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Pro-inflammatory CX3CR1^{+}CD59^{+}TL1A^{+}IL-23^{+} monocytes are expanded in patients with Ankylosing Spondylitis and modulate ILC3 immune functions

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Abstract

Objective: Gut derived ILC3 have been demonstrated to participate in AS pathogenesis. $CX3CR1^{+}$ mononuclear phagocytes (MNP) have been demonstrated to modulate ILC3 function in the gut. The aim of this study was to study the role of pro-inflammatory $CX3CR1^{+}CD59^{+}$ MNP in modulating ILC3 function in AS patients.

Methods: MNP subsets in blood were analysed by flow cytometry in AS patients and controls. Tissue presence of $CX3CR1^{+}CD59^{+}$ cells was confirmed by confocal microscopy. Expression of pro-inflammatory chemokine CX3CL1, CCL2 and decoy receptor D6 was

studied. Peripheral CX3CR1^{+}CD59^{+} cells were co-cultured with ILC3 and changes in their frequency evaluated by flow cytometry. Transcriptomic analysis of circulating CX3CR1 monocytes was also performed.

Results: D6 deficiency and CCL2 over-expression was observed in AS inflamed tissues. In the gut pro-inflammatory CX3CR1^{+}CD59^{+} MNP population was expanded, correlated with the presence of bacteria and produced high levels of TL1A and IL-23. CD11b^{+}CD11c^{+}MHC class II^{+} MNP, predominantly expressing CX3CR1, were also expanded in naïve SKG relative to BALB/c mouse small intestine. Gut-derived CX3CR1^{+}CD59^{+}CCR9^{+}TL1A^{+}IL-23^{+} MNP frequency was significantly higher in AS peripheral blood and synovial fluids. CCR9^{+}CX3CR1^{+}CD59^{+} monocytes were also expanded in AS synovial and bone marrow samples. Transcriptomic analysis of isolated CX3CR1^{+}CD59^{+} monocytes demonstrated a specific pro-inflammatory profile in AS. Isolated pro-inflammatory CX3CR1^{+}CD59^{+} MNP from AS induced the expansion and activation of ILC3.

Conclusions: Pro-inflammatory CX3CR1^{+}CD59^{+}TL1A^{+}IL-23^{+} MNP are expanded in AS patients displaying a specific pro-inflammatory transcriptomic profile. Given the ability of these cells in supporting ILC3 expansion, they may promote a sustained pro-inflammatory status in AS.

Keywords: CX3CR1^{+} monocytes, IL-23, TL1A, gut inflammation, ILC3

Subclinical gut inflammation has been demonstrated to occur in a significant percentage of patients with Ankylosing Spondylitis (AS) and is possibly one of the most important sites where interleukin (IL)-23 production takes place.[1] Intestinal IL-23 appears to be responsible for the induction of innate immune responses such as expansion and activation of type 3 innate lymphoid cells (ILC3) in AS.[2] Despite their mucosal origin, gut-derived $\alpha4\beta7^{+}$ILC3, are expanded in the peripheral blood, synovial fluid and bone marrow (BM) of patients with AS, suggesting their active recirculation between the gut and the site of active inflammation.[2] However, the factors influencing the maintenance of ILC3 in an activated state in extra-intestinal sites are not clear.

Recently $CX3CR1^{+}$ mononuclear phagocytes (MNP), strategically located near murine and human gut epithelium, have been demonstrated to produce high levels of IL-23 and TL1A efficiently supporting IL-22 production from ILC3.[3] Although $CX3CR1^{+}$ MNP have been considered as resident macrophages, recent evidence suggests that during intestinal inflammation, they may acquire migratory potential and move to secondary lymphoid organs, initiating immune responses.[4]

In mice, the intestinal replenishment of the $CX3CR1^{+}$ phagocytes come from newly arrived and recently divided $Ly6C^{hi}CCR2^{+}$ monocytes.[5] The trafficking of murine $Ly6C^{+}$ monocytes is regulated by the tissue expression of atypical chemokine receptor D6.[6] D6 is a decoy and scavenger receptor for most inflammatory CC chemokines, including CCL2, and helps prevent tissue inflammatory reactions.[7] Mice lacking D6 expression in the non-hematopoietic compartment display a significant increase of pro-inflammatory monocytes in the peripheral blood and in secondary lymphoid tissues in a CCR2-dependent manner.[6] Thus the net CX3CR1 mediated immune response likely is affected by the balancing effects of CCL2 and D6.

The immunological behaviour of CX3CR1$^+$ MNP in AS patients has not yet been investigated. Here we report the tissue frequency and distribution of intestinal pro-inflammatory CX3CR1$^+$ MNPs, and their distribution in the peripheral blood and inflamed tissues of AS patients. Finally, we performed a transcriptomic analysis of circulating CXCR1$^+$ monocytes from AS and evaluated their role in ILC3 activation.

Methods

Ethical statement

The study was conducted according to the Declaration of Helsinki and Italian legislation. Consent was obtained from all enrolled subjects after the nature of the investigation was explained and in accordance with the approved protocol from the institute review board at the University of Palermo (5/2014 16042014) and the University Health Network, Toronto.

Patients

Twenty-five consecutive HLA-B27 AS patients, fulfilling the modified New York criteria [8] with active disease defined as an AS disease activity score (ASDAS) [9] of 2 or more were enrolled in this study. We also enrolled patients affected by Crohn's disease (CD) (n=5) with active disease as control disease. All patients underwent ileocolonscopy independent of the presence of gastrointestinal symptoms. Twenty healthy individuals, matched by sex and age, without evidence of intestinal disease, undergoing ileocolonoscopy for diagnostic purposes were enrolled as healthy controls. Multiple (five) adjacent ileal mucosal biopsies were obtained from each patient and control to a total of 125 biopsies in AS, 25 in CD and 100 in controls. Gut specimens obtained from patients with AS were histologically divided into three groups as previously described,[2] normal gut histology, acute and chronic inflammation. Synovial samples and synovial fluids were also obtained from additional 5 patients with peripheral spondyloarthritis with active disease at the time of sample collection,

from 5 patients with osteoarthritis and from 5 patients with Rheumatoid arthritis (RA). Bone marrow samples were obtained from 5 AS patients with active disease and hypergammaglobulinemia and 5 controls without any history of inflammatory joint disease, undergoing BM biopsy for diagnostic purpose. In both patients and controls the number of plasma cells was <10% also displaying a normal phenotype ($CD38^{+}CD56^{-}CD19^{+}$). Baseline characteristics of patients and controls are specified in Table 1.

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

Total RNA was extracted by using the Qiagen RNeasy Mini kit, with on-column DNase I digestion as previously described.[2] Quantitative TaqMan real-time PCR, sets of primers and probes were obtained from Applied Biosystems (Foster City, CA) (see supplemental table 1). Both 18S and GADPH were used as housekeeping genes, giving comparable results. GADPH was used for the final results shown. Samples were run in triplicate using the Step-One Real-Time PCR system (Applied Biosystems) and relative changes in gene expression between controls and patients were determined using the ΔΔCt method as previously described.[2] Final values were expressed as fold of induction.

Immunohistochemistry and confocal microscopy analysis

Immunohistochemistry for D6, CCL2 and CX3CL1 and confocal microscopy analysis was performed on paraffin-embedded sections of gut, synovial and BM samples as previously described.[2] Tonsils samples were considered as positive controls. A list of primary and secondary antibodies used is provided in supplementary table 2. Quantification of labelled cells was assessed by two independent investigators (FC and AR) by manually counting the positive 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. The intra-rater agreement and the inter-rater agreement calculated by the Cohen's K coefficient for the two observers were 0.8 and 0.76, respectively. For more

details see supplemental methods. In order to detect bacteria, the highly sensitive Warthin-Starry silver/nitrate-based staining method, Gram staining and an antibody directed against bacterial lipopolysaccharide (LPS) were used as previously described [10]. For more details see supplemental methods.

ELISA for CX3CL1 and CCL2

Serum was collected in all patients and controls for ELISA. 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 CX3CL1 and CCL2 were analysed in sera employing sandwich-ELISA kits (Abcam, Cambridge for CX3CL1 and R&D for CCL2) following the manufacturer's instructions (measurable concentration range of 0.25 to 60 ng/mL for CX3CL1 and 31.2 - 2000 pg/mL for CCL2). All results were analysed using a five parameter-logistic (5PL) function for fitting standard curves obtained from recombinant protein standards.

Isolation of lamina propria, peripheral blood and synovial fluid mononuclear cells

Lamina propria mononuclear cells (LPMCs), peripheral blood mononuclear cell (PBMCs) and synovial fluid mononuclear cells (SFMCs) were respectively isolated from the gut, the peripheral blood and synovial fluid of patients with AS, RA and healthy controls as previously described.[2] For the phagocytosis assay, $CX3CR1^{+}CD59^{+}$ monocytes were FACS sorted by using CD14, CX3CR1 and CD59 specific antibodies. Fore more details see supplemental methods. A list of the antibodies used is provided on the online supplemental table S1.

Flow cytometry analysis of surface and intracellular antigens

Different monocyte subsets were analysed by flow cytometry in the peripheral blood, gut, synovial fluids of AS patients and controls. LPMC and PBMCs were isolated respectively from the gut biopsies and peripheral blood of all patients and controls as previously

described by GG with no access to histological and clinical data.[2] Paired SFMCs were also obtained from 5 AS patients, who underwent to ileocolonoscopy, and 5 OA patients. A list of the antibodies used is provided in supplemental table 1. 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.

Cell cultures

In order to evaluate the role of $CX3CR1^+$ in regulating the differentiation of ILCs type 3, $CX3CR1^+$ MNP were isolated from the peripheral blood of AS patients by using CX3CR1 microbeads (positive selection) and magnetic-activated cell sorting (MACS) techniques (Miltenyi Biotech GmbH, Bergisch Gladbach, Germany) according to the manufacturer's instructions. $CD45^+lin^-$ mononuclear cells were enriched in ILC3 precursors. $CD45^+lin^-$ mononuclear cells were isolated from tonsils of normal donors as previously described using lineage-marker (negative depletion with antibodies to CD5, CD45R (B220), CD11b, Gr-1 (Ly-6G/C), 7-4 and Ter-119) and c-kit microbeads (positive selection) and MACS (Miltenyi Biotech GmbH, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Isolated $CX3CR1^+$ MNP and CX3CR1- MNP were then cultured with isolated $CD45^+$ lin− precursor-enriched mononuclear cells (1:1), and the percentage of ILC3 was assessed by flow cytometry. All cultures were set up in triplicate and cells were used for flow cytometric analyses.

Transcriptomic analysis

PBMCs from 7 AS patients and 5 HC were isolated by density gradient centrifugation after layering blood on Ficoll-Paque (GE Healthcare) and $CX3CR1^+CD59^+$ monocytes were FACS sorted by using CD14, CX3CR1 and CD59 specific antibodies. Total RNA was isolated by

using TRIzol (Thermo Fisher Scientific) and further purified by using RNeasy MinElute cleanup kit (Qiagen). RNA samples were assessed on a RNA 6000 Pico chip (Agilent Technologies) by using the Agilent Bioanalyzer to determine sample RIN and quantified by the Qubit RNA HS assay kit (Life Technologies). All samples used had RIN values greater than 8. For more details see supplemental methods.

Isolation and staining of murine intestinal lamina propria cells

SKG and BALB/c mice housed under SPF conditions and/or injected with with 1,3-β- glucan (curdlan) at The University of Queensland Translational Research Institute (TRI) Animal Facility. Approval for all experiments was obtained from The University of Queensland animal ethics committee. Lamina propria Lymphocytes (LPL) were isolated from naïve BALB/c and SKG small intestine (SI) after dissecting Peyer's patches and fat removal. For more details see supplemental methods.

Statistical analysis

Student's t-test or the nonparametric Mann-Whitney test was used to calculate the statistical significance between groups. Paired samples were analysed with the Wilcoxon signed-rank test. Spearman's rank correlation was used to calculate the correlation between different variables in AS. *p* values less than 0.05 were considered significant.

Results

CX3CR1^{+}CD59^{+} monocytes producing IL-23 and TL1A are expanded in the gut of AS patients and correlated to presence of ileal bacteria

We first studied the frequency and distribution of different MNP subsets in the gut of AS and CD patients and HC. Accordingly to our previous demonstration, [11] in the inflamed ileum of AS patients, despite a global expansion of MNP (figure 1A-B), we observed a significant down-regulation of CD14 expression (Figure 1A-B) compared to healthy controls (supplemental Figure 1A). Due to the low levels of CD14 monocyte expression in AS gut, we could not separately analyse classical, intermediate and non-classical monocytes. Since Ly6 is a murine marker, for the further characterization of monocytes we used CD59, the human homologue.[12] Pro-inflammatory CX3CR1^{+}CD59^{+}, but not resident CX3CR1^{+}CD59^{-} (data not shown) monocytes were expanded in the gut of AS patients (Figure 1C-D) compared to CD patients (Figure 1D and supplemental figure 1B) being undetectable in controls (data not shown). The majority of inflammatory CX3CR1^{+}CD59^{+} monocytes produced both TL1A and IL-23 and expressed CCR9 (figure 1C,D). IL-23, TL1A and its receptor Death receptor 3 (DR3), expression was confirmed in AS ileal samples by RT-PCR (Figure 1E-G). Interestingly, the CX3CR1^{+}CD59^{+} monocyte frequency was significantly correlated with the percentage of intestinal ILC3 (Figure 1H) and with the bacterial scores (Figure 1I). The tissue distribution of CX3CR1^{+}CD59^{+} monocytes, studied by confocal microscopy analysis demonstrated their location close to crypt intestinal epithelial cells (Figure 1J-M) and to tissue CD3^{-}Thy1^{+}Tbet^{+} ILC3 (Figure 1N-O).

CD11b^{+}CD11c^{+}MHC class II^{+} MNP, predominantly expressing CX3CR1 are expanded in naïve SKG small intestine

BALB/c^{W163C} ZAP70-mutant (SKG) mice develop IL-23-dependent SpA-like disease after intraperitoneal administration of beta-1,3-glucan (curdlan), with peripheral and axial arthritis, enthesitis, psoriasiform inflammation of the skin and Crohn's-like ileitis [13-14]. Although

naïve SKG mice are healthy, they have fecal microbial dysbiosis relative to control BALB/c mice, and IL-23 is constitutively expressed in the ileum [13]. In naïve SKG mice, predisposed to SpA development, we compared the frequency of the small intestinal MNP relative to BALB/c controls. MNP were $CD11b^{+}CD11c^{+}$MHC class II^{+}, and expressed either CX3CR1 (intestinal macrophages) or CD103 (dendritic cells) (Figure 2A, B) [3]. MNP were significantly expanded in SKG relative to BALB/c small intestine. Expanded cells were predominantly $CX3CR1^{+}$, while $CD103^{+}$ MNP were significantly less frequent (Figure 2C). Interestingly, SKG mice challenged with Curdlan showed a significant further expansion of $CX3CR1^{+}$ MNP (Figure 2 C).

D6, CCR2 and CCL2 expression in the gut, bone marrow and synovia of AS patients

In mice, $CX3CR1^{+}$ monocytes appear to be derived from circulating $Ly6C^{+}CCR2^{+}$ monocytes in a D6-dependent manner.[5] Considering the role of D6 in controlling the trafficking of murine $Ly6C^{+}$ monocytes, we subsequently evaluated the expression of D6 in different inflamed tissues of AS patients. A global reduction of D6 expression was observed in the gut (independently of the degree of intestinal inflammation) (Figure 3A-D), bone marrow (Figure 3E-G) and synovial samples (Figure 3H-J) of AS patients compared to controls. Along with the reduced levels of D6, significantly increased expression of CCL2 and CCR2 was observed in AS gut (Figure 3K-L and Supplemental Figure 2A-C), synovia (Figure 3M-N and Supplemental Figure 2D-F) and BM (Figure 3O-P and supplemental Figure 2G-I) samples. Interestingly, the reduced D6 expression in AS inflamed tissues was paralleled by increased levels of CCL2 in AS serum compared to controls (data not shown).

$CD14^{++}CD16^{+}CCR9^{+}CX3CR1^{+}CD59^{+}$ monocytes are expanded in the peripheral blood, synovial fluid, synovial and bone marrow samples of AS patients

CCR9 has been shown before to be a gut homing marker [15,16]. We studied whether tissue D6 deficiency could be accompanied by an increased frequency and tissue distribution of gut derived $CD14^{++}CD16^{+}CCR9^{+}CX3CR1^{+}CD59^{+}$ cells in the peripheral blood and inflamed

tissues of AS patients. As shown in figure 2, an increased frequency of $CD14^{++}CD16^{+}CX3CR1^{+}CD59^{+}CCR9^{+}IL\text{-}23^{+}TL1A^{+}$ cells was observed in the peripheral blood (Figure 4B-C) of AS patients, being undetectable in controls (data not shown). Since their expression of CCR9, a marker of intestinal homing, we assumed that that these cells were of gut origin. The frequencies of circulating $CD14^{++}CD16^{+}CX3CR1^{+}CD59^{+}CCR9^{+}IL\text{-}23^{+}TL1A^{+}$ were significantly and directly correlated with the frequencies of circulating ILC3 (Figure 4D), with the disease activity as assessed by BASDAI (Figure 4E) and C-reactive protein (CRP) (Figure 4F). Interestingly, isolated $CD14^{++}CD16^{+}CX3CR1^{+}CD59^{+}CCR9^{+}IL\text{-}23^{+}TL1A^{+}$ from the peripheral blood, displayed in AS a more pronounced phagocytic activity compared to controls (Figure 4G). A significant expansion of $CD14^{++}CD16^{+}CCR9^{+}CX3CR1^{+}CD59^{+}IL\text{-}23^{+}TL1A^{+}$ was also demonstrated in AS synovial fluids (Figure 4H, I) and RA (Supplemental Figure 1C), being undetectable in controls (data not shown). Confocal microscopy analysis confirmed the presence of either scattered or aggregated $CX3CR1^{+}CD59^{+}$ cells also in the context of inflamed AS synovial (Supplemental Figure 3 A-E) and bone marrow tissues (Supplemental Figure 3G-M). Similar to the gut, a close spatial relationship between $CX3CR1^{+}$ cells and $CD3^{-}Tbet^{+}$ cells was observed in the inflamed AS tissues (Supplemental Figure 3F and N), suggesting a role of these cells in modulating ILC3 responses.

CX3CL1 levels are increased in the inflamed tissues but not in the peripheral blood of AS patients

As we found an expansion of $CX3CR1^{+}$ monocytes in the peripheral blood and in the inflamed tissues of AS patients, we measured the expression of its cognate ligand CX3CL1 by ELISA, RT-PCR and immunohistochemistry. Compared to controls, significant CX3CL1 over-expression was observed in AS gut (Figure 5A-D), synovial (Figure 5E-G) and bone marrow samples (Figure 5H-J). In AS gut and bone marrow samples, CX3CL1 expression was essentially observed in endothelial cells and inflammatory mononuclear cells. CX3CL1 vascular expression predominates in AS synovial samples. Contrary to tissue expression, serum levels of CX3CL1 were similar in AS patients and controls (data not shown).

Isolated CX3CR1^{+}CD59^{+} cells drive ILC3 expansion

Since CX3CR1^{+} cells have been demonstrated to expand ILC3 in the gut, we next evaluated the role of isolated CX3CR1^{+}CD59^{+} MNPs in modulating ILC3 expansion and activation. Isolated intestinal and circulating CX3CR1^{+}CD59^{+} MNPs were co-cultured with isolated peripheral ILC3. Despite ILC3 have been described to express the transcription factor RORc, we have previously demonstrated that in patients with AS these cells are almost totally Tbet^{+}[2]. As shown in figure 6, in the presence of intestinal (Figure 6A-C) or circulating (Figure 6D-F) CX3CR1^{+} MNPs we observed a significant activation of Lyn^{-}Tbet^{+}NKp44^{+} ILC3 producing IL-22, indicating a direct role of these cells in modulating ILC3 expansion.

Transcriptomic analysis of peripheral CD14^{+}CX3CR1^{+}CD59^{+} cells in AS patients

We finally investigated the transcriptome of CD14^{+}CX3CR1^{+}CD59^{+} monocytes, isolated from PBMC, to understand the differences in the biological processes and cellular pathways between AS and HC. To generate the specific gene expression profile, we focused on genes satisfying the following criteria: (a) showed ≥ 1 Fragments Per Kilobase of transcript per Million mapped reads (FPKM) of 1 or greater in at least one of the sample across all samples and (b) were differentially expressed, with a fold change of 1.3 or greater between AS and HC. We identified a total of 159 genes that were differentially expressed out of the 23620 genes identified from RNA-Seq. A total of 104 genes including TNF, CXCL2, JAK3, MAP3K8, ERAP2, MAP1LC3B, ATF4, NAIP, ZFP36L1 were significantly up-regulated in AS whereas 55 genes including TNFSF14, IL7R, granzyme A, granzyme B, granulysin, IL-2R and IL-5R were significantly down-regulated in AS (Supplemental Figure 4A). Granzymes are part of the recently described cytotoxic gene signature in the 'Mono4' population of monocytes (cytotoxic monocytes).[17] Pathway analysis including biological processes and immune system processes functions was performed with all differentially expressed genes. The up-regulated genes were enriched for pathways related to chronic inflammation response, positive regulation of chemokine production and positive regulation of

inflammatory response (Supplemental Figure 4B). Conversely, the down-regulated genes were enriched for pathways related to leukocyte chemotaxis, leukocyte and mast cells mediated immune responses (Supplemental Figure 4C).

Discussion

This study supports the concept that a subset of pro-inflammatory $CX3CR1^{+}CD59^{+}$ monocytes, producing IL-23 and TL1A are expanded in the inflamed gut of AS patients and of SKG mice and in the sites of active inflammation in AS, sustaining the activation of ILC3. In addition, we provide transcriptomic analysis of isolated $CX3CR1^{+}CD59^{+}$ MNP that showed a specific pro-inflammatory signature in AS compared to healthy controls.

Gut-derived ILC3, producing IL-17 and IL-22, are expanded in the peripheral blood and inflamed tissues of AS patients. [2] However, the factors involved in their expansion and maintenance in an activated status are not completely clear. Recently, $CX3CR1^{+}$ intestinal MNP have been demonstrated to efficiently support IL-22 production from ILC3 both in mice and humans.[3] Murine MNP can be classified into two distinct populations, inflammatory MNP ($CX3CR1^{+}Ly6^{+}$) and resident MNP ($CX3CR1^{+}Ly6^{-}$cells).[18] In humans classical ($CD14^{++}CD16^{-}$) monocytes resembling $Ly6^{+}$ monocytes, intermediate ($CD14^{++}CD16^{+}$) monocytes playing pro-inflammatory roles and non-classical ($CD14^{+}CD16^{++}$) patrolling monocytes have been demonstrated.[18] In the gut of AS patients, despite a global expansion of MNP we observed a decreased frequency of $CD14^{+}$ cells. As CD14 is down-regulated in intestinal MNP [19] to avoid dangerous immune responses, we speculate that the decreased levels of CD14 in AS patients could be related to differences in bacterial ileum colonization from controls [10]. Despite the CD14 down-regulation, the frequency of $CX3CR1^{+}CD59^{+}$ MNP was significantly increased in the gut of AS patients and correlated with the bacterial scores. These $CX3CR1^{+}CD59^{+}$ cells were characterized by the intense production of IL-23 and TL1A and aided the expansion of ILC3. Hence, we believe that

these cells are the human counterparts of murine CX3CR1^{+}Ly6c^{+} pro-inflammatory MNP. The CX3CR1^{+}CD59^{+} MNP were located close to intestinal crypt epithelial cells and to lamina propria ILC3. In mice, CX3CR1^{+} MNP are essential in maintaining LP MNP populations and preventing translocation of commensal bacteria to mesenteric lymph nodes. In naïve SKG mouse intestine characterised by intestinal dysbiosis and IL-23 over-expression, MNP expressing CX3CR1 were also increased, suggesting recruitment of dysbiosis-associated inflammatory monocytes to the intestine, prior to the development of disease. After Curdlan challenging, the frequency of MNP expressing CX3CR1 was further increased suggesting a role in triggering Crohn's-like ileitis and SpA in the SKG model. These observations, together with the evidence that in AS the frequency of CX3CR1^{+}CD59^{+} MNP significantly correlated with the percentages of intestinal ILC3, might indicate the importance of these cells in bridging immune responses from the intestinal lumen to the innate immune system.

In mice, the intestinal replenishment of the CX3CR1^{+} MNP derives from newly arrived Ly6C^{hi}CCR2^{+} monocytes in a D6-dependent manner.[5] D6 is a decoy and scavenger receptor for most inflammatory CC chemokines and recent evidences indicated that after D6 engagement, CCL2 is rapidly internalized and degraded suggesting that this receptor acts a chemokine- scavenging decoy receptor.[20-21] Thus a deficiency of D6 can lead to excess pro-inflammatory chemokine (eg. CCL2) accumulation. D6 deficient mice have a delay in the clearance of inflammatory CC chemokines and under inflammatory conditions display a significant increase in Ly6C^{high} MNP in secondary lymphoid organs.[6] Along with the increased frequency of tissue and circulating CX3CR1^{+}CD59^{+} MNP in AS, we observed a significant decreased expression of D6 in the inflamed gut, synovial and bone marrow tissues. D6 deficiency was paralleled by an increase in tissue and systemic expression of CCL2 as well as an increase in CX3CR1^{+}CD59^{+} cells. These findings seem to support a role for D6 deficiency in increased MNP mobilization to the inflamed AS tissues via the control of CCR2 ligand concentrations in the tissues and in the bloodstream.

CX3CR1^{+} MNP have been long considered as resident macrophages. However, recent evidence suggests that they may acquire migratory potential to secondary lymphoid organs and initiate immune responses.[4] Although the majority of intestinal CX3CR1^{+}CD59^{+} cells lie in the CD14(low) population, we decided to not analyse these cells in the AS peripheral blood and synovial fluids. Human CD14(low) monocytes are in fact considered patrolling monocytes with weak phagocytic ability and not producing pro-inflammatory cytokines.[22] We were interested to study the intermediate population of monocytes that are activated in the gut that are able to produce pro-inflammatory cytokines. In AS patients, CD14^{++}CD16^{+}CX3CR1^{+}CD59^{+}IL-23^{+}TL1A^{+} pro-inflammatory MNP were expanded in the peripheral blood and synovial fluids. A significant percentage of these cells expressed the marker of intestinal homing CCR9,[15] possibly indicating their intestinal origin. Importantly, the frequencies of circulating CD14^{++}CD16^{+}CX3CR1^{+}CD59^{+}CCR9^{+}IL-23^{+}TL1A^{+} monocytes were significantly correlated with the frequencies of circulating ILC3 as well as with disease activity. The presence of CX3CR1^{+}CD59^{+} cells was also confirmed in synovial tissues and bone marrow obtained from AS patients where they were in close proximity to tissue ILC3. This spatial association may indicate an important role of these cells in supporting ILC3 activation and expansion in extra-intestinal sites as well. This hypothesis is supported by our demonstration that isolated CD14^{++}CD16^{+}CX3CR1^{+}CD59^{+} MNP from the gut and peripheral blood of AS patients activated IL-17 and IL-22 producing ILC3. CX3CL1 is the chemokine controlling the tissue migration of CX3CR1^{+} cells.[23] Despite the expansion of CX3CR1^{+} cells in AS peripheral blood we did not observe any significant increase in circulating CX3CL1. However, CX3CL1 expression was high in the AS inflamed gut, synovial and bone marrow tissues indicating its role in tissue migration of CX3CR1-expressing inflammatory MNP.

It is noteworthy, that transcriptomic analysis performed on AS patients and controls demonstrated a specific pro-inflammatory phenotype of AS CX3CR1^{+}CD59^{+} monocytes. Among the 159 genes that were differentially expressed in AS patients from controls, 104

genes were up regulated in AS. Among these genes, pro-inflammatory cytokines and chemokines such as TNF, CXCL2 and LT4 and intracellular mediator of cytokine signalling such as JAK3 and MAP3K8 were significantly over-expressed in AS. We also found a significant over-expression of genes involved in innate immune pathways such as inflammasomes (NAIP) and autophagy (MAP1LC3B and ATF4), in the misfolding of HLA-B27 (UBE2J1), and in osteoblast and osteoclast activation. The role of $CX3CR1^+$ monocytes on bone homeostasis is not known. It was beyond the scope of the current study to explore the link between $CX3CR1^+$ monocytes and spinal ankylosis. Pathway analysis, finally, demonstrated the enrichment of genes related to chronic inflammation, positive regulation of chemokine production and positive regulation of inflammatory responses.

In conclusion, this study provides the first evidence that migrating MNP acquire in the gut of AS patients a pro-inflammatory $CX3CR1^+$ phenotype and re-circulate via the peripheral blood to inflamed AS tissues in a CX3CL1-dependent manner. $CX3CR1^+$ monocytes, displaying a specific pro-inflammatory transcriptomic phenotype in AS patients, actively participate in the activation and expansion of ILC3.

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Competing Interest: None declared

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Figure legends

Figure 1. CX3CR1+CD59+ monocytes are expanded in AS gut. A-B: percentages of monocytes and of CD14+ monocytes in the gut of AS patients and controls. C: gating strategy and representative dot plot showing CX3CR1+CD59+IL-23+TL1A+CCR9+ monocytes in the gut of a patient with AS. D: percentages of CX3CR1+CD59+IL-23+TL1A+CCR9+ monocytes in AS patients and controls. E-G: relative m-RNA levels of IL-23p19 (E), TL1A (F), DR3 (G) were assessed by RT-PCR in the ileal samples obtained from all the AS patients and HC. The percentage of CX3CR1+CD59+IL-23+TL1A+CCR9+ was significantly and directly correlated with percentage of gut ILC3 (H) and bacterial scores (I). J-K: representative confocal microscopy images of CX3CR1 (J), CD59 (K) and CD68 (L) in AS gut. M: merge triple staining showing CD68/CX3CR1/CD59 co-localization in AS gut. N-O: representative confocal microscopy analysis showing co-localization of CX3CR1+ cells with CD3- (Cyan) Tbet+ (Red) ILC3 in AS gut (N); O: CD3 positive staining in the same section. J-O: original magnification x400.

Figure 2. CX3CR1+ and CD103+ MNPs in SPF BALB/c and SKG gut. (A) Flow cytometric plots depicting gating strategy of the LPL (B). Representative flow cytometric plots of CX3CR1+ and CD103+ MNP from BALB/c and SKG lamina propria. Proportions of cell populations are shown in numeric values adjacent to gated boxes. (C). Frequency of MNP (MHC-II+CD11b+CD11c+ cells gated on live CD45.2+ cells), CX3CR1+ and CD103+ MNP cells from the BALB/c and SKG mice in specific pathogen free conditions (SPF) and after injection with 1,3-β- glucan (curdlan) analyzed as in A. Statistical analysis was done according to Mann-Whitney U test considering mean ± SEM for each bar. Each dot in B represents an individual mouse. * $P<0.05$, ** $P<0.005$.

Figure 3. Figure 3. D6 and CCL2 expression in AS patients. A: relative m-RNA levels of D6 was assessed by RT-PCR in the ileal samples obtained from all patients and controls. B-C: representative images showing D6 immunostaining in the gut of AS patients (B) and

controls (C). D: number of D6+ cells/infiltrating cells in the gut of AS patients and controls. E-F: representative images showing D6 immunostaining in the bone marrow of AS patients (E) and controls (F). G: number of D6+/infiltrating cells in the BM of AS patients and controls. H-I: representative images showing D6 immunostaining in the synovial samples of patients with SpA (H) and controls (I). J: number of D6+/infiltrating cells in the synovial samples of patients with SpA and controls. K, M, O: representative images of CCL2 in the gut (K), synovial (M) and bone marrow (O) samples of AS patients. L, N, P: number of CCL2+/infiltrating cells cells in the gut (L), synovium (N) and bone marrow (P) of patients and controls.

Figure 4. CX3CR1+CD59+ monocytes are expanded in AS peripheral blood and synovial fluids. A: representative dot plots showing the gating strategy in the peripheral blood of AS patients. B: representative dot plot showing CX3CR1+CD59+IL-23+TL1A+CCR9+ monocytes in the peripheral blood of a patient with AS. C: percentages of CX3CR1+CD59+IL-23+TL1A+CCR9+ monocytes in the peripheral blood of AS patients and controls. D: percentages of circulating CX3CR1+CD59+IL-23+TL1A+CCR9+ monocytes was correlated with the percentages of circulating ILC3. E-F: percentages of circulating CX3CR1+CD59+IL-23+TL1A+CCR9+ monocytes was correlated in AS with the disease activity levels evaluated by the BASDAI (E) and with C-reactive protein (F). G: Phagocytosis index among isolated CX3CR1+CD59+ MNP. H: representative dot plot showing CX3CR1+CD59+IL-23+TL1A+CCR9+ monocytes in the synovial fluid of a patient with AS. I: percentages of CX3CR1+CD59+IL-23+TL1A+CCR9+ monocytes in the synovial fluids of AS and RA patients and controls.

Figure 5: CX3CL1 expression in AS inflamed tissues. A: relative m-RNA levels of CX3CL1 were assessed by RT-PCR in the ileal samples obtained from all the AS patients and HC. B-C: representative images showing CX3CL1 immunostaining in the gut of AS patients (C) and controls (B). D: number of $CX3CL1^{+}$/infiltrating cells in the gut of AS patients and controls. E-F: representative images showing CX3CL1 immunostaining in the

synovial samples of patients with SpA (F) and controls (E). G: number of $CX3CL1^{+}$/infiltrating cells in the synovial samples of patients and controls. H-I: representative images showing CX3CL1 immunostaining in the bone marrow of patients with AS (I) and controls (H). J: number of $CX3CL1^{+}$/infiltrating cellscells in the bone marrow of patients with SpA and controls

Figure 6: CX3CR1+ MNP drives ILC3 expansion. A-C: isolated CX3CR1+ (A) and CX3CR1-(B) cells were isolated from the gut of AS patients and co-cultured with isolated peripheral Lyn−Tbet+NKp44+ ILC3. There was significant expansion of IL22+Tbet+ ILC3 with co-culture with both CX3CR1+ (A-C) cells and CX3CR1- cells (B-C) compared to no co-culture. D-F: isolated CX3CR1+ (D) and CX3CR1- (E) cells were isolated from the peripheral blood of AS patients and co-cultured with isolated peripheral Lyn−Tbet+NKp44+ ILC3. There was significant expansion of IL22+Tbet+ ILC3 with co-culture with CX3CR1+ (D-F) cells but not with CX3CR1- cells (E-F) compared to no co-culture. C, F: Percentages of ILC3 cells after co-culture with intestinal (C) and peripheral (F) CX3CR1+ and CX3CR1- cells.

Supplemental Figure 1. Supplemental Figure 1. Representative dot plots showing CX3CR1+ monocytes in the gut of HC (A) and CD patients (B) and synovial fluids (C) of RA patients.

Supplemental Figure 2: CCR2 expression in the inflamed tissues of AS patients. A: relative m-RNA levels of CCR2 was assessed by RT-PCR in the ileal samples obtained from all patients and controls. B-C: representative images showing CCR2 immunostaining in the gut of healthy controls (B) and AS patients (C). D: number of CCR2+ mononuclear cells (MNC)/infiltrating cells in the gut of AS patients and controls. E-F: representative images showing CCR2 immunostaining in the synovial samples of healthy controls (E) and SpA patients (F). G: number of CCR2+MNC/infiltrating cells in the synovial tissues of AS patients and controls. H-I: representative images showing CCR2 immunostaining in the bone marrow

samples of healthy controls (H) and AS patients (I). J: number of CCR2+MNC/infiltrating cells in the bone marrow of samples of patients with AS and controls. B-C, E-F, H-I: original magnification x400

Supplemental Figure 3. CX3CR1+CD59+ are expanded in SpA inflamed synovial tissues and AS bone marrow tissues and are located in close proximity to ILC3. A-C: representative confocal microscopy images of CX3CR1 (A), CD59 (B) and CD68 (C) in SpA synovial tissues. D: merge triple staining showing CX3CR1/CD59/CD68 co-localization in SpA synovia. E: number of CX3CR1+CD59+CD68+ cells in patients and controls. F: merge triple staining of CX3CR1+ cells with CD3- (Cyan) Tbet+ (Red) ILC3 in SpA synovial tissues. CD3+ positive cells are observable in another portion of the same sections. G-I: representative confocal microscopy images of CX3CR1 (G), CD59 (H) and CD68 (I) in AS bone marrow tissues. L: merge triple staining showing CX3CR1/CD59/CD68 co-localization in AS bone marrow. CD3+ positive cells are observable in another portion of the same sections. M: number of CX3CR1+CD59+CD68+ cells in patients and controls. N: merge triple staining of CX3CR1+ cells with CD3- (Cyan) Tbet+ (Red) ILC3 in AS bone marrow tissues. A-D, F-L, N original magnification x 600.

Supplemental Figure 4: Transcriptomic analysis of circulating CX3CR1+ AS monocytes. A: RNA-sequencing was done on flow-sorted CX3CR1+ monocytes. Differential gene expression between AS patients and Healthy controls was studied. Up-regulated (green to red) and down-regulated transcripts (yellow to blue) are shown as relative expression levels. B: Gene Functional enrichment network analysis among the genes highlighted in a. Network visualization of selected top enriched biological processes up-regulated (left) and down-regulated (right) in AS patients. Each significantly enriched network ($P<0.05$) is represented with a circle and representative genes of the network. The node size represents the number of genes assigned to a biological process. C: hierarchical cluster analysis of gene expression in AS patients and controls