Alma Mater Studiorum – Università di Bologna

DOTTORATO DI RICERCA IN

Scienze Biomediche e Neuromotorie

Ciclo XXXII

Settore Concorsuale: 05/E1

Settore Scientifico Disciplinare: BIO/10

THE ROLE OF CD8+CCR4+ T-CELLS IN AXIAL SPONDYLOARTHRITIS

Presentata da: Ylenia Silvestri

Coordinatore Dottorato

Prof. Pietro Cortelli

Supervisore

Prof. Flavio Flamigni

Co-supervisore

Prof.ssa Mariagrazia Uguccioni

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Esame finale anno 2019

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Author’s declaration

I declare that, the data presented in this thesis were obtained in experiments performed at the Institute for Research in Biomedicine, Bellinzona (Switzerland).
Except where explicit reference is made to the contribution of others, this thesis is the result of the own work and has not been submitted for any other degree at the University of Bologna or any other institution.

Ylenia Silvestri

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Abstract

Axial spondyloarthritis (AxSpA) is an inflammatory rheumatic disease predominantly affecting the axial skeleton with remodeling of skeletal structures.

The inflammatory infiltrate of T-cells in the structural lesions has been found to contribute to bone remodeling, but consensus relating the functional contribution of different T-cell subsets to axSpA pathogenesis has not been reached yet.

In this scenario, aim of the project was to characterize circulating T-cells and their homing markers from axSpA patients in order to identify cellular populations that could migrate to inflamed tissues and be implicated in axSpA development and perpetuation.

Taking advantage of a multicolor flow cytometry approach for immunophenotyping, we found an altered proportion of circulating naïve and memory T-cells in axSpA patients, and a skew in the T_C2/T_C1 ratio in favor of CD8+ T-cells expressing the chemokine receptor CCR4. Since CCL17 and CCL22, the two ligands for CCR4, are found to be elevated in the sera of axSpA patients, we investigated in details the role of CD8+CCR4+ T cells in axSpA.

Our data showed that circulating CD8+CCR4+ T cells display an effector memory phenotype and express homing markers for tissues that are target of the disease. Noteworthy, CD8+CCR4+ T-cells from axSpA patients were activated, expressed markers of proliferation and acquired a cytotoxic phenotype, as demonstrated by the increased production of granzyme and perforin. In line with their cytotoxic phenotype, we observed a higher frequency of CX_3CR1 positive cells, compared to healthy donors.

CD8+CCR4+ T cells from axSpA patients upregulate the transcription of genes involved in bone mineralization and downregulate genes involved in osteoclast differentiation, indicating their possible involvement in bone remodeling. Furthermore, CD8+CCR4+ T-cells stimulated with PMA and ionomycin were able to produce and release TNF and IL-8, two cytokines involved in osteoclastogenesis, indicating that CD8+CCR4+ T-cells after stimulation would be able to promote osteoclasts differentiation and neutrophils recruitment.

Taken together our data suggest that CD8+CCR4+ T cells might exert a pathogenic role in axSpA, by releasing mediators of tissue damage, bone remodeling and recruitment of other pro-inflammatory cells.

Chapter 1: General introduction

1.1 Axial spondyloarthritis

1.1.1 Definition and classification

1.1.1.1 Spondyloarthritis

Spondyloarthritis (SpA) is a generic term indicating a group of chronic inflammatory rheumatic diseases that share common clinical, genetic and pathophysiological features.

Originally, SpA was considered a variant of rheumatoid arthritis (RA), but in 1974 Moll and colleagues established a new classification for diseases characterized by the absence of rheumatoid factor, an autoantibody typically found in RA, introducing the concept of seronegative spondyloarthrities [1].

Nowadays, this group of chronic inflammatory diseases affecting the axial skeleton and/or the peripheral joints, and the entheses (regions where a tendon, ligament, or joint capsule attaches to the bone) is termed SpA.

According to the Assessment of SpondyloArthritis international Society (ASAS) classification criteria defined in 2009 [2;3], SpA patients are divided into two groups which differ depending on the main location of the affected joints:

- axial SpA (axSpA) patients, characterized by back pain as dominant symptom;
- peripheral SpA patients, characterized by inflammation of the peripheral joints and/or the entheses (known as enthesitis).

According to the ASAS classification criteria the following diseases are included in the SpA family: non-radiographic axial SpA (nr-axSpA), radiographic axial SpA (r-axSpA), also termed Ankylosing Spondylitis (AS), psoriatic arthritis (PsA), reactive arthritis (ReA), arthritis associated with inflammatory bowel disease (SpA-IBD), undifferentiated peripheral SpA (uSpA) (Figure 1).

All these diseases have been documented to be strongly associated with the human leukocyte antigen (HLA)-B27 gene and a similar pattern of inflammation (asymmetrical, often oligoarticular). Moreover, SpA patients manifest arthritis of the peripheral joints, sacroiliitis (inflammation of the sacroiliac joints) and/or spondylitis, enthesitis, dactylitis (inflammation of the fingers), and uveitis (inflammation of the uvea, which is the middle layer of the eye). Patients who do not meet any of these diagnostic criteria but show similar clinical manifestations are categorized as undifferentiated SpA patients.

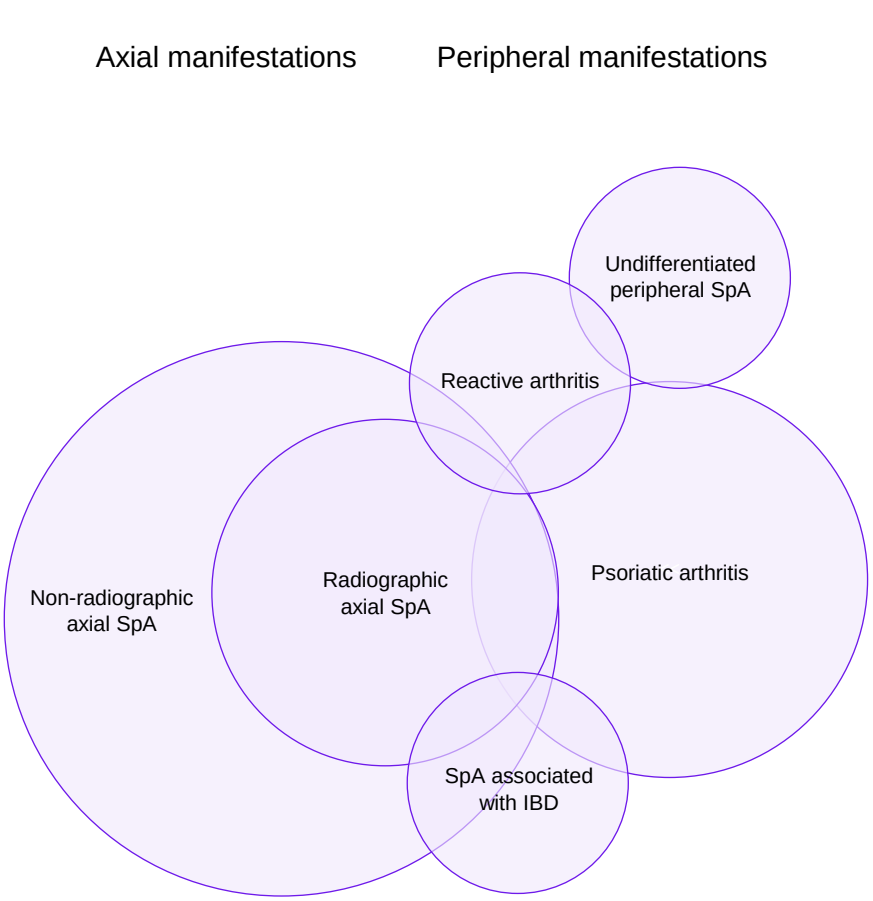


Figure 1. Spectrum of spondyloarthritis and overlap between the different forms. Adapted from Proft and Poddubnyy, 2018 [4].

SpA (Spondyloarthritis), IBD (Inflammatory Bowel Disease)

1.1.1.2 Axial spondyloarthritis

The term axSpA covers both the early inflammatory phase of the disease in which there is no structural damage in the sacroiliac joints (non-radiographic disease) and the later stages in which structural damage is visible on X-ray scans of the sacroiliac joints and of the spine (radiographic disease) (Figure 2) [4].

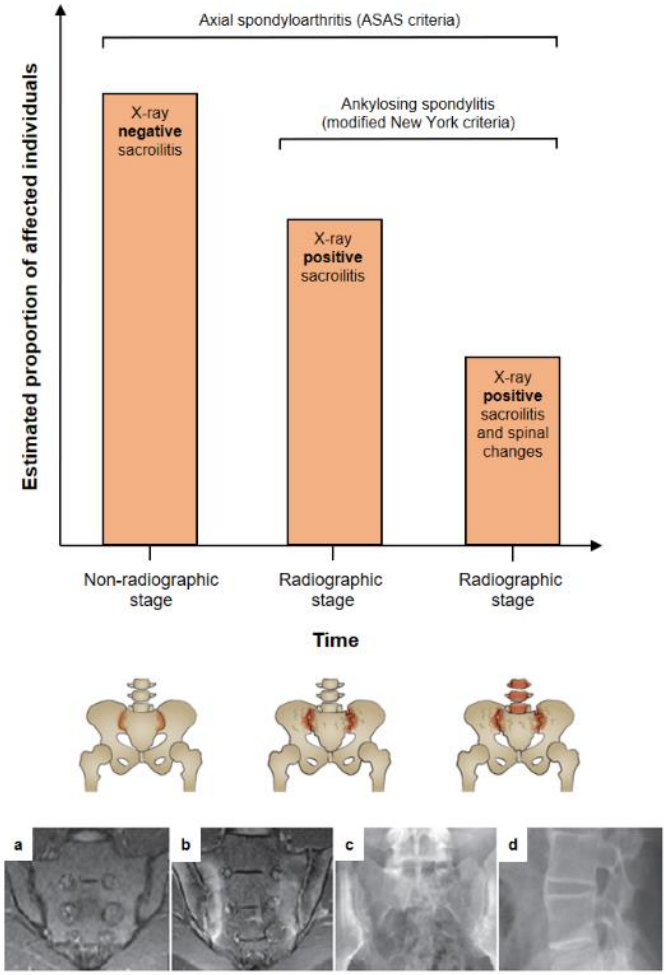
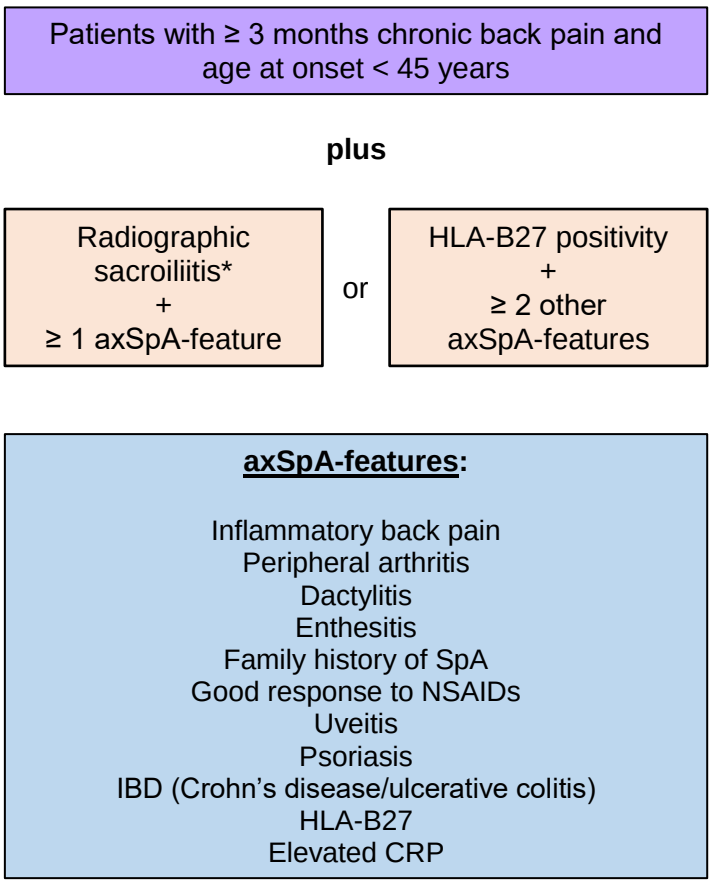


Figure 2. Spectrum of axial spondyloarthritis. Adapted from Sieper et al., 2015 [4;5].

Absence of abnormalities on MRI of the sacroiliac joints in patients with non-radiographic disease (**a**), inflammation of the sacroiliac joints detected by MRI before the occuring of structural damage (**b**), structural changes visible by X-ray including sclerosis, erosion and new bone formation (**c**), syndesmophytes of the spine characteristic of spinal involvement in axial spondyloarthritis (**d**).

ASAS (Assessment of Spondyloarthritis international Society), MRI (Magnetic Resonance Imaging)

According to the ASAS criteria, patients can be classified as axSpA patients either by using the imaging evaluation of the signs of active sacroiliitis in Magnetic Resonance Imaging (MRI) with at least one other SpA feature or by using clinical evaluation, where the presence of HLA-B27 is mandatory, together with at least two axSpA features (Figure 3), allowing the classification of the SpA patients at early stage of disease, when the structural damage in the sacroiliac joints has not yet occurred.



*according to modified New York criteria or active sacroiliitis on magnetic resonance imaging according to the ASDAS consensus definition.

Figure 3. Assessment of Spondyloarthritis international Society (ASAS) classification criteria for axial spondyloarthritis. Adapted from Proft and Poddubnyy, 2018 [4].

axSpA (axial Spondyloarthritis), HLA-B27 (Human Leucocyte Antigen-B27), NSAIDs (Nonsteroidal Anti-Inflammatory Drugs), IBD (Inflammatory Bowel Disease), CRP (C-Reactive Protein)

1.1.2 Epidemiology

The prevalence of axSpA ranges between 0.1% and 1.4% globally, showing geographic differences that can be explained in part by the prevalence of the HLA-B27 antigen positivity, the most important risk factor for the development of axSpA. In Europe, the prevalence of axSpA has been reported as 0.24% [6]. The disease often initiates before the age of 30 and predominantly occurs in men, with a male:female ratio of 3:1, with men generally experiencing a more progressive form of the disease. However, women experience greater negative impact on quality of life [7].

1.1.3 Clinical features

AxSpA is an inflammatory rheumatic disease predominantly affecting the axial skeleton (sacroiliac joints and spine). As compared with other rheumatic diseases, in axSpA the inflammation is linked to the remodeling of skeletal structures in a pathological and impedimental way (Figure 4A).

The main hallmark of axSpA is the new bone formation inside the ligaments of intervertebral joints (syndesmophytes) and inside the sacroiliac joints, resulting in an ankylosing of the spine. In the advanced stages of the disease, disability is mainly the result of spinal ankylosis, in which syndesmophyte formation bridges the bony fusion of the adjacent vertebrae. This clinical feature leads to an impaired function of the spine, with stiffness, limited motility, decreased thoracic excursions during respiration and thoracic kyphosis [8]. Ankylosis could involve the whole spine leading the radiographic picture of a bamboo-like spine (Figure 4B).

AxSpA is a complex disease, as indicated by the unclear relationship between inflammation and pathological bone remodeling [9;10], and the wide range of articular and extra-articular manifestations.

AxSpA patients are commonly affected by bone marrow inflammation (osteitis) and edema, and in approximately 30% of patients also by peripheral arthritis of large joints, with an asymmetrical and oligoarticular pattern [11;12].

Inflammation in axSpA is believed to first occur within the sacroiliac joints [13], but despite the site affected, in axSpA the inflammation is associated with reduced bone marrow cellularity, loss of synovium and cartilage, enthesitis and syndesmophyte formation [14]. Synovitis is succeeded by pannus and connective tissue formation, leading to the destruction of local cartilage and bone [14]. Following these destructive processes, new bone formation occurs, leading to a loss of joint function.

In addition to the obvious involvement of the joints, the increased levels of erythrocytes sedimentation rate (ESR), C-reactive protein (CRP), interleukin (IL)-6, IL-12, transforming growth factor β (TGF-β), IL-17A, and IL-23 in axSpA patients compared to controls, suggest the presence of a systemic inflammatory status [15;16].

AxSpA patients are also commonly affected by extra-articular manifestations (EAMs) such as: inflammatory bowel disease (IBD, disorders that involve chronic inflammation of digestive tract), uveitis (inflammation of the uvea, the middle layer of pigmented vascular structures of the eye), and psoriasis (long-lasting autoimmune disease characterized by patches of abnormal skin) with 5%, 25% and 10% of axSpA patients presenting these EAMs respectively [12;17;18]. Moreover, heart, lungs or kidneys involvement is also associated to axSpA, even though these organ manifestations are less common (Figure 4C).

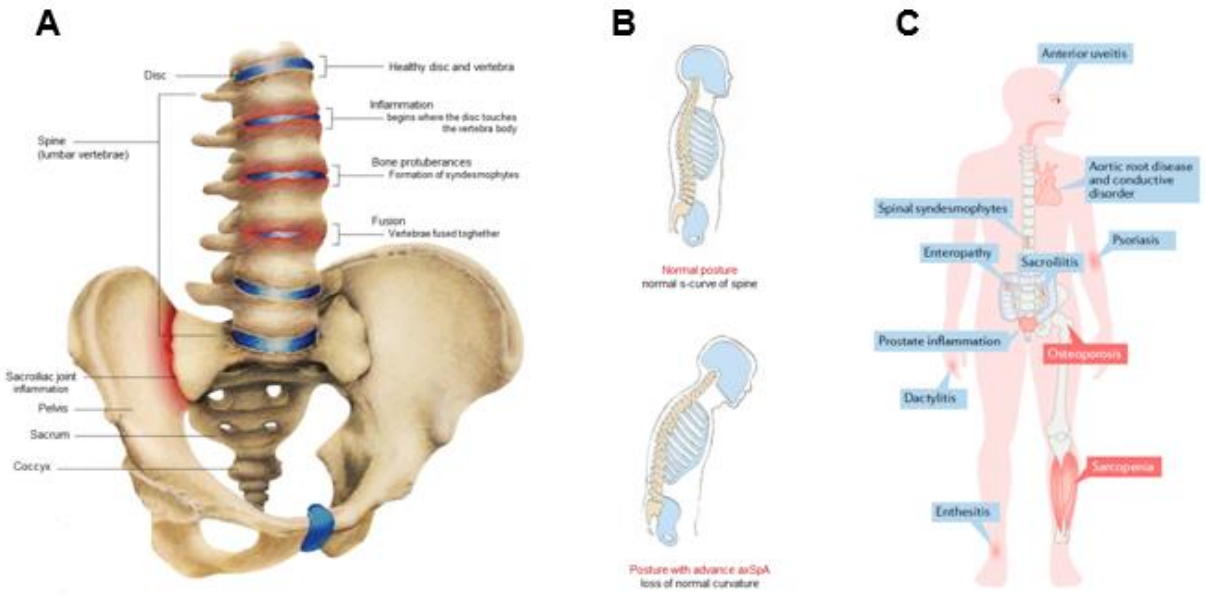


Figure 4. Anatomical sites of axial skeleton affected by axial spondyloarthritis (**A**), postural changes of the spine in axial spondyloarthritis (**B**), articular and extra-articular manifestation of axial spondyloarthritis (**C**).

1.1.4 Disease Activity Score (ASDAS)

The ASAS and the Outcome Measures in Rheumatology (OMERACT) endorse the Ankylosing Spondylitis Disease Activity Score (ASDAS) as a measure of axSpA disease activity with cut-offs [19;20]. This index combines several disease activity variables including the patient's assessments of the disease symptoms (back pain, duration of the morning stiffness, activity of the disease, pain and swelling of the peripheral joints) with a numerical rating scale from 0 to 10, and the laboratory data of CRP, mg/l or ESR, mm/hr [21;22].

The three cut-offs selected to classify the activity states are (Figure 5): <1.3 between "inactive disease" and "low disease activity", <2.1 between "moderate disease activity" and "high disease activity", and >3.5 between "high disease activity" and "very high disease activity" [20;23].

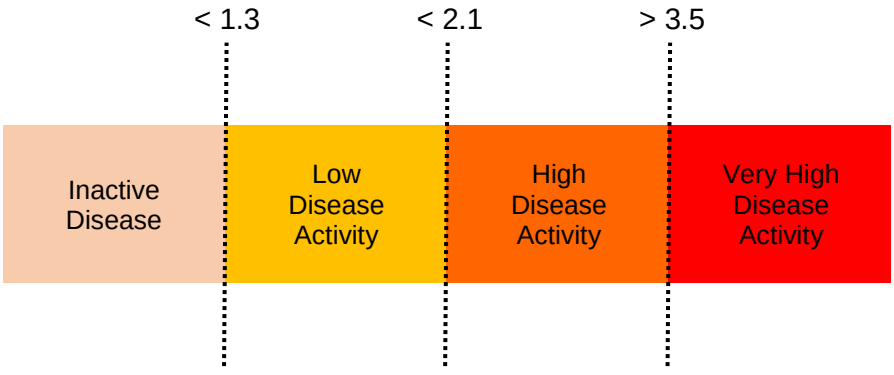


Figure 5. Cut-offs for disease activity states.

1.1.5 Treatments

Currently, all the available therapies primarily aim to alleviate the inflammatory symptoms and to prevent subsequent clinical deterioration. The first-line treatment requires the use of nonsteroidal anti-inflammatory drugs (NSAIDs) to reduce inflammation and pain, and the physiotherapy to improve spinal mobility [24]. NSAIDs have been shown to retard syndesmophytes formation [25], however some patients do not tolerate long-term use of NSAIDs due to gastrointestinal, renal and cardiovascular toxic side effects [26].

Conventional disease modifying anti-rheumatic drugs (DMARDs) have been shown to be effective on peripheral arthritis and other extra-articular manifestation of axSpA, such as psoriasis, uveitis, and IBD, but have no proven efficacy for the axial features [27-29].

At present, patients not responding to NSAIDs in terms of controlling symptoms progression, are treated with tumor necrosis factor inhibitors (TNFi), which target TNF preventing TNF engagement to the TNF receptor by binding circulating TNF or TNF receptor [30;31].

Simponi (Golimumab), Remicade (Infliximab), Humira (Adalimumab), Cimzia (Certolizumab) and Enbrel (Etanercept), have been shown to decrease inflammatory lesions on MRI imaging [32-38] and induce a rapid inflammatory remission [33;34;37;39-43]. Despite the effectiveness of TNF blockers, these treatments only partially retard but do not prevent radiographic progression [44-47]. TNFi have been shown to be effective also on peripheral arthritis, psoriasis and uveitis [34;48-50].

An improved understanding of the involvement of the IL-23/IL-17 axis in the pathogenesis of axSpA has led to the recent introduction of drugs targeting IL-17A, such as Secukinumab, that has been shown to be effective on axial symptoms but also on the spectrum of extra-articular manifestations [51-53]. However, in contrast to IL-17A inhibition, anti-IL-23 inhibitors failed to demonstrate efficacy in axSpA [54-56].

Despite the advances in disease treatment, approximately 50% of patients remain unresponsive to current therapies [57;58] highlighting the need of further investigations of the pathways involved in the pathogenesis of axSpA.

Categories	Examples
NSAIDs	COX-2 inhibitors, Ibuprofen, Naproxen, Etodolac, Diclofenac, Etoricoxib
DMARDs	Sulphasalazine, Methotrexate, Azathioprine, leflunomide
TNFi	Golimummab, Infliximab, Adalimumab, Certolizumab, Etanercept

Table 1. Drugs commonly used in the treatment of axial spondyloarthritis.

NSAIDs (Nonsteroidal Anti-Inflammatory Drugs), DMARDs (Disease Modifying Anti-Rheumatic Drugs), TNFi (Tumor Necrosis Factor inhibitor)

1.2 Pathogenesis of axial spondyloarthritis

Analogously to most of the autoimmune/autoinflammatory diseases, the clinical features of axSpA results from a complex interplay among genetic predisposition and environmental triggers. Several studies have suggested that the inflammation in axSpA initiates in the enthesis [59] as a consequence of the action of IL-23, which activates resident T-cells [60]. The elevated expression of IL-23 in these patients could be the result of multiple concomitant processes including the presence of susceptibility genes, microbial triggers due to the variations in the gut microbiome, and the activation of the immune response as a consequence of the biomechanical stress in the enthesis.

1.2.1 Genetics: role of HLA-B27

AxSpA has a strong genetic component, and several studies of familial aggregation estimate that the genetic background contribute to 80-90% of the susceptibility to the radiographic form of axSpA (AS) [17]. In particular, all SpA are characterized by the genetic association with HLA-B27 antigen, a major histocompatibility complex molecule (MHC) class I discovered in 1973 [61;62], that contributes to 30% of the heritability of the disease. The strong concordance rate between monozygotic (50-75%) versus dizygotic (15%) twins confirms that familial aggregation is more related to genetic rather than to environmental factors [63]. The association between HLA-B27 and axSpA is confirmed by the presence of the gene in 80-90% of patients with AS [64], opposed to 8-10% in the general population, and by the spondyloarthritis-like disease spontaneously developed by HLA-B27 transgenic rats [65]. To date the genetic association between HLA-B27 and AS is one of the strongest associations described, therefore it is not surprising that the presence of HLA-B27 is a diagnostic marker in suspected axSpA patients (Figure 6).

The association between HLA-B27 and axSpA is complex and several HLA-B27 haplotypes are associated with the disease susceptibility, for example HLA-B*2705, HLA-B*2701-08, HLA-B*2710, HLA-B*2714-15, and HLA-B*2719 [17;66-73], indicating that axSpA is highly heritable.

Although HLA-B27 expression is strongly associated with axSpA, only a small proportion of people in the general population who harbor HLA-B27 develops axSpA, indicating that the presence of HLA-B27 alone is not causative; actually the presence of HLA-B27 accounts for less than 50% of the genetic risk associated with the disease [17], suggesting the contribution of other genes.

Several other susceptibility genes for the development of axSpA were found by genome-wide association studies (GWAS) (Table 2), mainly including genes that are involved in immune responses, suggesting their potential role in the increase of autoimmune responses.

Gene	Function	Associated with			References
		axSpA	Psoriasis	IBD	
HLA-B60	MHC class I	Yes	..	..	[74]
HLA-DR1	MHC class II	Yes	..	..	[74]
IL-23R	Receptor of IL-23	Yes	Yes	Yes	[17;74-79]
STAT3	Signaling pathway of IL-23	Yes	Yes	Yes	[17;63;74-77;80;81]
ERAP1	MHC class I peptide loading	Yes	No	No	[17;74-77]
KIRs	NK cell activation	Yes	..	..	[17;67;82]
ANTXR2	Formation of capillaries	Yes	..	..	[17;74]
TNFSF15	Proliferation of T_H17 cells	Yes	..	Yes	[17;74]
RUNX3	CD8+ T cells maturation	Yes	Yes	Yes	[83]
IL-1	T_H1 and T_H17 differentiation	Yes	..	..	[17;84]

Table 2. Several additional genes that may contributes to axial spondyloarthritis pathogenesis.

HLA (Human Leukocyte Antigen), IL (Interleukin), STAT (Signal Transducer and Activator of Transcription), ERAP (Endoplasmic Reticulum Aminopeptidase), KIR (natural Killer cell Immunoglobulin-like Receptor), NK (Natural Killer), ANTXR (Anthrax Toxin Receptor), TNFSF (Tumor Necrosis Factor Superfamily), RUNX (Runt-related transcription factor)

The exact role of HLA-B27 in axSpA pathogenesis has not been clearly established yet, however, three major hypotheses have been proposed to explain its involvement.

1.2.1.1 Arthritogenic peptides

The MHC class I molecule HLA-B27 presents peptides to CD8+ T-cells. The formation of the complex between MHC class I and the peptides occurs in the endoplasmic reticulum (ER), in which peptides are trimmed by endoplasmic reticulum aminopeptidase 1 and 2 (ERAP1 and 2) and loaded on the MHC class I. The association between some single-nucleotide polymorphism (SNP) of ERAP1 and axSpA is known in literature [17;74-77] and it is restricted to HLA-B27 positive cases [83]. Therefore, aberrant peptide trimming and presentation might have a role in the development of axSpA.

The arthritogenic peptide hypothesis proposes the involvement of HLA-B27 in the presentation of peptides from microbiological agents that can induce immune responses that cross-react with self-antigen [85]. Autoantibodies occur in patients with axSpA that are able to react against protein involved in connective tissue matrix assembling, ossification and bone remodeling, such as glypican 3 and 4, chondromodulin 1, osteoglycin and osteonectin [86].
Moreover, proteoglycan (PG)-specific T-cells have been reported in AS patients [87] and the responses of cytotoxic T-cells against chondrocytes could be enhanced by elevated levels of the pro-inflammatory molecule interferon (IFN)-γ [88]. In AS patients, a higher amount of T-cells infiltration is found in histological sections of areas containing cartilage compared to the areas where the cartilage is almost absent, suggesting the ability of T-cells to react with the proteins of the cartilage matrix [89]. However, the finding that HLA-B27 transgenic rats, lacking CD8+ T-cells, still develop a SpA-like disease has posed questions on the validity of this hypothesis [90].

1.2.1.2 Free heavy chain homodimers

MHC class I molecules such as HLA-B27 are associated with β2-microglobulin (β2m) and are expressed on the cell surface for the interaction with CD8+ T-cells [91]. HLA-B27 shows a tendency to form disulfide-bounded heavy chain homodimers and this atypical form is not complexed to β2m [92]. In axSpA patients, both the atypical and the canonical form of HLA-B27 are expressed [92-96]. In this case, CD8+ T-cells are not activated, but the activation of natural killer (NK) cells and CD4+ T-cells through killer cell immunoglobulin-like receptors (KIRs) still occurs. KIRs activation may promote the survival of these cell populations leading to a perpetuation of the inflammatory response. In line with this hypothesis, atypical HLA-B27-expressing antigen presenting cells (APCs) have been shown to stimulate the proliferation and the survival of activated KIR3DL2+ CD4+ T-cells capable of IL-17 secretion [95]. Moreover, KIR3DL2+ NK cells and CD4+ T-cells have been shown to be expanded in the peripheral blood and synovial fluid of axSpA compared to healthy donors (HD) [82;95]. The atypical HLA-B27 molecules formed in ER that fail to reach the cell surface may also be involved in the ER stress. Therefore, atypical HLA-B27 molecules could promote the inflammation in axSpA patients through several pathways.

1.2.1.3 Misfolding

The misfolding hypothesis is related to the propensity of HLA-B27 to form intracellular homodimers within the ER [96], leading the accumulation of misfolded HLA-B27 molecules [97;98]. It has been reported that approximately 70% of newly synthetized HLA-B27 heavy chains are misfolded within the ER [97;98] and the HLA-B27 tendency to misfold is unique among MHC class I molecules [97].

The ER is able to degrade the misfolded proteins through endoplasmic-reticulum-associated protein degradation (ERAD) [97], but when the aggregation of misfolded proteins occurs, the degradation ability of ER is lost and ER stress pathways are activated. These pathways are collectively known as unfolded-protein response (UPR) [97]. The UPR activation has been linked to cytokine dysregulation, promoting lL-23, lFNβ, and lL-1α production, and lL-23/IL-17 axis activation [99-102]. In transgenic rats over-expressing HLA-B27, UPR activation is prominent [100;103]; moreover, it has been shown that overexpression of human β2m is able to reduce UPR as well as HLA-B27 misfolding, supporting the relationship between HLA-B27 misfolding and UPR activation. However, the overexpression of β2m, despite a reduction of UPR activation, exacerbates arthritis and spondylitis in this animal model [104]. The ER stress leads to an increased production of IL-23 by myeloid cells, through the activation of the receptor recognition patterns (RRPs) [102]; moreover translational studies with patient derived cells expressing HLA-B27 at physiologic levels have provided evidence that ER stress and UPR activation can occur in peripheral blood cells [105].
Contrasting data on the role of UPR on the promotion of IL-23 expression are present in the literature, and several reports have indicated the UPR hypothesis as unlikely. For example, Ciccia et al. demonstrated that HLA-B27 misfolding occurs in the gut of patients with axSpA, but it is linked to the activation of autophagy rather than to the induction of UPR [106]. Nonetheless, Neerinckx et al. did not find a higher IL-23 expression and autophagy activation in the synovium and PBMCs of HLA-B27 positive axSpA patients, suggesting that the production of IL-23, possibly driven by autophagy, could be a tissue-specific phenomenon [107;108].

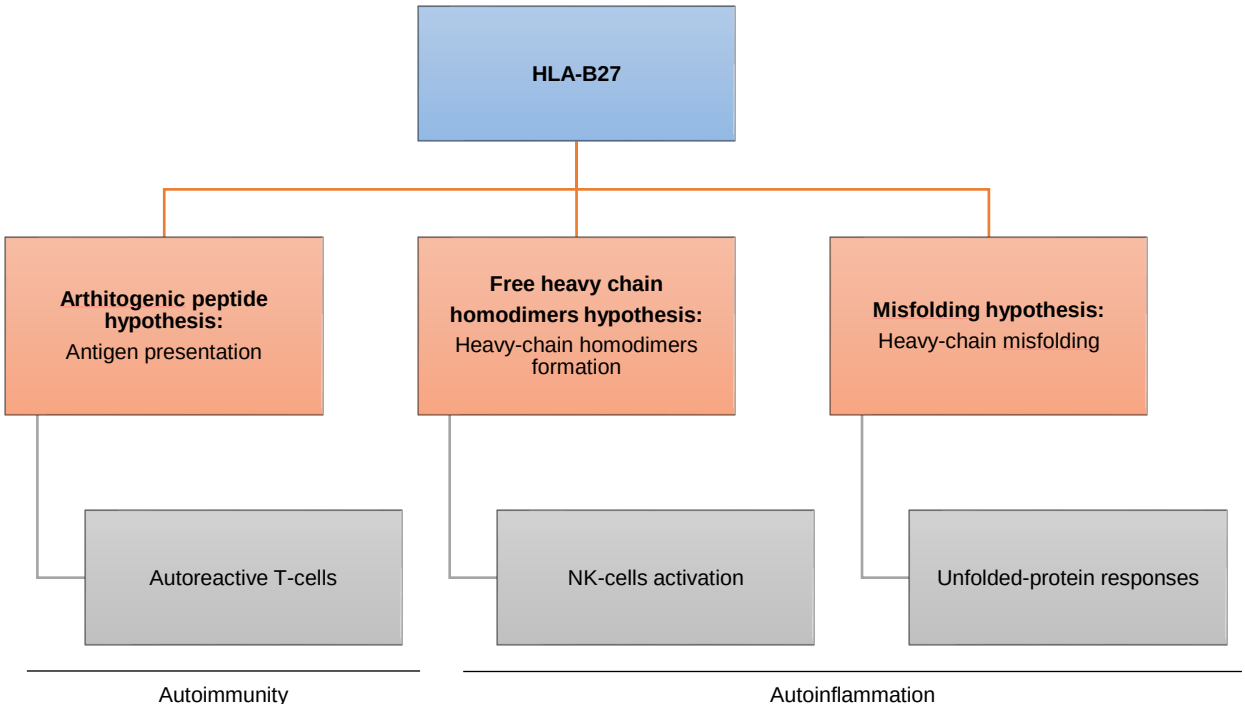


Figure 6. The three main hypotheses on the potential roles of HLA-B27 in triggering the pathogenesis of axial spondyloarthritis. Adapted from Dougados and Baeten, 2011 [109].

1.2.2 Microbial triggers

AxSpA is clinically, genetically and pathophysiologically related to barrier dysfunction diseases such as psoriasis and IBD [5], in which the damage of dermal and mucosal surfaces leads to an altered exposure of immune system cells to commensal and pathogenic microorganisms. In axSpA, the tight junctions between intestinal epithelial cells seems to be prone to an increased permeability [110;111] and this process could increase the foreign antigens presentation [8], triggering chronic inflammation.

In axSpA patients, the clinical association with bowel diseases is well established, considering that 5% of patients with axSpA develop IBD [12] and 70% might have subclinical bowel inflammation [112]. Supporting the role of intestinal inflammation in axSpA pathogenesis, HLA-B27 transgenic rats failed to exhibit gut and joint pathology in absence of intestinal flora, but, at the same time, skin inflammation was unaffected [113], providing evidence for the interplay between the intestinal environment, specifically the gut flora, and axSpA development. Furthermore, it has been showed in axSpA patients that over 10% of the gene pathways are shared between IBD and axSpA [114] and the severity of subclinical gut inflammation correlates with the involvement of sacroiliac joints [115].

In contrast to ReA, which is known to be trigged by the intestinal presence of several pathogens, as Salmonella, Shigella and Yersinia, the infectious trigger for axSpA has not been yet identified, although associations between infection and AS pathogenesis have been suggested. For example, 93% of inflamed AS patients were infected with Klebsiella-Enterobacter that show an immunological cross-reactivity with HLA-B27+ lymphocytes [116] and levels of anti-Klebsiella pneumoniae antibodies in serum and synovial fluid of AS patients were elevated compared to healthy controls and RA patients [117;118]. Actually several investigators have failed to establish an association between Klebsiella infections and axSpA pathogenesis [119], however other variations in gut microbiome, as the presence of Lachnospiraceae and Bacteroidaceae, and the decrease of Ruminococcaceae and Rikenellaceae have been described [120].

In conclusion, alterations in gut microbiome could induce an increased production of IL-23 that can interact with T-cells residing in the enthesis, causing local inflammation and bone remodeling through the release of IL-22 and IL-17 [60]. Nevertheless, the role of IL-23 in this whole context is still unclear.

1.2.3 Biomechanical stress

AxSpA mainly affects tissues that are exposed to considerable mechanical stress, such as sacroiliac joints, spine and entheses. The extended involvement of entheseal sites in axSpA led to the hypothesis that enthesitis is the key pathophysiological feature of the disease [121]. The high stress concentration in entheses increases the possibility of microdamage [122;123], which might results in a stimulus for the immune cells from synovium and bone marrow to interact with collagen and proteoglycans, the potential autoantigens present in cartilage and entheses [124;125]. In comparison with mechanical-induced enthesitis, bone marrow edema, which implies inflammation, is more severe in HLA-B27 positive patients suggesting that the presence of HLA-B27 aggravates the inflammation induced by the initial mechanical stress [126].

There is a strong evidence that the disease has site specific localization in the skeleton of axSpA patients dependent on bone stressing [127] and that the target sites of early axSpA exhibit MRI patterns of bone edema, linked to skeletal stressing, similar to normal subjects [121]. Therefore, tissue damage and repair processes occur in normal subject exactly in the same anatomical sites of axSpA patients. However, axSpA patients are characterized by new bone formation, a process with underlying mechanisms not yet fully understood, even if the downstream effect of IL-23 signaling on IL-22 is known to drive skeletal stem cells [128]. Therefore, the peri-fibrocartilageneous bone and the entheses represent the primary sites and tissue targets where innate and adaptive reactions occur initially as a repair process, but in later phases can result in remodeling effects including bone edema, osteitis and new bone formation that may culminate in the ankylosis of the spine [129].

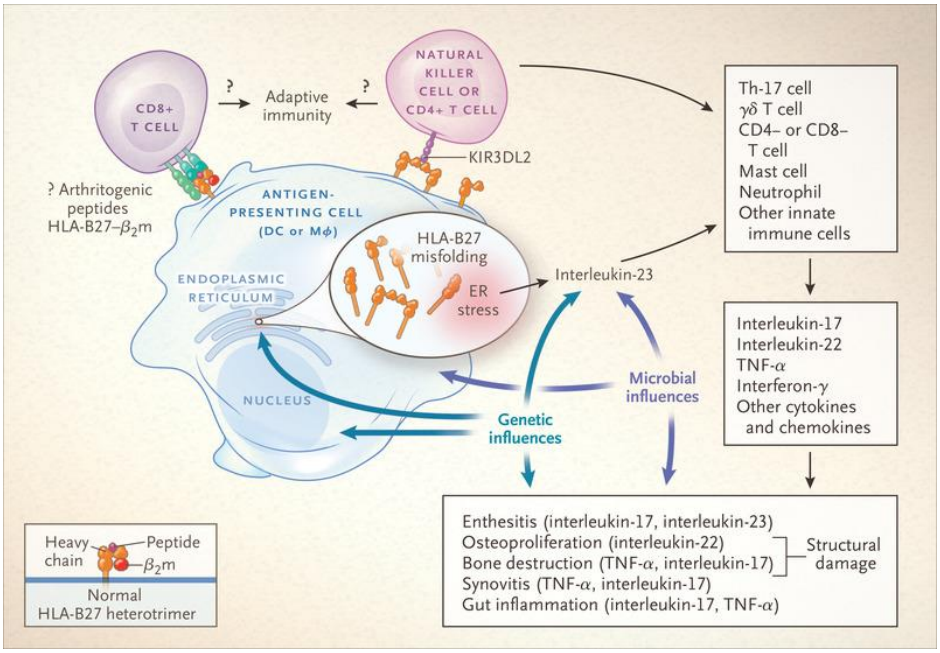


Figure 7. Pathogenic mechanisms in axial spondyloarthritis. Adapted from Taurog et al., 2016 [130].

1.3 The adaptive immune response in axial spondyloarthritis

The immune system orchestrates the defense mechanisms against pathogenic organisms, through the mobilization of effector immune cells. The immune response is mediated by the early reactions of the innate immunity and the later responses of adaptive immunity. The innate immune system provides the early line of defense by cellular and biochemical defense mechanisms that are in place even before the infection occurs, leading to a rapid response. The innate immunity could eradicate potentially harmful antigens by secreting molecules that initiate inflammation and cell recruitment. The cells that participate to the innate immunity are NK cells, mast cells, eosinophils, basophils, and phagocytic cells, such as monocytes, macrophages, neutrophils and dendritic cells.
In contrast, the adaptive immune response comprises T-cells and B-cells, stimulated by the exposure to antigens, and it is essential for the generation of long-lived immunological memory. The defining characteristics of the adaptive immunity are the specificity for distinct molecules and the ability to remember the antigens, in order to have a more vigorous response after a repeated exposure. Failure in the regulation of innate and adaptive responses could results in the development of chronic inflammation and autoimmune or autoinflammatory diseases.
Autoimmune diseases are characterized by the presence of serum autoantibodies, detected before the development of clinical manifestations, and by B- and T-cell selection defects that lead to aberrant responses to autoantigens [131]. Conversely, autoinflammatory diseases have no detectable autoantibodies and are characterized by a strong activation of the innate immune system [132]. Several rheumatic diseases including axSpA do not show a full overlap with the common features of autoimmune or autoinflammatory disease [133]. Indeed, axSpA is not considered to be typically associated with autoantibodies, but their presence has been described, though their pathogenicity has not been established yet [134]. On the basis of clinical features, axSpA was suggested to be a mixed pattern-disease [135], with the immune system having a fundamental role in its pathogenesis.

1.3.1 T-cells subpopulations

T-cells mediate the cellular immunity in adaptive immune responses. T-cells consist of functionally distinct populations that coordinate the defense mechanisms required to eradicate an infection. After antigen encountering, T-cells can secrete immunomodulatory molecules or induce the lyses of infected cells. Moreover, among T-cells, regulatory T-cells (Treg) modulate the immune response, by inhibiting aberrant and damaging immune responses against self-antigen or commensal bacteria. T-cells are characterized by the expression of CD3 molecule and they can be divided in two subpopulations depending on the expression of CD4 or CD8 molecule.

During antigen processing, protein antigens in the cytosol or internalized from the extracellular environment are converted into peptides by APCs cells and loaded onto MHC molecules for the antigen presentation to T-cells. The antigen receptor of MHC-restricted CD4+ and CD8+ T-cells is a heterodimer consisting of two transmembrane polypeptide chains covalently linked to each other by a disulfide bridge between extracellular cysteine residues, called T-cell receptor (TcR).

Depending on the type of subunits that compose the TcR, T-cells can be classified as αβ or γδ. The majority of T-cells express TcRαβ. The variable region of TcR specifically recognizes and binds a restricted set of peptide-MHC complexes.

During their development, T-cells, that express their own unique TcR, are selected on their capability to bind MHC-peptide complexes in the thymus. Once selected, CD4+ T-cells interact with MHC class II molecules, whilst CD8+ T-cells interact with MHC class I expressing cells. Antigen-inexperienced T-cells are known as naïve T-cells (T_N), characterized by the expression of CD45RA, CCR7 and CD62L and the lacking of CD45RO. Due to the expression of CD62L, they reside and recirculate throughout secondary peripheral lymphoid organs, until they are activated by APC expressing the correct MHC-peptide complex. Following the activation, CD4+ and CD8+ T-cells differentiate into effector populations that participate in primary and secondary immune responses. The induction of the adaptive immune response is central in the development of immunological memory. Memory cells may survive in a functional quiescent or slowly cycling state without stimulation by an antigen. The presence of these memory T-cells permits rapid, specific and improved adaptive responses towards previously encountered antigens. In humans, memory cells are identified by their expression of low levels of CD45RA and CD25, and high expression of CD45RO.

Functionally distinct memory T-cells can be differentiated based on their homing properties and functions:

- Central memory T-cells (T_{CM}), that express the chemokine receptor CCR7 and CD62L, enabling peripheral lymph node homing. They have a limited capacity to perform effector function when they encounter the antigen, but they can proliferate and generate many effector cells;
- Effector memory T-cells (T_{EM}), that are negative for CCR7 and CD62L expression, permitting their migration to peripheral sites. After antigenic stimulation, T_{EM} produce effector cytokines or become cytotoxic, but, compared to T_{CM} have a lower proliferative potential. The expression of other surface molecules promote their migration into sites of infection anywhere in the body;
- Effector memory T-cells RA (T_{EMRA}), that are terminally differentiated T_{EM} re-expressing CD45RA, but that lacks both CCR7 and CD62L [136;137]. These effector cells appear in the late phase of the immune responses and are more represented in the CD8+ compartment.

Therefore, the effector subset can rapidly respond to a repeated exposure to an antigen, but the complete eradication of the infection requires a large number of T_{EM} generated from T_{CM}.
Within the inflamed joints of axSpA patients, new blood vessel formation promotes the infiltration of inflammatory immune cells [138]. The populations associated with entheseal inflammation have been described: macrophages appear to dominate, but also infiltrating lymphocytes were observed [59]. T-cells that infiltrate at sites of entheses are mostly represented by CD8+ T-cells [139].
CD3+ lymphocytic aggregates, including both increased interstitial CD4+, and CD8+ lymphocytes, and B-cells, have been also observed in facet joints obtained from AS patients suffering of ankyloses of the lumbar spine [140]. The presence of macrophages and T-cells as infiltrating cells indicates that both branches of the immune system, innate and adaptive, are involved in the pathogenesis of axSpA [89].

1.3.2 CD4+ T-cells

After the antigenic stimulation, activated CD4+ T helper cells (T_H) proliferate and differentiate into effector cells whose functions are mediated largely by the secretion of immunomodulatory molecules known as cytokines, which are responsible for many of the cellular responses of innate and adaptive immunity and function as the messenger molecules of the immune system. The cytokines secreted by T_H stimulate proliferation and differentiation of T-cells themselves and activate other cells populations, such as B-cells and macrophages, driving activation of surrounding cells and cell recruitment. The nature of the cells that are recruited and activated is determined by the subset of CD4+ T-cells that are induced in the immune response. In general, T_H1 cells produce IFN-γ and are able to activate dendritic cells, macrophages and NK cells, T_H2 produce IL-4, IL-5 and IL-13 and activate eosinophils, T_H17 produce IL-17 and IL-22 and activate primarily neutrophils. T_H17 cells can switch to T_H1^* cells (or ex-T_H17 or T_H1 non classical), that lose the ability to produce IL-17 and acquire the ability to produce IFN-γ [141;142].
The cytokines produced by these cells determine their effector functions and roles in diseases. These different subsets of T_H have distinct homing phenotypes that direct them to migrate into different sites of inflammation. In fact, T_H1 express CCR5 and CXCR3, which can bind chemokines produced in tissues during innate immune responses. In contrast, T_H2 express CCR3, CCR4 and CCR8, which recognize chemokines that are highly expressed at sites of helminth infection or allergic reactions, particularly in mucosal tissue. T_H17 express CCR4 and CCR6, which binds the chemokine CCL20, a chemokine produced in various tissues following bacterial and fungal infections. After the switching of T_H17 in T_H1^*, the cells maintain the expression of CCR6 and gain the expression of CXCR3 [141;142].

The percentage of CD4+ T-cells subsets and their roles in the pathogenesis of axSpA is still subject of debate. Differences in percentage of T_H1 and T_H1/T_H2 ratio in HD and axSpA patients are not solidly established, but an imbalance in T_H1/T_H2 ratio could give rise to IFN-γ enhancement, which may cause inflammation in axSpA [143]. CCR4 is the chemokine receptor expressed by T_H2 cells and an increase in the percentage of CD4+CCR4+ T-cells in axSpA correlating with the disease score has been demonstrated [144].

The expression of CCR4 leads to the migration of T_H2 cells in response to macrophage-derived chemokine (MDC/CCL22) and thymus and activation-regulated chemokine (TARC/CCL17).

These two chemokines have been detected in a higher amount in serum of axSpA patients compared to HD [145], indicating the potential role of T_H2 in axSpA pathogenesis. Recent findings show that the IL-23/IL-17 pathway has an important role in axSpA pathogenesis, and several studies show that IL-17, IL-23 and the percentage of T_H17 are increased in axSpA patients [146-148]. Targeting T_H17 responses is a promising approach for the treatment of axSpA, and monoclonal antibodies against IL-17, such as secukinumab, have shown an effective outcome to the treatment of axSpA [149-151]. Recently IL-22 has been shown to be involved in human mesenchymal stem cells (MSC) osteogenesis [128], giving rise to the hypothesis of a role for T_H17 in the new bone formation, a typical feature of axSpA.

1.3.3 CD8+ T-cells

Activated CD8+ cytotoxic T-lymphocytes (CTLs) are able to kill cells after MHC-peptide complex recognition by secreting granules that can lyse cells in a contact dependent manner. To be efficiently recognized by CTLs, target cells must express MHC class I complexed to a peptide and the intracellular adhesion molecule 1 (ICAM-1). The interaction between MHC-peptide complex and CTL-expressed TCR results in the initiation of biochemical signals that activate the CTLs. The principal mechanism of CTL-mediated target cell killing is the delivery of cytotoxic molecules stored in cytoplasmic granules to the target cell by exocytosis. The cytotoxic granules of CTLs include granzyme (GRZ) A, B, and C and perforin (PRF). GRZs are serine proteases that cleave proteins after aspartate residues and PRF is a membrane-perturbing molecule homologous to the C9 complement protein. The main function of PRF is to facilitate delivery of the GRZs into the cytosol of the target cell. Once in the cytoplasm, GRZs cleave various substrates, including caspases, thus inducing the apoptosis of the cells. CTLs can induce cell death also by using a granule-independent mechanism mediated by the interaction between Fas ligand (FasL), expressed by CD8+ T-cells, and the death receptor Fas, expressed by the target cells. This interaction results in an activation of caspases and, consequently, in the apoptosis of the target cell.

Similarly to CD4+ T-helper cells, CD8+ cytotoxic T-cells (T_C) can be classified in distinct subpopulations, characterized by specific homing markers and expressing different cytokines. T_C1 cells are defined as CD8+ T-cells that preferentially express CXCR3 and secrete IFN-γ, but not IL-4 or IL-5; T_C2 preferentially express CCR4 and secrete IL-4, IL-5 and IL-13, but not IFN-γ, and T_C17 mainly express CCR6 and are able to secrete IL-17 and IL-22 [152]. In HD T_C2 do not have a cytotoxic profile [153;154] and could have a protective role in autoimmune or autoinflammatory diseases through the production of IL-4, IL-5 and IL-10. Although CTLs can typically target viral infected or cancerous cells, they can also attack self-antigens and cause organ-specific autoimmune or autoinflammatory diseases. For example, it has been shown that peptide derived from type II and IV collagen in cartilage could stimulate CD8+ T-cells in axSpA patients, leading to cartilage destruction [155].

In an inflammatory milieu, chondrocytes can act as APC presenting cartilage antigens to CD8+ T-cells and it has been shown that, following the enhancement of IFN-γ in the inflamed joints, MHC-I was upregulated on the surface of chondrocytes that become more susceptible to CTLs [88]. The production of inflammatory cytokines, such as TNF-α, IFN-γ and IL-17 by the different subtypes of CD8+ T-cells can support the chronic immune responses in axSpA patients.

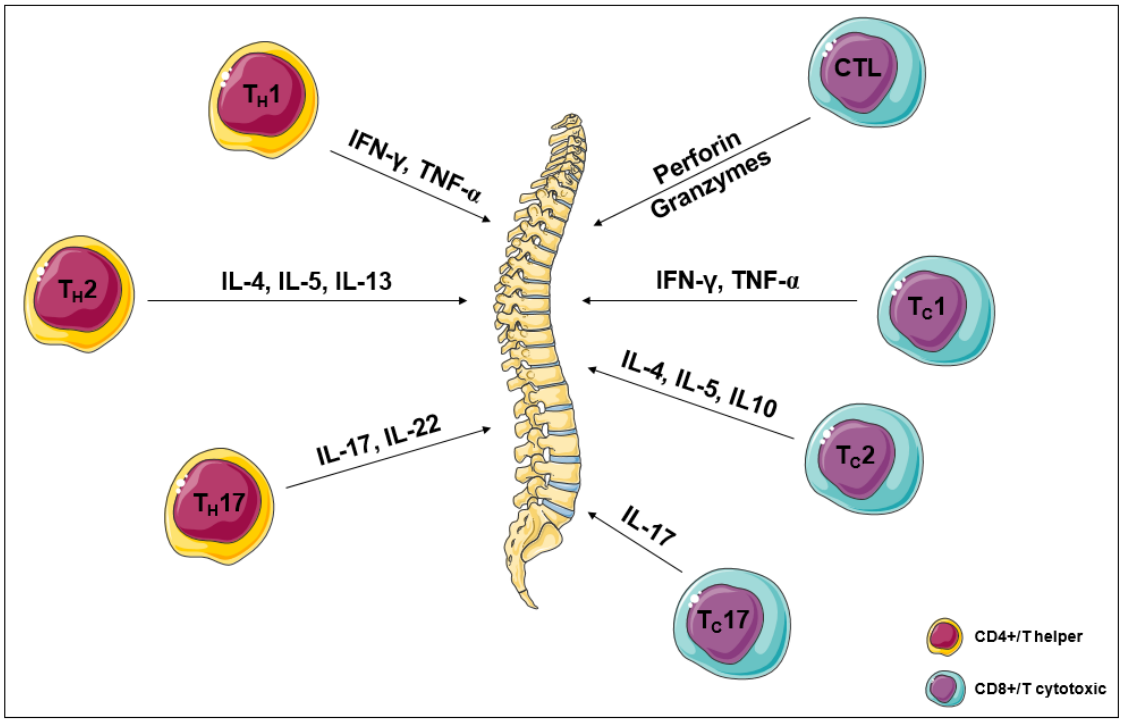


Figure 8. T-cells involved in the initiation, progression and regulation of axial spondyloarthritis.

T_H (T helper), T_C (T cytotoxic), IFN (Interferon), TNF (Tumor Necrosis Factor), IL (Interleukin)

1.3.4 Cytokines and chemokines

Cytokines are a large and heterogeneous group of secreted proteins produced by many types of cells that mediate innate and adaptive immune responses. Cytokines are not usually stored and after cellular activation, they are synthetized and rapidly secreted in a burst of release when needed. One cytokine can act on diverse cell types and have multiple biological effects; conversely, multiple cytokines may have the same action. In addition, one cytokine can stimulate or inhibit the production of others, and it can antagonize one another or produce additive or synergic effects.

Most cytokines act close to where they are produced, but when cells secrete a large amount of cytokines they may enter in the circulation and act at a distance from the site of production.

Several cytokines, both with pro-inflammatory and anti-inflammatory properties, have been found at high concentrations in the sera of axSpA patients, indicating how the immune-system actively tries to control a persistent inflammation.

Among others, TNF-α and IL-8 have been found to be elevated in the serum of axSpA patients [156-158]. TNF-α axis, involved in the acute phase of systemic inflammation, is a key inflammatory pathway in axSpA, as evidenced by the clinical efficacy of TNF inhibitors. However, the understanding of the role of this pathway in the disease is fairly limited. T_H1 and T_C1 cells are able to produce TNF-α and IFN-γ contributing to the disease as discussed above. Sera levels of IL-8, a pro-inflammatory cytokine able to induce the migration of target cells in the sites of infection and to stimulate phagocytosis, have been shown to be strongly correlated with ASDAS in axSpA patients [159]. Other cytokines associated with the disease include those associated with the T_C17 and T_H17 phenotypes, such as IL-17 and IL-22, as discussed above. The cytokines produced by T_H2 and T_C2, such as IL-4, IL-5, IL-13 and IL-10, are classified as anti-inflammatory cytokines. Unlike RA, which is well characterized by pro-inflammatory cytokines secretion, an increase in anti-inflammatory cytokines in patients' serum has been reported in axSpA [160;161].

In the group of cytokines, we can distinguish chemokines, a large family of structural homologous cytokines that stimulate leukocyte movement and regulate their migration (chemotaxis) from the blood to the tissues. The receptors for chemokines belong to the seven-transmembrane, guanoside triphosphate (GTP)-binding (G) protein-coupled receptors (GPCR) superfamily. The G proteins stimulate cytoskeletal changes and polymerization of actin and myosin filaments, resulting in increased cell motility.

These signals change the conformation of cell surface integrins and increase the affinity of the integrins for their receptors. Chemokine receptors may be rapidly downregulated by exposure to the chemokine, a mechanism that leads to the termination of the response. Different combination of chemokine receptors are expressed on different type of leukocytes, which results in a distinct pattern of migration. Chemokines are essential for the recruitment of circulating leukocytes from blood vessels into extravascular sites, towards their chemical gradient.

Chemokines can be classified as either homeostatic or inflammatory. Homeostatic chemokines, such as CCL19 and CCL21, are expressed in secondary lymphoid organs (SLOs) or in peripheral tissues, where they organize lymphoid structures in order to facilitate the adaptive immune response and recruit immune cell populations in peripheral tissues to maintain the homeostasis. In contrast, inflammatory cytokines, such as CCL5 and CCL3, allows the migration of cells towards sites of inflammation, driving their participation in the immune response. For these reasons, the chemokine receptors profile of immune cell populations influences migration patterns, cell phenotype and consequent functions.

Receptor	Chemokine
CCR1	CCL3, CCL5, CCL7, CCL10, CCL14, CCL15, CCL16, CCL23
CCR2	CCL2, CCL7, CCL12, CCL13, CCL16
CCR3	CCL15, CCL5, CCL7, CCL11, CCL13, CCL8, CCL24, CCL26, CCL2, CCL28, CXCL9, CXCL10, CXCL11
CCR4	CCL17, CCL22
CCR5	CCL3, CCL4, CCL5, CCL8, CCL14
CCR6	CCL20
CCR7	CCL19, CCL21
CCR8	CCL1
CCR9	CCL25
CXCR1	CXCL6, CXCL8, CXCL1
CXCR2	CXCL1, CXCL6, CXCL8, CXCL2, CXCL3, CXCL5, CXCL7
CXCR3	CXCL9, CXCL10, CXCL11
CXCR4	CXCL12
CXCR5	CXCL13
CX_3CR1	CX_3CL1

Table 3. Chemokine receptors expressed by T-cells.

SLOs (Secondary Lymphoid Organs), BM (Bone Marrow)

1.4 Hypothesis and aims

The immune system plays a fundamental role in the pathogenesis of axSpA and the inflammatory infiltrate in the structural lesions contributes to tissue damage through the release of inflammatory cytokines and mediators of bone remodeling. Specifically, T-cells have been shown to exert pro-osteogenic effects on osteoblasts precursors, providing evidence of the contribution of T-cells to catabolic processes and to anabolic responses of the bone [162-164].

Despite a relative abundance of information about inflammatory tissue infiltrate, consensus relating the functional contributions of the immune populations to axSpA pathogenesis has not yet been reached.

Alterations in the frequency of circulating T-cell subpopulations in patients with axSpA have been reported, suggesting a systemic imbalance in CD4+ T cell subsets that favors cells of the T_H17 lineage [146-148]. Little is known about the role of CD8+ subsets in the pathogenesis of axSpA, but there is evidence of an involvement of CD8+ T-cells in cartilage destruction [88;155]. Whether these changes are due to an impaired trafficking to tissues or to an uncontrolled expansion of specific leukocytes subsets still needs to be determined.

Taking advantage of the chemokine receptors profile of immune cell populations, which influences their migration patterns, cell phenotype and subsequent function, aim of this work is to identify cellular populations involved in disease development and perpetuation.

In particular, we have characterized circulating T-cells that could migrate to articular and extra-articular sites of inflammation. Moreover, we performed a thorough characterization of CD8+ T-cells expressing CCR4, a chemokine receptor that leads to the migration of these cells to the skin and to the joint, two districts known to be affected by axSpA.

Chapter 2: Materials and methods

2.1 Patients recruitment and ethical approval

The study was approved by the Ethical Committees of the Canton Ticino (license number: CE-3065) and of the Canton Zurich (license number: EK515). Informed consent from each subject was obtained before enrolment into the study, and all samples were rendered anonymous.

A total of 65 axSpA patients 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 study enrolment. Clinical and demographic information of each patient at the time of study enrolment was provided by the clinicians.

AxSpA patients included 52 r-SpA, fulfilling the modified New York 1984 [165] criteria, and 13 nr-axSpA classified according to fulfilment of the ASAS classification 2009 criteria [2;3].

For all axSpA patients the activity of the disease was assessed by the Ankylosing Spondylitis Disease Activity Score (ASDAS) [19;20].

Demographic and clinical characteristics of axSpA patients enrolled in the study are shown in Table 4.

Patients characteristics	**Total**	**Female**	**Male**
Gender *(n)*	65	21	44
Age *(mean ± SD)*	42.2 ± 10.4	44.5 ± 10.5	41.2 ± 10.2
HLA-B27 positive % *(n)*	86%	62%	98%
Radiographic axSpA % *(n)*	80%	71%	84%
IBD % *(n)*	5%	5%	4%
Psoriasis % *(n)*	3%	5%	2%
Uveitis % *(n)*	29%	19%	34%
TNFi therapy % *(n)*	55%	38%	64%
ASDAS *(mean ± SD)*	1.8 ± 0.9	2.0 ± 0.9	1.7 ± 0.9
ASDAS *(range)*	0.2 - 4.1	0.6 - 3.8	0.2 - 4.1

Table 4. Demographic and clinical features of axial spondyloarthritis patients included in the study.

SD (Standard Deviation), ASDAS (Ankylosing Spondylitis Disease Activity)

As controls, 11 RA patients and 40 healthy individuals were enrolled in the study. For the HD blood leukocytes were obtained from buffy coats samples received from the Central Laboratory of Swiss Red Cross (Basel, CH), and derived from spontaneous donations.

RA represents the paradigm of rheumatic autoimmune diseases [166], while axSpA is considered a “mixed-pattern disease”, ranked in between autoimmune and autoinflammatory diseases [133].

RA is characterized by symmetric and erosive arthritis typically affecting small joints [167], while axSpA affects principally the sacroiliac joints and the spine, and can also affect peripheral joints and entheses [57]. In RA, bone involvement is characterized by erosions, whereas in axSpA bone involvement also includes signs of new bone formation [167-169]. axSpA patients predominantly show anabolic damage that leads to the development of syndesmophytes in the spine.

We decided to use RA patients as controls, in addition to HD, in order to understand if the results obtained in axSpA patients are typical of this disease or are present also in other rheumatic diseases. All RA patients fulfilled the American College of Rheumatology 1987 revised criteria for the classification of RA [170], and disease activity was assessed by using the 28-joint Disease Activity Score (DAS28) [171] on the day of peripheral blood collection.

Demographic and clinical characteristics of RA patients enrolled in the study are shown in Table 5.

Patients characteristics	**Total**	**Female**	**Male**
Gender *(n)*	11	10	1
Age *(mean ± SD)*	55.2 ± 11.4	55.2 ± 12.0	55
DAS28 *(mean ± SD)*	3.8 ± 1.3	3.9 ± 1.5	3.9
DMARDs and biologics % (n)	82%	80%	100%
NSAIDs % (n)	9%	10%	0%

Table 5. Demographic and clinical features of rheumatoid arthritis patients included in the study.

SD (Standard Deviation), DAS28 (Disease Activity Score 28), DMARDs (Disease modifying antirheumatic drugs), NSAIDs (Nonsteroidal anti-inflammatory drugs)

2.2 Blood collection and cell isolation

Sixteen milliliters of whole blood from each patient were collected in BD Vacutainer® CPT™ Cell preparation tubes containing sodium citrate as anticoagulant (362782, BD Biosciences, San Jose, CA). Blood from HD was provided as buffy-coats.

Serum was collected, aliquoted and stored at -80°C until usage.

Targeted Human Antigen	Fluorochrome	Clone	Isotype	Source
CCR4	PE-Cy7	L291H4	Mouse IgG1	BioLegend
CCR5	FITC	45523	Mouse IgG2b	R&D Systems
CCR6	PE	11A9	Mouse IgG1	BD Biosciences
CCR7	PE-Cy7	G043H7	Mouse IgG2a	Biolegend
CCR7	Brilliant Violet 421	G043H7	Mouse IgG2a	BioLegend
CCR9	APC	112509	Mouse IgG2a	R&D Systems
CD27	V500	M-T271	Mouse IgG1	BD Biosciences
CD28	PE	CD28.2	Mouse IgG1	BioLegend
CD3	APC-Cy7	SP34-2	Mouse IgG1	BD Biosciences
CD38	Brilliant Violet 421	HB-7	Mouse IgG1	BioLegend
CD4	PerCP-Cy5.5	L200	Mouse IgG1	BD Biosciences
CD45RA	FITC	ALB11	Mouse IgG1	Beckman Coulter
CD8	APC	B9.11	Mouse IgG1	Beckman Coulter
CD8	BV786	RPA-T8	Mouse IgG1	BD Biosciences
CD8a	Pacific Blue	HIT8a	Mouse IgG1	BioLegend
CLA1	Brilliant Violet 421	HECA-452	Rat IgM	BD Biosciences
CX_3CR1	PE	2A9-1	Rat IgG2b	BioLegend
CXCR3	Alexa Fluor 488	1C6/CXCR3	Mouse IgG1	BD Biosciences
CXCR3	PE	1C6/CXCR3	Mouse IgG1	BD Biosciences
Granzyme B*	Pacific Blue	GB11	Mouse IgG1	BioLegend
HLA-B27	APC	REA176	Recombinant Human IgG1	Miltenyi Biotec
HLA-DR	V500	G46-6	Mouse IgG2a	BD Biosciences
IL-2*	PerCP-eFluor 710	MQ1-17H12	Rat IgG2a	eBioscience
Integrin β7	PE	FIB504	Rat IgG2a	BioLegend
Ki-67*	Alexa Fluor 488	Ki-67	Mouse IgG1	BioLegend
PD-1	Brilliant Violet 785	EH12.2H7	Mouse IgG1	BioLegend
Perforin*	APC	B-D48	Mouse IgG1	BioLegend

*Ab for intracellular staining

Table 6. Antibodies used for cytofluorimetric analysis.

Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood by using Ficoll-Hypaque density centrifugation within 24h from blood withdrawal. CD8+ and CD4+ T-cell enrichment from total PBMCs was performed either using positive immunoselection procedure (130-045-201, CD8 and 130-045-101, CD4 MicroBeads, Miltenyi Biotec, Germany) according to the manufacturer's instructions, or through cell sorting (ref. paragraph 2.3.2).

2.3 Flow cytometric analysis

The different panels of antibodies (Abs), used to stain specific markers in the present study are represented in table 7.

Panel	APC-Cy7	PerCP-Cy5.5	Pacific Blue	APC BV786	BV785 BV786	FITC AF488	PE-Cy7	PE	V500 AF430
A	CD3	CD4	CD38	HLA-B27		Ki67	CCR4	CCR6	HLA-DR
B	CD3	CD4	CD8			CXCR3	CCR4	CCR6	
C	CD3	CD4	CD8	CCR9		CD45RA	CCR7	CXCR3	
D	CD3	CD4	CD8			CD45RA	CCR7	CCR6	
E	CD3	CD4	CLA1	CD8			CCR4	Integrin-B7	
F	CD3	CD4	CCR7	CD8	PD-1	CD45RA	CCR4	CD28	CD27
G	CD3	CD4	CCR7	CD8	PD-1	CD45RA	CCR4	CX_3CR1	CD27
H	CD3	CD4		CD8		CCR5	CCR4		
I	CD3	CD4	CD8				CCR4		
L		CD4	CD8				CCR4		
M		CD4			CD8		CCR4	CX_3CR1	
N		IL-2	PRF	GRZB	CD8		CCR4	CX_3CR1	

Table 7. Panels of antibodies used for cytofluorimetric analysis.

The samples were acquired with BD LSRFortessa (BD Biosciences, San Jose, CA. USA), and the results were analyzed with FlowJo software (Tree Star). Relative mean fluorescence intensity (rMFI) was calculated as the ratio between stained and unstained samples.

2.3.1 Surface staining

For surface staining (Panels A-I), total PBMCs were incubated for 30 min at 4°C with the appropriate combination of Abs (Table 7) in PBS supplemented with 1% FBS. After incubation, unbound antibodies were removed by washing the cells with PBS supplemented with 1% FBS, and samples were acquired immediately or fixed in 1% PFA for later acquisition.

2.3.2 Cell sorting

After surface staining (Panel L or M), CD8+CCR4+ and CD8+CCR4- T-cell subsets were isolated using cell sorter BD FACSAria III (BD Biosciences, San Jose, CA, USA). As for HD, cell sorting was performed on CD8+ T-cells enriched by positive immune selection. For axSpA patients, in order to minimize the loss of cells, cell sorting was performed starting from total PBMCs.

2.3.3 Intracellular staining

After surface staining, cells were fixed and permeabilized with Cytofix/Cytoperm kit (554714, BD Biosciences, San Jose, CA, USA). Fixation and permeabilization were performed for 20 min at 4°C in Cytofix/Cytoperm Buffer and intracellular staining was performed by incubating fixed cells for 30min at 4°C in 1x Perm/Wash Buffer, using the appropriate combination of Abs specific for Ki67, Perforin, Granzyme B and IL-2. After incubation, unbound antibodies were removed by washing the samples with 1x Perm/Wash Buffer.

For intracellular detection of Perforin, Granzyme B and IL-2 (Panel N), sorted CD8+CCR4+ and CD8+CCR4- T-cells were incubated in U-bottomed 96-well plates for 5h at 37°C in a 5% CO_2 humidified atmosphere in RPMI-1640 (42401-018, Life Technologies Corporation, Carlsbad, CA, USA) supplemented with 10% Fetal Bovine Serum (FBS) (10270-106, Life Technologies Corporation, Carlsbad, CA, USA), 1% Penicillin Streptomycin (Pen Strep) (15070-063, Life Technologies Corporation, Carlsbad, CA, USA) and 1% GlutaMAX-I (35050-038, Gibco™, Dublin, Ireland) (R10 Medium) and stimulated with 50ng/mL Phorbol 12-myristate 13-acetate (PMA) (P1585, Sigma, Saint Louis, MO, USA) and 1µg/mL Ionomycin (I0634, Sigma, Saint Louis, MO, USA). After 2.5h, Brefeldin A 10µg/mL (B5936, Sigma, Saint Louis, MO, USA) was added to the wells to inhibit intracellular protein transport enhancing the detection of intracellular cytokines.

2.4 Cytokine and chemokine detection

CD8+CCR4+ T-cells were incubated for 6h at 37°C in a 5% CO_2 humidified atmosphere at a density of $1x10^6$ cell/mL in R10 Medium with or without 50 ng/mL PMA and 1 µg/mL Ionomycin.

Supernatants were collected in order to determine the concentration of released cytokines. Quantification of IL-6, IL-8, IL-10, IL-12, IL-1β and TNF in serum and supernatants was determined by using Cytometric Bead Array (CBA) - Human Inflammatory Cytokines Kit (551811, BD Biosciences, San Jose, CA, USA), that allows the determination of the indicated human cytokines simultaneously. Acquisition was perform with FACSCanto II (BD Biosciences, San Jose, CA), and the concentration was calculated from the MFI according to a standard curve of each cytokine.

2.5 RNA sequencing

2.5.1 RNA isolation and purification

After sorting, CD8+CCR4+ T-cells from HD and axSpA patients were stored in TRIzol Reagent (15596026, Life Technologies Corporation, Carlsbad, CA, USA) at -80°C until RNA extraction was performed. Total RNA was extracted with Zymo-Spin IC Columns (C1004-50, Zymo Research, Tustin, CA, USA) and Direct-zol RNA MiniPrep Kit (R2050, Zymo Research, Tustin, CA, USA) according to the manufacturer's instructions.

2.5.2 Library preparation

The quality of the isolated RNA was determined with a Qubit (1.0) Fluorometer (Life Technologies, Carlsbad CA, USA) and a Fragment Analyzer (Agilent, Santa Clara, CA, USA). Only those samples with a 260nm/280nm ratio between 1.8–2.1 and a 28S/18S ratio within 1.5-2 were further processed. TruSeq Stranded mRNA (Illumina, Inc., San Diego, CA, USA) was used in the succeeding steps. Briefly, total RNA samples (100-1000 ng) were polyA enriched and then reverse-transcribed into double-stranded cDNA. The cDNA samples were fragmented, end-repaired and adenylated before ligation of TruSeq adapters containing unique dual indices (UDI) for multiplexing. Fragments containing TruSeq adapters on both ends were selectively enriched with PCR. The quality and quantity of the enriched libraries were validated using Qubit (1.0) Fluorometer and the Fragment Analyzer (Agilent, Santa Clara, CA, USA).
The product is a smear with an average fragment size of approximately 260 bp. The libraries were normalized to 10 nM in Tris-Cl 10 mM, pH 8.5 with 0.1% Tween 20.

2.5.3 Cluster generation and sequencing

Novaseq 6000 (Illumina, Inc, San Diego, California, USA) was used for cluster generation and sequencing according to standard protocol. Sequencing was paired end at 2 X150 bp or single end 100 bp.

2.5.4 Bioinformatics analysis

RNA sequencing reads were aligned with the STAR-aligner [172]. The Ensembl human genome build GRCh38 was used as reference. Gene expression values were computed with the function featureCounts from the R package Rsubread [173]. Differential expression was computed using the generalized linear model implemented in the Bioconductor package DESeq2 [174].

2.6 Statistical analysis

Data were analyzed using Prism version 7.0 software (GraphPad, La Jolla, CA, USA) and presented as mean ± SEM. The statistical significance between two groups was determined using a non-parametric two-tailed Mann–Whitney U test or a paired t test. The statistical significance between more than two groups was evaluated using a Kruskal-Wallis test followed by Dunnett's multiple comparison adjustment. Correlation analysis between two groups of values was performed using the Spearman's correlation rank test, determining r- and p-value. Values were considered statistically significant when probability (P) values were equal or below 0.05 (*), 0.01 (**), 0.001 (***), 0.0001 (****).

Chapter 3: Results

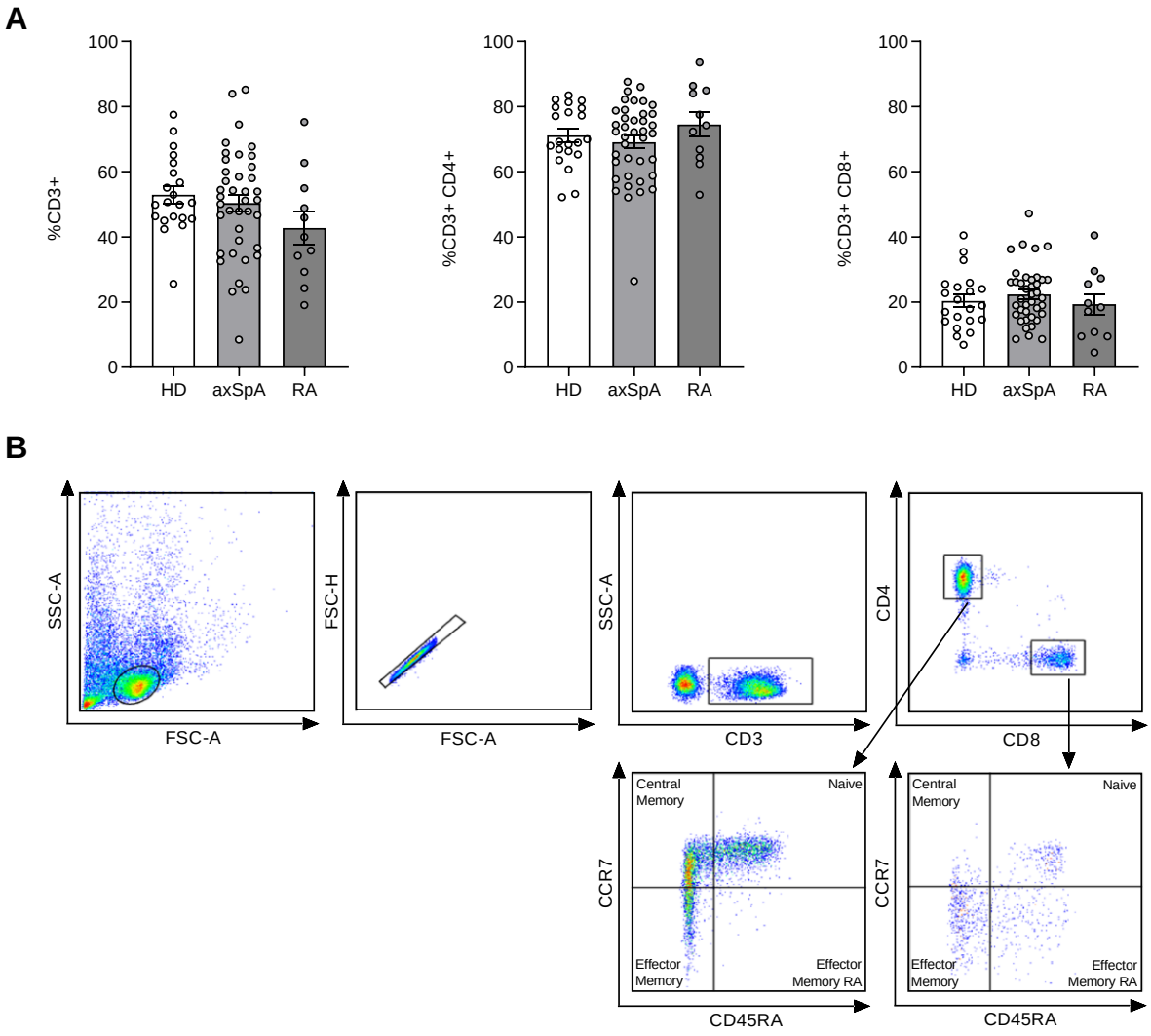
In the present study we have characterized circulating T-cells, potentially migrating to articular and extra-articular sites of inflammation, which could be involved in axSpA pathogenesis.
In particular, the study presented includes two major sections:

- Analysis of the phenotype and the chemokine receptors profile of circulating CD4+ and CD8+ circulating T-cells of axSpA patients.
- Evaluation of circulating CD8+ T-cells expressing the chemokine receptor CCR4, and their functional characterization.

3.1 Characterization of circulating T-cells

3.1.1 The percentage of T_N is increased whereas the percentage of T_{EM} is decreased in axSpA

In order to characterize circulating T-cells, we first analyzed the percentage of circulating CD3+, CD4+ and CD8+ T-cells from peripheral blood in our cohorts of HD, axSpA and RA patients and found no differences in the three groups (Figure 9A).
Then we performed a further characterization of these cells, and evaluated the memory phenotype of CD4+ and CD8+ T-cells, based on the expression of the chemokine receptor CCR7 and the naïve marker CD45RA. The expression of CCR7 and CD45RA can be used to divide CD4+ and CD8+ T-cells into naïve and different memory subpopulations. CCR7 is a chemokine receptor that controls homing to SLOs, and its expression could be used to divide human memory T-cells into two functionally distinct subsets. CCR7- memory cells (T_{EM} and T_{EMRA}) express receptors for migration to inflamed tissues and display immediate effector function. In contrast, CCR7+ memory cells (T_N and T_{CM}) express lymph node homing receptors and lack immediate effector function [136]. Representative FACS gates are shown in Figure 9B. As shown in Figure 9C, we observed an increased frequency of circulating T_N and a decreased frequency of T_{EM} in our cohorts of axSpA and RA patients, both in CD4+ and in CD8+ compartments, whereas we did not find any difference in the percentage of T_{CM} among the groups.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%CD3+ CD8+
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axSpA
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B
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CD8
CCR7
CD45RA
Central Memory
Naive
Effector Memory
Effector Memory RA
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6.0%
34.5%
34.0%
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TN
TCM
TEM
TEMRA

Figure 9. Percentage of T-cell populations and their memory phenotype.

(**A**) The percentage of CD3+, CD4+ and CD8+ T-cells were identified in peripheral blood of HD (*n=21*), axSpA (*n=38*) and RA patients (*n=11*) through the expression of the respective marker. (**B**) Gating strategy for the identification of memory subtypes through the expression of cell surface markers CCR7 and CD45RA in CD4+ and CD8+ T-cells. Doublets were excluded using the FSC-H vs FSC-A gates. (**C**) Comparison of the frequencies of naïve (CCR7+CD45RA+), central memory (CCR7+, CD45RA-), effector memory (CCR7-CD45RA-) and effector memory RA (CCR7-CD45RA+) CD4+ and CD8+ T-cells in HD (*n=21*), axSpA (*n=39*) and RA (*n=11*) patients. Values are expressed as means ± SEM, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

HD (Healthy Donor), axSpA (axial Spondyloarthritis), RA (Rheumatoid Arthritis), T_N (Naïve T-cells), T_{CM} (Central Memory T-cells), T_{EM} (Effector Memory T-cells), T_{EMRA} (Effector Memory RA T-cells)

3.1.2 Higher frequency of CCR5+ T-cells in axSpA

In order to characterize the phenotype of T-cells present in the blood stream that could reach the sites of inflammation in axSpA patients, we evaluated the expression of several activation markers: HLA-DR/CD38 (early activation), PD-1 (late activation/exhaustion), and CCR5, a chemokine receptor expressed on activated T-cells [175] that leads the migration of T-cells to inflamed tissues. In axSpA patients, no differences were found in the percentage of positive cells to HLA-DR/CD38 and PD-1, whereas a significantly higher frequency of both CD4+ and CD8+ T-cells expressing CCR5 was observed (Figure 10A). In RA patients, CD8+ T-cells displayed an activated phenotype, with a higher frequency of HLA-DR/CD38+ and CCR5+ expressing cells (Figure 10A).

To evaluate the proliferation status of CD4+ and CD8+ T-cells, we analyzed the percentage of Ki67 positive cells (Figure 10B). We did not detect significant differences among the three groups in CD4+ T-cells, whereas the cells positive to Ki67 expression are significantly decreased in CD8+ T-cells from axSpA and RA with respect to HD.

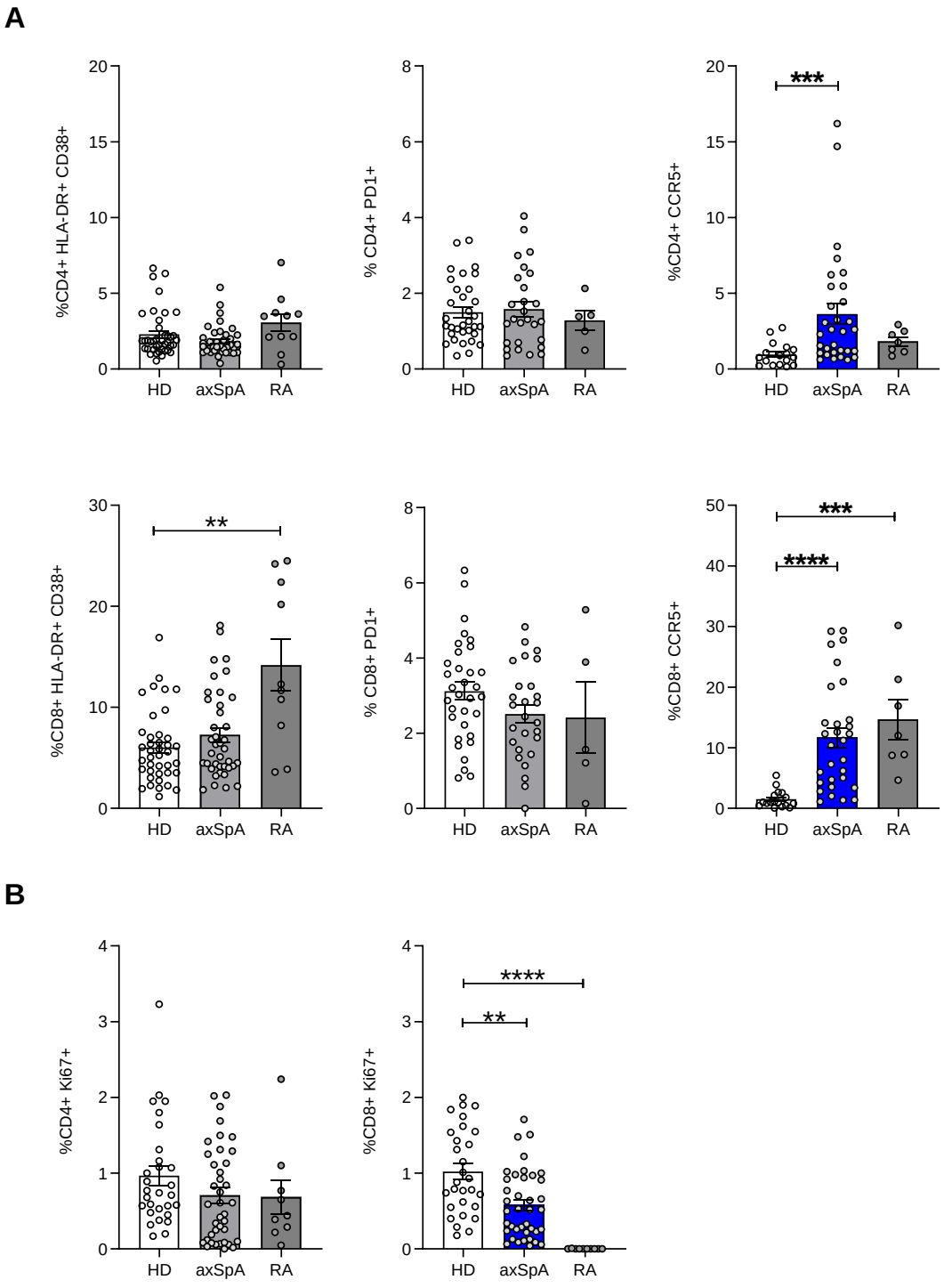


Figure 10. Evaluation of activation and proliferation markers.

(**A**) Circulating T-cells double positive for HLA-DR and CD38 (HD *n=40*, axSpA *n=39*, RA *n=11*) are considered early activated and cells positive for the expression of PD-1 are considered late activated/exhausted (HD *n=33*, axSpA *n=27*, RA *n=5*). The frequency of CCR5 was evaluated in HD (*n=17*), axSpA (*n=31*) and RA (*n=7*). (**B**) Percentage of Ki67 positive cells was used to evaluate the cell proliferation status in HD (*n=29*), axSpA (*n=39*) and RA (*n=10*) patients. Values are expressed as means ± SEM, **p < 0.01, ***p < 0.001, ****p < 0.0001.

HD (Healthy Donor), axSpA (axial Spondyloarthritis), RA (Rheumatoid Arthritis), HLA-DR (Human Leukocyte Antigen-DR), PD-1 (Programmed cell Death protein 1)

3.1.3 CD4+ T-cells display a phenotype prone to migrate to lymphoid tissues in axSpA

In order to further dissect T-cells capacity to migrate at different sites, we evaluated the expression of homing receptors. As the Figure 11A shows, and in line with the increase of T_N observed in axSpA (Figure 10C), we found a significant increased percentage of CCR7+ cells on total CD4+ and CD8+ T-cells. Of note, comparing CD4+ and CD8+ T-cells, we found that the percentage of CD4+ CCR7+ is higher than CD8+ CCR7+ T-cells. This result indicates that circulating CD4+ T-cells display a phenotype prone to migrate to lymphoid organs, while CD8+ T-cells have the capability to migrate to lymphoid and peripheral non-lymphoid tissues.

In addition, we analyzed the percentage of CCR6+ T-cells in CD4+ and CD8+ compartments. CCR6 is the chemokine receptor that drives the homing to mucosal sites. As shown in Figure 11B, we found a significant reduction of CD8+CCR6+ T-cells in RA patients compared to HD cohort.

Furthermore, we analyzed the frequency of the cutaneous lymphocyte antigen 1 (CLA1) and of the chemokine receptor CCR4. The expression of CLA1 defines a subset of human peripheral T-cells characterized by skin-homing capability [176], and CCR4 is the chemokine receptor that drives the homing to normal or inflamed skin. Figure 11C shows a decrease in the percentage of CLA1 positive cells both in CD4+ and in CD8+ T-cells that reaches the significance only in CD8+ T-cells of RA patients. In circulating CD4+ and CD8+ T-cells, we did not appreciate any difference in the percentage of CCR4+ cells in axSpA patients compared to HD.

In addition to lymphoid and skin homing markers, we analyzed the frequency of the gut homing marker integrin β7 that facilitates the retention of effector and memory lymphocytes in the gut epithelial layer via interactions with E-cadherin, and of CCR9, the chemokine receptor that drives the homing to the small intestine. As shown in Figure 11D, the frequency of the gut-homing marker integrin β7 positive cells in CD4+ T-cells and positive cells to CCR9 in CD8+ T-cells were enhanced in RA patients, while the frequency of CCR9 in CD4+ T-cells from axSpA patients was significantly decreased.

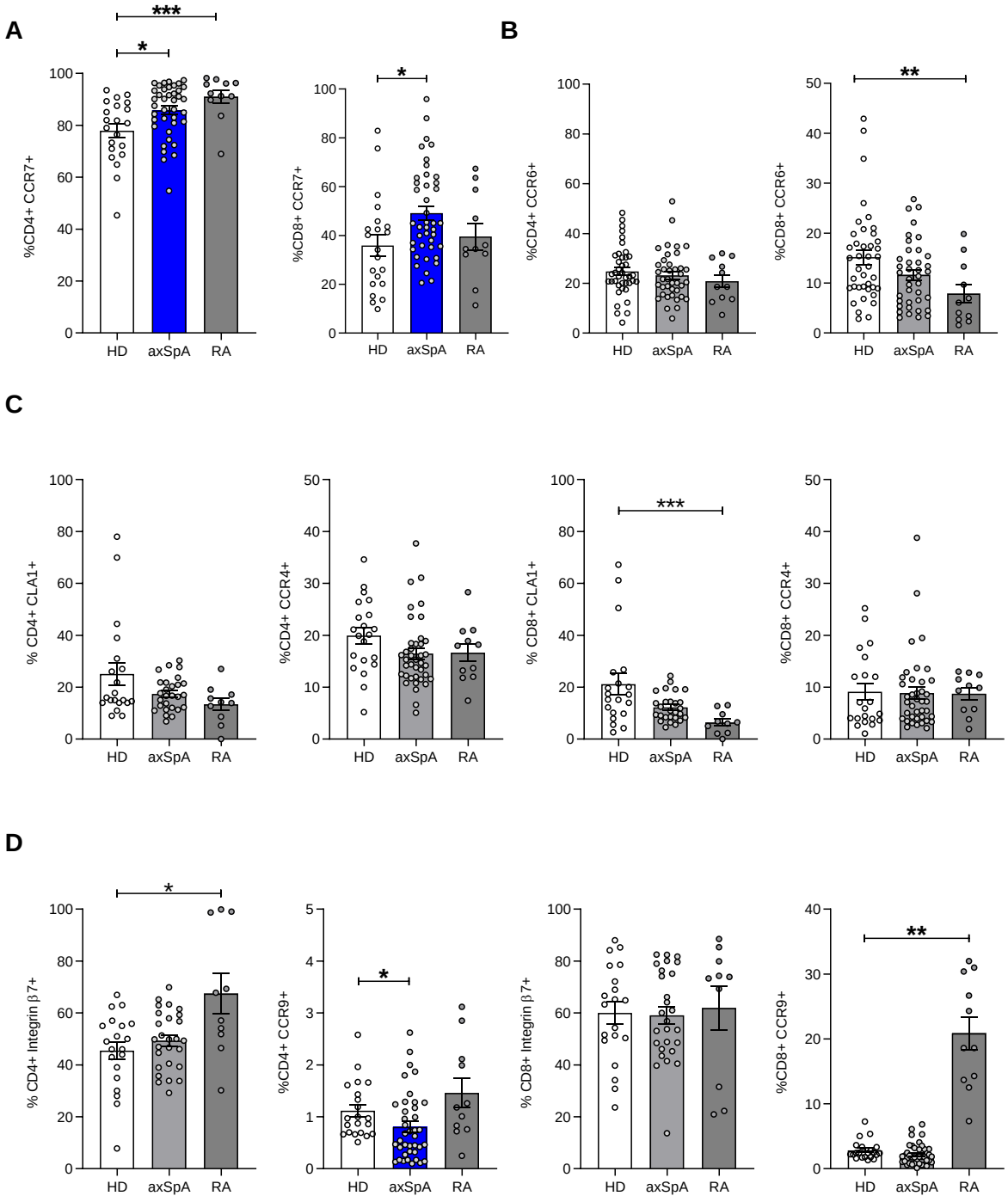


Figure 11. CD4+ and CD8+ T-cells homing markers expression.

(**A**) Percentage of CCR7+ CD4+ and CD8+ T-cells in HD (*n=21*), axSpA (*n=39*) and RA (*n=11*) patients. (**B**) Percentage of CCR6+ CD4+ and CD8+ T-cells in HD (*n=40*), axSpA (*n=39*) and RA (*n=11*) patients. (**C**) Frequency of skin-homing marker CLA1 in HD (*n=20*), axSpA (*n=29*) and RA (*n=10*) patients and frequency of the chemokine receptor CCR4 in HD (*n=21*), axSpA (*n=39*) and RA (*n=11*) patients. (**D**) Frequency of the gut-homing marker integrin β7 in HD (*n=20*), axSpA (*n=27*) and RA (*n=10*) patients and frequency of the chemokine receptor CCR9 in HD (*n=21*), axSpA (*n=39*) and RA (*n=11*) patients. Values are expressed as means ± SEM, *p < 0.05, **p < 0.01, ***p < 0.001.

HD (Healthy Donor), axSpA (axial Spondyloarthritis), RA (Rheumatoid Arthritis), CLA (Cutaneous Lymphocyte Antigen)

3.1.4 T_H1 and T_C1 subsets are decreased in axSpA patients

In addition to the expression of CCR4 and CCR6 (Figure 11), the analysis of the expression of CXCR3 allows clustering of the different T_H and T_C subpopulations. Therefore, the expression of CCR4, CXCR3 and CCR6 was used to distinguish different subsets of CD4+ T-cells: T_H1 (CCR6-CXCR3+), T_H2 (CCR6- CCR4+), T_H17 (CCR6+ CCR4+) and T_H1^* (CCR6+ CXCR3+). For the CD8+ T-cells compartment, the division in different subtypes is based on the cytokine production and a preferential chemokine receptors expression. Even if a consensus in literature has not yet been reached, for this work we decided to divide CD8+ T-cells into different subtypes according to the expression of chemokine receptors: T_C1 (CXCR3+), T_C2 (CCR4+) and T_C17 (CCR6+). As shown in Figure 12A, the percentage of circulating T_H1 cells was significantly decreased in axSpA patients compared to HD, whereas the percentage of T_H17 and T_H1^* cells were significantly decreased in RA patients. For the CD8+ T-cells compartment (Figure 12B), it was possible to appreciate a significant decrease in T-cells expressing CXCR3 (T_C1) in axSpA patients and a significant decrease in T_C17 in RA patients.

3.1.5 The T_C2 subset is increased in axSpA

In addition to T-cell subsets differences, imbalances in T-cell subset ratios have been shown to be present in RA [177] and in axSpA [143]. An imbalance in the IL-23/IL-17 axis has been documented in axSpA, and recently anti-IL17 antibodies have been introduced in the clinic [51]. We have further analyzed the ratios between the different subsets of CD4+ and CD8+ T-cells in our cohorts in order to confirm and expand the previous results. As the Figure 13A shows, despite the fact that we did not find any difference in the frequency of circulating T_H17 cells or T_C17 cells in our cohort of axSpA patients, a skew toward the IL-17 axis could be observed in T_H17/T_H1 and in T_C17/T_C1 ratios ($p=0.03$). Furthermore, we found that the T_C2/T_C1 ratio is significantly higher in axSpA patients compared to HD ($p=0.01$). Of note, by dividing the axSpA patients according to their disease status (Figure13B), we found that T_C2/T_C1 ratio is significantly higher in patients with active disease (ASDAS ≥ 2.1).

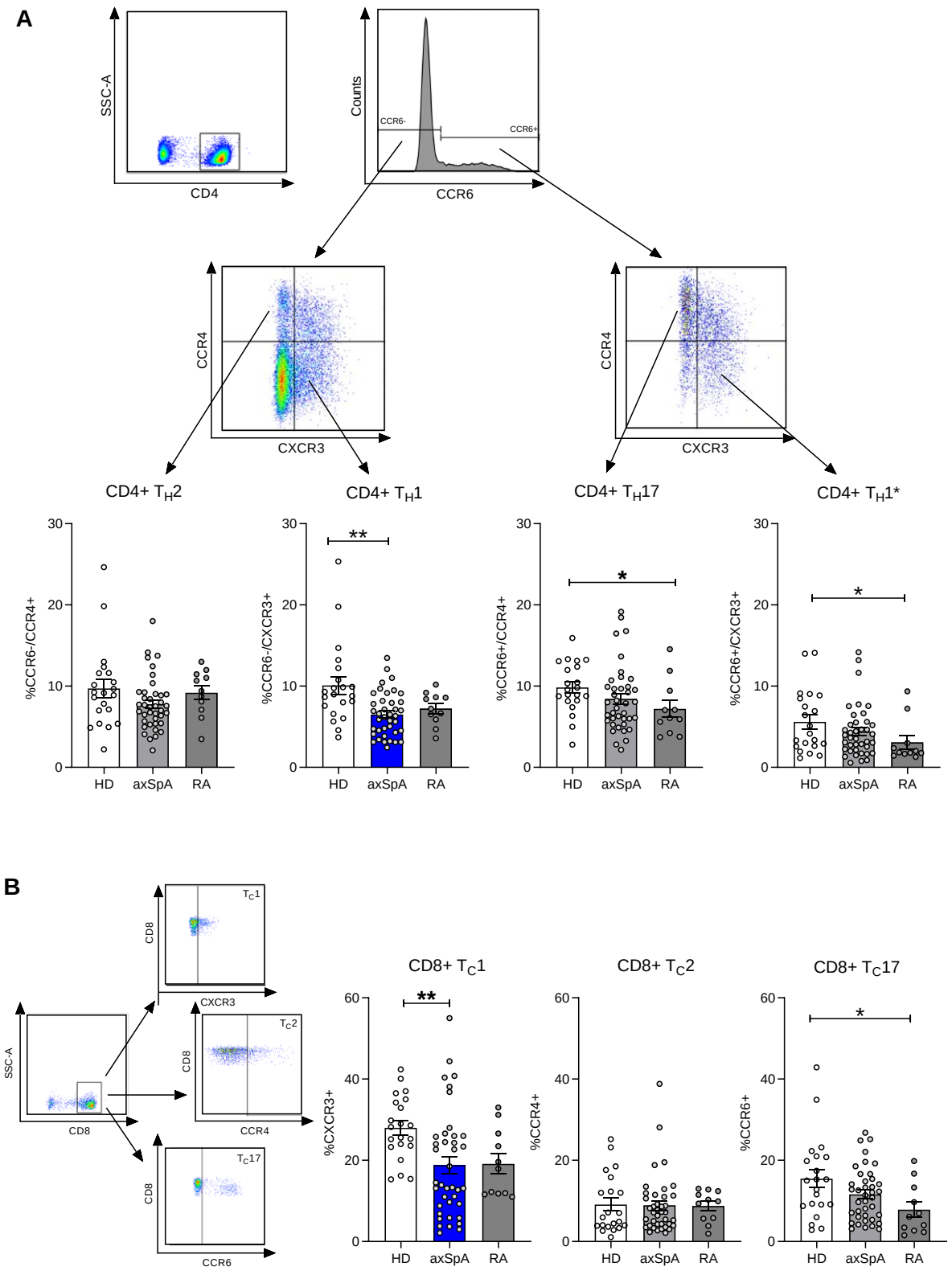


Figure 12. T-cell subsets.

(**A**) Gating strategies and percentage of CD4+ T_H1, T_H2, T_H17, T_H1* T-cells and (**B**) CD8+ T_C1, T_C2, T_C17 T-cells in HD (*n=21*), axSpA (*n=39*) and RA (*n=11*). Values are expressed as means ± SEM, *p < 0.05, **p < 0.01.

HD (Healthy Donor), axSpA (axial Spondyloarthritis), RA (Rheumatoid Arthritis), T_H (T helper), T_C (T cytotoxic)

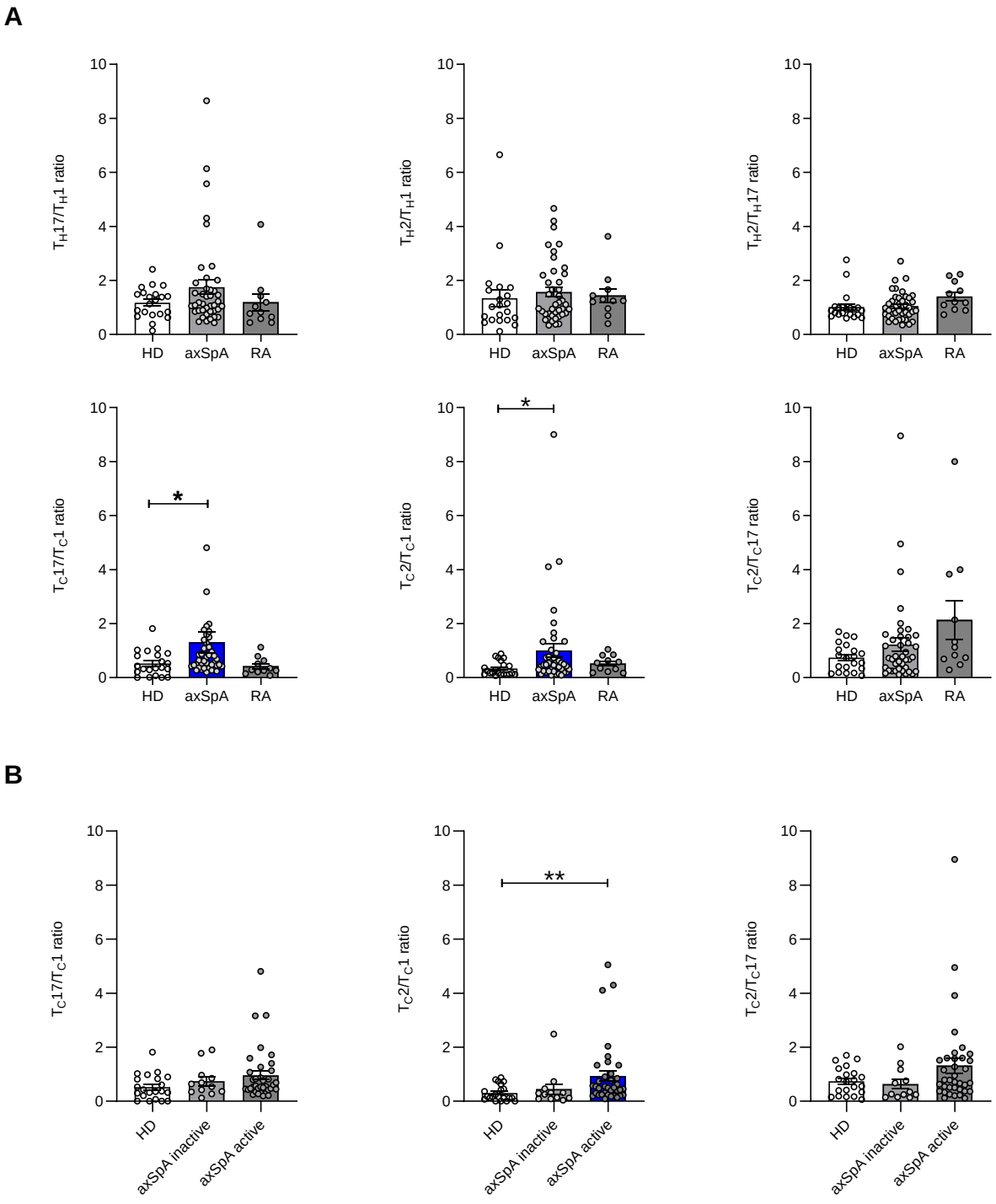


Figure 13. T-cell subsets ratios.

(**A**) T_H17/T_H1, T_H2/T_H1, T_H2/T_H17, T_C17/T_C1, T_C2/T_C1 and T_C2/T_C17 ratios in HD (*n=21*), axSpA (*n=48*) and RA (*n=11*). (**B**) T_C17/T_C1, T_C2/T_C1 and T_C2/T_C17 ratios in HD (*n=21*) and axSpA patients with inactive (*n=12*) and active (*n=36*) disease status. Values are expressed as means ± SEM, *p < 0.05, **p < 0.01.

HD (Healthy Donor), axSpA (axial Spondyloarthritis), RA (Rheumatoid Arthritis), T_H (T helper), T_C (T cytotoxic)

3.2 Characterization of circulating CD8+CCR4+ T-cells

As showed in Figure 13, an imbalance in the ratio of T_C2/T_C1 occurs in axSpA patients that, together with cytokines production, could contribute to disease progression. Therefore, we have further characterized circulating CD8+ T-cells expressing the chemokine receptor CCR4+, also in comparison with CD4+CCR4+ T-cells.

3.2.1 CD8+CCR4+ T-cells are more activated and proliferative

To understand the possible role of circulating T-cells that express CCR4, we analyzed their memory phenotype (Figure 14A). Interestingly, we found that the majority of circulating CD8+CCR4+ T-cells were $T_{EM,}$ whereas CD4+CCR4+ T-cells were T_{CM} (data not shown). These data show the possible different function of circulating CD8+CCR4+ and CD4+CCR4+ T-cells; in fact, CD8+CCR4+ T-cells, being primarily T_{EM}, are prone to migrate in peripheral tissues where they can accomplish their effector function, while CD4+CCR4+ T-cells can recirculate in lymphoid tissues thanks to the expression of CCR7. We did not find any difference regarding CD8+CCR4+ or CD4+CCR4+ T-cells from axSpA in comparison with T-cells from HD, while a significant decrease of T_{EM} and an increase of T_{EMRA} was observed in RA patients. Furthermore, analyzing the expression of CD27 and CD28 as a marker of the cell differentiation status, we found that CD8+CCR4+ T_{EM} cells are prevalently early differentiated in HD (71.32±10.54%), axSpA (67.69±14.09%) and RA patients (66.44±19.60%), with no differences between the groups analyzed (data not shown).

Following evaluation of activation/proliferation markers of CD8+CCR4+ T-cells (Figure 14B and Figure 14C), we detected an increase in the percentage of PD-1, CCR5 and Ki67 positive cells, indicating that this subset is activated and proliferates in axSpA. Similar results in the percentage of CCR5+ and Ki67+ T-cells were obtained when analyzing CD8+CCR4+ T-cells in RA patients. The significant increase in the percentage of PD-1+ and Ki67+ observed in CD8+CCR4+ T-cells from axSpA was observed also in the CD4+CCR4+ compartment (data not shown, PD-1 $p \leq 0.0001$ and Ki67 $p=0.0015$).

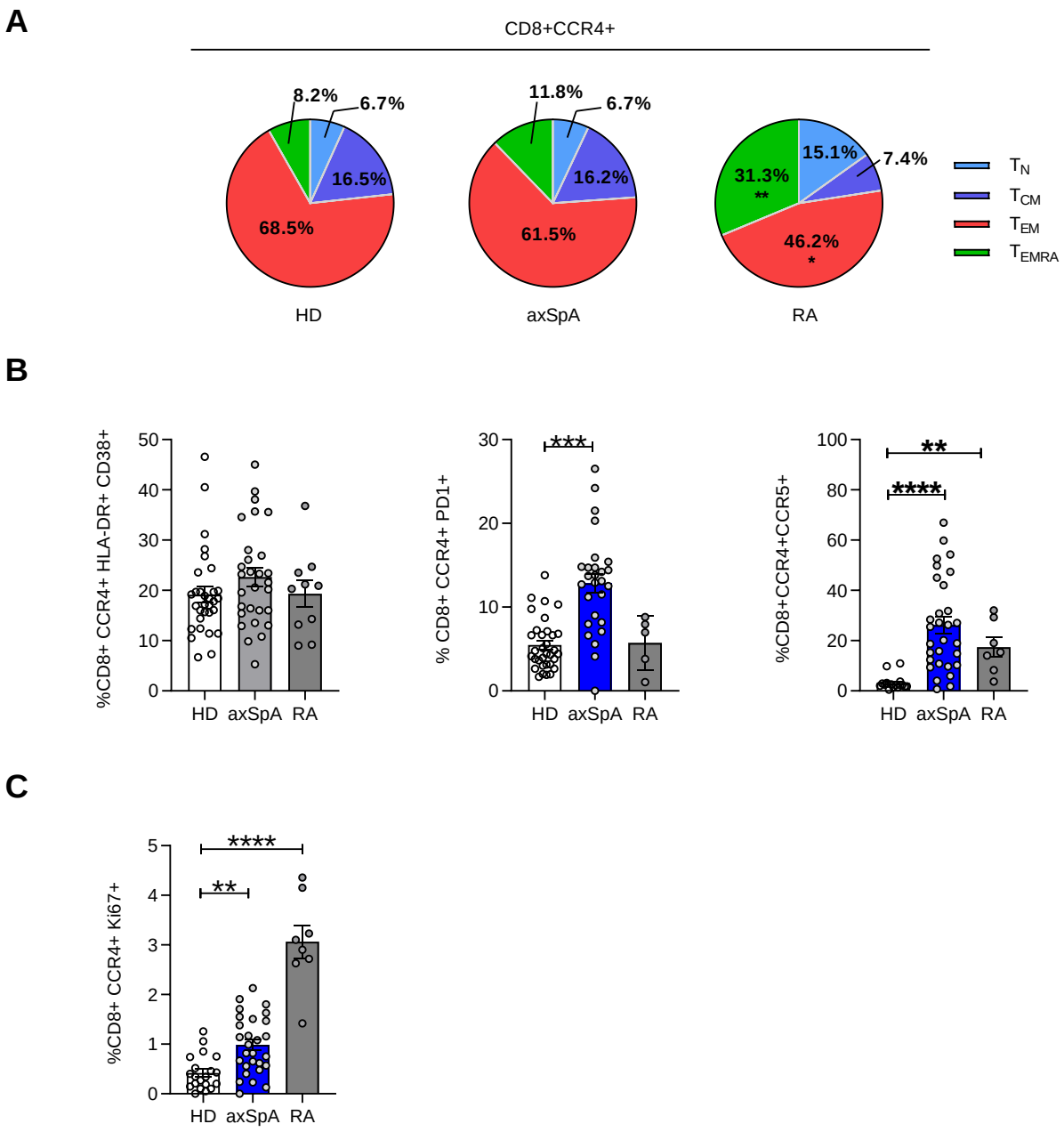


Figure 14. CD8+CCR4+ T-cells characterization.

(**A**) Comparison of the frequencies of naïve (CCR7+CD45RA+), central memory (CCR7+, CD45RA-), effector memory (CCR7-CD45RA-) and effector memory RA (CCR7-CD45RA+) CD8+CCR4+ T-cells in HD (*n=36*), axSpA (*n=26*) and RA (*n=5*). (**B**) Percentage of circulating CD8+CCR4+ T-cells double positive for HLA-DR and CD38 in HD (*n=31*), axSpA (*n=29*) and RA patients (*n=10*). CD8+CCR4+ T-cells positive for the expression of PD-1 in HD (*n=33*), axSpA (*n=27*) and RA (*n=5*) patients. (**C**) Percentage of CD8+CCR4+ Ki67+ T-cells in HD (*n=20*), axSpA (*n=28*) and RA (*n=8*) patients. Values are expressed as means ± SEM, **p < 0.01, ***p < 0.001, ****p < 0.0001.

HD (Healthy Donor), axSpA (axial Spondyloarthritis), T_N (Naïve T-cells), T_{CM} (Central Memory T-cells), T_{EM} (Effector Memory T-cells), T_{EMRA} (Effector Memory RA T-cells), HLA-DR (Human Leukocyte Antigen-DR), PD-1 (Programmed cell Death protein 1)

3.2.2 Circulating CD8+CCR4+ T-cells can migrate to different tissues

CCR4 is required for the migration to the skin under steady state and inflammatory conditions [178-180], but the cells that express CCR4 could migrate also in different tissues thanks to the expression of other chemokine receptors on their surface. For this reason, we decided to evaluate the percentage of cells positive for CCR7 (homing to lymphoid tissues), CCR6 (homing to mucosal sites), CXCR3 and CX_3CR1 (homing to inflammatory sites) (Figure 15A). The frequency of CD8+CCR4+ T-cells CCR7+ was not different in axSpA and RA patients, while the percentage of CCR6+ cells was significantly reduced in CD8+CCR4+ T-cells from axSpA and not in cells from RA patients, compared to HD. Regarding the expression of chemokine receptors that sustain the homing to inflammatory tissues, we found significantly more cells expressing CX_3CR1 in axSpA, and less expressing CXCR3 in RA patients.

Respecting the frequency of the skin-homing marker CLA1 and the gut-homing marker integrin β7 (Figure 15B), we found that circulating CD8+CCR4+ T-cells can express both markers, with no difference in their percentage in axSpA and RA, compared to HD.

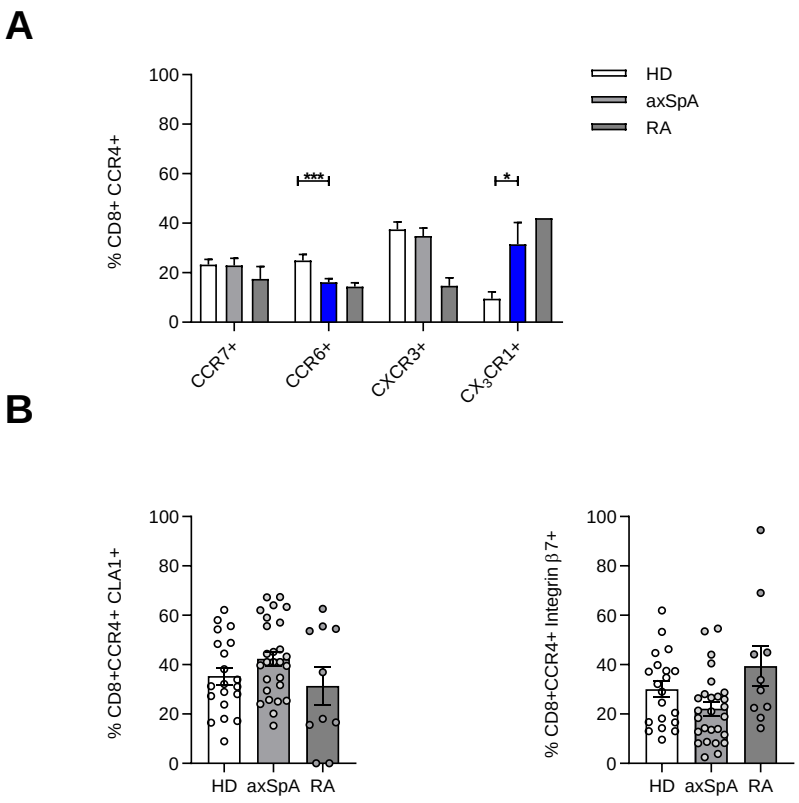


Figure 15. CD8+CCR4+ T-cells homing markers expression.

(**A**) Percentage of chemokine receptors CCR7, CCR6, CXCR3 and CX_3CR1 expressed on the surface of CD8+CCR4+ T-cells. (**B**) Skin- and gut-homing markers, CLA1 and integrin β7, frequency in CD8+CCR4+ T-cells from HD (*n=18*), axSpA (*n=27*) and RA (*n=10*) patients. Values are expressed as means ± SEM, *p < 0.05, ***p < 0.001.

HD (Healthy Donor), axSpA (axial Spondyloarthritis), CLA (Cutaneous Lymphocyte Antigen)

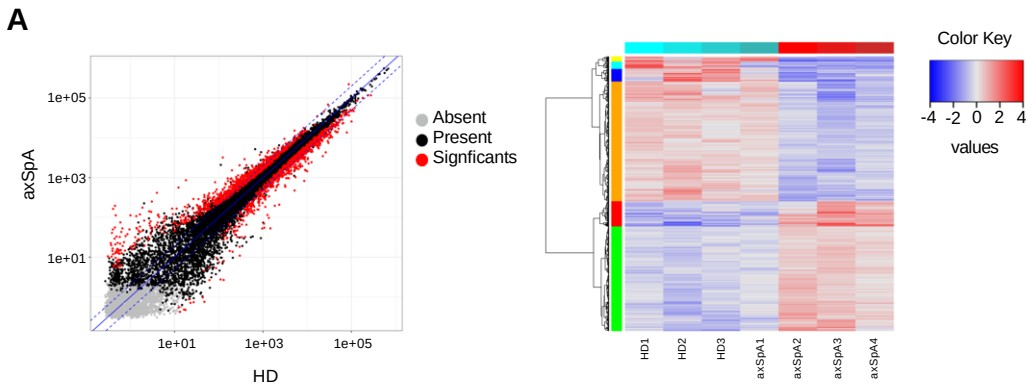
3.2.3 CD8+CCR4+ T-cells are cytotoxic in axSpA and might have a role in bone remodeling

We finally investigated the transcriptome of CD8+CCR4+ circulating T-cells, in order to understand if the differences observed by cytofluorimetric analysis would indicate differences in biological processes and cellular pathways between HD and axSpA. In Figure 16A are represented the comparison of average expression and the clustering of significant features obtained from the RNA sequencing analysis of HD and axSpA naïve for TNF inhibitor therapy. Of note, as the clustering analysis shows, we appreciated a distinct gene expression pattern for HD and axSpA patients except for axSpA1, the unique patient with inactive disease included in the analysis. To generate the specific gene expression profile, we focused on genes differentially expressed, with a fold change of 1.0 or greater between HD and axSpA. We identified a total of 1788 genes that were differentially expressed out of the 21386 genes identified from RNA sequencing. A total of 940 genes including GRZH, GRZB, MEF2C, TMEM119, SMAD3, IL-8 and CX_3CR1 were significantly up-regulated in CD8+CCR4+ T-cells from axSpA patients, whereas 848 genes including CSF1, TNF, IL-23A, IFN-γ, FOS and IL-2 were significantly down-regulated. Pathway analysis including biological processes and immune system functions was performed with all differentially expressed genes.

The up-regulated genes were enriched for pathways related to cytotoxic functions (Figure 16B1) and bone mineralization (Figure 16B3). Conversely, the down-regulated genes were enriched for pathways related to positive regulation of osteoclast differentiation (Figure 16B2).

In order to confirm the cytotoxic profile of CD8+CCR4+ T-cells identified from the RNA sequencing, we stimulated CD8+CCR4+ and CD8+CCR4- T-cells with PMA and ionomycin (Figure 16C). In line with data from the literature [154], we found that CD8+CCR4+ T-cells are not cytotoxic in HD, but, interestingly, these cells are able to produce GRZ and PRF in axSpA patients, and this production is higher in patients with active disease (ASDAS ≥ 2.1). A low percentage of CD8+ T-cells negative for the expression of CCR4 are cytotoxic after stimulation also in HD, but differences in GRZ and PRF production between HD and axSpA were not observed. A lower percentage of IL-2 producing cells was observed in CD8+CCR4+ T-cells from axSpA compared to HD. The percentage of CD8+CCR4- T-cells producing IL-2 was similar in HD and axSpA.

To better understand the possible role of CD8+CCR4+ T-cells in the regulation of osteoclast differentiation, we evaluated the release of two osteoclastogenic cytokines, TNF and IL-8 (Figure 16D), after cells stimulation with PMA and ionomycin. Our data shows that CD8+CCR4+ T-cells were able to produce and release TNF and IL-8, and IL-8 production was significantly increased in T-cells from axSpA patients.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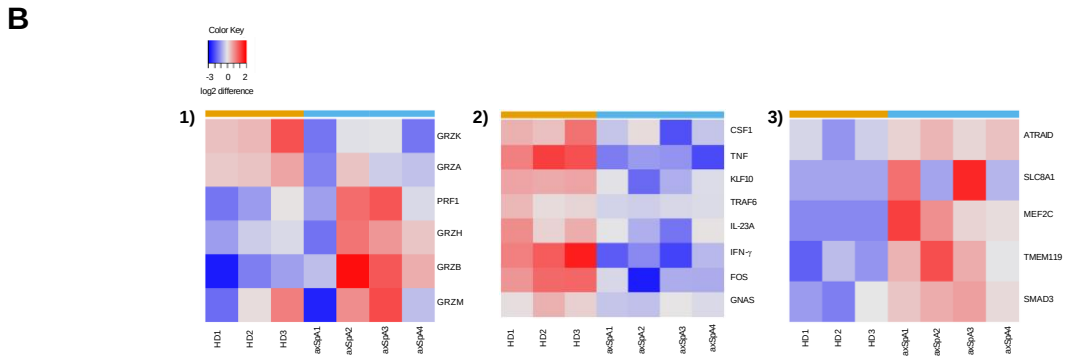
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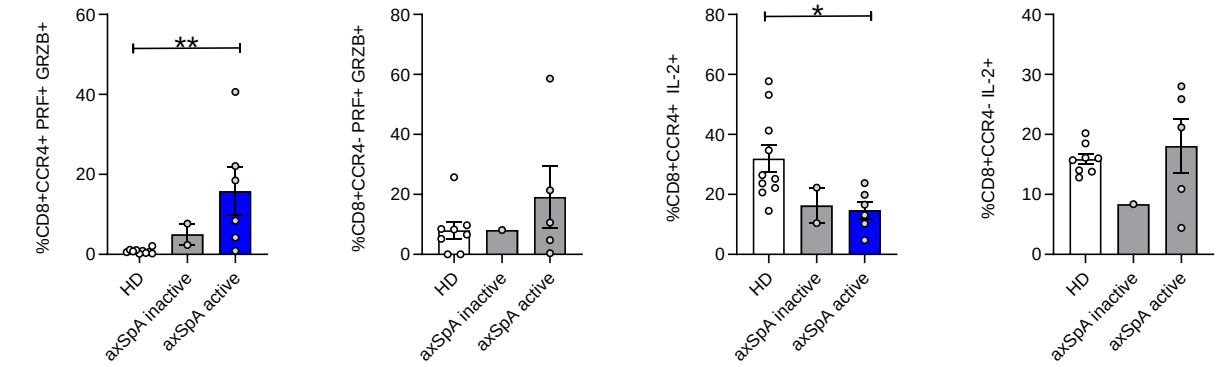
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%CD8+CCR4+ PRF+ GRZB+
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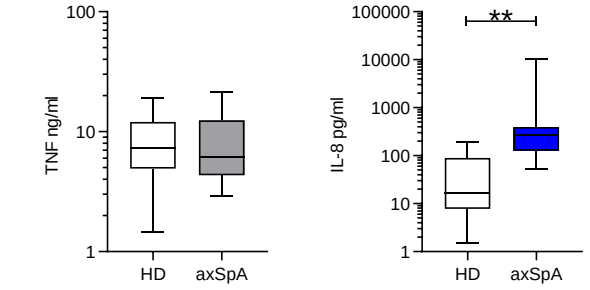
D
TNF ng/ml
IL-8 pg/ml
**
HD
axSpA

Figure 16. Transcriptome and functional analysis of CD8+CCR4+ T-cells.

(**A**) Comparison of average expression and clustering of significant features obtained from RNA sequencing analysis of *n=3* HD and *n=4* axSpA patients. Differences between mean values are considered significant when $p < 0.01$. (**B1**) Heatmaps of genes involved in cytotoxic functions, (**B2**) positive regulation of osteoclast differentiation and (**B3**) bone mineralization. (**C**) GRZ, PRF and IL-2 production after stimulation by CD8+CCR4+ circulating T-cells in HD (*n=10*), axSpA patients with inactive (*n=2*) and active disease status (*n=5*) and by CD8+CCR4- T-cells in HD (*n=8*), inactive (*n=1*) and active disease status (*n=6*). (**D**) Detection of IL-8 and TNF-α in the supernatant of CD8+CCR4+ T-cells stimulated with PMA and ionomycin from HD (*n=6*) and axSpA patients (*n=9*). Box plots: minimum to maximum. Values are expressed as means ± SEM, *$p < 0.05$, **$p< 0.01$.

HD (Healthy Donor), axSpA (axial Spondyloarthritis), log (logarithm), GRZ (Granzyme), PRF (Perforin), CSF (Colony Stimulating Factor), TNF (Tumor Necrosis Factor), KLF (Krueppel-Like Factor), TRAF (TNF Receptor Associated Factor), IL (Interleukin), IFN (Interferon), GNAS (Guanine Nucleotide binding protein Alpha Stimulating), ATRAID (All-Trans Retinoic Acid Induced Differentiation factor), SLC (Solute Carrier), MEF (Myocyte Enhancer Factor), TMEM (Transmembrane Protein)

Chapter 4: Discussion

4.1 General discussion

AxSpA is an inflammatory rheumatic disease predominantly affecting the axial skeleton with remodeling of skeletal structures in a pathological and impedimental way.

The immune system plays a fundamental role in the pathogenesis of axSpA and the inflammatory infiltrate in the structural lesions contributes to tissue damage through the release of inflammatory cytokines and mediators of bone remodeling. Among the immune cells, T-cells have been shown to exert pro-osteogenic effects on osteoblasts precursors, contributing to catabolic processes and to anabolic tissue responses of the bone [162-164].

Despite a relative abundance of information about inflammatory tissue infiltrate, consensus relating the functional contributions of the immune populations to axSpA pathogenesis has not yet been reached.

The main sites of inflammation and aberrant bone processes in axSpA are within the spine, the sacroiliac joints and enthesis [57]. However, since access to spinal tissue is limited, most studies investigate axSpA pathogenesis employing extra-articular and peripheral tissues and focus on synovial fluid and blood samples.

In this project, we have taken advantage of the chemokine receptors profile of immune cell populations, which influences their migration capacity, phenotype and subsequent function, and characterized circulating T-cells from axSpA patients in order to identify cellular populations that can migrate to inflammatory tissues where they can exert a role in disease development and perpetuation.

While analyzing the memory phenotype of T-cells, we found an increase of T_N and a decrease of T_{EM}. Goronzy et al. proposed that a prematurely altered thymic function in RA induces a compensatory expansion of peripheral T_N cells that could results in a contracted and distorted repertoire, possibly promoting T-cells with autoreactive potential [181]. In line with this hypothesis, we observed an increased percentage of circulating T_N cells in our cohort of RA patients and, interestingly, we also found a similar increase in axSpA. In contrast, the study by Fessler et al. showed that an early thymic failure and a premature senescence of naïve and memory T-cells was not associated to an increase of T_N in axSpA [182]. However, to the best of our knowledge this is the only study about thymic failure in axSpA. While the percentage of T_N was increased in our cohorts of axSpA and RA patients, the percentage of T_{EM} was decreased. Since T_{EM} exert their functions migrating to multiple peripheral tissue sites, this decrease could be due to their migration from the blood stream to the periphery in order to reach the sites of inflammation, or it may represent an impairment in T_{EM} generation. In conclusion, the different distribution of circulating T_N and T_{EM} in axSpA and RA patients of our cohort could be due to an altered thymus functionality, to peripheral migration of T_{EM}, or to an impairment in their generation.

In axSpA patients, a higher frequency of both CD4+ and CD8+ circulating T-cells expressed CCR5, a chemokine receptor whose expression is restricted to activated lymphocytes. The increase of CCR5+ T-cells was not associated to the expression of other activation markers or to proliferation. In RA patients we found a significant increase only in the percentage of CD8+ positive for the expression of HLA-DR/CD38 and CCR5 activation markers, and a decrease in the proliferation rate as showed by the percentage of Ki67+ cells. The increase of CCR5+ T-cells is particularly interesting, since a selective accumulation of these cells into the inflamed joints of RA patients has been described [183].

The analysis of the chemokine receptors and homing markers to peripheral tissues did not reveal an altered pattern in axSpA T-cells compared to HD. In RA patients, the frequencies of CD8+ T-cells expressing CCR6 and CLA1 were decreased and the percentage of cells positive to CCR9 was increased. Furthermore, CD4+ T-cells that express integrin β7 were increased, in line with the ability of T-cells to adhere to rheumatoid synovium [184].

It has been proposed that an unbalanced distribution of circulating T-cell subsets plays a role in the pathogenesis of axSpA, but contrasting data on relative percentages and role of CD4+ and CD8+ T-cells subsets in axSpA are present in the literature. An increased percentage of T_H1 and a significant higher T_H1/T_H2 ratio in AS patients were reported by several groups [143;146-148;185]. Yang et al. have found an increased percentage of CD4+ that express CCR4+, the chemokine receptor expressed by T_H2 T-cells. This percentage positively correlates with the disease score [144]. For the CD8+ compartment, the percentage of T_C1 and T_C1/T_C2 ratio have been found increased in the blood stream of AS patients [143]. In our cohort of axSpA patients, we observed a significant decrease of T_H1 and T_C1 circulating T-cells in the blood stream. The differences between our results and what has been reported in literature could be due to the characteristic of the cohort of patients. In particular our cohort includes patients with non radiographic and radiographic (AS) forms of axSpA. Furthermore, in our cohort we have patients with different grade of disease severity, consequently receiving different pharmacological treatments. Since both T_H1 and T_C1 are characterized by the expression of CXCR3, a chemokine receptor that drives the migration of T-cells to inflamed tissues, the decrease in their frequency in the circulation may be the results of their migration into peripheral inflamed tissues. Indeed, elevated levels of CXCL10, a ligand of CXCR3, are found in the sera of AS patients and they positively correlates with the disease score [145]. Recent findings show that the IL-23/IL-17 pathway has an important role in axSpA pathogenesis, and several studies show that IL-17, IL-23, T_H17 and T_H17/Treg ratio are increased in axSpA patients [143;146-148]. In our cohort, we found no differences in T_H17 and T_C17 percentage, but a higher T_C17/T_C1 ratio, suggesting a possible involvement of T_C17 in axSpA pathogenesis. The ability of these cells to produce IL-17 still remains to be assessed.

Of note, we found an imbalance in the T_C2/T_C1 ratio in favor of T_C2 T-cells subtype in axSpA with active disease, which has prompted us to evaluate the role of CD8+CCR4+ in the pathogenesis of axSpA.

The main findings on T-cell subset distribution observed in our cohort of axSpA patients are listed in Table 8.

	HD (Mean ± SEM)	**axSpA** (Mean ± SEM)	**RA** (Mean ± SEM)
CD4+ T_N	41.45 ± 3.41	**51.79 ± 3.35 ****	**55.67 ± 4.63 ***
CD4+ T_{EM}	20.17 ± 2.64	*12.28 ± 2.37 **	*0.94 ± 0.39 ***
CD4+ T_{EMRA}	1.94 ± 0.42	*1.14 ± 0.41 **	**8.03 ± 2.45 ***
CD8+ T_N	28.57 ± 3.88	**43.24 ± 4.93 *****	34.03 ± 5.19
CD8+ T_{EM}	35.10 ± 3.29	*27.43 ± 4.11 **	25.89 ± 3.92
CD4+ CCR5+	0.97 ± 0.18	**3.63 ± 0.68 *****	1.83 ± 0.29
CD8+ CCR5+	1.51 ± 0.35	**11.68 ± 1.63 ******	**14.67 ± 3.33 *****
CD8+ Ki67+	2.30 ± 0.24	*1.83 ± 0.16 ***	**3.06 ± 0.55 ******
CD4+ CCR7+	77.89 ± 2.66	**85.78 ± 1.62 ***	**91.04 ± 2.55 *****
CD8+ CCR7+	35.91 ± 4.30	**49.18 ± 2.91 ***	39.58 ± 5.41
CD4+ CCR9+	1.12 ± 0.12	*0.80 ± 0.10 **	1.46 ± 0.28
CD4+ T_H1	10.08 ± 1.09	*6.52 ± 0.44 ***	7.24 ± 0.60
CD8+ T_C1	27.99 ± 1.71	*18.78 ± 2.09 ***	19.12 ± 2.51
T_C17/T_C1 ratio	0.52 ± 0.10	**1.30 ± 0.38 ***	0.42 ± 0.09
T_C2/T_C1 ratio	0.33 ± 0.06	**1.00 ± 0.25 ***	0.52 ± 0.09

Table 8. Main findings in T-cell subsets.

In **bold** significantly increased, in *italics* significantly decreased.

HD (Healthy Donor), axSpA (axial Spondyloarthritis), RA (Rheumatoid Arthritis), T_N (Naïve T-cells), T_{EM} (Effector Memory T-cells), T_{EMRA} (Effector Memory RA T-cells), T_H (T helper), T_C (T cytotoxic)

Given the expression of CCR4, these cells are able to migrate to normal and inflamed cutaneous sites in response to CCL17 and CCL22 [186;187]. Elevated levels of these two chemokines are found in the sera of axSpA patients [145], and are known to be mainly produced by cell lineages closely related to osteoclasts [187]. Several lines of evidence indicate that CCL22 expression is upregulated in the synovial fluid obtained from patients with RA [188;189], and is associated to the presence of CCR4+ mononuclear cells in their synovial membrane, supporting a role for the CCR4 axis in attracting T-cells into the joints [188]. While no data are available regarding the expression of these two chemokines in axSpA joint infiltrates, dense cellular infiltrates containing CD8+ T-cells are found in the synovial portion of the sacroiliac joints of axSpA patients [190]. Moreover, an involvement of CD8+ T-cells in cartilage destruction has been described [88;89;155]. Collectively, these evidences suggest that CCR4+ T-cells infiltrate inflamed joints even in axSpA.

The majority of circulating CD8+CCR4+ were T_{EM} that can migrate to peripheral target tissues in order to accomplish their effector functions. We found that in RA CD8+CCR4+ T-cells were increased in T_{EMRA} at the expense of the T_{EM} pool. This could be due to the presence of a chronic antigen stimulation that induce the differentiation of T_{EM} in T_{EMRA}, the most differentiated memory subtype [191]. It is still matter of debate whether in axSpA a chronic antigen exposure is at the basis of the pathology [134], and in our cohort, we did not find an alteration in the percentage of T_{EMRA}, nor in the total CD8+ or in CD8+CCR4+ T-cells.

Interestingly, we showed that circulating CD8+CCR4+ T-cells are activated and proliferative, as indicated by the increased percentage of PD-1, CCR5 and Ki67 positive cells in axSpA.

As discussed above, given the expression of CCR4, these activated and proliferative T-cells can migrate to the skin and to the joint; however we found that, thanks to the expression of other chemokine receptors they could reach other peripheral sites. In particular, our results show that CD8+CCR4+ T-cells express both CLA1 and Integrin β7 indicating their ability to reach both the skin and the gut. Interestingly, we observed a decreased percentage of CD8+CCR4+ co-expressing CCR6 T-cells in the blood stream, whereas CX_3CR1+ cells were increased. In recent years, several studies have demonstrated that CD8+ T-cells expressing the CX_3CR1 receptor possess a unique signature, and that this chemokine receptor can be used to distinguish CD8+ T-cells with cytotoxic effector function from those with proliferative capacity [192;193]. Interestingly, CX_3CR1 has been also found to be expressed by pro-inflammatory monocytes that are expanded in AS patients [194]. The production of CX_3CL1, the ligand of CX_3CR1, is induced by inflammatory stimuli in several cell types including osteoblasts. Moreover, CX_3CL1 expression has been found to be upregulated in gut, synovium and bone marrow (BM) of AS patients [194].

In line with the literature [154], our data show that CD8+CCR4+ T-cells were not cytotoxic in HD.

According to the increased percentage of CX_3CR1+ T-cells, however, CD8+CCR4+ T-cells acquired cytotoxic functions in axSpA, in particular in patients with active disease. In parallel, the production of IL-2, an interleukin that regulates inflammatory responses and cell proliferation, was decreased in patients with active disease.

The key functional markers expressed by CD8+CCR4+ T-cells are summarized in Table 9.

	HD (Mean ± SEM)	**axSpA** (Mean ± SEM)	**RA** (Mean ± SEM)
CD8+CCR4+ PD1+	5.47 ± 0.53	**12.84 ± 1.14 *****	5.73 ± 1.44
CD8+CCR4+ CCR5+	3.20 ± 0.68	**26.13 ± 3.36 ******	**17.42 ± 3.92 ****
CD8+CCR4+ Ki67+	0.42 ± 0.08	**0.98 ± 0.11 ****	**3.06 ± 0.32 ******
CD8+CCR4+ CCR6+	24.94 ± 2.44	*16.15 ± 1.48* ***	14.36 ± 1.63
CD8+CCR4+ CX_3CR1+	9.51 ± 2.77	**31.47 ± 8.85 ***	42
CD8+CCR4+ PRF+GRZ+	0.79 ± 0.18	**13.10 ± 4.74 *****	

Table 9. Main findings in CD8+CCR4+ T-cells.

In **bold** significantly increased, in *italics* significantly decreased.

HD (Healthy Donor), axSpA (axial Spondyloarthritis), RA (Rheumatoid Arthritis), PD-1 (Programmed cell Death protein 1), GRZ (Granzyme), PRF (Perforin), IL (Interleukin)

It is noteworthy that the transcriptome analysis performed on axSpA patients and HD indicated that CD8+CCR4+ might play a role in bone remodeling. In fact, up-regulated genes were enriched in pathways related to bone mineralization and down-regulated genes were enriched for pathways related to positive regulation of osteoclast differentiation. Interestingly, CD8+CCR4+ T-cells stimulated with PMA and ionomycin were able to produce and release TNF and IL-8, two cytokines also involved in osteoclastogenesis. In particular, IL-8 release was higher in axSpA patients. These data indicate that CD8+CCR4+ T-cells have a role in bone metabolism, specifically they could inhibit the osteoclasts differentiation and stimulate the mineralization phase, but after a generic stimulation they would be able to release TNF and IL-8, promoting neutrophils recruitment and osteoclasts differentiation.

4.2 Conclusion

Taking advantage of a multi-color flow-cytometric analysis of circulating lymphocytes, we found an altered proportion of naïve and memory CD4+ and CD8+ T-cells in axSpA and RA patients, and a skew in T-cell subset ratios in favor of CD8+ T-cells expressing the chemokine receptor CCR4 in axSpA. There is evidence of an involvement of CD8+ T-cells in cartilage destruction has been reported [88;155], but little is known about the role of CD8+ subsets in the pathogenesis of axSpA [143].

We found that circulating CD8+CCR4+ T-cells are effector memory cells that in axSpA show a proliferative phenotype and express marker of late activation. These features are of particular interest, since total CD8+ T-cells in axSpA do not display a similar phenotype, thus suggesting an antigen-driven proliferation of the CCR4+ T-cell subset. An oligoclonal expansion of circulating CD8+ T-cells has been described in axSpA [195], nonetheless a common or a specific tissue antigen giving rise to the amplification of CD8+ T-cells carrying a specific T_CR has not been identified.

Thanks to the expression of several homing markers, the CD8+CCR4+ T-cell subset we identified has the ability to recirculate through several inflammatory districts involved in axSpA.

We show that this subset releases TNF and IL-8 upon PMA and ionomycin stimulation, two cytokines that are found to be increased in the sera of axSpA patients. Noteworthy and differently from what described in allergy [196], these cells acquire a cytotoxic phenotype in axSpA as demonstrated by the increased production of GRZ and PRF, by the co-expression of the CX_3CR1 receptor and a parallel decrease in IL-2 production.

Moreover, the transcriptome analysis of circulating CD8+CCR4+ T-cells indicate the possible involvement of these cells in bone remodeling, by promoting bone mineralization and inhibiting osteoclast differentiation.

Taken together our data suggest that CD8+CCR4+ T-cells, recirculating in inflammatory sites, might exert a pathogenic role in axSpA through multiple mechanisms of action. In particular, we propose that CD8+CCR4+ T-cells release cytotoxic mediators, molecules involved in bone remodeling and pro-inflammatory cytokines, which elicit tissue damage and favor new bone formation and the recruitment of different leukocytes at sites of inflammation.

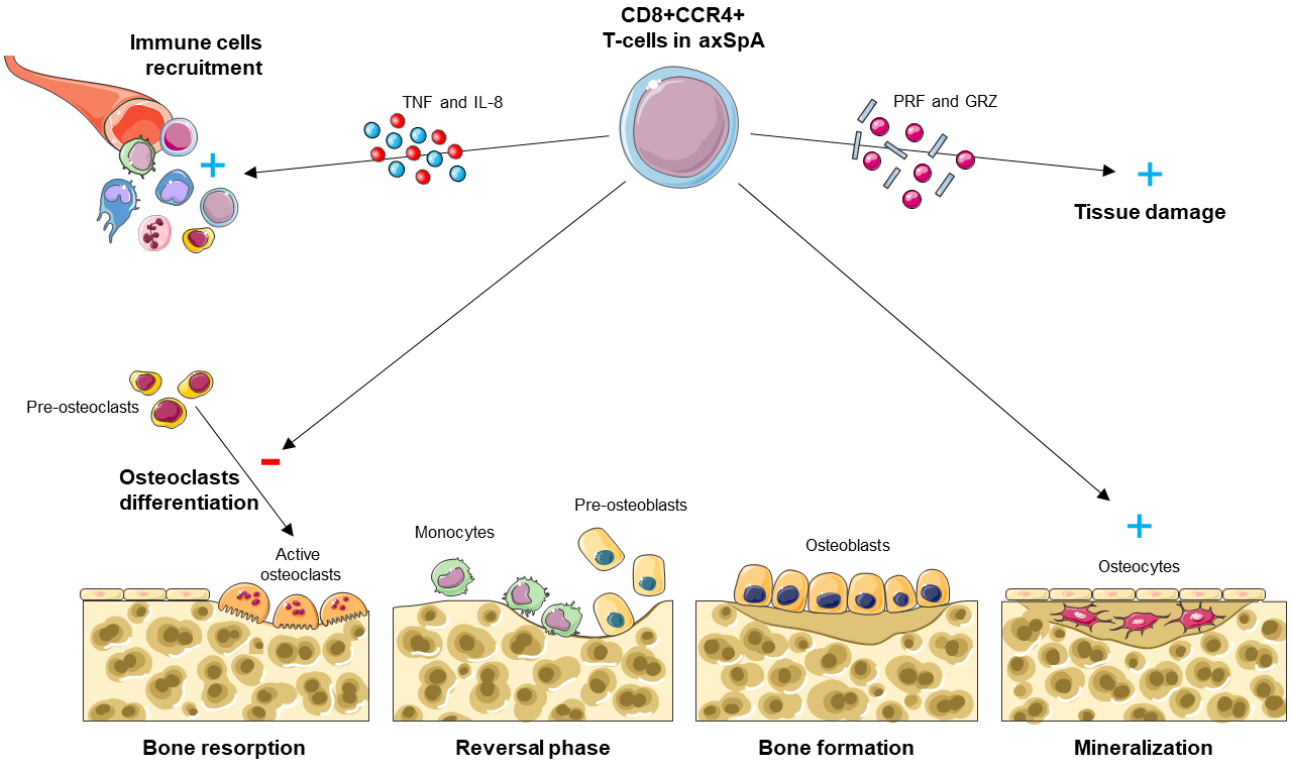

Figure 17. Suggested mechanisms of action of CD8+CCR4+ T-cells in axial spondyloarthritis.

4.3 Future prospects

Our study calls for a deeper characterization of T-cells and their crosstalk with immune cells that can reach inflamed joints/enthesis, in order to understand their role in axSpA development and perpetuation.

In axSpA a significantly lower diversity of T_CR repertoire is observed in peripheral blood. In addition, this repertoire is more restricted in patients with severe disease, suggesting that the T_CR repertoire diversity might be associated with the clinical severity of the disease [197]. A restricted T_CR repertoire results from T-cell clonal expansions. Our data indicate that CD8+CCR4+ T-cells in axSpA patients are activated and proliferative, and it is tempting to speculate that this phenotype is due to an antigen-driven stimulation. Therefore, the analysis of the T_CR repertoire in CD8+CCR4+ T-cells could give an indication on the clonal diversity of this T-cell subset and confirm our hypothesis.

The transcriptome signature of these cells reveals their possible involvement in bone remodeling; besides, the effect of CD8+CCR4+ T-cells in osteoclasts differentiation and in bone mineralization can be dissected by *in vitro* co-culture studies.

Alterations in several innate immune-related pathways have been characterized in axSpA [198], indicating the importance of the innate immune system in this inflammatory disease.

For this reason, it is also important to investigate the interaction between this subset of T-cells and the innate immune system. We found that circulating CD8+CCR4+ T-cells from axSpA patients, can produce large amount of IL-8, which in turn could induce neutrophil recruitment into inflammatory sites. In axSpA patients, the percentage of circulating neutrophil expressing receptor activator of nuclear factor kappa-B ligand (RANKL) is higher when compared to HD [199], indicating their activation and their capability to promote osteoclastogenesis [200;201].

Furthermore, Ciccia et al. showed that pro-inflammatory CX_3CR1 monocytes are able to sustain the activation of type 3 innate lymphoid cells (ILC3) [194]. ILC3 are expanded in gut, peripheral blood, synovial fluid and BM of axSpA patients [202] and their activation is sustained by CX_3CR1+ monocytes through the production of IL-23 and TL1A. Since we found an increased percentage of CX_3CR1 positive cells in CD8+CCR4+ T-cells, it would be interesting also to evaluate their possible interaction with ILC3.

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Chapter 5: Appendix

5.1 List of abbreviations

A

Abs	Antibodies
AF	Alexa Fluor
ANTXR	Anthrax Toxin Receptor
APC	Allophycocyanin
APC	Antigen Presenting Cell
AS	Ankylosing Spondylitis
ASAS	Assessment of Spondyloarthritis international Society
ASDAS	Ankylosing Spondylitis Disease Activity Score
axSpA	axial Spondyloarthritis

B

BM	Bone Marrow
bp	base pair
BV	Brilliant Violet
β2m	β2-microglobulin

C

CBA	Cytometric Bead Array
CLA1	Cutaneous Lymphocyte Antigen1
CRP	C-Reactive Protein
CTL	Cytotoxic T-Lymphocytes
Cy	Cyanine

D

DAS28	28-joint Disease Activity Score
DMARD	Disease Modifying Anti-Rheumatic Drug

E

EAM	Extra-Articular Manifestation
ELISA	Enzyme-Linked Immunosorbent Assays
ER	Endoplasmic Reticulum
ERAD	Endoplasmic-Reticulum-Associated protein Degradation
ERAP	Endoplasmic Reticulum Aminopeptidase
ESR	Erythrocyte Sedimentation Rate

F

FasL	Fas Ligand
FBS	Fetal Bovine Serum
FITC	Fluorescein Isothiocyanate
G	
GPCR	G Protein-Coupled Receptors
GRZ	Granzyme
GTP	Guanoside Triphosphate
GWAS	Genome-Wide Association Studies
H	
HD	Healthy Donor
HLA	Human Leukocyte Antigen
5HPF	5 high power fields
I	
IBD	Inflammatory Bowel Disease
ICAM-1	Intracellular Adhesion Molecule 1
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ILC3	Type 3 innate lymphoid cells
K	
KIR	natural Killer cell Immunoglobulin-like Receptor
M	
MDC	Macrophage Derived Chemokine
MHC	Major Histocompatibility Complex
MRI	Magnetic Resonance Imaging
MSC	Mesenchymal Stem Cell
N	
NK	Natural Killer
NSAID	Nonsteroidal Anti-Inflammatory Drug
nr-axSpA	non-radiographic axial Spondyloarthritis
O	
OMERACT	Outcome Measures in Rheumatology

P

PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate-Buffered Saline
PE	Phycoerythrin
Pen Strep	Penicillin Streptomycin
PerCP	Peridinin Chlorphyll Protein
PFA	Paraformaldehyde
PMA	Phorbol 12-Myristate 13-Acetate
PRF	Perforin
PsA	Psoriatic Arthritis

R

RA	Rheumatoid Arthritis
RANKL	Receptor activator of nuclear factor kappa-B ligand
ReA	Reactive Arthritis
rMFI	relative Mean Fluorescence Intensity
RRP	Receptor Recognition Pattern
RUNX	Runt-related transcription factor

S

SLO	Secondary Lymphoid Organ
SNP	Single-Nucleotide Polymorphism
SpA	Spondyloarthritis
STAT	Signal Transducer and Activator of Transcription

T

TARC	Thymus and Activation Regulated Chemokine
T_C	Cytotoxic T-cell
T_{CM}	Central Memory T-cell
TcR	T-cell Receptor
T_{EM}	Effector Memory T-cell
T_{EMRA}	Effector Memory T-cell RA
TGF-β	Transforming Growth Factor-β
T_H	Helper T-cell
T_N	Naïve T-cell
TNF	Tumor Necrosis Factor
TNFi	Tumor Necrosis Factor inhibitor

TNFSF	Tumor Necrosis Factor Superfamily
Treg	Regulatory T-cell

U

UDI	Unique Dual Indices
UPR	Unfolded-Protein Response
uSpA	undifferentiated Spondyloarthritis

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