Hypermotility of CD8$^+$ T cells in Ankylosing Spondylitis

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Summary

Ankylosing Spondylitis (AS) is a debilitating and progressive inflammatory rheumatic disease representing the prototype of Spondyloarthropathies (SpA). It affects the axial spine but may also involve extra-articular sites. The most important factor associated to AS is the Human Leucocyte Antigen (HLA)-B27 gene, encoding for a HLA class I molecule, whose main role is to present antigens to $CD8^+$ T lymphocytes. GWAS (Genome Wide Association Studies) studies have identified other genes potentially implicated in the development of AS which regulate the functions of $CD8^+$ T lymphocytes. Taking into account these observations, it is reasonable to investigate the contribution of this cell population in the maintenance of the inflammatory state in AS patients. In this work we have analysed the chemotactic properties of $CD8^+$ T lymphocytes. Interestingly, we have observed an intrinsic hypermotility of $CD8^+$ T cells of patients with AS, which extended to patients with PsA, another disorder of the SpA cluster, in absence of chemotactic stimuli compared to Healthy Donors (HD) and patients with Rheumatoid Arthritis (RA). Such spontaneous migration would appear to be related to a "senescent/inflammescent" phenotype in which there is evidence of CD28 loss and expression of CD57 markers as well as shortening and attrition of telomeres. The future objective of this work will be the transcriptome analysis and the functional characterization of this cellular population to assess the cytolytic activity (perforin, granzyme B and granulysin production) and the secretion of pro-inflammatory cytokines (IFNγ, TNFα, IL17).

Synopsis

This work aimed to define the migratory properties of $CD8^+$ T lymphocytes in the context of Ankylosing Spondylitis (AS), the most common inflammatory autoimmune disease belonging to the cluster of Spondyloarthropathies (SpA). Lymphocyte migratory events are important in maintaining the homeostasis of the organism; in particular, each immune cell moves to specific sites through a complex system consisting of chemokines, receptors and adhesion molecules. Altered trafficking of lymphocytes could contribute to the development of different pathologies, such as autoimmune diseases, chronic inflammatory diseases and cancer. Our hypothesis is that pathogenic $CD8^+$ T cells, moving/migrating to and from several inflamed sites, depicting the clinical picture of AS, could contribute to the maintenance and the progression of inflammation. Indeed, the disease mainly affects the axial skeleton and the sacroiliac joints, but the inflammation can also extend to other extra-articular sites such as the skin (psoriasis), the eyes (uveitis) and the intestine (IBD). Moreover, the involvement of $CD8^+$ T cells is supported by the genetic association studies. In fact, besides the strongest risk factor, the Human Leucocyte Antigen (HLA)-B27, involved in the presentation of (viral and/or self) peptides to $CD8^+$ T lymphocytes, GWAS (genome wide association studies) studies have disclosed several other factors including ERAP1 and 2, that shape the peptidome presented by HLA-I molecules and RUNX3, TBX21, ZMIZ1, EOMES which modulate the $CD8^+$ T cell development, differentiation, function and counts. All together these AS-associated genes point out the antigen presentation to $CD8^+$ T cells and the functions of these cells as putative pathogenic events in the occurrence of the disease. For this study, a cohort of patients affected by AS has been recruited and compared with patients with Rheumatoid Arthritis (RA), an unrelated chronic inflammation condition, patients with Psoriatic Arthritis (PsA), another disease belonging to the SpA group and Healthy Donors (HD). Starting from Peripheral Blood Mononuclear Cells (PBMC), through positive selection, we isolated $CD8^+$ and $CD4^+$ T cells that we used to perform migration experiments. $CD8^+$ T lymphocytes were stimulated with CXCL9, CXCL10, CXCL11, three inflammatory chemokines sharing the CXCR3 receptor; CXCL12, a homeostatic chemokine ligand of CXCR4 receptor and CCL20, a chemokine inducing the homing to mucosal sites and ligand of CCR6. These migration assays showed no relevant differences in the absolute number of migrated cells among the four cohorts, meaning that all cells are responsive to the specific chemoattractant stimuli. However, the power of the migration if expressed as Migration Index (MI) was lower in $CD8^+$ T cells from AS and PsA patients compared to HD. Surprisingly, these chemotaxis data were profoundly influenced by the basal motility of $CD8^+$ T cells. Indeed, $CD8^+$ T cells derived from AS and PsA patients displayed a spontaneous hyper-migratory potential in absence of any chemokine stimulation. Interestingly, this intrinsic hypermotility has proved to be a hallmark of $CD8^+$ T cells not shared by the $CD4^+$ T counterpart. The immune phenotype analysis of $CD8^+$ T cells in terms

of naïve/memory subsets indicated the Effector Memory (especially in patients with AS) and TEMRA (*T Effector Memory Re-expressing CD45RA*) as the lymphocyte subsets mostly implicated in this spontaneous hypermotility. Assuming that the enrichment of these two subsets in the migrated cells could be related to an accelerated/premature T cell senescence due to the immunological stress dictated by the chronic inflammation, we investigated the senescent profile of migrated versus non-migrated cells using CD28 and CD57 markers. In fact, the combination of these two markers is classically exploited to identify four $CD8^+$ T subpopulations: non-activated/early-activated $CD28^+CD57^-$, activated $CD28^+CD57^+$, activated/early-senescent $CD28^-CD57^-$ and terminally differentiated-senescent-like $CD28^-CD57^+$ T cells. Interestingly, this spontaneous hypermigration was pertained to a senescent/overstimulated cell phenotype since the majority of migrated cells were $CD8^+$ $CD28^-$ $CD57^+$ T lymphocytes. Based on the tempting idea that these hyper-migrating T cells possessing senescence features could be pathogenic because of a high cytotoxic potential, we analysed the expression of fractalkine receptor CX3CR1 which, when highly expressed, identifies a population of long-lived effector memory cells with cytolytic properties. As expected, we found an enrichment of $CD8^+$ T lymphocytes, CX3CR1 positive, in the migrated cells and a trend of higher expression of this receptor in the total $CD8^+$ T cells from patients compared to HD. To further define the senescence profile of such $CD8^+$ T cells, we performed real-time PCR analysis to investigate the telomere length in migrated versus non-migrated cells. Accordingly to their terminally differentiated-senescent immune-phenotype, migrated $CD8^+$ T cells possessed shorter telomeres compared to non-migrated ones. Afterwards, we considered another aspect related to cell senescence, i.e. the telomere DNA dysfunction that we analysed by Immunofluorescence (IF)-Fluorescence in situ hybridization (FISH) analysis. Interestingly, the migrated $CD8^+$ T lymphocytes displayed a higher telomeric attrition compared to non-migrated counterpart and, of note, $CD8^+$ T cells derived from AS and PsA patients presented a higher number of telomeric damages per nucleus compared to HD, suggesting a premature senescence in the migrated cells especially from patients. Overall, this study highlighted a subset of $CD8^+$ T cells endowed with spontaneous hypermotility, enriched in $CD28^-$ $CD57^+$ $CX3CR1^+$ effector memory/TEMRA cells and showing senescence hallmarks like as telomere shortening and damage. It can be speculated that these cells are the outcome of a chronic antigen stimulation and that they could invade the tissues creating damages with their high cytotoxic potential in SpA patients.

In conclusion, these data suggest a link between lymphocyte basal hypermotility, cellular senescence and chronic inflammation in a cluster of human autoimmune diseases. In the future, it will be interesting to establish a cause-effect relation between these processes.

Aim of the work

Given the genetic background of AS which points to an involvement of pathogenic $CD8^+$ T lymphocytes and the clinical picture affecting multiple sites, the focal point of this work has been to assess the contribution of this subset in spreading and sustaining the inflammation in AS patients. . To this aim, patients affected by AS, Psoriatic Arthritis (PsA), another disease of SpA cluster, Rheumatoid Arthritis (RA), as additional cohort of an unrelated chronic inflammatory disease and Healthy Donors (HD) have been enrolled. The migratory properties of $CD8^+$ T lymphocytes have been tested towards a panel of chemokines involved in general homing (CXCL12), in the recruitment of T lymphocytes into inflamed sites (CXCL9, CXCL10 and CXCL11) and in the intestinal and skin mucosa (CCL20). The interesting feature emerging from this study has been an intrinsic basal hypermotility that characterizes exclusively the $CD8^+$ T lymphocytes of subjects affected by SpA (AS and PsA) which does not extend to $CD4^+$ T cells. The first goal of the current work has been the characterization of the subset involved in the basal hypermotility discriminating the cells in terms of naïve, central memory, effector memory and TEMRA subsets. The results obtained prompted us to deepen the immunophenotype analysis by assessing the cellular senescence by checking CD28 and CD57 markers and the inflammescent phenotype through the analysis of the CX3CR1 expression. To establish whether the $CD8^+$ T cell hypermotility was related to a senescent/over stimulated phenotype, an analysis of telomere length and telomere dysfunction has been performed.

In conclusion, this study has revealed an enrichment of terminally differentiated $CD8^+$ T cells able to move in absence of chemotactic stimuli in patients with chronic inflammatory autoimmune disorders of SpA group, like as AS and PsA. These spontaneously hypermotile $CD8^+$ T cells are characterized by a senescent/inflammescent phenotype with shorter telomeres and telomere attrition. Further analyses (RNAseq and time lapse microscopy) are ongoing to gain insight into the relevance of these cells in the pathogenesis of SpA.

Introduction

Ankylosing Spondylitis (AS) is a chronic inflammatory disease that mainly affects the axial skeleton inducing to a progressive vertebral ankylosis, but the clinical picture of patients may present other sites of inflammation: skin, eyes, gut (*Raychaudhuri and Deodhar, 2014*). The causes of the disease are still unknown but several genetic association studies prompt us to direct research into specific fields of investigation. The strongest susceptibility factor associated with AS is the Human Leucocyte Antigen (HLA)-B27, an HLA class I gene involved in the antigen presentation to $CD8^+$ T lymphocytes (*Lin and Gong, 2017*). In addition to HLA-B27, GWAS studies (Genome-Wide Association Study) have highlighted other AS-associated factors: TBX21, EOMES, IL7R, RUNX3 and ZMIZ1, all of which are involved in the differentiation, activity and frequency of $CD8^+$ T lymphocytes (*Vecellio et al., 2018*). Such studies suggest that this population is potentially involved in the pathogenesis of AS (*Cortes et al., 2013*). $CD8^+$ T lymphocytes are usually classified into four main subpopulations: naïve, central memory (T_{CM}), effector memory (T_{EM}) and T_{EMRA}. Naïve T cells are non-activated cells that have never encountered antigens. They circulate in peripheral blood and secondary lymphoid tissues and are characterized by the expression on the membrane of the following molecules: CD27, CD28, CD45RA, CD62L and CCR7 (*Carvalheiro et al., 2013*). T_{CM}s are $CD45R0^+$ memory cells that constitutively express CCR7 and CD62L, two receptors required for extravasation through the high endothelium venules and necessary for the migration to the T areas of the lymphoid organs. They mainly produce IFN-γ, IL-2, TNF-α and are commonly found in the lymph nodes and in the peripheral circulation. T_{EM}s are cells that have lost the constitutive expression of CCR7, they are heterogeneous for CD62L expression, and they show characteristic production of a set of chemokines and adhesion molecule receptors required for the homing in inflamed tissues (*Sallusto et al., 2004*). T_{EMRA}s are a subpopulation of effector memory cells expressing CD45RA, CCR5 and CXCR3. They show a robust cytotoxic phenotype with abundant release of perforins and granzymes, but they have a poor proliferative capacity, consistently with their terminally differentiated state (*Pang et al., 2012*). Following the activation process of $CD8^+$ T lymphocytes, they differentiate into cytotoxic (CTL) and memory lymphocytes.

Resolute conditions of inflammation take to an increase of the immune system aging, resulting in a state of immuno-senescence (*Tedeschi et al., 2022*). The cellular senescence or the persistent antigenic stimulation of $CD8^+$ T lymphocytes leads to the progressive downregulation on their cell surface of CD28 (*Pangrazi et al., 2020*). The downregulation of this marker is accompanied by the upregulation of CD57, described as a marker of terminally differentiated cells (*Brenchley et al, 2003*). $CD8^+$ $CD28^-$ $CD57^+$ T lymphocytes have been extensively studied in the case of chronic viral infections, such as in the case of HIV, HCMV, EBV, hepatitis C virus infections where an increase of these cells has been observed as well

as in the case of autoimmune diseases, including multiple sclerosis, ankylosing spondylitis and rheumatoid arthritis (*Strioga et al., 2011*). The expression of CD57 on the surface of $CD8^+$ T cells correlates with the production of cytotoxic molecules such as granzyme A, granzyme B and perforin, suggesting that despite being a senescent population it still possess cytotoxic features (*Chattopadhyay et al., 2009*). Downregulation of CD28 is associated with lower replication capacity and telomeric shortening (*Larbi et al., 2014*) but this is not a rule. Indeed, in CMV^+ and HIV^+ subjects a population of $CD8^+$ T_{EM} was identified and defined as "inflammescent", characterized by a high expression of the fractalkine receptor CX3CR1, $CD57^+$ and $CD28^-$ which, although senescent is able to proliferate, *in vitro*, under stimulation by the inflammatory cytokine IL-15. (*Morris et al., 2020*).

Probably, $CD8^+$ CD28- T cells undergo to a limited rate of cellular cycles due to the activation of other co-stimulation pathways that may occur during persistent antigenic stimulation such as chronic infections, autoimmune diseases and tumors (*Tedeschi et al., 2022*). CX3CR1, as mentioned before, is the receptor of fractalkine (also known as CX3CL1), a molecule existing in the soluble form and as transmembrane protein expressed on different cells type as well as endothelial cells, intestinal endothelial cells, osteoclasts, astrocytes (*Kim et al., 2011*). The receptor is expressed on the membrane of many immunological cells and the axis CX3CR1-CX3CL1 is very important for the recruitment and the extravasation of lymphocyte during inflammation (*Tanaka et al., 2020*). Effective immune surveillance is based on the correct differentiation of T cells and on lymphocytic migratory events through the bloodstream, lymphoid organs and non-lymphoid tissue (*Fu et al., 2016*). The mechanisms underlying the lymphocyte movement are finely regulated by adhesion molecules, their corresponding ligands, chemokines and chemokine receptors (*Ebert et al., 2005*). The circulation of T cells is defined by stochastic movements and randomly oriented trajectories. Two patterns of motility have been identified: random diffusion-type movement (Brownian-type), adopted by activated T lymphocytes that migrate to peripheral tissues; super-diffuse random movement (Levy-type), adopted by naïve T lymphocytes during the recirculation between blood and secondary lymphoid organs. In addition, T cells in some circumstances and for short periods can also exhibit ballistic migration: they can follow an almost straight trajectory (*Krummel et al., 2016*). Lymphocyte trafficking is of fundamental importance in the pathogenesis of various autoimmune diseases. The recruitment of autoreactive lymphocytes into inflamed tissues is characterizing for several persistent autoimmune conditions. Therefore, a better understanding of the molecular events involved in cell migration is required, since, as mentioned above, these are essential for lymphocyte homing (*Comerford et al., 2014*).

Results

In order to assess a putative role of $CD8^+$ T cells in sustaining the inflammation in patients with AS, we investigated their migratory properties by comparison to a cohort of Healthy Donors (HD) and patients affected by Rheumatoid Arthritis (RA) and Psoriatic Arthritis (PsA), as additional references of chronic inflammatory conditions. Clinical and demographic data of patients recruited in this study are summarized in Table 1.

	AS	RA	PsA	HD
Sex (M/F)	71 M; 29 F	15 M; 41 F	19 M; 17 F	30 M; 30 F
Age (yrs)	51 ± 1,4	56 ± 1,9	57 ± 2,9	49 ± 2,1
HLA-B27+ (%)	80%	16%	31%	2%
CRP	5 ± 1,3	2 ± 0,6	3 ± 0,8	n / a
ESR	20 ± 1,9	22 ± 3	14 ± 2,5	n / a
BASDAI	3 ± 0,26	n / a	3 ± 0,3	n / a
DAS28	n / a	3 ± 0,2	n / a	n / a
DAPSA	n / a	n / a	10 ± 2,1	n / a
Biologics (%)	76%	79%	86%	n / a
NSAIDs (%)	10%	0%	0%	n / a
DMARDs (%)	10%	3%	14%	n / a
None (%)	8%	5%	6%	n / a

Table 1. Clinical data of patients

*Mean ± SEM

AS, Ankylosing Spondylitis; RA, Rheumatoid Arthritis; PsA, Psoriatic Arthritis; HD, Healthy Donors; CRP: C-reactive protein; ESR, Erythrocyte Sedimentation Rate; BASDAI, Bath Ankylosing Spondylitis Disease Activity Rate; DAS28, Disease Activity Score 28; DAPSA, Disease Activity in PSoriatic Arthritis; NSAIDs, Non-Steroidal Anti-Inflammatory Drugs; DMARDs, Disease-Modifying Anti-Rheumatic Drugs; None, non in therapy; n/a, not applicable.

Frequencies of CD3+, CD3+ CD4+ and CD3+ CD8+ T lymphocytes in PBMC derived from patients with AS, RA, PsA and HD

Firstly, we analysed the frequency of $CD3^+$, $CD3^+$ $CD4^+$ and $CD3^+$ $CD8^+$ T cells in PBMC of 70 AS,47 RA, 32 PsA patients and 49 HD by flow cytometry analysis. Our results showed that

the percentage of $CD3^+$ T lymphocytes in AS patients was lower in respect to that of RA and PsA patients (p value < 0.05 and p value < 0.01 respectively) (Fig.1). Moreover, the percentage of $CD3^+$ $CD4^+$ T cells was higher in RA patients than in HD (p value < 0.05) while no statistically relevant difference in the percentage of $CD3^+$ $CD8^+$ T cells was found among the four cohorts analysed (Fig.1).

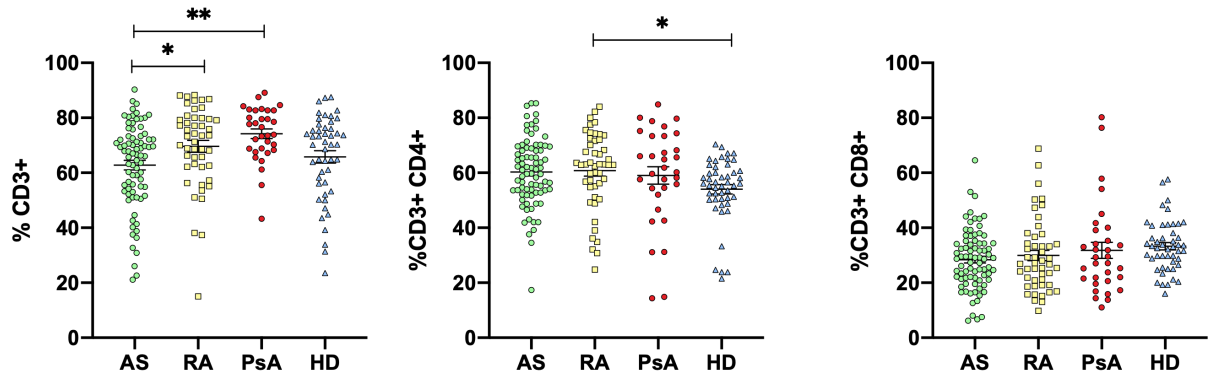

Figure 1. Percentage of $CD3^+$, $CD3^+$ $CD4^+$, $CD3^+$ $CD8^+$ T cells in PBMC from AS, RA, PsA patients and HD. The first panel on the left shows the percentage of $CD3^+$ T cells in PBMC in the four cohorts. The panel in the middle shows the percentage of $CD3^+$ $CD4^+$ T cells, while the third panel shows the percentage of $CD3^+$ $CD8^+$ T lymphocytes. Statistically significant differences emerged in the percentage of $CD3^+$ T cells between AS and RA patients and AS and PsA patients and in the percentage of $CD3^+$ $CD4^+$ T cells between RA patients and HD (Kruskal-Wallis test *p value < 0.05; **p value < 0.01).

$CD8^+$ T cells from AS patients are able to migrate upon specific chemokine stimulation although with a lower power compared to HD

Starting from PBMC by means of positive selection, we isolated $CD8^+$ or $CD4^+$ T cells that we used to perform migration experiments in presence or absence of chemokine stimuli.
We treated $CD8^+$ T cells from subjects of the four cohorts above described with three inflammatory chemokines sharing CXCR3 as receptor: CXCL9 at 100 nM (42 AS, 29 RA, 20 PsA patients and 26 HD); CXCL10 at 100 nM (40 AS, 28 RA, 22 PsA patients and 25 HD) and CXCL11 at 300 nM (30 AS, 21 RA, 13 PsA patients and 22 HD) (Fig.2). Moreover, we analysed the migration of $CD8^+$ T cells induced by CXCL12 (100 nM), a homeostatic chemokine, ligand of CXCR4 receptor in 39 AS, 22 RA, 14 PsA patients and 30 HD and by CCL20 (300 nM), a chemokine inducing the homing to mucosal sites and ligand of CCR6 in 30 AS, 21 RA, 13 PsA patients and 22 HD (Fig.3).

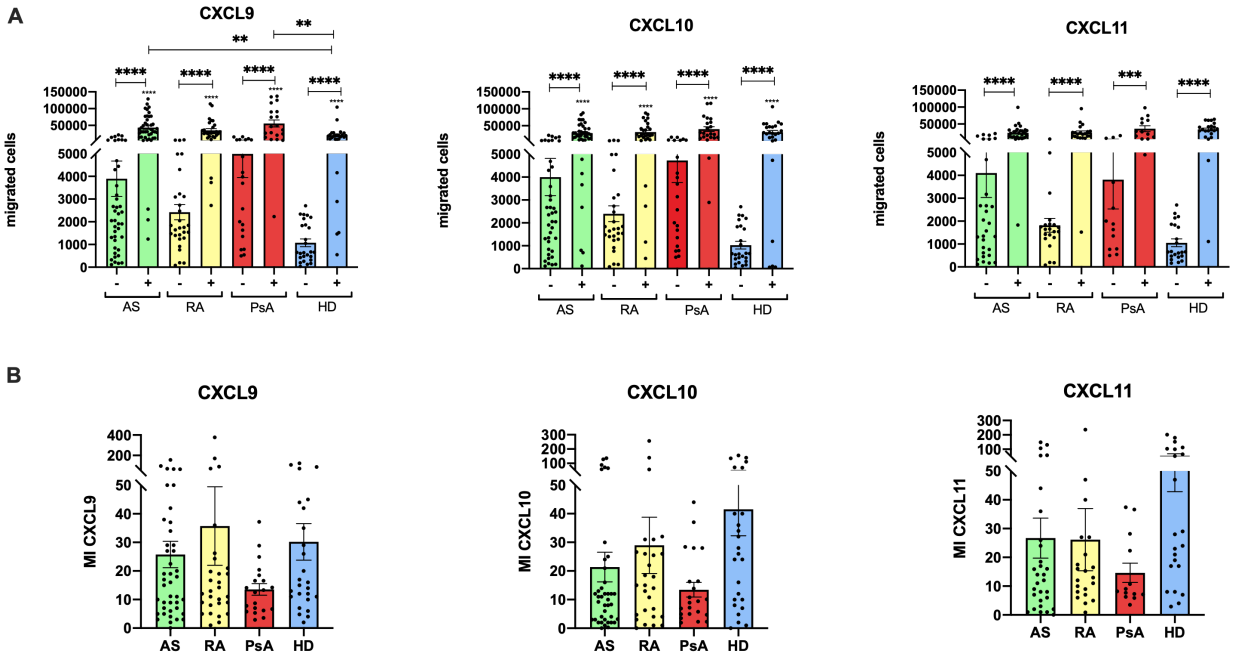

Figure 2. Migration of CD8⁺ T cells upon stimulation with the inflammatory chemokines CXCL9, CXCL10 and CXCL11. A) The upper panels show the number of migrated CD8⁺ T cells in AS, RA, PsA patients and HD with or without CXCL9, CXCL10 or CXCL11 chemokine. In each cohort analysed emerges a significant difference between the basal migration and the migration towards the chemotactic stimulus. The number of migrated cells towards the CXCL9 is higher in AS and PsA patients compared to HD. B) In the lower panels the ability of the cells to migrate towards the selected chemokines is expressed as Migration Index (see materials and methods section). No difference emerges among the four cohorts analysed (Wilcoxon test ***p value < 0.001; ****p value < 0.0001; Kruskal-Wallis test **p value < 0.01).

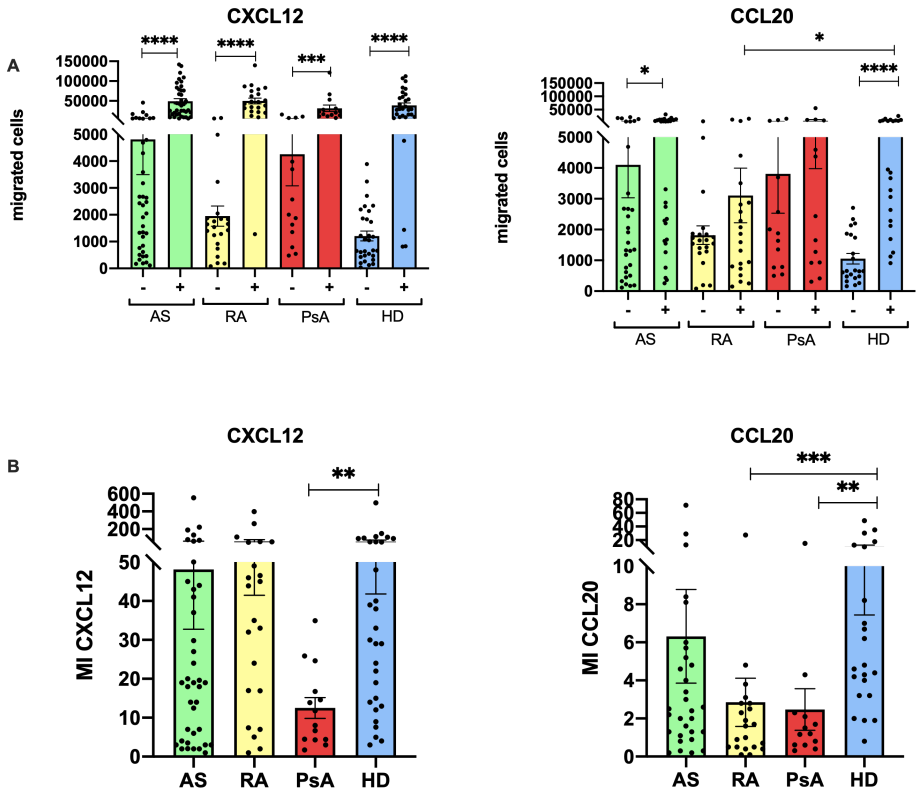

Figure 3. Migration of CD8⁺ T cells upon stimulation with CXCL12 and CXCL20 chemokines. A) The upper panels show the number of migrated CD8⁺ T cells in AS, RA, PsA patients and HD with or without CXCL12 or CCL20 chemokine. In each cohort emerges a statistically significant difference between the basal migration and the migration towards the chemotactic stimulus. B) Lower panels display the Migration Index towards the selected chemokines. The number of migrated cells in RA toward CCL20 is lower than in HD (A). The MI of CD8⁺ T cells from RA and PsA patients towards the CCL20 (B) is lower compared to HD. The MI correlated to CXCL12 chemotaxis is lower in PsA patients compared to HD (Wilcoxon and Kruskal-Wallis test *p value < 0.05; **p value < 0.01; ***p value <0.001; ****p value < 0.0001).

In Figure 2 and 3, the migration of CD8⁺ T cells towards the selected chemokines has been reported both as number of migrated cells (migrated cells normalized on the input) and as capability of the cells to migrate, expressed by the Migration Index (MI) (n° migrated cells with chemokines/n° migrated cells at basal level).

In general, CD8⁺ T cells from all groups were able to move towards the specific chemoattractants as shown by the panels A of Figure 2 and 3. However, the power of the response, expressed as MI, appeared, in several cases, to be lower in the cohorts of patients, especially those affected by AS and PsA (Fig. 2B and 3B).

In particular, considering the absolute number of $CD8^+$ T cells moving towards CXCL9 chemokine, a higher motility of cells from AS and PsA patients compared to HD control group emerged (Fig. 2A). However, when the migration activity was expressed as MI no difference was found among the cohorts and, even, $CD8^+$ T cells from AS and PsA patients resulted to give a lower response to all three inflammatory chemokines (CXCL9, CXCL10 and CXCL11) (Fig. 2B).

Such observation could be extended to the CXCL12 and CCL20 stimulation where a lower MI characterized $CD8^+$ T cells of PsA patients, despite these cells were able to migrate with a comparable cell number in respect to HD. (Fig. 3A-3B). By contrast, $CD8^+$ T cells derived from RA patients were less able to migrate towards CCL20 either in terms of number of migrated cells or MI compared to HD (Fig. 3).

To explain these data, we have taken into account the cell membrane expression of the corresponding chemokine receptors (CXCR3, CXCR4 and CCR6) but no significant correlations have been found (Supplementary Fig. S1).

$CD8^+$ T cells, but not $CD4^+$ T cells, from AS and PsA patients show a high spontaneous motility

Chemotaxis data shown above when expressed in terms of MI, were profoundly influenced by the basal motility of $CD8^+$ T cells. Interestingly, $CD8^+$ T cells from patients with AS and PsA were endowed with a higher basal motility compared to HD. We evaluated the spontaneous motility of $CD8^+$ T cells from 100 AS, 58 RA, 33 PsA patients and 63 HD (Fig.4). Notably, $CD8^+$ T cells of AS and PsA patients were characterized by a pronounced basal hypermotility compared to HD with a statistically significant difference (p value < 0.0001 and p value < 0.001 respectively). No relevant difference from the comparison of CD8+ T cell migration between RA and HD cohorts arised.

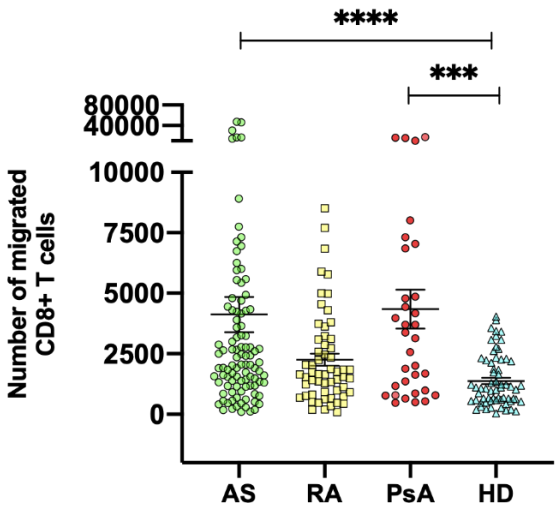

Figure 4. Basal hypermotility of CD8⁺ T cells of AS and PsA patients. In absence of chemotactic stimuli, the migration of CD8⁺ T cells derived from AS patients (n=100) and PsA patients (n=33) is more pronounced than that of CD8⁺ T cells from RA (n=58) (no relevant difference) and HD (n=63) (Kruskal-Wallis test ***p value < 0.001; ****p value < 0.0001).

To assess whether such hypermotility found in patients with AS and PsA was an exclusive feature of CD8⁺ but not of CD4⁺ T subset, we conducted migration experiments to compare the basal migration of CD8⁺ and CD4⁺ T cell using the same cohorts of cases and controls. From these experiments performed on 50 AS, 29 RA, 26 PsA patients and 24 HD, it emerged that this spontaneous hypermotility featured by CD8⁺ T cells of patients with AS and PsA did not extend to CD4⁺ T cells (Fig.5). In parallel, CD4⁺ T cells stimulated with chemokines attractants (CXCL10 and CXCL12) were able to migrate (Fig. S4).

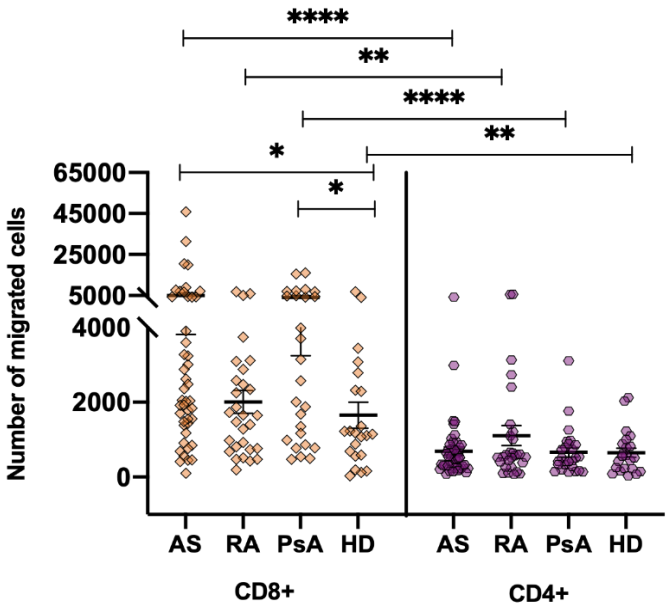

Figure 5. Basal migration of CD8⁺ and CD4⁺ T cells. The figure shows the number of spontaneously migrated CD8⁺ T cells on the left and CD4⁺ T cells on the right in 50 AS, 29 RA, 26 PsA and 24 HD. CD8⁺ T lymphocytes in AS and PsA patients have a higher basal motility than those in HD. In each group of cases and controls the number of migrated CD4⁺ T cells is lower compared to that of migrated CD8⁺ T cells. The motility of CD4⁺ T cells is comparable between the cohorts analysed (Mann-Whitney test, **p value < 0.01; ****p value < 0.0001; Kruskal-Wallis test *p value < 0.05).

Migrated CD8⁺ T cells of AS patients are enriched in Effector Memory and TEMRA subsets

Next, we compared the immune profile of migrated and not migrated CD8⁺ T cells in absence of chemokine stimuli from 51 AS, 26 RA, 22 PsA patients and 30 HD by flow cytometry analysis (Fig.5). To this aim, we considered the expression of two markers, CCR7 and CD45RA, through which it is possible to distinguish four subsets of CD8⁺ T cells: naïve (CCR7⁺/CD45RA⁺), central memory (CCR7⁺/CD45RA⁻), effector memory (CCR7⁻/CD45RA⁻) and TEMRA (CCR7⁻ CD45RA⁺). Analysing the relative frequencies of each subset before and after migration, it emerged, as expected, that naïve cells decreased in migrated CD8⁺ T cells in all cohorts analysed (Fig. 6A); central memory cells decreased in migrated CD8⁺ T cells of AS, RA and PsA subjects (Fig.6B); effector memory cells raised only in migrated CD8⁺ T cells of AS patients (Fig. 6C) and TEMRA cells increased in migrated CD8⁺ T cells of AS, RA and PsA patients (Fig. 6D).

These data suggest that a subset of experienced and terminally differentiated CD8⁺ T cells are able to randomly migrate independently of a specific chemotactic gradient. Therefore, it

could be speculated that these cells fuel the inflammatory state reaching peripheral sites and injuring them by their cytotoxic functions.

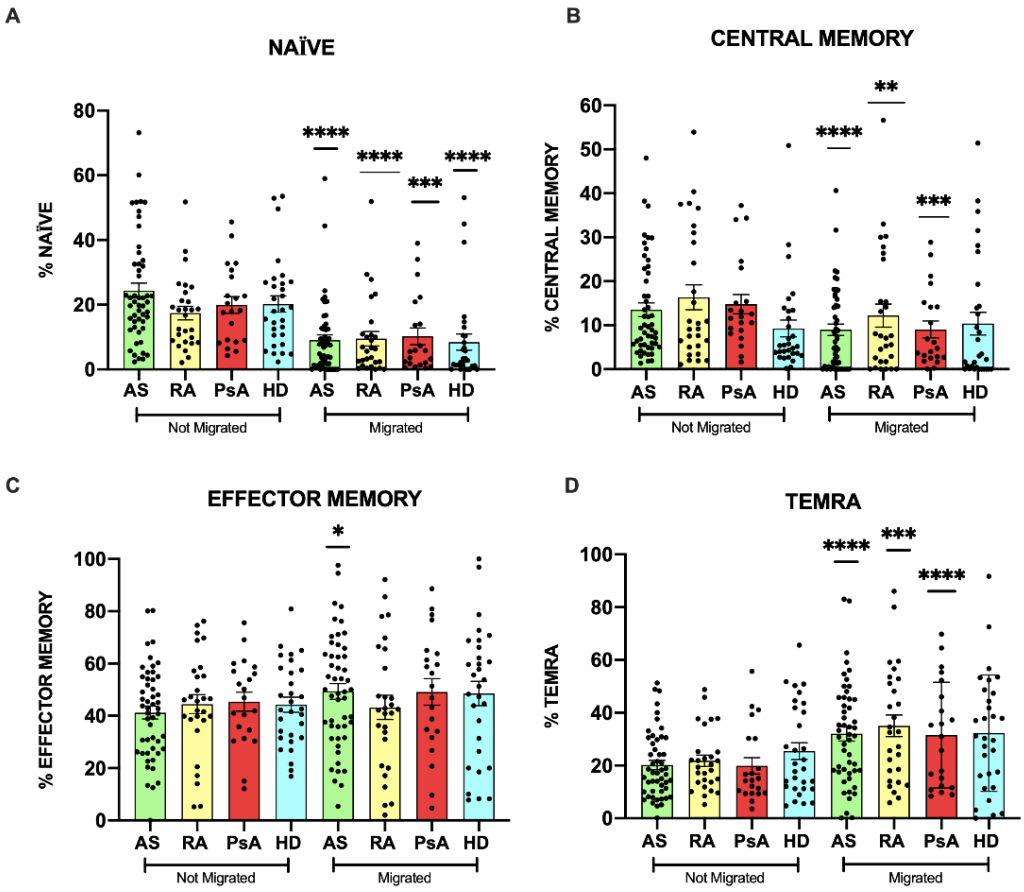

Figure 6. Characterization naïve, central memory, effector memory and TEMRA in not migrated and migrated CD8⁺ T cells. The analysis of migrated and not migrated CD8⁺ T cells in absence of chemotactic stimuli have been tested in 51 AS, 28 RA, 21 PsA individuals and 30 HD. The analysis shows the involvement in the migration of effector memory cells only in AS patients (C) and TEMRA subset in all patients analysed but not in HD. Instead, CD8⁺ T naïve (A) and central memory (B) cells decrease after basal migration, the former in all the cohorts and the latter only in patients with a significant difference (Wilcoxon test migrated vs not migrated cells from the same cohort of individuals, *p value < 0.05; **p value < 0.01, ***p value < 0.001; ****p value < 0.0001).

Migrated CD8⁺ T cells are enriched in CD28⁻CD57⁺subset with features of "inflammescent" cells by expressing CX3CR1 marker

Considering the experienced/differentiated state of the migrated CD8⁺ T cells in terms of naïve/memory immune phenotype, we further dissected the senescent profile of the

spontaneously migrated versus not migrated CD8⁺ T cells in the four cohorts under study, by analysing CD28 and CD57 expression. In fact, these markers are classically exploited to identify four CD8⁺ T cell subpopulations: non-activated/early-activated CD28⁺CD57⁻, activated CD28⁺CD57⁺, activated/early-senescent CD28⁻CD57⁻ and terminally differentiated-senescent-like CD28⁻CD57⁺ T cells. For this set of flow cytometry experiments 51 AS, 32 RA, 28 PsA patients and 21 HD have been analysed (Fig.7). Consistently with the enrichment of effector memory and TEMRA cells, our data clearly showed a decrease of the percentage of CD8⁺ CD28⁺ CD57⁻ in all four cohorts upon migration (Fig.7A) with a parallel increase in the percentage of CD8⁺ CD28⁻ CD57⁺ T cells in migrated cells compared to not migrated ones (Fig.7D). Hence, the CD8⁺ T cells that spontaneously migrate are undoubtedly senescent cells with effector features.

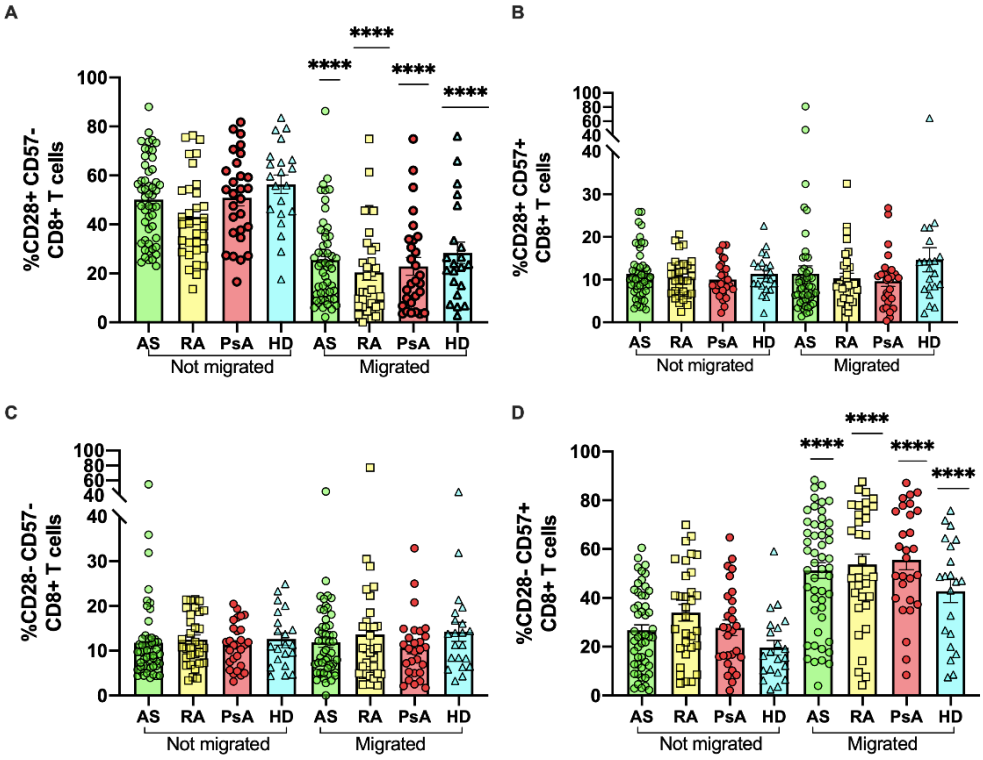

Figure 7. Characterization of migrated versus non-migrated CD8⁺ T cell subsets by CD28 and CD57 as senescence markers. The figure shows the flow cytometry analysis with two markers of CD8⁺ T cells senescence (CD28 and CD57) in 51 AS, 32 RA, 28 PsA patients and 21 HD subjects. CD8⁺ CD28⁻ CD57⁺ T cells, described as senescent, are enriched after the migration in all the cohorts analysed (D). On the contrary, the 'youngest' non-activated subset or at the early stage of activation (CD8⁺ CD28⁺ CD57⁻ T cells) is less frequent after migration in all subjects analysed with statistically significant differences (A). No

significant variation in the frequency of the other two subsets (B and C) has been observed (Wilcoxon test, ****p value < 0.0001).

Furthermore, we analysed the expression of fractalkine receptor CX3CR1 which, when highly expressed, identifies a population of long-lived effector memory cells with cytolytic properties (*Böttcher et al., 2015; Gerlach et al., 2016; Nishimura et al., 2002*), in migrated versus non-migrated $CD8^+$ T cells from 29 AS, 19 RA, 21 PsA subjects and 8 HD (Fig.8). The results shown in Figure 8 point out an enrichment of $CX3CR1^+$ cells in the migrated $CD8^+$ T cell subpopulation with statistically relevant difference between migrated and non-migrated cells in AS, RA, PsA patients (p value < 0.0001) and in HD cohort (p value < 0.01) (Fig.8).

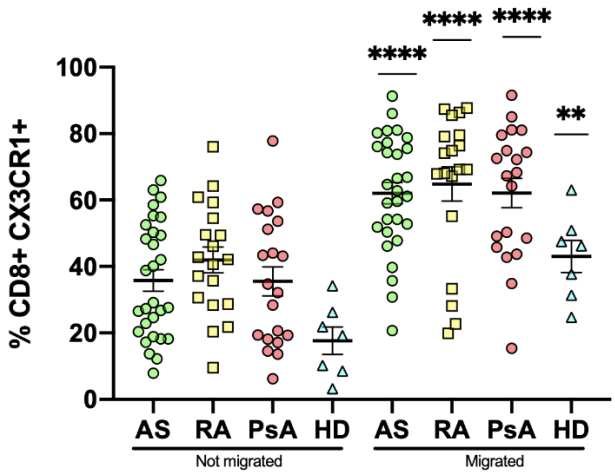

Figure 8. Comparative expression analysis of CX3CR1 in not migrated versus migrated $CD8^+$ T lymphocytes. The graph shows the percentage of non-migrated and migrated $CD8^+$ $CX3CR1^+$ T cells: this cellular subset increases in all the cohorts analysed in the migrated ones. It is possible to observe in the group of not migrated a trend of higher percentage of $CD8^+$ $CX3CR1^+$ T cells in the patients (AS, RA and PsA) compared to HD (Wilcoxon test, **p value < 0.01; ****p value < 0.0001).

We next reconsidered the change in the percentage of $CX3CR1^+$ cells upon migration within the "oldest" subset of $CD28^-$ $CD57^+$ $CD8^+$ T cells (i.e. the terminally differentiated senescent-like phenotype) (Fig.8.1A) and the "youngest" subset of $CD28^+$ $CD57^-$ $CD8^+$ T cells (i.e. non-activated/early-activated phenotype) (Fig.8.1B). We observed a general increase of $CXCR1^+$ cells in the migrated subsets both within the $CD28^-$ $CD57^+$ $CD8^+$ T cells and the $CD28^+$ $CD57^-$

$CD8^+$ T cells of all cohorts analysed. Nevertheless, only for patients with AS and for HD the comparison of migrated with non-migrated cells yielded statistically significant differences (* p value < 0.01; **p value < 0.001) (Fig. 8.1A and 8.1B).

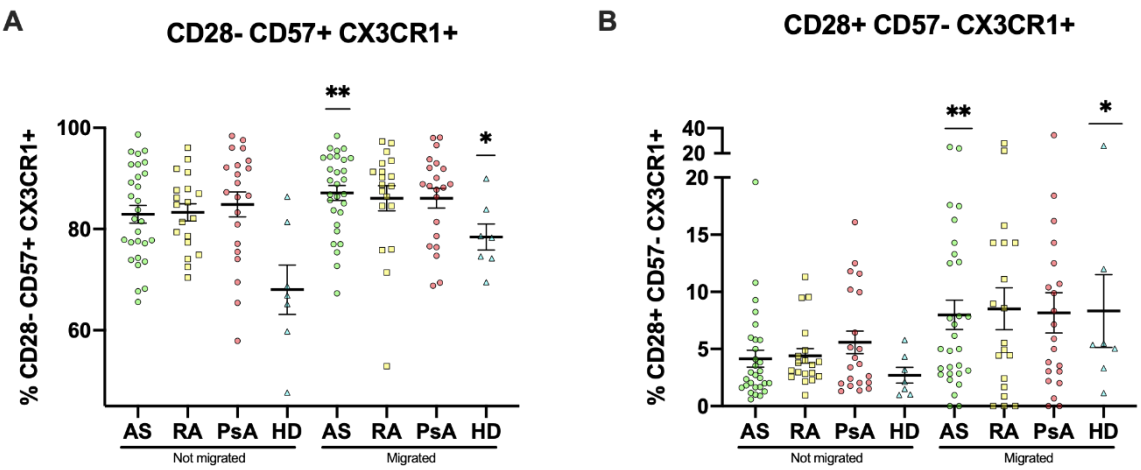

Figure 8.1. Analysis of the "oldest" and the "youngest" subsets expressing CX3CR1. A) The panel shows the percentage of migrated and not migrated $CD8^+$ $CD28^-$ $CD57^+$ T cells expressing CX3CR1 (the oldest subset). B) This panel shows the percentage of migrated and non-migrated $CD8^+$ $CD28^+$ $CD57^-$ T lymphocytes expressing CX3CR1 (the youngest subset). It is noteworthy the different percentage of cell subset in A (a high number of $CD8^+$ $CD28^-$ $CD57^+$ T cells is $CXCR1^+$) in respect to cell subset in B (a small number of $CD8^+$ $CD28^+$ $CD57^-$ T is $CX3CR1^+$). From both panel A and B emerge a trend of $CX3CR1^+$ cell enrichment in migrated $CD8^+$ T cell subsets for all the cohorts analysed. However, the increase of $CX3CR1^+$ $CD28^-$ $CD57^+$ $CD8^+$ T cells and $CX3CR1^+$ $CD28^+$ $CD57^-$ $CD8^+$ T lymphocytes in migrated fraction reaches statistically relevance in AS patients and in HD cohort (Wilcoxon test, *p value < 0.05; **p value < 0.01, respectively).

The "inflammescent" migrating $CD8^+$ T cells display telomere shortening

Our previous data prompted us to take an in-depth look at the senescence profile of spontaneously migrated versus non-migrated $CD8^+$ T cells evaluating the telomere length by Real Time PCR analysis. For this purpose, we extracted the DNA from migrated and not migrated $CD8^+$ T cells from 15 AS, 9 RA, 9 PsA patients and 13 HD (Fig.9). The length of telomeres was reported as T/S ratio indicating the number of copies of telomere repeats (T) on a control gene expressed in single copy (S; β-globin). In line with the observed senescent state, in all groups investigated, the migrated cells were characterized by shorter telomeres

compared to non-migrated counterpart. The differences in T/S ratio were statistically significant only within the cohorts of AS patients (p value < 0.01) and HD (p value < 0.05).

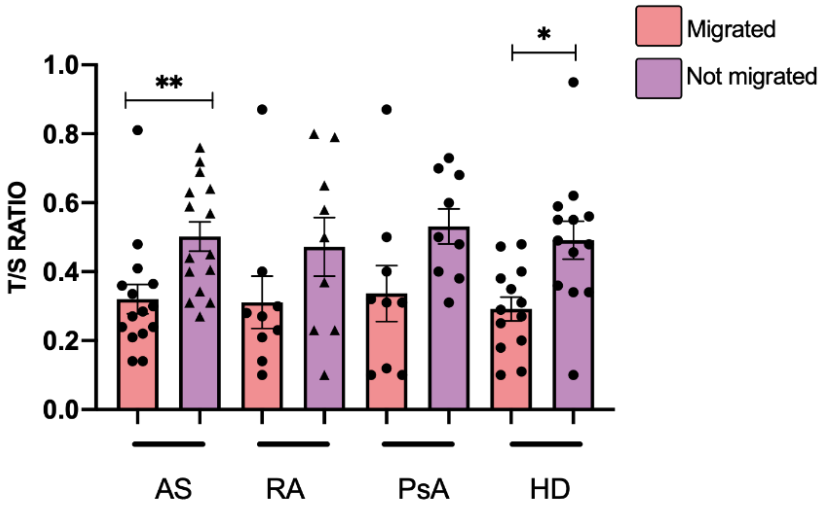

Figure 9. Analysis of telomere length. The analysis of telomere length in migrated and non-migrated $CD8^+$ T cells of 15 AS, 9 RA, 9 PsA patients and 13 HD highlights a reduced T/S ratio in migrated cells. This indicates a telomere length shortening in migrated cells with a significant difference in AS patients and HD controls (Wilcoxon test, *p value < 0.05; **p value < 0.01).

The correlation between the age of the subjects analysed and the telomere length in the subsets of migrated and non-migrated cells from the four cohorts showed a statistically significant negative correlation in non-migrated cells from HD cohort (*p value < 0.05; Spearman r -0,5851). In contrast, no correlation between T/S ratio of migrated cells and the age of the subjects hold true in all cohorts analysed (no significative difference detected) (Fig.10.1). This means that the shortening of the telomeres in migrated cells is probably an intrinsic feature correlated to a specific phenotype rather than a consequence of the age.

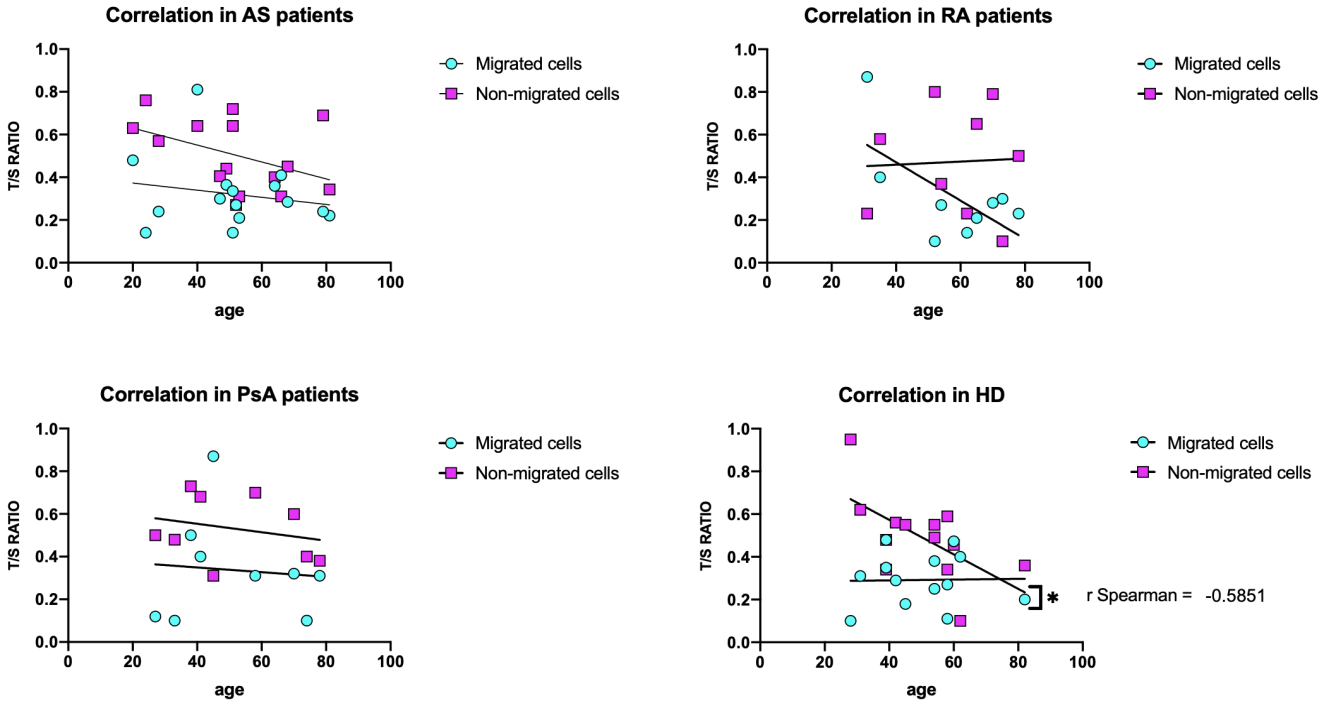

Figure 9.1. Correlation between T/S ratio and age in the four cohorts. In these panels emerge a trend of negative correlation between the T/S ratio of non-migrated cells and the age in all the four cohorts; only in HD cohort the correlation has a significant p value with a r Spearman -0,5851 (*p value < 0.05). By contrast, there is no correlation between the T/S ratio of migrated cells and the age of the subjects in all the groups analysed.

Detection of telomere damages in non-migrated and migrated CD8⁺ T cells

Afterwards, we investigated the telomere DNA dysfunction in migrated compared to non-migrated cells by Immunofluorescence (IF)-Fluorescence in situ hybridization (FISH) analysis in 5 AS, 4 RA, 3 PsA patients and 3 HD (Fig.10).

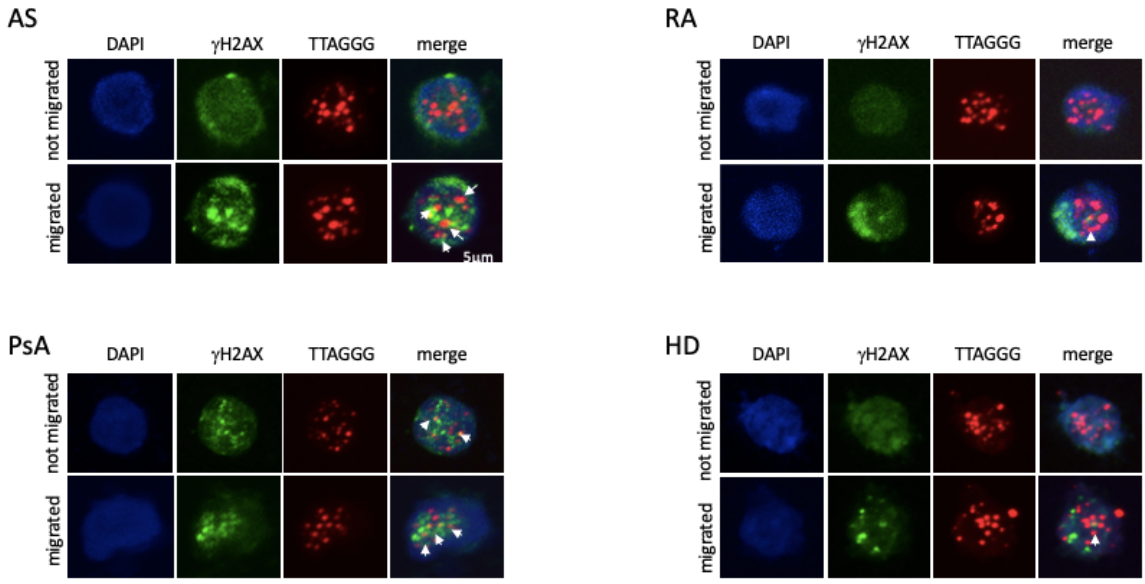

Figure 10. IF-FISH analysis of non-migrated and migrated CD8⁺ T cells. Each panel shows the IF-FISH analysis of not migrated and migrated CD8⁺ T cells in one representative out of 5 AS, 4 RA, and 3 PsA patients and in one representative subject out of 3 HD. Cells were stained with an anti-γH2AX antibody or hybridized with the Cy3-conjugated telomeric DNA probe recognizing the "TTAGGG" human telomeric sequence to detect DNA damages and telomeric regions, respectively. DAPI was used to detect cell nuclei. The merge highlights the damages in the telomeric regions (TD). AS and PsA patients display a higher number of merged spots compared to RA patients and HD, indicating higher telomeric dysfunction.

To this aim, we detected the signals from γH2AX, a classical marker of DNA damage and repair, by using a specific mAb, and from the telomeric DNA, hybridized with a fluorescent peptide nucleic acid (PNA)-telomere probe. The phosphorylation of H2AX allows the recruitment of DNA repair machinery and it is used as marker of the DNA double strand breaks (DSBs) (*Kuo and Yang, 2008)*. In all the cohorts analysed, a greater percentage of γH2AX foci in the whole nucleus, reported as DNA damage response activation (DDR) (Fig.10.1A), as well as in the telomeric regions (TD) (Fig.10.1B) was detectable in migrated CD8⁺ T cells compared to non-migrated counterpart. However, the differences were statistically significant only in the cohort of patients with AS (p value < 0.01) (Fig.10.1A and B). Very interestingly, the estimation of telomere damage spot number per cell revealed a more pronounced telomere dysfunction in migrated CD8⁺ T lymphocytes from patients with AS and PsA, both belonging to the Spondylarthritis cluster, than in those from RA patients and HD controls (Fig.10.1C). All together these data suggest a clear correlation between the

spontaneous hypermotility of CD8+ T cells and the occurrence of a more pronounced telomere attrition.

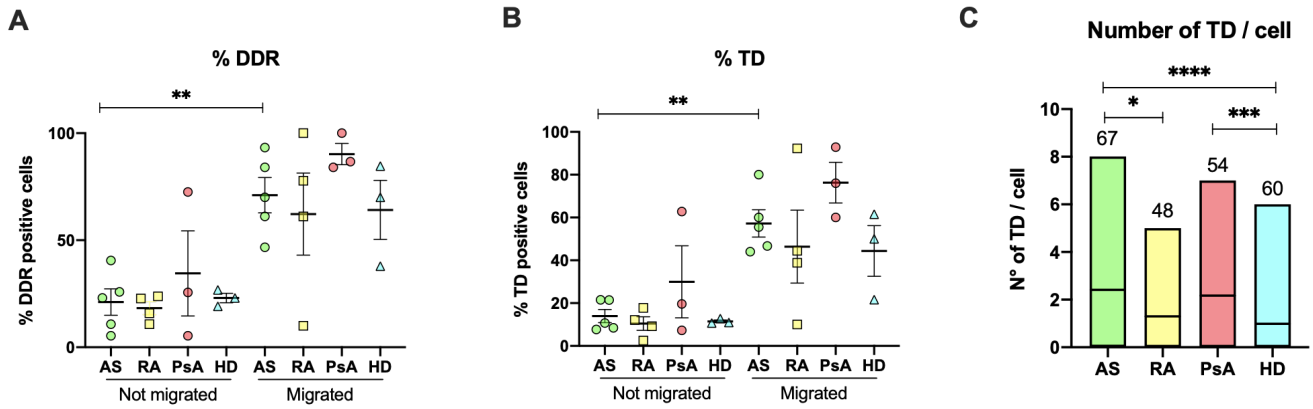

Figure 10.1. Analysis of DNA Damage Response activation (DDR) and Telomeric Damage (TD) in non-migrated versus migrated CD8+ T cells. The panels A and B show the analysis of the percentage of positive non- migrated and migrated $CD8^+$ T cells to γH2AX, as DNA damage marker, in the whole DNA (% DDR) and in the telomeric regions (%TD), respectively. In both panels, a higher percentage of DDR and TD in migrated $CD8^+$ T cells of AS patients (Mann-Whitney test, **p value < 0.01) has been found. In the panel C it has been analysed the number of Telomeric Damage (TD) per cell considering the group of migrated $CD8^+$ T lymphocytes. In $CD8^+$ T cells derived from 5 AS patients it has been counted the number of damages in 67 cells, in those derived from 4 RA patients 48, in those from 3 PsA patients 54 and in those from 3 HD 60 cells. Patients with AS show a higher number of TD per cell compared to RA patients and HD; also PsA patients possess a higher TD per cell compared to those of HD (Kruskal-Wallis test, *p value < 0.05; ***p value < 0.001; ****p value < 0.0001).

Discussion

Genetic association data and the clinical picture characterizing patients affected by AS have supported our interest in the study of $CD8^+$ T lymphocytes as possible cell population involved in fuelling the inflammatory state typical of AS disease (*Cortes et al., 2013*). In fact, GWAS studies highlighted several genetic factors associated to AS risk like as the HLA-B27, ERAP1 and ERAP2, EOMES, IL7R, RUNX3 and ZMIZ1 that, directly or indirectly, impact the $CD8^+$ T subset in terms of counts, differentiation pattern and function (*Evans et al., 2011; Navarro and De Castro 2013*; *Keidel et al. 2013 Vecellio et al., 2019)*. Moreover, the complex clinical condition of AS patients shows a spread of the inflammation involving the vertebral spine, main site of inflammation, peripheral joints and several tissues like as eyes, skin, intestine (*Rogler et al.,2021*). Notably, 70% of patients with AS possess subclinical inflammation at bowel level (*Costello et al., 2015*). This clinical evidence leads to consider the gut-joints axis as pivotal in AS disease, determining a continuous traffic of innate and adaptive cells between tissues and blood. The T lymphocytes, in fulfilling their immune surveillance function, must circulate between blood, lymphoid organs and tissues through various migratory events, all coordinated by an elaborate system of cytokines, receptors and adhesion molecules (*Fu et al., 2016*). An alteration of migratory events could cause an impaired homeostasis inducing conditions of chronic inflammation such as in AS. The need to focus our study on the migratory properties of $CD8^+$ T cells stems from all above considerations.

In the first place, we analysed the percentage of $CD3^+$, $CD3^+$ $CD4^+$ and $CD3^+$ $CD8^+$ T cells in PBMC from patients with AS, RA, PsA and HD. Data from literature about the frequency of these cellular subsets in AS patients are controversial. Indeed, a recent study reported no differences in the absolute number and in the percentage of $CD8^+$ T cells in the blood of AS patients compared to controls (*Toussirot et al., 2018*). In contrast, Gracey et al. found a lower percentage of $CD8^+$ T cell in the blood of AS patients compared to HD (*Gracey at al., 2020*) suggesting also an altered cytotoxic profile of these cells. On the contrary, in a very recent study based on single cell RNA-seq of PBMC in patients with AS, Xu and colleagues described $CD8^+$ T lymphocytes as one cell cluster over-represented in patients with AS (*Xu et al., 2021*). In our cohorts, we have not found differences in the frequency of T cell subsets comparing AS patients to HD, although a trend of higher percentage in $CD3^+$ $CD4^+$ T cells and conversely, of lower percentage in $CD3^+$ $CD8^+$ T cells emerged (Fig.1).

In the four cohorts under study, we have analysed the chemotactic capability of $CD8^+$ T cells carried out in presence or absence of chemokine stimuli. The chemokines used for

chemotaxis were the CXCL9 mediating lymphocyte infiltration in inflamed sites (*Tokunaga et al., 2018*), CXCL10 involved in pro-inflammatory and anti-angiogenic mechanisms (*Gotsch et al., 2007*) and CXCL11 that exerts a strong chemotactic power on activated T cells (*Erdel et al., 1998*). Moreover, we included in the study the homeostatic chemokine CXCL12 and the CCL20, an important chemokine for the homing of T cells in mucosal tissues (*Schutyser et al., 2003*). In general, the stimulation with this panel of chemokines induced the migration of $CD8^+$ T cells with no relevant difference in the number of migrated cells among the four cohorts. However, the power of the migration, expressed as Migration Index (MI), was lower in the patient groups compared to healthy subjects (Fig.2 and 3). In particular, $CD8^+$ T cells of RA patients show a lower MI and a lower number of migrated cells when stimulated by CCL20 compared to HD (Fig.3). In some autoimmune diseases like as psoriasis, IBD and RA an important role of the CCL20-CCR6-axis in the inflammatory process has been highlighted (*Meitei et al., 2021*). In subjects affected by RA a significant increase of CCL20 in the synovial fluid has been found and, also, an increase of $CD4^+$ $CD45RO^+$ $CCR6^+$ memory T cells in the peripheral blood (*Ruth et al., 2003*). Additionally, it has been observed a reduced severity of RA disease upon administration of a monoclonal antibody against CCR6, probably because a minor percentage of $CD4^+$ T cells is recruited to the joints (*Hirota et al., 2007*). We have found no differences between RA and HD groups both in the percentage of $CD8^+$ $CCR6^+$ T cells and in rMFI of the receptor (Fig. S1). Therefore, the lower ability of $CD8^+$ T cells observed in RA patients to move towards CCL20 might be due to the fact that the majority of $CD8^+$ $CCR6^+$ T cells have already been recruited to the inflamed joints.

Interestingly, the apparently weaker migration power of $CD8^+$ T cells from AS and PsA patients, expressed as MI, was strongly influenced by the basal motility of these cells. Unexpectedly, $CD8^+$ T cells of AS and PsA patients disclosed a higher spontaneous motility, independent of a specific chemokine, compared to HD (p value < 0.0001 and p value < 0.001 respectively) (Fig.4). Notably, this $CD8^+$ T cell basal hypermotility characterizes the cohorts of patients within the same cluster of SpA but not patients with RA, a rheumatic autoimmune disorder with a different genetic and pathogenic background. This suggests that although the $CD8^+$ T lymphocytes could be involved in SpA as well as in RA their features and recruitment to the involved tissues could have distinct peculiarities. Moreover, it is reasonable to envisage a scenario where potentially pathogenic $CD8^+$ T cells could move into several tissues without a specific chemotactic signal and spread *in situ* damage.

In line with this idea, it was important to better clarify the immune profile of such "hypermigrating" $CD8^+$ T cells; to this aim, we performed flow cytometry analysis

using CCR7 and CD45RA markers to discriminate the cells in terms of naïve ($CCR7^+/CD45RA^+$), central memory ($CCR7^+/CD45RA^-$), effector memory ($CCR7^-/CD45RA^-$) and T_{EMRA} ($CCR7^-$ $CD45RA^+$) (Fig.6). This analysis displayed that the subsets involved in this spontaneous migration were mainly effector memory, especially in the AS patients, and T_{EMRA} cells in the AS, RA and PsA cohorts (Fig.6C and D). As expected, naïve cells resulted less prone to migrate in all cohorts as well as central memory cells in the groups of patients (Fig.6A and B). Therefore, in all groups analysed, our data indicated the effector cells (T_{EMRA} and T_{EM} subsets), exerting potent effector functions after activation, as the cells more capable of such spontaneous migration. However, despite the condition of chronic inflammation is a common feature of all three diseases (AS, PsA and RA), the higher basal migration appears as a hallmark only of AS and PsA patients.

In the light of the chronic inflammation condition characterizing AS and PsA patients and considering that this basal hypermotility affected terminally differentiated cells, it was conceivable to hypothesize a connection between this spontaneous migration and a cellular senescent immunophenotype. Therefore, we have undertaken, by flow cytometry, an analysis with two markers of cellular senescence: CD28 and CD57. Indeed, $CD8^+$ T cells exposed to a sustained activation state due to a persistent antigenic stimulation, loss the expression of CD28 and gain the expression of CD57 (*Pangrazzi et al., 2020*). In patients with AS and Behçet's disease (BD) a higher number of circulating $CD8^+$ $CD28^-$ cells has been correlated with the disease status and not with the age (*Tedeschi et al., 2022; Schirmer et al., 2002; Yang et al., 2018*). These senescent/overstimulated cells have a high cytotoxic potential. In fact, in the $CD8^+$ $CD28^-$ T population increased levels of T-bet and EOMES were highlighted, responsible of the production of granzyme B, granzyme H and perforin (*Fann et al., 2005*). By our comparative analysis of migrated versus non-migrated $CD8^+$ T cells, $CD8^+$ $CD28^-$ $CD57^+$ T lymphocytes have proved to be the major population of migrated group of all cohorts analysed. On the opposite, $CD8^+$ $CD28^+$ $CD57^-$ T cells resulted less prone to migrate (Fig.7). The enrichment in $CD8^+$ $CD28^-$ $CD57^+$ T lymphocytes within the group of migrated cells, confirmed our hypothesis about a putative link between cellular senescence and elevated basal migratory ability which could be helpful to these cells for infiltration in the body sites.

It is important to point out that the immune phenotype of migrating $CD8^+$ T cells is the same in all groups analysed; however, what makes the difference, in AS and PsA patients compared to HD and RA patients, is the relevant higher proportion of these hyper-motile and senescent $CD8^+$ T cells.

Along these lines, we have found an enrichment of $CD8^+CX3CR1^+$ T lymphocytes in migrated cells of all cohorts under study (Fig. 8). We also observed a trend of higher percentage of cells positive for this receptor comparing patients and HD. The fractalkine receptor CX3CR1 was recently found to be a good marker of T-cell differentiation, where $CX3CR1^+$ $CD8^+$ T cells represented the progeny of $CX3CR1^-CD8^+$ T cells and exhibited a terminally differentiated phenotype with a robust cytotoxic potential in anti-viral immunity (*Bottcher et al., 2015; Gerlach et al., 2016*).

Our interest for the expression of CX3CR1 on $CD8^+$ T cells stemmed from the idea that cells with higher basal migration potential might be antigen over-stimulated cells and could therefore have characteristics of both inflammatory and senescence cells. A population of "inflammescent" $CD8^+$ T_{MEM} $CX3CR1^+$ $CD57^+$ $CD28^-$ lymphocytes has been described in a paper by Morris and colleagues (*Morris et al., 2020*) who have found in HIV and CMV infected individuals this subset of $CD8^+$ T cells able to expand *in vitro* with stimulation via the cytokine IL-15. Although this population expresses the characteristic markers of senescence, it is a subset that retains proliferative and cytotoxic capabilities, contributing to inflammation (*Morris et al., 2020*). In the spontaneously migrated $CD8^+$ T cells versus the non-migrated ones, we found an increase of cells positive for CX3CR1 both within the $CD28^-$ $CD57^+$ subset (the "oldest" one) which was the most abundant as well as in the $CD28^+$ $CD57^-$ subset (the "youngest" one) which was poorly represented. This enrichment occurred in all cohorts analysed (Fig.8.1) whereas the increase was particularly relevant in AS patients and HD (p value < 0.05 and p value < 0.01 respectively) suggesting a crucial role of CX3CR1 in the spontaneous migration phenomenon. Since AS patients show an altered microbiome in which there is an increased of some bacterial species and a decreased of others (*Cardoneanu et al., 2021, Scalise et al., 2021, Breban et al., 2019)* the enrichment of immunosenescent, hypermotile $CD8^+$ T cells could be linked to a chronic activation of $CD8^+$ T cells caused by pathobionts or even pathogens, as well as in the case of the chronic stimulation induced by CMV which caused expansion of immunosenescent cells (*Pawelec et al., 2014*).

Moreover, the hypermotility appears as a hallmark of $CD8^+$ T lymphocytes population; indeed, the migration analysis of $CD4^+$ T cells did not display the same behaviour (Fig.5), although experimental and clinical data strongly sustain they implication in AS disease (*Smith, 2015*). Certainly, when stimulated, $CD4^+$ T cells move on the chemoattractant in all cohorts analysed without differences (Fig.S2). Probably, the phenomenon is linked to $CD8^+$ T cell depending on the higher sensitivity to the age-variation in comparison to $CD4^+$ T cells (*Czesnikiewicz-Guzik et al., 2008*).

Since the analysis of the immunophenotype has revealed a senescent profile of the spontaneously migrated $CD8^+$ T cells from all cohorts, we further investigated this aspect by a different experimental approach, measuring telomere length by real time PCR. Our results have shown a telomere shortening of migrated cells of AS patients and HD in comparison to not migrated T lymphocytes (p value < 0.001 and p value < 0.05 respectively) (Fig.9). In patients with PsA and RA we also detected a lower T/S ratio in the migrated versus non-migrated cells but in these two cases probably because of the lower cohort power the statistical significance was not reached. Notably, the comparison of telomere length of non-migrated $CD8^+$ T cells deriving from patients in respect to HD did not point out significant differences, apart from a slightly greater telomeric length in AS patients versus HD. Surely, all cohorts need to be expanded to make these results more robust. However, these results are not surprising, since other studies have described a greater telomeric length of PBMCs from patients affected by AS and PsA than healthy controls (*Heba et al., 2021; Tamayo et al., 2010*). The increase in telomere length could be linked to a higher telomerase activity (*Tamayo et al., 2010*). On the other hand, a shorter telomere length was found in the $CD4^+$ and $CD8^+$ T lymphocytes from patients with axial Spondylarthritis under age of 35 (*Fessler et al., 2016*) which suggested, together with telomerase insufficiency, a premature T-cell subset senescence in such autoimmune disorders.

The rate of telomeric shortening has certainly increased with age but it is also due to additional phenomena among which conditions of prolonged cellular stress and chronic inflammation (*Heba et al., 2021*). Therefore, we correlated the T/S ratio with the age of the subjects analysed in each cohort (Fig.9.1). As expected, we found a trend of negative correlation between T/S ratio in non-migrated $CD8^+$ T cells and the age in all groups analysed; therefore, the higher the age, the shorter the telomere length. However, the correlation resulted statistically significant only in HD cohort (p value < 0.05) (Fig.9.1). Certainly, these results were influenced by the low number of subjects analysed. Nevertheless, it can be speculated that the lack of statistically significant correlation between telomere length and the age in AS, RA and PsA patients might be due to the elevated variability in the activation of telomerases and accelerated ageing process occurring in inflammatory conditions and autoimmune diseases (*Punder et al., 2019*). Interestingly, no correlation emerged between T/S ratio of migrated cells and the age of the individuals in all groups tested (Fig.9.1) hinting that, in this case, the telomere length is independent of the age and, probably, dependent on the enrichment in experienced, senescent, perhaps inflammescent cells.

Defects of the DNA damage repair pathway have been shown to drive telomere shortening and ageing in T cells of RA patients (*Li et al., 2016*). Nevertheless, even in

presence of functional DNA damage response, telomeres are well known hypersensitive sites of replication and oxidation dependent DNA damage (*Gilson and Geli, 2007; Foquerel et al., 2019; Barnes et al., 2022*). Moreover, in senescent T cells, telomere attrition *per se* is accompanied by the loss of structures deputed to telomere capping which exposes terminal DNA to be recognized by the DNA damage response pathway (*Chebel et al*., 2009). Therefore, we performed IF-FISH analysis on migrated versus non-migrated $CD8^+$ T cells to detect the DNA double strand breaks (DSBs) in the telomeric regions by an anti-γH2AX mAb and fluorescent (PNA)-telomere probe (Fig.10). H2AX is a histone variant mainly phosphorylated by the ataxia telangiectasia mutated (ATM) protein kinase (*Burma et al., 2001*). Once phosphorylated, γ-H2AX represents the first signal for the recruitment of DNA repair machinery which can be used as a biomarker of DNA damage since γH2AX foci represent DSBs with a ratio of 1: 1 (*Kuo and Yang, 2008*).

Coherently with this, we found that AS migrated $CD8^+$ T cells, in which there was evident telomere attrition, displayed a higher percentage of positivity to γH2AX foci compared to not migrated cells, with a statistically relevant difference (p value < 0.01; Fig.10.1A). In addition, in the same cohort we observed an increase of the percentage of cells showing telomeric damages (TD) in migrated versus not migrated cells (p value < 0.01; Fig 10.1B). By looking at a single cell level, $CD8^+$ T cells of AS and PsA patients showed a higher number of TD per nucleus compared to that of HD and RA patients (Fig.10.1.C). Besides, we also detected the presence of few non-telomeric DNA damages per nucleus, meaning that other DNA sites could be damaged in migrating AS patient derived $CD8^+$ T cells (Fig.10.1A). Although further analysis is required to strengthen the statistical data significance, telomeric damage nicely correlated with the telomere shortening and the hypermotility of the cells in AS patients, suggesting a potential link between telomere integrity and hypermotility of the cells, that could involve a signalling pathway across the nuclear lamina and the cytoskeletal architecture of the cell, that will be our next step of investigation.

Therefore, we can speculate that the hypermotility that we have described in this study could imply a higher ability of $CD8^+$ T cells to invade the inflamed tissues. Accordingly, in $CD4^+$ T cells of patients with RA a deficit in MRE11A has been described, which impairs the DNA repair machinery, thus determining telomere fragility and elevated ability of cells to invade synovial tissues (*Li et al., 2016, Weyand et Goronzy, 2021; Zhao et al., 2022*). It is not a fortuitous case that the spontaneous hypermotility only characterizes $CD8^+$ T cells of AS and PsA patients in whom we have found a higher number of telomeric damages per cell.

Overall, this study highlights a particular behaviour distinguishing the $CD8^+$ T lymphocytes in AS and PsA patients. The putative antigen overstimulation of these cells leads to basal hypermotility accompanied to a senescent/inflammescent phenotype sustained by telomere shortening and attrition. These inflammescent and hypermotile $CD8^+$ T cells could be favoured in reaching and move in the inflamed tissues rising the inflammation. These data encourage us to pursue the study analysing the transcriptome profile of these CD8+ T cells, the quality of the movement by time lapse microscopy and their cytotoxic/effector functions.

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Supplementary

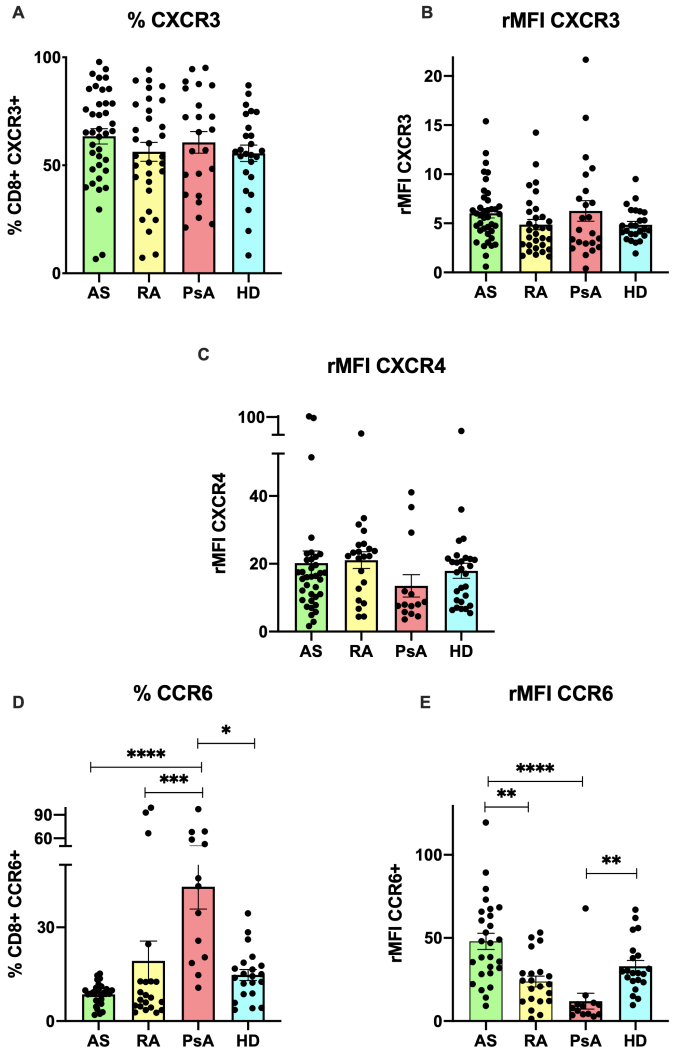

Figure S1. Analysis of chemokine receptors. A and D panels report the percentage of CD8⁺ T cells positive for CXCR3 (39 AS, 31 RA, 23 PsA and 25 HD) and CCR6 (28 AS, 21 RA, 13 PsA and 21 HD) respectively. In the panels B, C and E, the rMFI (relative Mean Fluorescence Intensity) of CXCR3 (B), CXCR4 (C) (36 AS, 22 RA, 14 PsA and 28 HD), CCR6 (E) on CD8+ T cells is shown. The rMFI of CXCR4 is higher in CD8⁺ T cells of RA patients compared to PsA subjects (C). The percentage of CD8⁺ CCR6⁺ T cells is higher in PsA patients compared to all other cohorts (D). The rMFI of CCR6 is higher in AS patients compared to RA and PsA patients; and in HD compared to PsA subjects (Krusal-Wallis test; *p value < 0.05; **p value < 0.01; ***p value < 0.001; ****p value < 0.0001).

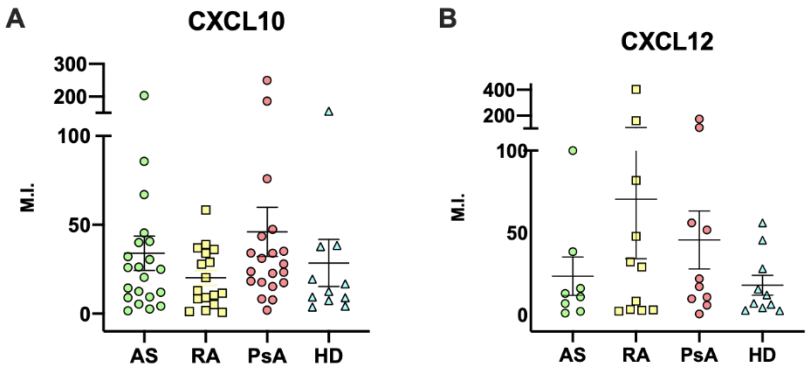

Figure S2. Migration of CD4⁺ T cells upon chemokine stimulation. The two panels show the Migration Index of CD4⁺ T cells upon stimulation with the inflammatory chemokine CXCL10 (A) in 21 AS, 17 RA, 20 PsA patients and 11 HD and the homeostatic chemokine CXCL12 (B) in 8 AS, 11 RA, 10 PsA patients and 10 HD. No significant difference emerged among all cohorts analysed (Kruskal-Wallis test).

Methods

Patient cohorts

For this study were recruited 254 individuals, including 100 Ankylosing Spondylitis (AS) patients, 58 Rheumatoid Arthritis (RA) patients, 33 subjects affected by Psoriatic Arthritis (PsA) and 63 Healthy Donors (HD) provided by the University Medicine Unit of the SM Hospital Goretti of Latina, the University of Cagliari (Department of Medical Sciences and Public Health-Rheumatology Unit) and the University of Rome La Sapienza (Center for Transfusion Medicine, Policlinico Umberto I).

The study received the approval by the Ethics Committee of the University of Cagliari (365/09/CE), by the U.O. MEDICINA INTERNA UNIVERSITARIA — ASL LATINA (0018614/2019) and by the University of Rome La Sapienza (Center for Transfusion Medicine, Policlinico Umberto I) (Rif. 6893). All subjects provided written informed consent prior to enrolment.

Isolation of PBMCs from peripheral blood

Peripheral blood mononuclear cells (PBMCs) derived from patients affected by AS, RA and PsA and HD individuals were isolated on density gradient from venous blood samples in sodium citrate. Each blood sample was layered on a Lympholyte solution (Cederlane Laboratories) and centrifuged for 30 minutes at 2000 rpm. At the end of centrifugation, the lympho-monocyte ring formed between the supernatant, consisting of plasma, and the underlying Lympholyte phase, granulocytes and erythrocyte pellets was recovered. The PBMCs were then washed twice with PBS 1X (Phosphate Buffered Saline, Euroclone).

Isolation of $CD8^+$ and $CD4^+$ T lymphocytes

The positive selection of $CD8^+$ T and $CD4^+$ T lymphocytes was carried out starting from PBMCs, using the "$CD8^+$ T Cell Isolation" and "$CD4^+$ T Cell Isolation" kits and columns for LS depletion (Miltenyi Biotec), according to the manufacturer's instructions. These kits allow to isolate human $CD8^+$ T and $CD4^+$ T lymphocytes from other mononuclear cells (B, NK and dendritic cells) by positive immuno-selection, incubating the PBMCs with magnetic beads (Microbeads-20 µl of beads / 10^8 cells) for 20 minutes on ice.

Subsequently, the cells were washed in MACS buffer (10% FBS, EDTA 2mM, PBS 1X), 10 minutes at 1100 rpm and finally resuspended in MACS buffer. At this point, the magnetic separation of the cells was carried out using the LS columns, previously equilibrated by the addition of 3 ml of MACS buffer. Once isolated, the $CD8^+$ and $CD4^+$ T lymphocytes were resuspended at 10^6 cells/ml density in a culture medium consisting of RPMI (Euroclone) supplemented with: 5% of FBS (Fetal Bovine Serum, Biochrom), Glutamine 2mM (Euroclone), Amphotericin B 2.5 µg/ml (Euroclone), Pen/Strep 100U/ml/100 µg/ml

(Euroclone). After an overnight culture, migration, flow cytometric characterization and telomeres length analysis were performed.

Transwell migration assay

The migration assay or chemotaxis allows to evaluate *in vitro* ability of cells to migrate in response to stimuli. The assays were carried out using 96-well plates (Transwell, Corning), in which there are polycarbonate inserts, at a fixed distance from the base, which allows the subdivision of the well into two compartments, lower (basal) and upper (apical). Since the serum present in the culture medium could contain chemo-attracting molecules capable of altering the migration induced by a specific chemokine, $CD8^+$ T cells were washed for 10 minutes at 1100 rpm in the Chemotaxis Buffer (CB; buffer without serum and containing 0.5% BSA, 25 mM Hepes in RPMI) at room temperature, resuspended in CB $1.5x10^5$ cells/80µl and subjected to migration in the presence of the chemokines of interest; as a control of the basal movement, independent of the chemotactic action, the migratory capacity of cells was evaluated in the presence of CB alone. In the experiment, a further condition is considered, whose cells are not plated in the transwell but are used as a control of the actual number of cells plated in each well (INPUT). In the basal well, 235µl of CB are plated alone or containing the chemokines of interest (CXCL9, CXCL10 and CXCL12 100 nM; CXCL11 and CCL20 300 nM) while, in the apical one, $1.5x10^5$ $CD8^+$ were seeded making sure that cells constitute a monolayer above the membrane. Afterwards, the plate is left in the incubator (37°C 5% CO2) for an hour and a half, at the end of which it is possible to check the migration of the cells under a microscope. Subsequently, the cells are collected from the basal wells and centrifuged for 3 minutes at 3000 rpm. At this point, $CD8^+$ T lymphocytes are counted by cytometer (FACSCalibur, BD Biosciences) for 1 minute at constant speed and the number of migrated cells is calculated using the following formula: n° migrated cells x 150000 / n° input cells (pre-migration). The migratory capacity of the cells is reported as the Migration Index: n° of cells migrated in response to the chemokine / n° of cells migrated in the presence of CB alone.

Immunofluorescence

The expression of chemokine receptors was validated by incubating $CD8^+$ T lymphocytes isolated from PBMCs ($2x10^5$cells/condition) with the following antibodies: anti-CXCR3-, -CXCR4- and -CCR6-PE (BD Biosciences) for 30 minutes in ice.

Subsequently, the cells were washed and resuspended in 150 µl of FIX 1 (2% paraformaldehyde) and then analyzed by a flow cytometer (FACSCalibur BD Biosciences). The "naïve" and "memory" lymphocyte subpopulations were identified by incubation with the anti-CCR7-PE (BD Biosciences) and anti-CD45RA-FITC (Immunotools) mAbs, while the

CD28/CD57 subsets were identified by incubation with anti-CD28-FITC (BD Biosciences) and anti-CD57-APC (BD Biosciences) antibodies. Moreover, to evaluate the 'inflammescent' subset it has been included also an anti-CX3CR1-PE (BD Biosciences) specific antibody. After 30 minutes of incubation on ice, the samples were washed and resuspended in 150 μl of FIX1. They were then acquired with FACSCalibur and analyzed by FlowJo software (Tree Star Inc.).

DNA extraction from CD8⁺ T lymphocytes

The extraction of genomic DNA from the pellet of migrated and non-migrated cells was carried out using a special kit (QIAamp DNA Micro Kit, QIAGEN), according to the instructions given in the protocol provided by the company. The amount of DNA was subsequently determined using the Nanodrop (ThermoScientific).

Analysis of telomeres length by rtPCR

The analysis of telomeres length has been performed by rtPCR in samples of DNA extracted from $CD8^+$ T cells non-migrated and migrated without chemokine stimuli.

A quantitative PCR reaction was performed for each DNA sample relating to the repeats of telomeric ends and the constitutively expressed single copy gene (β-globin). The protocol for the two reactions is similar, it differs exclusively for the sequence of the primers used, one specific for the telomeric repeats and the others for the β-globin gene sequence. Reference DNA sample of known concentration (10 ng/μl; Human Male DNA, Thermoscientific) is serially diluted (dilution factor of 2) to yield 5 concentrations of DNA in 20 μl final volume (17 ng, 8.5 ng, 4.2 ng, 2.1 ng, 1 ng), required for subsequent normalization.

Each DNA sample is amplified in triplicate in a final volume of 20 μl containing:

- Sensimix 2x SYBR Hi-ROX (Bioline): 10 μl
- Primers (Eurofins):

Tel Fw (5′ – CGG TTT GTT TGG GTT TGG GTT TGG GTT TGG GTT TGG GTT – 3′) 0,2 μM

Tel Rev (5′ – GGC TTG CCT TAC CCT TAC CCT TAC CCT TAC CCT TAC CCT – 3′) 1,8 μM

β-globin Fw (5′ – CAA CTT CAT CCA CGT TCA CC – 3′) 0,5 μM

β-globin Rev (3′ – GCC ATC TAT TGC TTA CAT TTG C – 3′) 0,2 μM

- H2O distilled by volume

The Real-Time PCR amplification reaction is performed using the 7300 Real Time PCR System (Applied Biosystem) instrument. The amplification program includes the following cycles:

-Stage 1: Step 1: 95°C per 10′ (1x)

-Stage 2: Step 1: Step 1: 95°C per 15′′ (40x)

Step 2: 60°C per 1′ (40x)

-Stage 3: Step 1: 95°C per 15′′ (1x)

Step 2: 60°C per 1′ (1x)

Step 3: 95°C per 15′′ (1x)

Data are reported as T/S ratio indicating the number of copies of telomer repeats (T) on a control gene expressed in single copy (β-globin) normalized with a standard curve at known concentration of DNA.

ImmunoFluorescence and DNA-FISH

Migrated and non-migrated $CD8^+$ T cells were plated on poly-L-lysine coated 18x18 coverslips. Once adhered, cells were fixed with 2% formaldehyde, permeabilized with 0.1% triton X-100 in PBS1x and subsequently blocked in 5% BSA in PBS1x. Then samples were incubated with a Mab Anti-phospho-Histone H2AX (Ser139) (clone JBW301, Merk Millipore) followed by the by the anti-mouse IgG Alexa fluor 488 conjugate secondary antibody (Cell Signaling). Then samples were re-fixed in 2% formaldehyde, dehydrated with ethanol series (70, 90, 100%) air dryed and co-denaturated for 3 min at 80°C with a Cy3-labeled PNA probe, specific for telomeric sequences (TelC-Cy3, Panagene, Daejon, South Korea), and incubated for 2 h in a humidified chamber at room temperature in the dark. After hybridization, coverslips were washed with 70% Formamide, 10mM TrisHCl pH7,2, BSA 0.1%, and then in TBS/Tween 0.08%, dehydrated with ethanol series, and finally counterstained with DAPI (0,5 μg/ml, Sigma-Aldrich) and mounted on specimen slides in mounting medium (Gelvatol Moviol, Sigma Aldrich). Fluorescence signals were acquired by a Nikon Crest Spinning disk at 60X magnitude. Z-stacks were acquired at 0.3 mm steps and then processed with a NIS software.

Statistical analysis

Differences of percentage of $CD3^+$/$CD3^+CD4^+$/$CD3^+CD8^+$, Chemokine receptor expression, Migration Index (MI) values, number of migrated cells at basal level and with a chemokine

stimuli, subset frequencies (naïve/memory; CD28/CD57; CX3CR1), percentage of TD, DDR and TD per cell among the four cohorts were evaluated using the Kruskal-Wallis test, whose statistical significance is accepted for p value levels <0.05.

The comparison between the number of migrated $CD8^+$ and $CD4^+$ T lymphocytes was performed by Mann Whitney test. The frequencies of each subset (naïve / memory; senescent / exhausted; $CX3CR1^+$) before and after migration were compared using the Wilcoxon test. The percentage of TD and DDR before and after migration were compared using Mann Whitney test.

The comparison of the T/S Ratio between migrated and not migrated cells within the same group was carried out using the Wilcoxon test, while the comparison among the different cohorts was carried out using the Kruskal-Wallis test.

Results were analysed using GraphPad Prism software version 8.0 (GraphPad Software: San Diego, CA, USA).