present in the ileal samples of these rats, ileal samples from 5 HLA-B27 TG and 5 wild type (WT) rats were evaluated. HA-B27 rats displayed ileal inflammation characterized by IL-23 increased expression (Figure 6B), occludin down-regulation (Figure 6F) and the presence of adherent bacteria (Figure 6J). After antibiotic treatment caused a significant amelioration of signs of intestinal inflammation as previously described,[18] the reduction of IL-23 expression (Figure 6C-D), the normalization of occludin expression (Figure 6G-H) and the disappearance of adherent bacteria (Figure 6K-L).

Discussion

In this study we demonstrate that adherent and invading bacteria are present in the ileum of AS patients and are associated with the alteration of epithelial and gut-vascular barriers. The presence of leaky epithelium and endothelium in AS ileum is accompanied by the translocation of zonulin and bacterial products into bloodstream possibly inducing the modulation of the innate immune system in AS.

The intestinal microbiota plays a critical role in modulating the immune response of the gut.[19] The potential role of intestinal bacteria in the pathogenesis of gut inflammation in SpA patients has been highlighted by the identification of dysbiosis in different SpA subsets, including AS patients.[5, 20-21] However, the question of how dysbiosis can influence local and systemic immune responses in AS has not yet been explored.

In this study we confirm and expand our previous results [5] by demonstrating that Gram negative bacteria, essentially *Escherichia Coli and Prevotella spp*, and Gram^{+} bacteria are present in AS ileal samples, displaying both adherent and invasive behavior. Interestingly, the presence of invasive bacteria was associated with specific histologic alterations mainly characterized by the detachment of basal membrane from lamina propria, leading to the formation of vacuoles inside the villi and hemorrhagic extravasation. These histologic findings seem to be directly attributable to the presence of bacteria since similar histologic alterations have previously been reported in mice infected with enteropathogenic *Escherichia coli*.[22]

In the presence of pathogenic or non-pathogenic enteric bacteria, mammalian small intestines activate the zonulin pathway [3] that is involved in the regulation of the permeability of epithelial tight junctions.[4] In our study, tissue levels of zonulin were significantly up-regulated in AS ileal samples and accompanied by IL-8 over-expression and a profound reduced expression of tight junction proteins by epithelial cells. These

alterations were dependent of the degree of intestinal inflammation, associated with both adherent and invasive bacteria and apparently related to a reduced expression by epithelial cells. We, however, cannot exclude that loss of epithelial cells may also contribute to the reduced tight junction protein expression. Serum zonulin increase was also observed in AS patients with more pronounced gut inflammation, accompanied by an increased intestinal permeability evaluated by LA/MA urine ratio. Interestingly, isolated bacteria from AS ileal biopsies significantly up-regulated zonulin expression in cultured epithelial cells, apparently indicating a specific effect of AS associated bacteria. It is unclear whether these alterations are the cause or the consequence of intestinal dysbiosis. However, here we demonstrated that alterations of tight junctions, also present in HLA-B27 TG rats, are restored after antibiotic treatments and that antibiotics therapy reduced epithelium-adherent bacteria, suggesting that intestinal dysbiosis might be responsible for the impairment of the epithelial barrier. The reduced number of adherent intestinal bacteria we observed is consistent with previous studies demonstrating that antibiotic treatment reduces mucosal adherent bacteria in mice.[18]

Together with gut-epithelial barrier, a GVB has been recently demonstrated in mice and humans, that acts by preventing the entry into the bloodstream of microbiota-derived products.[12] The GVB shows adherens junctions and tight junctions that seem to be modulated or down-regulated, as demonstrated in our *in vitro* experiments, by zonulin. Increased zonulin expression was in fact accompanied by a significant down-regulation of endothelial tight junction proteins, such as occludin, and vascular adherens proteins such as VE-Cadherin and by the up-regulation of PV1, a marker of increased endothelial permeability.[23] The presence of a “leaky endothelium” was also confirmed by confocal microscopy experiments showing disorganized staining for CD31, occludin and GFAP and by the demonstration of increased serum levels of zonulin and bacterial products such as LPS, iFABP and LPS-BP in AS patients’ serum. Overall, our results point to a zonulin-

dependent epithelial and endothelial loss of barrier function. The fact that gene expression analysis cannot distinguish between pre-HP2 (alias zonulin) and Hp2 and that antibodies used for the IHC experiments may not be specific enough to exclusively detect zonulin and not mature HP2 may raise the possibility that HP2 rather than zonulin is upregulated. However, the decreased expression of tight junction protein and, most importantly the direct correlation between zonulin and LA/MA point clearly to the involvement of zonulin and not the mature HP2 that has never been reported to have an effect on barrier function. Zonulin has a CD163 binding motif identical to that present in mature haptoglobin 2 [9] that has been shown to bind the haptoglobin receptor CD163.[13] Therefore, it is conceivable that zonulin binds to CD163 as well as haptoglobin. The potential functional relevance of this binding in the regulation of monocytes behavior, however, has been not previously studied. Here we demonstrated that zonulin induces a significant *in vitro* expansion of $CD163^{+}cMAF^{+}$ monocytes, compatible with the M2 phenotype, and that these cells were expanded in the peripheral blood of AS patients. Macrophages play essential activities in homeostasis maintenance during different organism's conditions and may be polarized according to various stimuli into distinct populations. M2 macrophages are macrophages essentially involved in the pathogenesis of asthma, fibrosis, atopic dermatitis, cancer and granuloma formation.[24] Furthermore, an increased frequency of $CD163^{+}$M2 monocytes, producing IL-23, has been previously demonstrated to be expanded in the peripheral blood and inflamed gut and synovial tissues of AS patients.[25-26] The *in vitro* stimulation of AS PBMC with recombinant zonulin, was also accompanied by a significant expansion of $cMAF^{+}CD163^{+}$ M2 cells. We also observed the zonulin-dependent expansion of $c\text{-}MAF^{+}CD163^{-}$ cells. Beyond its role in modulating macrophage differentiation, c-MAF is also involved in the differentiation of Th17 cells [27-28] and we cannot exclude that zonulin might induce also the expansion of $c\text{-}MAF^{+}$ T cells.

We also studied the functional relevance of the increased circulating levels of bacterial products in AS. In the gut, the presence of high LPS concentrations down-regulates the monocyte expression of CD14, the receptor involved in the binding of LPS/LPS-BP complex.[15] Increased LPS levels, in AS, were accompanied by the down-regulation of CD14 on the surface of monocytes together with the reduced expression of HLA-DR. $CD14^{-}HLADR^{-}$ monocytes have been demonstrated to be functionally anergic [29] and this anergic phenotype was rescued, at least in part, by the co-incubation of these cells with LPS+sCD14 leading to an increased expression of IL-23.

In conclusion, in this study we provide the first evidence that adherent and invasive bacteria are present in the inflamed gut of AS patients and that these bacteria, through the release of zonulin, may induce a leaky gut epithelial and endothelial barrier, leading to the translocation of intestinal derived proteins into bloodstream, ultimately inducing systemic immune alterations that might participate in AS pathogenesis.

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Competing Interest: None declared

Figure legends

Figure 1. Invasive and adherent bacteria are present in the ileum of AS patients and are associated with alterations of tight junction proteins. A-D representative microphotographs showing adherent (A) and invasive (B-C) bacteria in AS but not in controls (D). E bacterial scores are directly correlated with with the number of infiltrating mononuclear cells. F-G: representative images showing Gram staining in AS patients demonstrating the presence of invading Gram+ bacteria. H-J: representative images showing immunohistochemistry for LPS in AS patients (H-I) and controls (J). K-M: histologic alterations are associated with the presence of bacteria such as hemorrhages (K-L) and detachment of epithelium from basal membrane (M). N: Cultures of isolated bacteria displayed mainly E Coli and Prevotella. O-R: relative m-RNA levels of Claudin1 (O), Claudin 4 (P), Occludin (Q) and Zonula occludens 1 (R) were assessed by quantitative RT-PCR in ileal samples obtained from all the patients and all the controls. Data are expressed a mean (SEM). A-D, F-J: original magnification x 250. Insert in A-C and F-G: original magnification x 630

Figure 2. Occludin, Claudin 4 and Zonulin 1 tissue expression is altered on AS patients and modulated by intestinal bacteria. A-B: representative imaging showing Occludin expression in the gut of AS patients (A) and controls (B). C: higher numbers of Occludin positive cells were observed in healthy controls compared to AS. D-E: representative imaging showing Claudin 4 expression in the gut of AS patients (D) and controls (E). F: higher numbers of Claudin 4 positive cells were observed in healthy controls compared to AS. G: relative m-RNA levels of Zonulin 1 were assessed by RT-PCR in the ileal samples obtained from all the AS patients and HC. H-I: representative imaging showing Zonulin 1 expression in the gut of AS patients (H) and controls (I). J: quantification of Zonulin 1 positive cells was performed in the ileal biopsies of all the patients and the controls showing higher numbers of Zonulin 1 positive cells in AS

patients. K: the number of zonulin positive cells was significantly and directly correlated with the number of IL-8 positive cells. L: Caco-2 cells were incubated with bacteria isolated from ileal biopsies obtained from 5 AS patients and the modulation of Zonulin expression assessed by RT-PCR. Data are expressed as mean (SEM) of 5 independent experiments. A-B: original magnification x 630. D-E: original magnification x 250. H-I: original magnification x 400. Data are expressed as mean (SEM)

Figure 3. Gut vascular barrier (GVB) in AS patients. A-C: relative m-RNA levels of VE-Cadherin (A), JAM-1 (B) and PV1 (C) were assessed by RT-PCR in AS and HC ileal samples. D-F and G-I: representative confocal microscopy images showing CD31 and Occludin co-localization in HCs (D-F) and AS (G-I). J-M and N-Q: representative confocal microscopy images showing PV1, CD31 and GFAP co-localization in HCs (J-M) and AS (N-Q). D-Q: original magnification x 400. Data are expressed as mean (SEM)

Figure 4. Serum levels of Zonulin in AS patients and in vitro effects of zonulin on HUVEC and peripheral monocytes. A-B: mRNA expression of Occludin (A) and VE-cadherin (B) was assessed in HUVEC cells treated or not with recombinant human zonulin by RT-PCR. Significant down-regulation of Occludin and VE-cadherin was observed in HUVEC after incubation with zonulin. C-D: Serum levels of zonulin were evaluated in 20 AS patients and 20 controls (C) and correlated with LA/MA ratio (D). E-H: PBMC obtained from 5 AS patients (E) and 5 controls (G) were incubated with recombinant zonulin and the percentage of CD163+cMAF+ cells evaluated by flow cytometry; percentages of AS (F) and controls (H) CD163+cMAF+ cells before and after zonulin stimulation. A-B: data are expressed as mean (SEM). C-D, F, H: data are expressed as individual data points.

Figure 5. Intestinal bacterial products translocate into AS bloodstream and modulate monocytes behavior. A-C: serum levels of LPS (A), LPS-BP (B) and iFABP (C) are increased in the sera obtained from AS patients compared to controls. D-F: percentages of CD14+ cells is reduced in PBMC from AS patients. D: representative dot

plot showing the percentage of CD14+ cells gated on CD45 region)among PBMC in AS patients and controls, E: representative histogram showing CD14 MFI in AS and HCs. F: percentages of CD14+ cells in AS patients and controls. G-H: percentage of HLA-DR+ cells is reduced in PBMC from AS patients. G: representative dot plot showing the percentage of HLA-DR+ cells gated on monocytes region among PBMC in AS patients and controls, H: percentages of CD14+ cells in AS patients and controls. I-K: effects of monocytes stimulation with LPS alone, sCD14 alone or sCD14+LPS on CD14+ (H) and CD14- monocytes. Combination of LPS+sCD14 increased IL-23 production only in CD14- cells (I-J). K: representative dot plots showing the gating strategy and the percentage of IL-23 expressing cells. Results are showed as mean (SEM)

Figure 6. Ileal inflammation and dysbiosis in HLAB27 transgenic rats is modified by antibiotic treatment. A-C: representative images showing IL-23 staining in ileal samples obtaining from WT rats (A), HLA-B27 TG rats (B) and HLA-B27 TG rats after antibiotics treatment (C). D: semiquantitative evaluation of IL-23+ cells. E-F: representative images showing IL-23 staining in ileal samples obtaining from WT rats (E), HLA-B27 TG rats (F) and HLA-B27 TG rats after antibiotics treatment (G). H: semiquantitative evaluation of IL-23+ cells. I-K: representative images showing Warthin starry staining for identifying bacteria in ileal samples obtaining from WT rats (I), HLA-B27 TG rats (J) and HLA-B27 TG rats after antibiotics treatment (K). Higher number of adhering and sometimes invading bacteria were observed in HLA-B27 rats (J and insert in J) L: semiquantitative evaluation of bacteria in rats ileal samples. A-C, E-G: I-K original magnification x250; J insert: original magnification x 630. Data are expressed as individual data points.

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.

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