





ORIGINAL RESEARCH

Patients with ankylosing spondylitis present a distinct CD8 T cell subset with osteogenic and cytotoxic potential

Veronica Martini ¹, Ylenia Silvestri,¹ Adrian Ciurea ², Burkhard Möller,³ Gabriela Danelon,¹ Flavio Flamigni,⁴ David Jarrossay,¹ Ivo Kwee,¹ Mathilde Foglierini,¹ Andrea Rinaldi,⁵ Valentina Cecchinato ¹, Mariagrazia Ugucioni ¹

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VM and YS contributed equally.

VM and YS are joint first authors. VC and MU are joint senior authors.

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For numbered affiliations see end of article.

Correspondence to

Dr Mariagrazia Ugucioni;
mariagrazia.ugucioni@irb.usi.ch

Dr Valentina Cecchinato;
valentina.cecchinato@irb.usi.ch

ABSTRACT

Objectives Ankylosing spondylitis (AS) is a chronic inflammatory rheumatic disease affecting mainly the axial skeleton. Peripheral involvement (arthritis, enthesitis and dactylitis) and extra-musculoskeletal manifestations, including uveitis, psoriasis and bowel inflammation, occur in a relevant proportion of patients. AS is responsible for chronic and severe back pain caused by local inflammation that can lead to osteoproliferation and ultimately spinal fusion. The association of AS with the human leucocyte antigen-B27 gene, together with elevated levels of chemokines, CCL17 and CCL22, in the sera of patients with AS, led us to study the role of CCR4⁺ T cells in the disease pathogenesis.

Methods CD8⁺CCR4⁺ T cells isolated from the blood of patients with AS (n=76) or healthy donors were analysed by multiparameter flow cytometry, and gene expression was evaluated by RNA sequencing. Patients with AS were stratified according to the therapeutic regimen and current disease score.

Results CD8⁺CCR4⁺ T cells display a distinct effector phenotype and upregulate the inflammatory chemokine receptors CCR1, CCR5, CX3CR1 and L-selectin CD62L, indicating an altered migration ability. CD8⁺CCR4⁺ T cells expressing CX3CR1 present an enhanced cytotoxic profile, expressing both perforin and granzyme B. RNA-sequencing pathway analysis revealed that CD8⁺CCR4⁺ T cells from patients with active disease significantly upregulate genes promoting osteogenesis, a core process in AS pathogenesis.

Conclusions Our results shed light on a new molecular mechanism by which T cells may selectively migrate to inflammatory loci, promote new bone formation and contribute to the pathological ossification process observed in AS.

INTRODUCTION

Ankylosing spondylitis (AS) is a chronic inflammatory rheumatic disease belonging to the group of seronegative spondyloarthritides (SpA), which is defined by radiographic signs of destructive pathologies in the sacroiliac joints and ankyloses.^{1 2} AS presents a

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Ankylosing spondylitis (AS) is a chronic autoimmune-inflammatory condition affecting mainly the axial skeleton, with local new bone formation. The mechanical stress present in the spine contributes to new bone formation by driving local inflammation and the release of several proinflammatory cytokines. Additionally, local inflammation initiates the production of chemokines and the recruitment of immune cells, including CD8⁺ T cells. However, how the recruited immune cells and the chemokine system contribute to local bone deposition is not fully understood.

WHAT THIS STUDY ADDS

⇒ The identification of a subpopulation of circulating high cytotoxic CD8⁺ T cells upregulating chemokine receptors and genes promoting the ossification process in patients with AS that might contribute to AS pathogenesis.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ Understanding the role of CD8⁺CCR4⁺ T cells and of the relevant chemokines might provide a rationale for the development of additional novel treatments for AS.

disease continuum including earlier and milder disease forms and affects up to 1% of the population worldwide, with an average onset around 20–30 years of age.^{3 4} The aetiology of AS is poorly understood, but genetic factors, such as the human leucocyte antigen (*HLA*)-B27 gene, and environmental triggers might contribute to the development of the disease. Enthesitis, the inflammation of the entheses, located at the attachment of ligaments and tendons to bone, is a hallmark of AS.⁵ These sites are prone to mechanical stress and microdamages that, in genetically predisposed subjects, might not be resolved

by tissue repair mechanisms but instead represent areas of chronic inflammation and subsequent new bone formation.⁶ The progressive remodelling at sites of spinal entheses of sacroiliac spinal joints ultimately leads to bone fusion (ankylosis).^{7,8} Peripheral disease manifestations (arthritis, enthesitis and dactylitis) and extra-musculoskeletal manifestations, such as anterior uveitis, psoriasis and inflammatory bowel disease, are present to different degrees in a significant proportion of patients.⁹

By driving local inflammation with the release of several proinflammatory cytokines, mechanical stress can contribute to new bone formation. It has been recently demonstrated that exposure to tumour necrosis factor (TNF), interleukin (IL)-1 β , IL-17, IL-22 and IL-23 aberrantly upregulates the calcium-sensing receptor in osteoblasts, a phenotype also observed in patients with AS, and promotes osteogenic differentiation both *in vitro* and *in vivo*.¹⁰ In addition, stimulation with low doses of TNF promotes the expression of the osteoinductive molecule Wnt in preosteoblasts.¹¹ IL-1 β and TNF have also been reported to upregulate other osteogenic molecules, such as bone morphogenetic proteins (BMPs), in mesenchymal cells.^{12,13} Currently available treatments, ranging from non-steroidal anti-inflammatories to TNF, IL-17 and Janus Kinase inhibitors, target the ongoing inflammation and aim to minimise symptoms.^{4,14-16}

Animal models of SpA, achieved by the dysregulated expression of TNF or IL-22 and IL-23, displayed new bone formation at the enthesis together with extra-articular manifestations.^{17,18} These models first proved the presence of T cells in regions thought to be populated solely by stromal cells. Studies in patients with AS demonstrated that the enthesis is populated by osteogenic precursors, chondrocytes and osteoblasts together with immune cells, while resident T cells, able to produce TNF and IL-17A, have also been reported in normal entheses.¹⁹⁻²¹ Chemokines, such as CXCL12, regulate the migration of both immune cells and bone cells to this site, suggesting that the chemokine system is also involved in AS pathogenesis.¹⁹

Alterations in chemokine and chemokine receptor expression, including CCL17, CCL22, CXCL10, CX3CL1, CCR4 and CX3CR1, have been reported in this context, even though their relevance in triggering the pathology has not been fully understood.²²⁻²⁷ Indeed, elevated plasma levels of the chemokines CCL17 and CCL22, known to be involved in different inflammatory conditions,^{28,29} and of circulating CD4⁺ T cells expressing the cognate receptor, CCR4, have been described in patients with AS, indicating altered immune cell trafficking via this axis.²²⁻²⁵ We therefore hypothesised that given the importance of HLA-B27 and the elevated levels of CCL17 and CCL22, CD8⁺ T cells expressing CCR4 might be involved in AS pathogenesis, with the potential of trafficking between different inflammatory sites.

In the present study, we show that CD8⁺CCR4⁺ T cells isolated from patients in an active state of the disease display an altered phenotype with a distinct cytotoxic and

transcriptomic profile, upregulating genes promoting ossification. Altogether, these results point to new molecular mechanisms by which T cells may promote new bone formation and sustain the pathological ossification process characteristic of AS.

METHODS

Ethical approval and patient recruitment

The study was approved by the Ethical Committees of the Canton Ticino (CE-3065) and of the Canton Zurich (EK515). Informed consent from each subject was obtained before enrollment in the study, and all samples were coded. A total of 76 patients with AS undergoing routine disease assessment at the University Hospitals of Zurich or Bern (CH) were enrolled in the study. Blood and sera from each patient were collected at the time of enrolment, and clinicians provided clinical and demographic information. All patients with AS fulfilled the modified New York 1984 criteria,² and disease activity was assessed by the Ankylosing Spondylitis Disease Activity Score (ASDAS).³⁰ Demographic and clinical characteristics of patients with AS enrolled in the study are shown in [table 1](#). Blood samples from healthy donors (HD) were received from the Central Laboratory of the Swiss Red Cross (Basel, CH), the Centro Trasmfusionale Lugano or from spontaneous donations, and usage was approved by the Ethical Committee of the Canton Ticino (CE-3428).

Blood collection and cell isolation

Sixteen millilitres of peripheral blood from each patient were collected in BD Vacutainer CPT cell preparation tubes (362782, BD Biosciences). Blood from HD was provided as buffy coats or was withdrawn and collected in BD Vacutainer CPT tubes.

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque density centrifugation within 24 hours of blood withdrawal. CD8⁺ T cell enrichment from total PBMCs was performed either using positive immunoselection (130-045-201, Miltenyi Biotec), according to the manufacturer's instructions, or through cell sorting using FACSaria III (BD Biosciences).

Flow cytometric analysis

Phenotyping

Freshly isolated PBMCs were used for surface staining and incubated for 30 min at 4°C with fluorochrome-conjugated antibodies (online supplemental table 1) in phosphate-buffered saline (PBS; D8537, Sigma-Aldrich) with 1% fetal bovine serum (10270106, Gibco, Life Technologies). Cells were then washed with PBS and immediately acquired or fixed in 1% (w/v) paraformaldehyde (158127, Sigma-Aldrich) in PBS for subsequent acquisition.

Cytokine and cytotoxic molecule production

To assess cytokines, perforin (PRF) and granzyme B (GZMB) production, CD8⁺CCR4⁺ T cells were sorted and left overnight in T cell medium (Roswell Park Memorial

Table 1 Characteristics of the patients with AS

Patient characteristics	Total	Active AS	Inactive AS
Number of patients	76	54	22
Gender (M/F)	M (54) / F (22)	M (37) / F (17)	M (17) / F (5)
Age (mean±SD)	42.9±12.1	45.3±12.0	37.4±10.2
Human leucocyte antigen-B27 ⁺ (%)	89.47%	88.9%	90.9%
Inflammatory bowel disease (%)	6.6%	7.4%	4.5%
Psoriasis (%)	3.9%	5.5%	0%
Uveitis (%)	27.6%	22.2%	36.4%
Tumour necrosis factor inhibitor (%)	53.9%	42.6%	81.8%
ASDAS (mean±SD)	1.92±1.00	2.35±0.87	0.86±0.23
ASDAS range	0.2–4.1	1.3–4.1	0.2–1.2

AS, ankylosing spondylitis; ASDAS, Ankylosing Spondylitis Disease Activity Score.

Institute-1640 medium (11875093) supplemented with 1% (v/v) glutaMAX-I (35050061), 1% (v/v) non-essential amino acids (11140068), 1 mM sodium pyruvate (11360088), 50 µM β-mercaptoethanol (31350010), penicillin (50 U/mL), streptomycin (50 µg/mL) (15070063), 1% (v/v) kanamycin (15160047) (all from Gibco, Life Technologies) and 5% (v/v) human serum (Swiss Blood Centre, Basel). Cells were then stimulated with 50 ng/mL phorbol 12-myristate 13-acetate (P1585, Sigma-Aldrich) and 1 µg/mL ionomycin (I0634, Sigma-Aldrich), resuspended in T cell medium, for 5 hours at 37°C in a 5% carbon dioxide-humidified atmosphere. After 2.5 hours, 10 µg/mL brefeldin A (B5936, Sigma-Aldrich) and 2 mM monensin (M5273, Sigma-Aldrich) were supplemented. Where indicated, directly conjugated anti-CX3CR1 antibody (online supplemental table 1) was added to the cells 30 min prior to fixation. Intracellular staining of cytokines, PRF and GZMB, was performed using fluorochrome-conjugated antibodies (online supplemental table 1) and Cytofix/Cytoperm kit (554714, BD Biosciences), according to the manufacturer's instructions. All samples were acquired on BD LSRFortessa (BD Biosciences), and the results were analysed with FlowJo software V.10.7.1 (Tree Star).

RNA sequencing

RNA isolation and purification

After sorting, CD8⁺CCR4⁺ T cells from six patients with AS and five HDs were stored in TRIzol Reagent (15596026, Life Technologies) at -80°C until RNA extraction was performed. Total RNA was extracted with Zymo-Spin IC Columns (C1004-50, Zymo Research) and the Direct-zol RNA MiniPrep Kit (R2050, Zymo Research) according to the manufacturer's instructions. RNA sequencing was performed using NEBNext Ultra Directional RNA Library Prep for Illumina (New England BioLabs) according to the manufacturer's instructions. The libraries were sequenced using NextSeq 500 (Illumina) or Novaseq 6000 (Illumina). Detailed data analysis information is provided in online supplemental file 1.

Statistical analysis

Data were analysed using Prism V.9.0 software (GraphPad) and presented as mean±SEM. The statistical significance between HD and AS groups was determined using the Kruskal-Wallis test with Dunn's correction for multiple comparisons. Simple linear regression analysis was performed to correlate the percentages of CD8⁺CCR4⁺ T_{EM} and the ASDAS for active patients with AS under TNFi treatment. Values were considered statistically significant when probability (p) values were equal or below 0.05 (*), 0.01 (**), 0.001 (***) and 0.0001 (****).

Patient and public involvement

Patients and/or the public were not involved in the design, or conduct, or reporting, or dissemination plans of this research.

RESULTS

Altered phenotype of CCR4⁺ T cells in active AS

Previous reports showing elevated levels of the ligands of CCR4 in the sera of patients with AS^{22,23} prompted us to study the role of CCR4-expressing T cells in disease development and progression. Patients with AS (table 1) (n=76) were classified according to their disease activity, measured as ASDAS,³⁰ at the time of inclusion in the study. Patients with an ASDAS below 1.3 were considered in the inactive phase of the disease (n=22, inactive AS), while patients with an ASDAS above or equal to 1.3 were assigned to the active status group (n=54, active AS). Blood samples from HDs (n=42) were used as a control. Flow cytometric analysis revealed no difference in the frequency of circulating CD4⁺CCR4⁺ and CD8⁺CCR4⁺ T cells in patients with AS, regardless of disease activity (figure 1A,B). In humans, the combined expression of CD45RA and CCR7 allows the distinction of naive T cells (CD45RA⁺ CCR7⁺) from antigen-experienced T cells, comprising central memory (T_{CM}, CD45RA⁻ CCR7⁺), effector memory (T_{EM}, CD45RA⁻ CCR7⁻) and effector memory re-expressing CD45RA (T_{EMRA}, CD45RA⁺

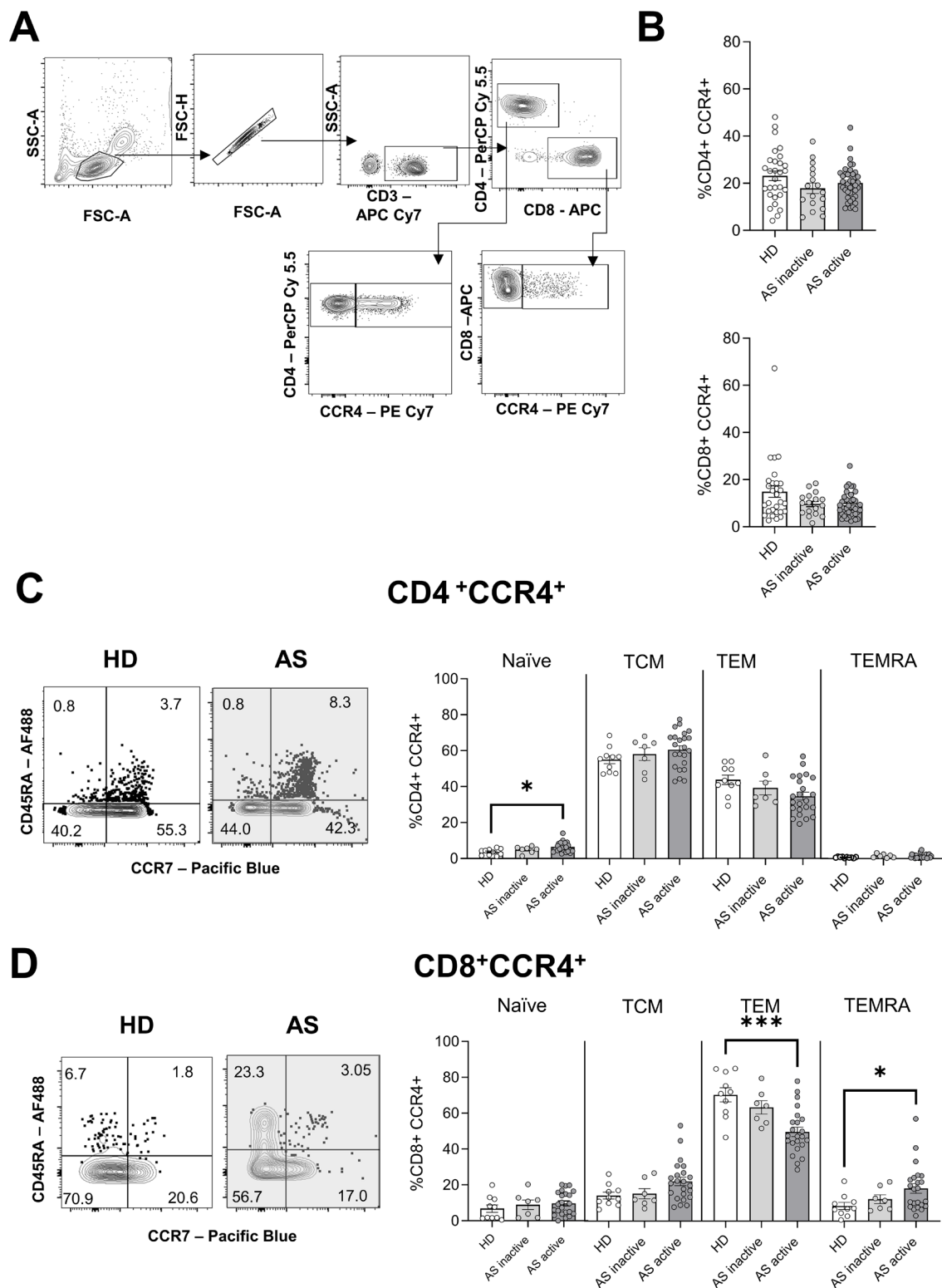


Figure 1 Phenotype of CCR4⁺ T cells from patients with AS and HD. (A) Gating strategy for the analysis of CCR4⁺ T cells. (B) Mean frequency \pm SEM of CD4⁺ (top) and CD8⁺ (bottom) T cells expressing CCR4 in HD and patients with active or inactive AS, according to Ankylosing Spondylitis Disease Activity Score. (C and D) Representative plot from the flow cytometric analysis showing the expression of CD45RA and CCR7 by CD4⁺CCR4⁺ (C) and CD8⁺CCR4⁺ (D) T cells. The numbers indicate the proportion of the population in each quadrant. Mean frequency \pm SEM of naïve (CD45RA⁺CCR7⁺), T_{CM} (CD45RA⁻CCR7⁺), T_{EM} (CD45RA⁻CCR7⁻) and T_{EMRA} (CD45RA⁺CCR7⁻) are represented for HD and patients with AS. Each symbol represents an individual. Kruskal-Wallis was used for the analysis, and asterisks indicate significant differences between the groups (* p <0.05, *** p <0.001). AS, ankylosing spondylitis; HD, healthy donor; T_{CM}, T cell central memory; T_{EM}, T cell effector memory; T_{EMRA}, T cell effector memory re-expressing CD45RA.

CCR7).^{31–33} CD4⁺CCR4⁺ T cells displayed a T_{CM} and T_{EM} phenotype, and a small but significant increase in the frequencies of naive cells was observed in patients with active disease (figure 1C). As no major differences in frequencies or phenotypes were found in CD4⁺CCR4⁺ T cells, we did not explore this population any further.

On the other hand, CD8⁺CCR4⁺ T cells from patients with active disease showed a significant reduction in T_{EM} (active AS vs HD, p=0.001) phenotype and a concomitant increase in T_{EMRA} (active AS vs HD, p=0.036) (figure 1D). To investigate whether these observed differences in T_{EM} and T_{EMRA} populations reflected an altered differentiation state, we assessed their CD27 and CD28 expression. During differentiation, CD27⁺CD28⁺ CD8⁺ T cells progressively lose both CD27 and CD28, upregulating cytotoxic molecules.^{34 35} Highly differentiated T_{EMRA}, lacking the expression of both CD27 and CD28, was predominant in both HD and patients with AS, with no differences between the groups (online supplemental figure 1A). CD8⁺CCR4⁺ T_{EM} were less differentiated, and the majority of cells expressed both receptors. Overall, these results indicate that the altered phenotype present in CCR4-expressing CD8⁺ T cells is not accompanied by differences in CD27 and CD28 expression.

The use of TNFi is widely adopted as a therapeutic strategy for the treatment of AS. To study the impact of TNFi therapy on the phenotype and function of CCR4⁺ T cells, patients with active AS were divided according to their therapeutic regimen at the time of enrolment in the study (table 1). CD8⁺CCR4⁺ T cells were reduced in patients treated with TNFi and presented a higher proportion of T_{CM} and a subsequent lower level of T_{EM} (online supplemental figure 1B). The reduction in T_{EM} levels was also present in the patients who did not receive TNFi (TNFi–), suggesting that this is a unique feature of active AS (online supplemental figure 1B). The significant reduction of CD8⁺CCR4⁺ T_{EM} in patients with active AS compared with controls follows the trend present in total CD8⁺ T cells (HD vs TNFi–: 46.4% vs 27.5%, p=0.0099; HD vs TNFi+: 46.4% vs 20.1%, p=0.0002). Of note, in patients with active AS under TNFi treatment, the number of circulating CD8⁺CCR4⁺ T_{EM} correlates with the ASDAS (r=0.859, p=0.006), while no correlation was observed in the TNFi– group (online supplemental figure 1C). The higher proportion of T_{EMRA} in CD8⁺CCR4⁺ T cells observed in patients with active AS was not dependent on TNFi treatment (online supplemental figure 1C).

These results showed that CD8⁺CCR4⁺ T cell frequencies are not altered in the blood of patients with AS; however, they exhibit a distinct phenotype. Moreover, the reduction in frequencies of T_{EM} appears to be independent of TNFi treatment.

CD8⁺CCR4⁺ T cells in AS exhibit distinct homing features

To further characterise the trafficking potential of CD8⁺CCR4⁺ T cells to inflamed tissues in AS, we investigated the expression of additional chemokine receptors

that guide leucocytes to specific tissues under homeostatic (CCR6 and CXCR4) or inflammatory (CCR1, CCR5, CXCR3 and CX3CR1) conditions. Increased expression of CCR1 was found in patients with inactive AS (figure 2A,B). Significantly higher levels of CCR5 were present in cells isolated from patients with AS, regardless of the disease activity (figure 2A,C). In patients with active AS, TNFi treatment was associated with decreased CCR5 expression to levels similar to the control group (figure 2C). In addition, CX3CR1 was upregulated in active AS and restored to normal levels in patients receiving TNFi treatment (figure 2A,D). By contrast, no differences between the groups were observed for CCR6, CXCR3 and CXCR4 (figure 2A).

The increased expression of inflammatory receptors prompted us to study the activation and exhaustion states of CD8⁺CCR4⁺ T cells. Recently activated T cells express both CD38 and HLA-DR and upregulate PD-1. On average, about 20% of CD8⁺CCR4⁺ T cells co-expressed both CD38 and HLA-DR molecules, but no significant differences were observed between healthy individuals and patients with AS (online supplemental figure 2A). Similar levels of PD-1 were present in CD8⁺CCR4⁺ T cells in both HD and patients with AS (online supplemental figure 2B). CCR4-expressing cells were also evaluated for their proliferation status based on the expression of Ki67, but no differences were found (online supplemental figure 2C).

To further characterise the migratory capacity of this T cell subpopulation, we assessed the expression of adhesion molecules promoting infiltration at the site of inflammation, selecting those involved in migration to lymphoid organs (CD62L) as well as to sites of AS extra-musculoskeletal manifestations: cutaneous lymphocyte-associated antigen 1 (CLA1), CD11a and integrin β7. In both inactive and active AS, a significant increase in CD62L expression was found in CD8⁺CCR4⁺ T cells (figure 2A,E). TNFi treatment did not appear to affect the high expression of this selectin. No significant differences in CLA1, CD11a or integrin β7 expression were found (figure 2A).

Together, these results suggest a unique trafficking capability of CD8⁺CCR4⁺ T cells in patients with AS mediated by the upregulation of CCR1, CCR5, CX3CR1 and CD62L.

CD8⁺CCR4⁺ T cells in AS have increased cytotoxic potential

CX3CR1 expression on memory CD8 T cells has been associated with cytotoxic effector functions.^{36 37} Therefore, we investigated the production of PRF and GZMB upon ex vivo stimulation by sorting CD8⁺CCR4⁺ T cells based on their expression of CX3CR1. Consistent with previous studies, higher expression of GZMB was present in CX3CR1⁺ T cells (figure 3A,B). CD8⁺CCR4⁺CX3CR1⁺ T cells from patients with inactive or active AS produced significantly higher levels of PRF and GZMB than those from HD. On the other hand, CX3CR1[–] cells only marginally produce PRF.

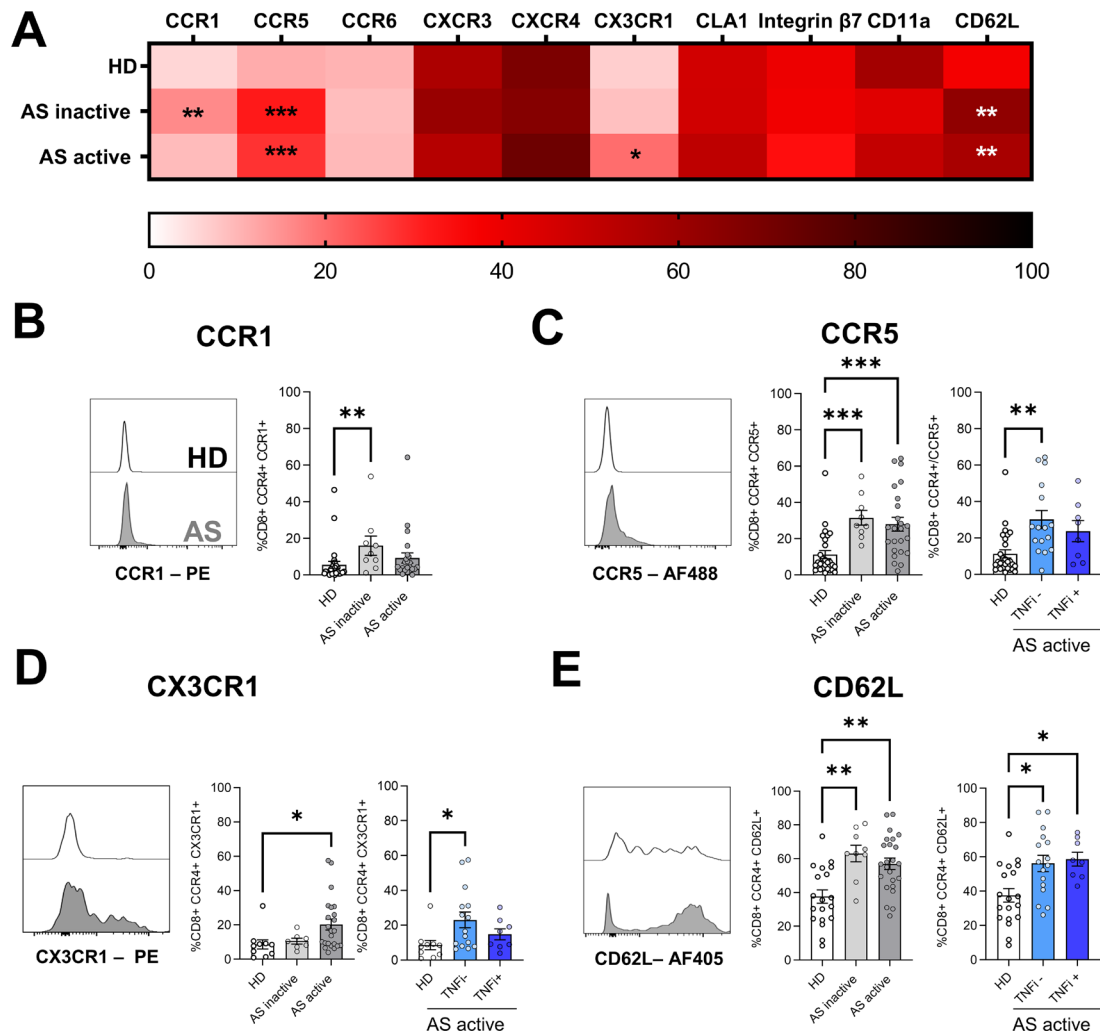


Figure 2 Expression of chemokine receptors and adhesion molecules in CD8⁺CCR4⁺ T cells. (A) Heatmap summarising mean frequencies of expression of the reported chemokine receptor and adhesion molecules in CD8⁺CCR4⁺ T cells. (B-E) Expression of significantly different receptors is represented as overlaid histograms (left) and mean frequencies \pm SEM (right) of CD8⁺CCR4⁺ T cells. Each symbol represents an individual, and Kruskal-Wallis test was used to compare the markers among the groups; asterisks indicate significant differences between the groups (* p <0.05, ** p <0.01, *** p <0.001).

In addition, several cytokines, both with proinflammatory and anti-inflammatory properties, are elevated in sera of patients with AS, among others, TNF, IL-17A and IL-23.³⁸ Overall, ex vivo stimulated CD8⁺CCR4⁺ T cells, isolated from patients with active AS, produced fewer cytokines, with a significantly lower proportion of interferon (IFN)- γ , TNF and IL-2 compared with HD (online supplemental figure 3). IL-4 and IL-17 were also reduced, while all groups equally secreted IL-22 and a minimal level of IL-23 (online supplemental figure 3). Together these data indicate that, among the CD8⁺CCR4⁺ T cells, generally presenting a low cytokine production, those expressing CX3CR1⁺ show an enhanced cytotoxicity.

CD8⁺CCR4⁺ T cells in AS upregulate gene promoting osteogenesis

Whole-transcriptome analysis was performed on sorted CD8⁺CCR4⁺ T cells from patients with active or inactive AS and from HD. The principal component analysis of the expressed genes highlighted that cells from patients

with AS clustered according to disease activity and were distinct from those derived from HD (figure 4A). Of all 12 334 genes detected, 777 genes were significantly differently expressed (false discovery rate \leq 0.05 and the absolute value of \log_2 fold change \geq 1) between active AS and HD. Genes involved in the cytolytic process (*SH2D1B*, *NRCAM* and *GZMB*) and expressed in non-senescent cells (*IRRN3*) were upregulated in active AS, while, among others, *TNF*, the nuclear receptor *NR4A1*, implicated in early TCR signalling,³⁹ and the chemokine receptor *CXCR5* were downregulated (figure 4B,C). On the other hand, 2161 genes were expressed differently in the active versus inactive AS and 1670 genes between inactive AS and HD. Gene set enrichment analysis of active AS versus HD identified 153 pathways differentially expressed. Interestingly, among the top 10 upregulated pathways in active AS, two were involved in the ossification process: positive regulation of ossification (Gene Ontology Biological Process (GOBP): 0045778, adjusted

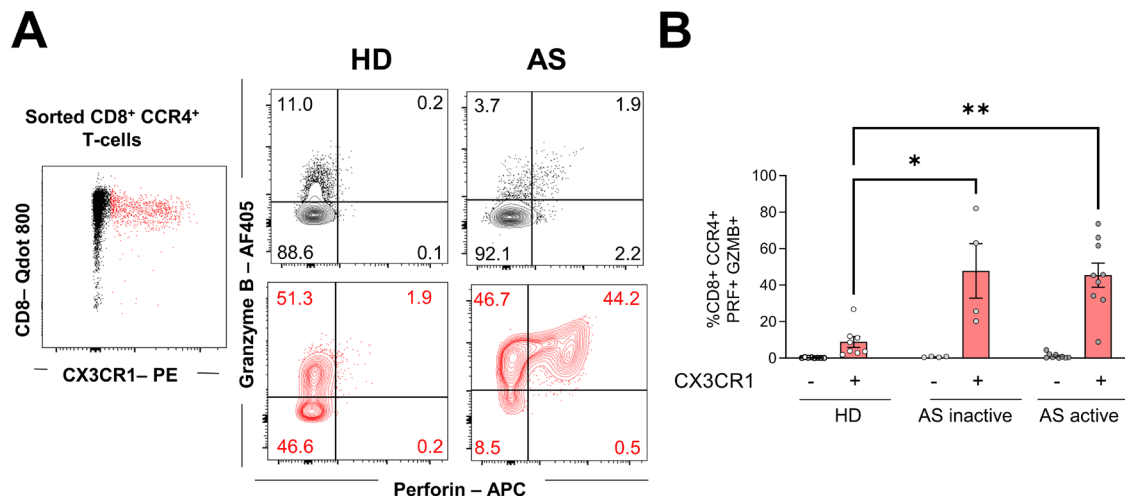


Figure 3 PRF and GZMB secretion by CD8⁺CCR4⁺ T cells. Sorted CD8⁺CCR4⁺ T cells were stimulated with phorbol 12-myristate 13-acetate and ionomycin for 5 hours and expression of CX3CR1, PRF and GZMB was assessed. (A) Representative plot showing CX3CR1⁻ (black) and CX3CR1⁺ (red) cells expressing PRF and GZMB. Numbers in each quadrant report percentages relative to the parental population. (B) Mean frequencies±SEM of PRF⁺GZMB⁺ cells relative to CD8⁺CCR4⁺ CX3CR1⁻ (black) or CX3CR1⁺ (red) cells. Each symbol represents an individual, and Kruskal-Wallis was used to compare PRF and GZMB production between groups within the CX3CR1⁺ or the CX3CR1⁻ populations. Asterisks indicate significant differences between the groups (*p<0.05, **p<0.01). GZMB, granzyme B; PRF, perforin.

p value (q)=0.013) and positive regulation of osteoblast differentiation (GOBP: 0045669, q=0.002) (figure 4D). Among these pathways, BMP receptors (*BMPRIA*, *BMPRI1B*, *ACVR2A* and *ACVR2B*), insulin growth factor 1 (*IGF1*), Wnt proteins (*WNT5*) and nephronectin (*NPNT*) genes were significantly upregulated in active AS compared with HD (figure 4E). Both pathways were not significantly altered in inactive AS compared with HD; however, some genes, including the transcription factor *RUNX2* and the BMP receptor *ACVR2A* remained upregulated (figure 4E). Interestingly, the expression of *BMPRI1B* and *IGF1* positively correlated with the expression of the fractalkine receptor gene *CX3CR1* (figure 4F).

Taken together, our results show that CD8⁺CCR4⁺ T cells from patients with active AS upregulate genes promoting ossification, which correlates with *CX3CR1* expression.

DISCUSSION

The genetic predisposition associated with HLA-B27, discovered 50 years ago,^{36 40} leads to the hypothesis that T cells may play an important role in AS pathogenesis via TCR-HLA-B27 engagement. Nevertheless, the precise mechanism by which T cells influence new bone formation remains poorly understood. In this study, we provide for the first time the characterisation of a unique subset of CD8⁺ T cells with osteoregulatory potential. These cells have the potential to travel to extra-musculoskeletal sites of inflammation, where they might cause damage thanks to enhanced production of GZMB and PRF. We have focused on the radiographic state (AS) of a disease continuum now known as axial SpA to also include earlier and milder disease forms.⁴¹ Spinal radiographic progression is minimal during the non-radiographic disease

state,⁴² and we targeted a population with demonstrated potential for further structural damage.

Contrasting data on the frequencies of circulating CCR4⁺ T cells and their correlation with disease activity have been reported.^{24 25} Our study expands the first characterisations of an altered CCR4⁺ T cells presence in the circulation of patients with AS and shows, in a larger cohort, that CCR4-expressing CD4⁺ and CD8⁺ T cells are not altered in frequencies, regardless of disease activity. Interestingly, we have found that patients with active disease under TNFi treatment displayed lower frequencies of CD8⁺CCR4⁺ T cells compared with HD, suggesting a potential egress of these cells from the circulation to the inflamed sites (online supplemental figure 1B). These data are corroborated by initial findings on the presence of a CCR4⁺ T cell subpopulation in the synovial fluid of patients with AS.⁴³

We have found high expression of CCR1 and CCR5 on CD8⁺CCR4⁺ T cells from patients with AS (figure 2A), and their ligands, CCL3, CCL4 and CCL5, have been reported to guide effector T cell migration during inflammation.^{44 45} These chemokines are also expressed in psoriatic skin⁴⁶ and in a mouse model of uveitis.⁴⁷ This suggests that, in AS, CD8⁺CCR4⁺ T cells have the potential to travel to sites of extra-musculoskeletal manifestations, including eyes, skin and gut, thanks to the upregulation of inflammatory chemokine receptors and the expression of CLA1 and integrin β7.

Chemokine–chemokine receptor interaction, in addition to its homing regulation, can also influence other T cell activities, such as proliferation, survival and activation.⁴⁸ CX3CR1 expression on antigen-experienced CD8⁺ T cells is associated with their ability to produce GZMB, irrespective of CD62L or CCR7 expression.^{36 49 50}

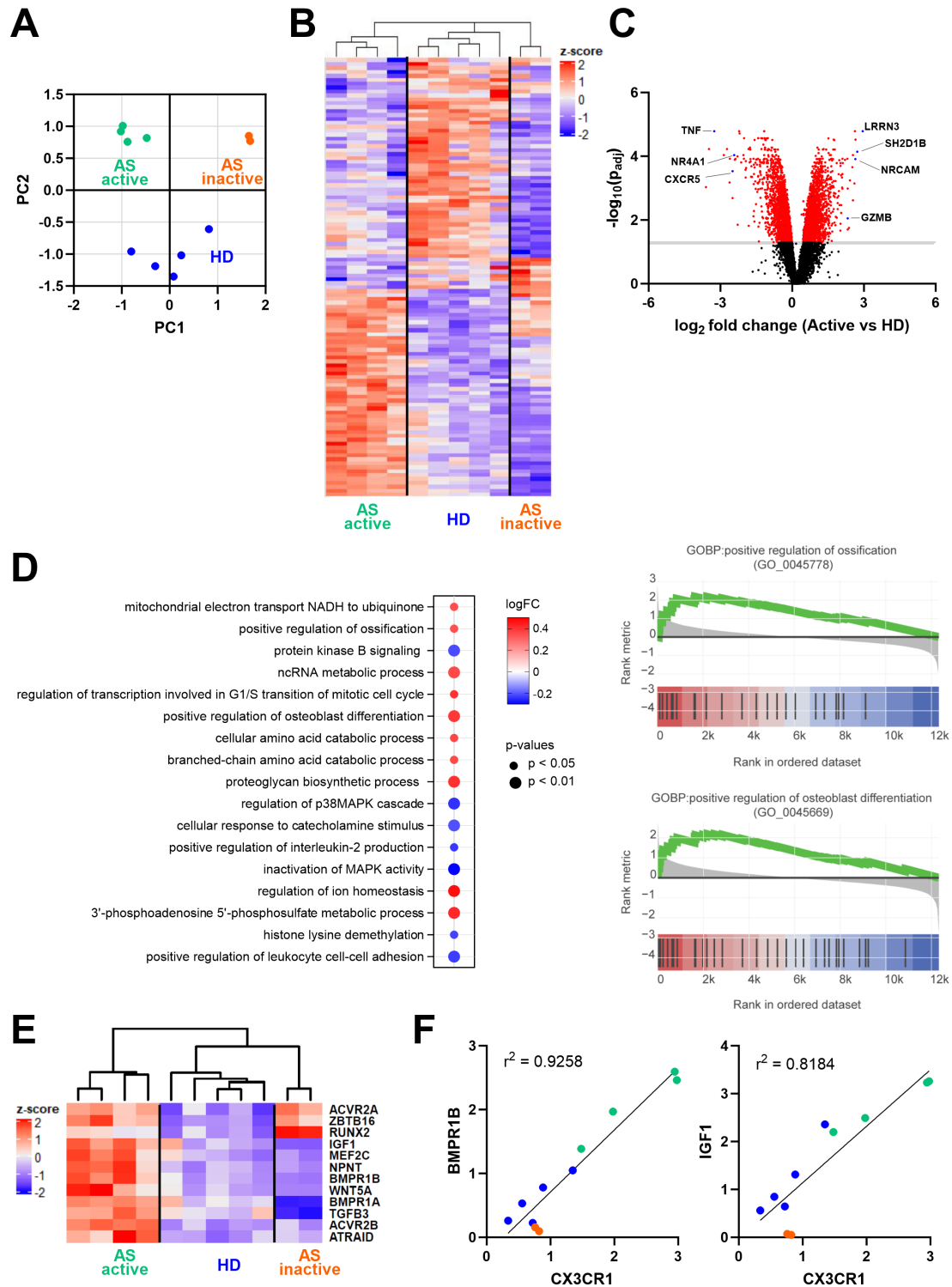


Figure 4 Gene expression analysis of sorted $CD8^+CCR4^+$ T cells. (A) Principal component analysis and sample clustering based on the whole transcriptome. (B) Heatmap showing normalised expression of the significantly differentially expressed genes between active AS and HD. (C) Volcano plot displaying differentially expressed genes between active AS and HD; in red are highlighted the significantly different genes ($-\log_{10}(p \text{ value adjusted}) > 1$). (D) The graph on the left shows the top 10 upregulated and the seven downregulated pathways identified in the gene set enrichment analysis between active AS and HD. Pathways were ordered based on the number of identified genes in the dataset. The graphs on the right show gene set enrichment analysis of the Gene Ontology Biological Process (GOBP) related to positive regulation of ossification and osteoblast differentiation in patients with active AS. The y-axis reports the fold change expression compared with control samples, while in the x-axis, black bars indicate the rank of genes in the gene set list. The green curve corresponds to the running statistics of the enrichment score. (E) The heatmap reports significantly upregulated genes promoting ossification in active AS. (F) Correlation of $CX3CR1$ and $BMPR1B$ or $IGF1$ gene expression detected in the RNA sequencing analysis. AS, ankylosing spondylitis; HD, healthy donor.

Patients with active AS presented a higher proportion of CD8⁺CCR4⁺ T cells expressing CX3CR1.

Interestingly, CD8⁺CCR4⁺CX3CR1⁺ T cells isolated from patients with AS, regardless of disease activity, produced both PRF and GZMB, in contrast to the exclusive production of GZMB registered in HD (figure 3B). This enhanced cytotoxic profile may reflect the enrichment of T_{EMRA}, a subset of CD8⁺ T cells known for their low proliferative rate, high cytotoxicity, sensitivity to apoptosis and association with chronic inflammation.^{51 52} The overexpression of cytotoxic molecules is in line with previous work highlighting the upregulation of *GZMH*, *GZMB* and *NKG7* on circulating CD8⁺ T_{EM} cells isolated from patients with AS.⁵³

Of note, elevated levels of CX3CL1 are present in the gut, bone marrow and synovium of patients with AS, but not in sera samples.^{26 53} CX3CR1 and CX3CL1 overexpression has been reported in other autoimmune diseases, and in rheumatoid arthritis (RA), this axis has been targeted by an antibody-based therapeutic intervention.⁵⁴ Synovial fibroblasts and synoviocytes, expressing CX3CL1, are able to promote CD4⁺ T cell adhesion in vitro, adhesion that is impaired by the addition of soluble chemokine.⁵⁵ Thanks to the presence of CX3CL1 on mature osteoblasts,⁵⁶ cells specialised in bone matrix deposition, we envision a similar interaction between CD8⁺CCR4⁺ T cells expressing CX3CR1 and osteoblasts in AS. Indeed, it has been shown that CD8⁺ T cells, as well as osteoblasts, are present in the enthesis and in the perientheseal bone.^{19 21}

Importantly, *CX3CR1* expression levels in CD8⁺CCR4⁺ T cells correlated with many of the ossification-promoting genes upregulated in patients with active AS (figure 4F). While the activity of these genes has been extensively studied on osteoblasts,⁵⁷ little is known about their role in leucocyte biology. IGF1 promotes the differentiation and acquisition of a proinflammatory and cytotoxic profile in both T and natural killer (NK) cells^{58 59} and induces chondrocyte and osteoblast proliferation and the deposition of extracellular matrix.⁶⁰ NPNT protein contains epidermal growth factor-like motifs, responsible for the regulation of osteoblast differentiation and angiogenesis in the bone,⁶¹ while its function on T cells and immune cell development has not yet been identified. BMPs induce regulatory T cell differentiation and promote an M2 phenotype in macrophages, while NK cells, activated by BMPs, secrete more IFN γ .⁶² The upregulation of *BMPRIA*, *B* and *ACVR2* in CD8⁺CCR4⁺ T cells from patients with active AS and the fact that their ligand, BMP-2, is found at high levels in the ossifying enthesis,⁶³ point to a role of this axis in AS pathogenesis. Of note, *ACVR2A* and *RUNX2*, but not *BMPRIA* and *BMPR1B*, all involved in BMPs/transforming growth factor- β signalling, were also upregulated in CD8⁺CCR4⁺ T cells from patients with inactive AS. Functional experiments will need to clarify if this difference reflects a ‘quiescent’ state of this pathway in patients with inactive AS that may be

‘reactivated’ by inflammatory cues, present during the active disease.

This report highlights, for the first time, a new pathway by which T cells could directly regulate new bone formation through the expression of ossification-related molecules. Other mechanisms by which T cells regulate bone growth and resorption have been previously proposed. Ahuja and colleagues reported the expression of CD40 and major histocompatibility complex-I on murine osteocytes and osteoblasts, hypothesising that CD8⁺ T cells, expressing CD40L, could provide these cells with a survival signal.⁶⁴ CD40–CD40L interaction has also been shown to indirectly induce bone loss in a model of hormone-driven osteoporosis.⁶⁵ A second mechanism, demonstrated in vitro, relies on cytokine production by bystander activated T cells and the subsequent release of BMP-2 by osteoblast precursors and their matrix mineralization.^{66 67} Our study shows that CD8⁺CCR4⁺ T cells from patients with AS produced low levels of IFN γ , TNF and IL-17 (online supplemental figure 3), suggesting that these cells do not rely on cytokine production to promote bone mineralisation. These data were further corroborated by the low level of TNF gene expression found by RNA sequencing (figure 4C). Other studies pointed at a minimal contribution of CD8⁺ T cells in AS for the production of major inflammatory cytokines, with decreased levels of IFN γ ⁺ and TNF⁺ CD8⁺ T cells both in circulation and in inflamed sites.^{68 69} On the other hand, excessive IL-17 production was demonstrated to be crucial in the pathogenesis of AS.¹⁵ Nevertheless, in line with our data, increasing evidence points to mucosal-associated invariant T cells and $\gamma\delta$ cells as major producers of IL-17 in AS, with limited production by CD8⁺ T cells.^{70 71} CD8⁺IL-17⁺ T cells are found in the synovial fluid of patients suffering from other inflammatory joint diseases, such as psoriatic arthritis, but are absent in RA.⁷² We cannot exclude that in the inflamed tissues of patients with AS, CD8⁺CCR4⁺ T cells might acquire a different cytokine expression profile.

Our study reports a CD8⁺ T cell subset in AS displaying both osteoregulatory and cytotoxic functions. These cells, due to their chemokine receptor expression pattern, have the potential to traffic to the bone, promoting ossification, and to sites of extra-musculoskeletal manifestations, where they could contribute to inflammation via tissue damage.

A better understanding of the crosstalk between immune cells and the bone in AS will be invaluable for establishing novel therapeutic approaches. Current treatments successfully target inflammation but only partially influence new bone formation. The present data suggest that targeting the chemokine system might represent an additional approach to effectively address this unmet medical need.

Author affiliations

¹Institute for Research in Biomedicine, Università della Svizzera italiana, Bellinzona, Switzerland

²Department of Rheumatology, University of Zurich, University Hospital Zurich, Zurich, Switzerland

³Department of Rheumatology and Immunology, Inselspital-University Hospital Bern, University of Bern, Bern, Switzerland

⁴Department of Biomedical and Neuromotor Sciences, University of Bologna, Bologna, Italy

⁵Institute of Oncology Research, Università della Svizzera italiana, Bellinzona, Switzerland

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ORCID iDs

Veronica Martini <http://orcid.org/0000-0002-6086-0358>

Adrian Ciurea <http://orcid.org/0000-0002-7870-7132>

Valentina Cecchinato <http://orcid.org/0000-0001-7415-8706>

Mariagrazia Ugucioni <http://orcid.org/0000-0002-9570-7011>

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