Macrophage phenotype in the subclinical gut inflammation of patients with ankylosing spondylitis

Francesco Ciccia1, Riccardo Alessandro2, Aroldo Rizzo3, Antonina Accardo-Palumbo3, Stefania Raimondo2, Francesca Raiata3, Giuliana Guggino1,2, AnnaRita Giardina1, Giacomo De Leo2, Guido Sireci2 and Giovanni Triolo1

Abstract

Objective. Long-term evolution of subclinical gut inflammation to overt Crohn’s disease (CD) has been described in AS patients. The aim of this study was to evaluate macrophage polarization occurring in the inflamed gut of patients with AS.

Methods. Twenty-seven HLA-B27+ AS patients, 20 CD patients and 17 normal controls were consecutively enrolled. Classic M1 (iNOS+IL-10−), resolution phase (iNOS+IL-10+), M2 and CD14+ macrophages were characterized by immunohistochemistry and flow cytometry. Quantitative gene expression analysis of IFN-γ, IL-4, IL-5, IL-33 and STAT6 was performed by real time PCR.

Results. Classic M1 macrophages were expanded in CD and AS, where resolution phase macrophages predominate. A large increase in CD163+ (M2) macrophages was observed in AS strictly correlated with the expression of IL-33, a Th2 cytokine involved in M2 polarization. Unlike in CD, CD14+ macrophages were virtually absent in the gut of AS patients and controls.

Conclusion. The absence of CD14+ macrophages together with the expansion of resolution phase and M2 macrophages is the immunological signature of subclinical ileal inflammation in AS.

Key words: ankylosing spondylitis, gut inflammation, M1 macrophages, M2 macrophages, resolution phase macrophages, interleukin 33.

Introduction

Subclinical gut inflammation occurs in up to 50% of patients with AS, with long-term evolution to overt Crohn’s disease (CD) occurring in 7–10% of AS [1–3]. Several immunological similarities between gut inflammation in AS and CD have been described [4–7], supporting the concept that this subgroup of AS patients may be considered a model of early immune alterations related to CD. Indeed, a lack of hyper-inflammation is observed in the gut of AS patients, mainly associated with tissue protective responses. Increased IL-23 expression, without a defined Th17 polarization [8], expansion of tissue-protective IL-22-producing NKP44+ natural killer (NK) cells [9] and increased frequency of IL-10-producing regulatory T (Treg) cells [10] characterize the immunological signature of subclinical gut inflammation in AS. Complex regulatory pathways are involved in the maintenance of intestinal immune homeostasis, including immune-regulatory cytokines and cellular elements such as dendritic cells and inhibitory macrophages, which represent the largest pool of tissue macrophages in the body [11].

In the last several years macrophages have been subdivided and categorized on the basis of their activity, location and cell surface marker expression. Environmental cues, derived from microbes, damaged tissues or activated lymphocytes, may induce the emergence of a spectrum of distinct functional macrophage phenotypes. Borrowing the Th1/Th2 nomenclature, prevalent M1 (classical/proinflammatory) or M2 (alternative/anti-inflammatory) macrophage activation have been
described, which represent extremes of a continuum in a universe of activation states [12]. Commitment of M1 macrophages may be determined by adaptive immunity through the stimulation of type 1 inflammatory cytokines (e.g. IFN-γ) and/or by innate immunity with microbial products (such as lipopolysaccharide) stimulating Toll-like receptors (TLRs) [12]. On the other hand, IL-4, IL-13, IL-21 and IL-33 are potent inducers of an alternative M2 form of macrophage activation (reviewed in [13]). M2 macrophages seem to exert a prevalent anti-inflammatory role through the production of IL-10 [14], but are also able to induce lung eosinophilia and inflammation up-regulating Ym1, arginase-1 and several chemokines. In fact, M1 and M2 macrophages display distinct chemokine profiles, with M1 macrophages expressing Th1 cell-attracting chemokines, such as CXCL9 and CXCL10, and M2 macrophages expressing the chemokines CCL17, CCL22 and CCL24 (reviewed in [13]). Together with M1 and M2 macrophages, another subset, the so-called resolution phase macrophages, which express markers typical of M1 macrophages (i.e. COX2 and iNOS) and produce high levels of M2 cytokines such as IL-10 [15], has recently been described, highlighting the ability of macrophages to change their phenotype during the course of inflammation.

Unlike blood monocytes or other tissue macrophages, intestinal macrophages are unresponsive to bacterial stimuli and do not up-regulate co-stimulatory molecules in response to TLR ligands or cytokines such as IFN-γ [16]. This state of unresponsiveness is thought to be dependent upon the reduced expression of TLRs and other functional receptors necessary for macrophage activation (i.e. CD14) [17]. In chronic inflammatory disorders as well as in CD this anergic phenotype is lost and CD14-expressing macrophages accumulate in the inflamed lamina propria where they are actively involved in the release of pro-inflammatory mediators [18], resulting in the induction of both Th1 and Th17 responses [19, 20]. In the present study we investigated the frequencies of the different macrophage subsets in the intestine of AS patients. In this regard M1, M2, resolution phase and CD14+ macrophages were studied and correlated with the expression of Th1 and Th2 cytokines.

**Methods**

**Patients and controls**

Ileal biopsies from patients with AS without symptoms of bowel inflammation, CD patients with active disease and normal controls were consecutively obtained. Demographic and clinical characteristics of patients and controls are summarized in supplementary Table S1, available as supplementary data at Rheumatology Online. The AS group consisted of 27 consecutive HLAB27-positive patients fulfilling the modified New York criteria [21]. Disease activity was evaluated using the BASDAI [22]. The CD group consisted of 20 patients and disease activity was evaluated using the CD activity index [23]. The control group consisted of 17 healthy subjects undergoing ileocolonscopy for routine evaluation. All patients provided written informed consent and the study was approved by the ethics committee of the University of Palermo.

**Histomorphological grading**

Tissue samples were processed as previously described [8] and the grade of inflammation was evaluated by an experienced pathologist (A.R.). Specimens from patients with AS were divided into three subgroups as previously described [8]: those with normal gut histology, those with acute inflammation and those with chronic inflammation. Acute inflammation was defined by the presence of neutrophils and/or eosinophils in the crypt and villus epithelium with preservation of normal architecture. Chronic inflammation was defined by alterations of the mucosal architecture, an increased mononuclear cell infiltrate and formation of basal lymphoid aggregates in the lamina propria.

**Antibodies and reagents**

The following primary and secondary antibodies were used: mouse anti-human CD68 (clone PGM-1, 1:200 dilution, DAKO, Denmark), mouse anti-human CD14 (clone TUK4, 1:100 dilution, DAKO), mouse anti-human CD163 (clone 10D6, 1:100 dilution, Novocastra, UK), rabbit anti-human iNOS (1:100 dilution, AbCam, UK), mouse anti-human IL-10 (1:100 dilution, R&D Systems, Abingdon, Oxford), rat anti-human CCL24 (1:100 dilution, Novus Biological, Littleton, CO, USA), rabbit anti-mouse Rhodamine Red (1:100 dilution, Invitrogen, Carlsbad, CA, USA), goat anti-rabbit FITC (1:100 dilution, Invitrogen), goat anti-rabbit Cy5 (1:100 dilution, Invitrogen), goat anti-rat Cy5 (1:100, Invitrogen).

**Immunohistochemistry**

Immunohistochemistry was performed on 5-μm-thick paraffin-embedded sequential sections as previously described [8]. The number of CD68+, iNOS+, CD163- and CD14-expressing cells was determined by counting the immune reactive cells on photomicrographs obtained from three random high-power microscopic fields (original magnification 400×) under a Leica DM2000 optical microscope using a Leica DFC320 digital camera. Results were reported as the mean (s.d.). To characterize resolution phase, M1 and M2 macrophages, double and triple stainings were performed on paraffin-embedded sections of human ileum. Sections were incubated with unlabelled anti-human-iNOS and IL-10 antibodies (resolution phase macrophages), iNOS, CD68 and CCL24 antibodies (M1 macrophages) and CD163, CD68 and CCL24 (M2 macrophages) and then treated with FITC- or Rhodamine Red- or Cy-5 conjugated anti-mouse or anti-rabbit antibodies plus RNasi (200 ng/ml) and counterstained using Toto-3 iodide (642/660; Invitrogen). Confocal analysis was used to acquire fluorescence staining.
Flow cytometry analysis of surface and intracellular antigens

Lamina propria mononuclear cells (LPMCs) were isolated from ileal biopsies of patients with AS (n = 5) or CD (n = 5) and from healthy controls (n = 5) as previously described [10]. The isolated cells were counted and checked for viability using 0.1% Trypan Blue (viability ranged from 90 to 94%). Fresh cell suspension was immediately analysed by flow cytometry for phenotypical characterization of macrophages. The isolated cells were stained with the following monoclonal antibodies (mAb): anti-human CD68-APC (BioLegend, San Diego, CA, USA), anti-human CD163-FITC (BioLegend), anti-human iNOS-PE (BD Biosciences, San Jose, CA, USA), anti-human CD14-PE (BioLegend), anti-human IL-10-FITC (BioLegend). Within an extended lymphocyte-monocyte light scatter gate, granulocytes as well as necrotic cells were excluded. The CD45-CD68+ cell population was gated and used to discriminate the iNOS+ from the CD163+ and CD14+ macrophages.

Intracellular cytokine staining for IL-10 of iNOS+ cells was performed as follows: after 2 h of stimulation without or with lipopolysaccharide (10 ng/ml), brefeldin A (10 μg/ml) was added to cell cultures. All cultures were set up in triplicate. After 24 h of culture, cell surface staining was then conducted with the appropriate antibodies. After 30 min, cells were thoroughly washed and samples were analysed by FACSCalibur (Becton Dickinson, USA). A minimum of 30 000 events were gathered from each sample. The acquired data were analysed using the CellQuest software program (Becton Dickinson).

RNA extraction and quantitative TaqMan RT-PCR for IFN-γ, IL-4, IL-5, IL-33 and STAT6 in ileal biopsies

The analysis of target gene expression in the intestinal biopsy samples with quantitative real-time PCR was performed as previously described [8]. For quantitative TaqMan real-time PCR, master mix and TaqMan gene expression assays for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) control and for IFN-γ (Hs00989291_m1), IL-4 (Hs00174122_m1), IL-5 (Hs01548712_g1), IL-33 (Hs01125942_m1) and STAT6 (Hs00598625_m1) were obtained from Applied Biosystems (Foster City, CA, USA). Samples were run in triplicate using the Step-One real-time PCR system (Applied Biosystems).

Statistical analysis

Statistical analysis of quantitative variables was performed using the Kruskal-Wallis non-parametric test, with Dunn’s post-test. Pearson’s correlation analysis was utilized to quantify the expression associations between the genes of interest. P-values <0.05 were considered significant.

Results

Histomorphological grading

On the basis of gut histology as defined above [8], we identified two main groups of AS patients: patients (group 1) displaying normal histology (n = 6) or minor inflammatory signs (acute lesions, n = 8) and patients (group...
2) with chronic inflammation (n = 13). As shown in supplementary Table S1, available as supplementary data at Rheumatology Online, the two groups of AS patients did not differ significantly by demographic characteristics, clinical presentation and medications used. A comparative analysis within AS patients was performed by subgrouping patients with AS with chronic inflammation vs those with acute inflammation or normal histologic findings.

Macrophage evaluation

Macrophages were evaluated and enumerated as CD68+ cells in ileal biopsies from AS and CD patients and controls by immunohistochemistry and flow cytometry. CD68+ cells were found to be significantly increased in the inflamed lamina propria of AS patients with chronic gut inflammation and CD patients compared with controls, with the CD samples displaying the greater number of CD68+ macrophages (Fig. 1). To characterize macrophage differentiation, M1, M2, resolution phase and CD14+ macrophages were subsequently evaluated. iNOS+ macrophages were significantly increased in the gut of chronic inflamed AS (Fig. 2A, D and G) and CD (Fig. 2B, E and G) patients when compared with controls (Fig. 2C, F and G). The M1 phenotype of iNOS-expressing macrophages was confirmed by confocal microscopy, demonstrating their co-expression of CXCL9 (an M1-associated chemokine) in chronic inflamed AS patients (Fig. 2H-K) and CD patients (data not shown). Macrophages expressing iNOS, classified as M1 macrophages, may be induced by adaptive immunity through IFN-γ. Evident overexpression (>10-fold increase) of IFN-γ was detected in patients with CD. On the other hand, no significant up-regulation of IFN-γ was observed in AS independent of the degree of intestinal inflammation and controls (Fig. 2L) (P < 0.001).

A subset of iNOS+ - and IL-10-expressing macrophages has been recently demonstrated to characterize the

![Fig. 2](image-url)  

**Fig. 2** iNOS+ macrophages are significantly expanded but differently activated in the ileum of AS and CD patients.

LPMCs from AS patients (n = 5), CD patients (n = 5) and healthy controls (n = 5) were stained with labelled antibodies as described in the Methods section. (A-C) Dot plot analysis of CD68+INOS+ cells among isolated LPMCs of one representative (A) AS patient, (C) CD patient and (E) healthy control. (D-F) Representative microphotographs showing iNOS+ cells in paraffin-embedded ileal samples from (D) AS and (E) CD patients and (F) healthy controls. Positive cells are stained brown by specific immunohistochemistry. (D-F Original magnification 250×). (G) Quantification of INOS+ cells in ileal biopsies from patients and controls. (H-K) M1 macrophages in the gut of AS patients. (H-J) Single staining iNOS (green), CD68 (red) and CXCL9 (blue), respectively. (K) Merged triple staining. (L) Relative mRNA quantification of IFN-γ was assessed by RT-PCR in the ileal biopsy specimens from patients and controls. Data are shown as mean (s.d.).
resolution phase of tissue inflammation [15]. Resolution phase macrophages were evaluated by FACS (CD68+iNOS+IL-10+ cells) and laser confocal microscopy (iNOS+IL-10+ cells) in the ileum of patients and controls. iNOS+IL-10+ resolution phase macrophages were significantly increased only in AS patients with chronic gut inflammation (Fig. 3E/C) when compared with CD patients (Fig. 3I/C) or controls (Fig. 3A/C).

CD163+ macrophages are significantly expanded and correlated with expression of IL-33 in the intestinal mucosa of AS patients

M2 (CD163+ cells) macrophages were found to be increased in the inflamed ileal specimens from AS patients displaying chronic gut inflammation (Fig. 4A, D and G) when compared with CD patients (Fig. 4B, E and G) and controls (Fig. 4C, F and G). The M2 phenotype of CD163-expressing macrophages was confirmed by confocal microscopy demonstrating their co-expression of CCL24 (an M2-associated chemokine) in chronic inflamed AS patients (Fig. 4H-M) and CD patients (data not shown).

The mRNA levels of IL-4, IL-5, IL-33 and STAT6, which are involved in M2 polarization, were also evaluated. IL-4, IL-5 and STAT6 were similarly up-regulated in the inflamed gut of both CD and AS patients with chronic gut inflammation (Fig. 5A-C). On the other hand IL-33, a potent inducer of M2 polarization [24], was significantly up-regulated only in the gut of chronic inflamed AS patients (>20-fold increase) (Fig. 5D) and directly correlated with the numbers of CD163+ cells (r=0.82, P=0.0046) (Fig. 5E), suggesting a role for IL-33 in the commitment of M2 macrophages in the gut of AS.

CD14+ macrophages are virtually absent in the subclinical gut inflammation of AS patients

Macrophages interact with bacteria and their products through CD14, a surface receptor involved in the detection of lipopolysaccharide and other associated molecular patterns [11]. Thus we examined whether the subclinical gut inflammation of AS was characterized by the
Fig. 4 CD163⁺ macrophages are significantly expanded in the ileum of AS and CD patients.

LPMCs from AS patients (n = 5), CD patients (n = 5) and healthy controls (n = 5) were stained with labelled antibodies as described in the Methods section. (A–C) Dot plot analysis of CD68⁺CD163⁺ cells in isolated LPMCs from one representative (A) AS patient, (B) CD patient and (C) healthy control. (D–F) Representative microphotographs showing CD163⁺ cells in paraffin-embedded ileal samples from (D) AS and (E) CD patients and (F) healthy controls. Positive cells are stained brown by specific immunohistochemistry. (D–F original magnification 250 ×). (G) Quantification of CD163⁺ cells in ileal biopsies from patients and controls. Data are shown as mean (s.d.). (H–K) Single staining for CD163 (red), CD68 (green) and CCL24 (blue), respectively. (K) Merged triple staining for CD163, CD68 and CCL24.

expression of CD14. As shown in Fig. 6, CD14⁺ macrophages were rarely detected in the gut of normal controls (Fig. 6A, D and G) and AS patients independent of the degree of intestinal inflammation (Fig. 6B, E and G). Conversely, greater numbers of CD14⁺ macrophages were observed in CD patients (Fig. 6C–G), suggesting that the presence of this subset of macrophages immunologically differentiates the gut of AS from that of CD patients.

Discussion

Quantitative and qualitative alterations in the pattern of secreted mediators produced by intestinal macrophages might represent a major factor contributing to the pathogenesis of gut involvement in both AS and CD. Macrophages are not a homogeneous population and different pathways of macrophage activation have been described in humans according to their phenotype and function [12]. Similar to the Th1/Th2 paradigm, macrophages have been classified in two main populations: classically activated macrophages (M1), which are the main source of soluble proinflammatory cytokines and are associated with a proinflammatory state, and alternatively activated macrophages (M2), which are associated with an anti-inflammatory state and/or Th2-mediated tissue inflammation [12]. Another subset of macrophages, the so-called resolution phase macrophages have been recently described [15]. These cells are neither classically nor alternatively activated, but possess aspects of both definitions consistent with an immune regulatory phenotype.

In non-inflamed mucosa, resident macrophages lack the expression of innate immune co-receptor CD14 and do not produce proinflammatory cytokines against commensal bacteria [17]. In the inflamed mucosa, as observed
in CD, expansion of CD14+ intestinal macrophages occurs and contributes to the pathogenesis of disease via the production of IL-23 and TNF-α and induction of Th1 and Th17 responses [18–20].

Impairment of M1-derived mediators and expansion of M2 macrophages has been demonstrated in the colon and synovial fluid of AS patients [7, 25]. How the different subsets of macrophages are represented in the inflamed ileum of AS patients is still unclear. In the present study we studied the frequency of M1, M2, resolution phase and CD14+ macrophages in the gut of AS patients with subclinical gut inflammation and investigated the transcript levels of cytokines and transcription factors.

Classically activated M1 macrophages (iNOS+IL-10+/CXCL-9+) were significantly expanded in the inflamed gut of both chronic inflamed AS and CD patients. Although a plethora of stimuli may induce M1 polarization, M1 macrophages may be induced in response to type 1 inflammatory cytokines (e.g. IFN-γ) and/or microbial products (e.g. lipopolysaccharide) through TLR stimulation [25, 26].

Analysis of M1 polarization in our study showed that adaptive immunity, mainly through the Th1 cytokine IFN-γ, seems to be the main inducer of M1 macrophage polarization in CD. The absence of a Th1 signature observed in the subclinical gut inflammation of AS patients in this and other reports may suggest a role of intestinal microbiota in driving the classic M1 macrophage response we demonstrated in this study.

Despite the increased presence of M1 polarized macrophages, in AS patients we observed a lack of excessive intestinal inflammation as demonstrated by the absence of intestinal signs and symptoms. The significant increase in iNOS+IL-10+ cells (defined as resolution phase macrophages) together with the large increase in the numbers of CD163+ ileal macrophages (M2 or alternatively activated) observed in our study in AS patients with chronic gut inflammation could counterbalance the activation of M1 macrophages, inhibiting the transition through a chronic inflammatory intestinal disease. M2 macrophages in particular are known to exert an anti-inflammatory role and to promote tissue repair and remodelling [14]. M2 polarization is mainly induced by Th2 cytokines such as IL-4 (through the activation of STAT6), IL-13 and IL-33 [14, 24]. M2-activated macrophages produce IL-10 and favour Th2-polarized T cell responses [14]. A cytokine milieu characterized by high levels of Th2-related cytokines and transcription factors such as IL-4, IL-13 and STAT6 was found in both AS and CD. On the other hand, significantly increased IL-33 expression was observed in AS patients, whereas no change was observed in CD. IL-33 is a cytokine of the innate immune system that is released
in response to bacterial and/or viral stimuli and is up-regulated in human inflammatory diseases [27-30]. The strong expression of IL-33 and its relationship with the numbers of CD163+ macrophages observed could suggest a key role of this cytokine in regulating macrophage polarization in the AS gut. In this regard, serum levels of IL-33 have been demonstrated to be elevated in AS patients and to enhance TNF-α production by peripheral blood mononuclear cells and inducing neutrophil migration [31].

In non-inflamed lamina propria, to maintain mucosal homeostasis, macrophages lack the expression of CD14, the innate immune co-receptor of TLR7 and TLR9 [32] and display prominently an anergic phenotype [17]. In the present study we confirm previous results that show that CD14+ macrophages are expanded in CD [18] and demonstrate that CD14+ macrophages are not present in the lamina propria of AS patients with subclinical gut inflammation. CD14+ macrophages have been implicated in disease pathogenesis via production of IL-23 and TNF-α[19, 20] and induction of Th1 and Th17 responses. Together with the absence of a clear Th1/Th17 response [14, 15], this behaviour appears to clearly differentiate the immunological signature of patients with AS from those with CD. The down-regulation of CD14 expression in AS could be explained by the high levels of intestinal TGF-β and IL-10 [10, 33], which are known to be involved in the down-regulation of the membrane-associated CD14 [34].

In conclusion, a balanced macrophage activation seems to be present in the gut of AS patients with chronic inflammation where CD14+ macrophages are absent and classic M1 macrophages are increased, presumably in response to the microbial flora. The M1 macrophage expansion may be counterbalanced by the increased numbers of resolution phase and M2 macrophages. The differences in the immunological behaviour observed in the gut of AS patients with normal histology and/or acute inflammation compared with those with chronic inflammation might represent a different evolutionary
step in a continuum of inflammatory processes. It is not possible, however, to exclude that acute inflamed and chronic inflamed intestine of AS patients may be a distinct pathologic disease. The absence of functional studies and the difficulty in isolating intestinal macrophages to demonstrate specific transcriptional profile differences prevents us from reaching any definitive conclusions. Further studies are also required to specifically address the micro-environmental cues conditioning the immunological changes that characterize the evolution of AS subclinical gut inflammation towards a clinically evident CD.

Rheumatology key messages

- Expansion of M2 and resolution phase macrophages predominates in the ileum of AS patients.
- IL-33 is overexpressed in the inflamed ileum of AS patients.

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Supplementary data

Supplementary data are available at Rheumatology Online.

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