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Original article

# Lymphopenia in primary Sjögren's syndrome is associated with premature aging of naïve CD4+ T cells

**Johannes Fessler[1,2], Patrizia Fasching[1], Andrea Raicht[3], Sabrina Hammerl[1], Jennifer Weber[1], Angelika Lackner[1], Josef Hermann[1], Christian Dejaco [1,4], Winfried B. Graninger[1], Wolfgang Schwinger[3] and Martin H. Stradner[1]**

## Abstract

**Objective.** To investigate peripheral lymphopenia, a frequent finding in primary Sjögren's syndrome (pSS) associated with higher disease activity and increased mortality.

**Methods.** Prospective, cross-sectional study of consecutive patients with pSS ($n = 66$) and healthy controls ($n = 181$). Lymphocyte subsets were analysed by flow cytometry, naïve ($CD45RA^+$) and memory ($CD45RO^+$) $CD4^+$ T cells were purified by MACS technology. *In vitro* proliferation and senescence-associated $\beta$-galactosidase (SABG) were assessed by flow cytometry. Telomere length and TCR excision circles (TREC) were measured by real-time PCR. Telomerase activity was analysed according to the telomeric repeat amplification protocols (TRAP).

**Results.** In pSS, lymphopenia mainly affected naïve $CD4^+$ T cells. We noted a lower frequency of proliferating naïve $CD4^+$ T cells *ex vivo* and decreased homeostatic proliferation in response to IL-7 stimulation *in vitro*. Furthermore, naïve $CD4^+$ T cells exhibited signs of immune cell aging including shortened telomeres, a reduction in IL-7R expression and accumulation of SABG. The senescent phenotype could be explained by telomerase insufficiency and drastically reduced levels of T-cell receptor excision circles (TRECs), indicating a history of extensive post-thymic cell division. TRECs correlated with the number of naïve $CD4^+$ T cells linking the extend of earlier proliferation to the inability to sustain normal cell numbers.

**Conclusion.** In pSS, evidence for increased proliferation of naïve CD4+ T cells earlier in life is associated with a senescent phenotype unable to sustain homeostasis. The lack of naïve CD4+ T cells forms the basis of lymphopenia frequently observed in pSS.

**Key words:** T cells, Sjögren syndrome, inflammation, autoimmunity

**Rheumatology key messages**

- Naïve $CD4^+$ T cells are the main population affected by lymphopenia in pSS patients.
- Naïve $CD4^+$ T cells of pSS patients feature altered homeostasis, increased replicative history and cellular senescence.
- These alterations in naïve $CD4^+$ T cells might occur already before disease onset.

## Introduction

Primary Sjögren's syndrome (pSS) is a chronic, systemic autoimmune disorder characterized by lymphocyte infiltration of exocrine glands such as salivary and lachrymal glands. Glandular inflammation and tissue damage ultimately lead to secretory dysfunction and symptoms of dryness including keratoconjunctivitis sicca and xerostomia [1, 2]. In addition, extra-glandular manifestations affecting the joints, lungs, the peripheral and central

[1]Department of Rheumatology and Immunology, Medical University of Graz, Graz, Austria, [2]Department of Neurology, Harvard Medical School, Brigham and Women's Hospital, Harvard, MA, USA, [3]Department of Pediatric Hemato-Oncology, Medical University of Graz, Graz, Austria and [4]Servizio di Reumatologia, Azienda Sanitaria dell'Alto Adige, Ospedale di Brunico, Brunico, Italy

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Correspondence to: Wolfgang Schwinger, Department of Pediatric Hemato-Oncology, Medical University Graz, Auenbruggerplatz, Graz 8036, Austria. E-mail: wolfgang.schwinger@medunigraz.at





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nervous system or the kidneys occur in a subset of patients [3].

The pathophysiology of pSS is multifaceted and environmental as well as genetic factors contribute to the disease. The presence of auto-antibodies, ectopic germinal centres, hypergammaglobulinemia, and the increased risk for the development of non-Hodgkin's lymphoma highlight a pivotal role of B cells in the pathogenesis of pSS [4]. The robust genetic association between HLA class II and pSS implies a crucial involvement of T cells. IFNγ as well as IL-17 producing T cells are present in inflamed salivary glands of pSS patients and are associated with tissue damage [5, 6]. Moreover, $CD4^+$ T cells can be activated by salivary gland epithelial cells and differentiate into T follicular helper cells interacting with B cells to promote survival and generation of high affinity antibodies [7]. Therefore, $CD4^+$ T cells and their interaction with B cells are critical for development of pSS and might be a suitable target for treatment [8, 9].

Given the importance of both B and T cells in the pathogenesis of pSS, it is interesting to note that peripheral lymphopenia is a frequent finding in pSS patients and was shown to be associated with higher disease activity and mortality [10]. In addition, patients with lymphopenia are at increased risk for non-Hodgkin's lymphoma [11]. Lymphopenia in pSS is known to be a result of decreased $CD4^+$ T cells [12]. In healthy individuals, T-cell production of the thymus as well as homeostatic proliferation of peripheral T cells compensate for naturally occurring T-cell loss. The reasons for insufficient compensation in pSS patients, however, is currently unknown. This study aims to identify the lymphocyte subsets responsible for lymphopenia and to characterize the cells affected.

## Methods

### Study population

We prospectively recruited 66 consecutive pSS patients fulfilling the revised American-European Consensus Group classification criteria of 2002, 50 consecutive SLE patients fulfilling the revised and updated 1982 ACR criteria as well as 181 healthy controls (HC) [13, 14]. Participants were devoid of chronic infections or malignant disease in any individual as determined by medical history, clinical and routine laboratory examinations. This study was approved by the Institutional Review Board of the Medical University Graz and written informed consent was obtained from each individual.

Medical history including comorbidities, medication and present symptoms were assessed in all patients. Blood samples were tested for ESR (range 0–10mm/1st h) and CRP (range 0–5 mg/l) levels. pSS patients were assessed using the EULAR Sjögren Syndrome disease activity index (ESSDAI) and the EULAR Sjögren Syndrome patient-reported index (ESSPRI). Data of spondyloarthritis (SpA) and RA patients were obtained from a study previously published [15].

### CMV IgG ELISA

Serum samples of pSS patients were collected and stored at −80°C. Determination of CMV IgG levels was performed by ELISA (NovaTec Immundiagnostica, Germany) according to manufacturer's instructions.

### Flow cytometry

FACS analysis of T-cell subsets was performed according to a common protocol [15]. In brief, erythrocytes were lysed and cells were incubated with antibodies against CD3 (Becton Dickinson, San Diego, USA), CD4, CD8, CD28, CD31, CD45, CD45RO (all Beckman Coulter, Brea, USA), CD19, CD197/CCR7, CD127, CD183/CXCR3, CD196/CCR6, CD194/CCR4 (all Miltenyi, Bergisch Gladbach, Germany) and CD38 and CD45RA (Becton Dickinson, San Jose, USA). A detailed gating strategy for T-cell subsets is given in Supplementary Fig. S1, available at *Rheumatology* online. For intracellular staining of Ki-67 (Becton Dickinson), cells were permeabilized with IntraPrep Reagents (Beckman Coulter) and measured within 2 h. These analyses were performed using Cytomics FC500 (Beckmann Coulter) and CxP software or a FACS Canto II (Becton Dickinson) using FACS Diva and FlowJo software (Becton Dickinson). Phosphorylated STAT5 was detected using a phospho-specific antibody (Cell Signalling Technology, Danvers, USA) after fixation and permeabilization with Phosflow perm/wash buffer (Becton Dickinson).

### Isolation of T-cell subsets

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density gradient centrifugation as previously described [16]. $CD4^+$ and $CD8^+$ T cells were obtained using negative selection MACS-kits according to manufacturer's instructions ($CD4^+$ T cell Isolation Kit and $CD8^+$ T cell Isolation Kit; Miltenyi). In a second step, $CD45RA^+$ and $CD45RO^+$ T cells were obtained via positive selection using CD45RA or CD45RO Microbeads (Miltenyi).

### Quantification of TRECS

T-cell receptor excision circles (TRECs) from PBMCs, naïve and memory $CD4^+$ and $CD8^+$ T cells were analysed as described previously [17]. In brief, DNA was extracted using QIAamp DNA Mini Kit (Qiagen, Chatsworth, CA, USA). TREC concentrations were determined by quantitative real-time PCR based on the coding TREC sequence using LightCycler 480 II (Roche, Vienna, Austria) and primers to amplify a DNA fragment 108 bp across the remaining recombination sequence δrec/ψalpha (5′-CACATCCCTTTCA ACCATGCT-3′ and 5′-GGTGCAGGTGCCTATGC-3′). For quantification, a synthetic internal standard (Ingenetix, Vienna, Austria) was used. PCR reaction ran with 0.25 μg DNA, primers and hybridisation-probe labelled with FAM-TAMRA (5′-ACACCTCTGGTTTTTGTA AAGGTGCCCAC-3′). To avoid bias by different numbers of naïve T cells, TRECs were calculated in relation to numbers of T cells.

### Proliferation assay

Naïve CD4$^+$ T cells were enriched from freshly isolated PBMCs using a negative selection MACS-kit (Naive CD4$^+$ T cell Isolation Kit II, Miltenyi) according to manufacturer's instructions. Cells were stained with CellTrace Violet Cell Proliferation Kit (Thermo Fisher Scientific, Waltham, USA) according to the manufacturer's protocol. 1*10$^6$ cells/ml were cultured for 14 days in the presence of IL-7 (3-30ng/ml, Miltenyi) in a round-bottom 96-well plate. Afterwards, cells were stained for Annexin V (Thermo Fisher Scientific, Waltham, USA), cellular viability (Fixable Viability Dye, Thermo Fisher Scientific) and phosphorylated STAT5 (Cell Signalling Technology) and analysed using a FACS Canto II (Becton Dickinson) and FACS Diva and FlowJo software (Becton Dickinson).

### Determination of telomere length

We measured telomere lengths using DNA from PBMCs, isolated naïve and memory CD4$^+$ and CD8$^+$ T-cell subsets by quantitative real-time PCR analysis and LightCycler FastStart DNA Master SYBR Green I (Roche) according to manufacturer's instructions. The following primers were used: 5′-CGGTTTGTTTGG GTTTGGGTTTGGGTTTGGGT TTGGGTT-3′ and 5′GGCTTGCCTTACCCTTACCCT TACC CTTACCCTTACCCT-3′. A single copy gene (36B4) (fw primer: 5′CCCATT CTATCATCAACGGGTACAA-3′; rev primer: 5′CAGCAAGTGGGAAGGTGTAATCC-3′) served for normalization. Ct-values of test samples and control single copy genes were calculated and $2^{\Delta Ct}$ value was determined [18].

### B-Galactosidase assay

Freshly purified PBMCs were stained with Cellular Senescence Flow Cytometry Assay (Cell Biolabs, San Diego, USA) according to the manufacturer's protocol. Afterwards, cells were stained according to our flow cytometry protocol.

### Telomerase assay

For determination of telomerase activity naïve and memory, CD4$^+$ and CD8$^+$ T cells were isolated and cultured in advanced Roswell Park Memorial Institute (RPMI) 1640 (Thermo Fisher Scientific, Waltham, USA) in the presence of 30 U/ml rIL-2 (Sigma, Vienna, Austria) and CD3/CD28 Dynabeads (bead: cell-ratio 1:1; Invitrogen). After 3 and 6 days 2 $\times$ 10$^5$ cells were harvested and telomerase activity was determined utilizing TeloTAGGG Telomerase PCR ELISAplus kit (Roche) according to Telomeric Repeat Amplification Protocols (TRAP) [19].

### Statistical analysis

All statistical analyses were performed using SPSS, v20.0 (Chicago, IL, USA). For data with normal distribution (according to Kolmogorov-Smirnov test), the mean (S.D.) is shown and we applied the Student's t-tests for comparisons. Otherwise, we indicated the median (range) and used the Mann–Whitney *U* test. Bonferroni's adjustment was applied in case of multiple testing (as stated). Correlation between variables was evaluated by the Spearman's rank correlation coefficient. A multiple regression model was performed exploring the possible influence of clinical and laboratory parameters (age, sex, disease activity, CMV status, smoking status, glucocorticoids treatment) on lymphocyte counts of pSS patients. Normality, linearity and homoscedasticity of all regression models were tested and significant co-linearity of predictors was excluded. For sensitivity analysis, we excluded high leverage cases as well as cases producing low/high difference in beta (DFBETAs) and/or large Cook values.

We included consecutive pSS patients' samples in our study to produce data that are generalizable to pSS patients visited in clinical routine rather than using matched samples. Therefore, we adjusted for differences regarding age and sex by statistical means and divided patients into five age groups: I: 18–35 years; II: 35.1–45 years; III: 45.1–55 years; IV: 55.1–65 years; and V: >65 years (see Supplementary Table S1, available at *Rheumatology*, for numbers and mean age in each group).

## Results

### Patients' characteristics

Clinical characteristics are depicted in Table 1. As expected, pSS patients were older and more commonly female than HCs. Most patients had a low disease activity while 11 (16.6%) patients exhibited moderate to high disease activity indicated by an ESSDAI of ≥5. Eighteen (27.3%) patients had a disease duration ≤2 years.

### Lymphopenia in pSS is characterized by reduced naïve CD4$^+$ T-cell count

To investigate which lymphocytic subsets are affected by lymphopenia in pSS patients, we conducted immune phenotyping of pSS patients and healthy controls by flow cytometry. As depicted in Fig. 1A, we found a decrease in absolute numbers of CD3$^+$ T cells, CD19$^+$ B cells as well as CD16$^+$CD56$^+$ NK cells in absolute numbers, which was more pronounced in younger individuals (≤45 years). ESSDAI correlated inversely with the number of CD3$^+$ T cells, but not with the number of CD19$^+$ B cells or CD16$^+$CD56$^+$ NK cells (Fig. 1B and data not shown). Because lymphopenia was mostly the result of T-cell deficiency, we performed a detailed analysis of T-cell subsets and observed a more pronounced reduction of CD4$^+$ T cells compared with their CD8$^+$ counterparts (Fig. 1C). Disease activity score ESSDAI correlated inversely with the number of CD4$^+$ T cells (Fig. 1D). Further sub-analysis of CD4$^+$ T cells revealed that primarily naïve T cells and to a lesser extent central memory (CM) T cells were affected, whereas effector memory (EM) and terminally differentiated effector memory (TEMRA) cells were comparable between cohorts (Fig. 1E). In contrast to healthy controls, the frequency of naïve CD4$^+$ T cells did not correlate with age in our

**Table 1** Patients' characteristics

| | HC | pSS | *P*-value |
|---|---|---|---|
| Number | 181 | 66 | |
| Age [years][a] | 47.9 (14.7) | 57.9 (12.7) | <0.001 |
| Female, *n* (%) | 90 (49.7) | 59 (89.4) | <0.001 |
| Disease duration [years][a] | n.a. | 5.7 (4.7) | |
| **Disease activity scores** | | | |
| ESSDAI[b] | n.d. | 1 (0–15) | |
| ESSPRI[a] | n.d. | 4.2 (2.2) | |
| **Laboratory data** | | | |
| ESR [mm/1$^{st}$h][b] | n.d. | 14 (0.6–77) | |
| CRP [mg/l][b] | n.d. | 1.7 (0.5–23.1) | |
| ANA positive, *n* (%) | n.d. | 56 (84.8) | |
| Anti-Ro positive, *n* (%) | n.d. | 56 (84.8) | |
| Anti-La positive, *n* (%) | n.d. | 38 (57.6) | |
| RF positive, *n* (%) | n.d. | 33 (50) | |
| Focus Score[b] | n.d. | 3 (0–4) | |
| IgG[b] | n.d. | 14.1 (6.4–38.8) | |
| CMV seropositive, *n* (%) | n.d. | 39 (63.9%)[c] | |
| Leukocyte count (per μl)[b] | 5550 (2100–12400) | 5000 (2300–10400) | 0.001 |
| Lymphocyte count (per μl)[b] | 1649 (800–3237) | 1345 (16–2109) | <0.001 |
| Lymphocyte frequency (%)[b] | 30 (10–52) | 27 (0.4–50.1) | 0.245 |
| **Current medication** | | | |
| Corticosteroids, *n* (%) | 0 | 13 (19.7) | |
| Dose [mg/d][a] | n.a. | 6.8 (2.4) | |

[a]Mean (S.D.). [b]Median (range). [c]61 patients tested. ANA: [>1:80]; CRP: [0–5 mg/l]; ESR: [0–30 mm/h]; ESSDAI: EULAR Sjögren Syndrome disease activity index; ESSPRI: EULAR Sjögren Syndrome patient-reported index; HCs: healthy controls; *n*: number; n.a.: not applicable; n.d.: dot determined; pSS: primary Sjögren Syndrome.

pSS cohort (Fig. 1F) but correlated significantly with ESR ($corr_{coeff} = -0.473$; $P = 0.011$). To investigate the interplay between clinical and laboratory parameters including age, sex, CMV serostatus, disease activity, glucocorticoid treatment and smoking status on lymphocyte counts of pSS patients, we performed multivariate regression analysis. However, none of the aforementioned variables was identified as a significant predictor of lymphocyte numbers in pSS patients.

## Homeostatic proliferation is reduced in pSS

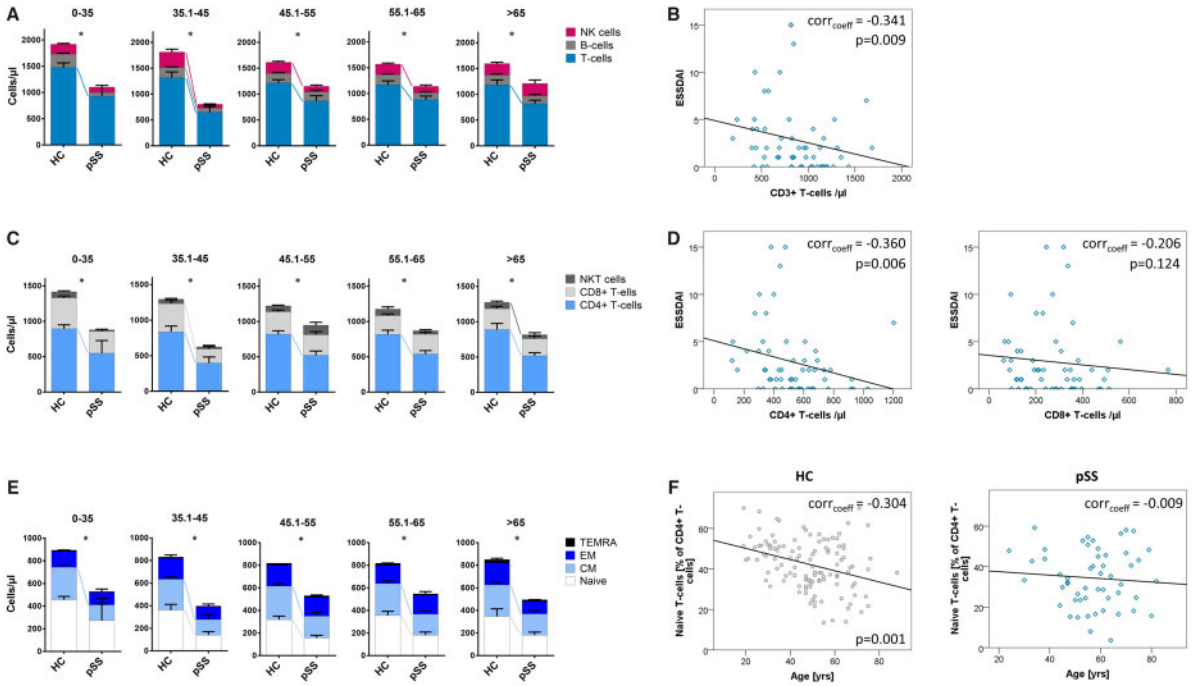
We aimed to investigate if the reduction of naïve $CD4^+$ T cells is attributable to a diminished thymic output or a defect in homeostatic proliferation. To assess the remaining thymic output, we analysed the frequency of $CD31^+$ recent thymic emigrates (RTE) among naïve $CD4^+$ T cells. Of note, the frequency of $CD4^+CD31^+$ RTEs correlated with the number of naïve $CD4^+$ T cells in HCs, but was similar comparing HCs and pSS patients, suggesting an alternative reason for naïve T-cell lymphopenia in pSS patients (Fig. 2A).

Homeostatic proliferation is the physiological response to lymphopenia in order to maintain a constant pool of T cells [20]. We analysed the frequency of $Ki67^+$ in naïve $CD4^+$ T cells as a marker for cell proliferation directly *ex vivo* and observed a substantially reduced T-cell proliferation in pSS patients compared with HC (Fig. 2B). Survival and homeostatic proliferation of naïve T cells are enhanced by IL-7 [20]. To further elucidate the ability of these cells to respond to homeostatic stimuli, we treated MACS-sorted naïve $CD4^+$ T cells with IL-7 and monitored their proliferation *in vitro*. Naïve $CD4^+$ T cells from HCs were more likely to proliferate and divided significantly more often than their pSS counterparts (Fig. 2C). We observed no difference in cell viability or rate of apoptosis (data not shown). When analysing CD127 expression, we found diminished expression in naïve $CD4^+$ T cells of pSS patients compared with HC (Fig. 2D) suggesting lower expression of the IL-7 receptor-α could be responsible for reduced homeostatic proliferation. However, downstream STAT5 phosphorylation was not reduced in naïve $CD4^+$ T cells of pSS patients compared with HC (data not shown).

## Senescent phenotype of naïve $CD4^+$ T cells in pSS

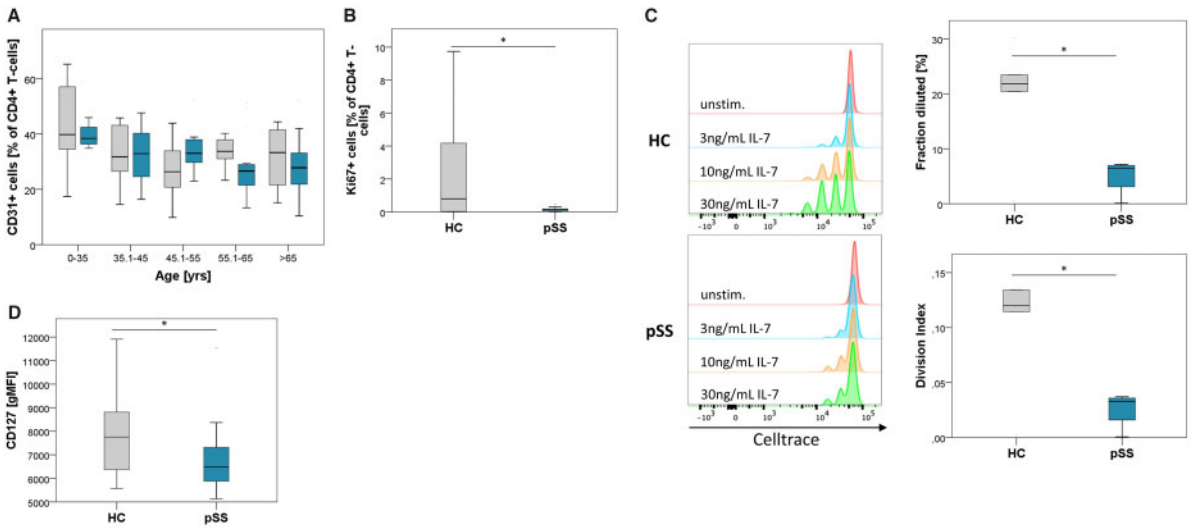
A decline in regenerative capacity and downregulation of CD127 are hallmarks of immune cell aging [21, 22]. To investigate whether the decreased proliferative capacity of naïve $CD4^+$ T cells in pSS is the result of an altered replicative age, we measured the telomere length of naïve T cells of pSS patients and HCs. We observed that naïve T cells from young pSS patients already possess shorter telomeres compared with naïve cells isolated from HCs (Fig. 3A). In addition, memory T cells showed the same changes. Of note, naïve as well as memory $CD4^+$ T cells of patients on corticosteroids had

**Fig. 1** Lymphopenia in pSS patients mainly affects naïve CD4$^+$ T cells



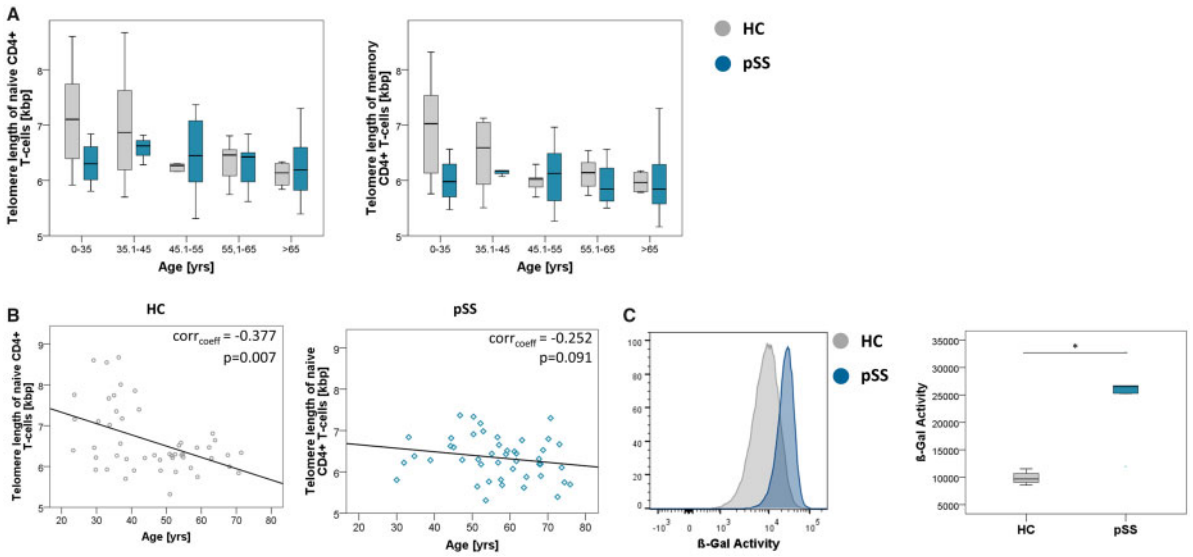

Graphs show (**A**) absolute numbers of CD3$^+$ T cells, CD19$^+$ B-cells and CD56$^+$ NK cells of pSS patients and HCs in age groups as displayed; (**B**) correlation of disease activity score ESSDAI with absolute numbers of CD3$^+$ T cells in pSS patients; (**C**) absolute numbers of CD4$^+$ and CD8$^+$ T cells as well as CD3$^+$CD56$^+$ Natural killer T cells of pSS patients and HCs in age groups as displayed; (**D**) correlations of disease activity score ESSDAI with absolute numbers of CD4$^+$ or CD8$^+$ T cells in pSS patients; (**E**) absolute numbers of naïve, central memory (CM), effector memory (EM) and terminally differentiated effector memory (TEMRA) CD4$^+$ T cells pSS patients and HCs in age groups as displayed; and (**F**) correlations of frequencies of naïve CD4$^+$ T cells with age of pSS patients and HCs. Lines between bars indicate significantly altered fractions. *$P$ <0.05.

**Fig. 2** Naïve CD4$^+$ T cells of pSS patients show signs of decreased homeostatic proliferation




Graphs show (**A**) frequencies of CD31$^+$ recent thymic emigrants (RTE) of pSS patients and HCs; (**B**) frequencies of Ki67$^+$ proliferating naïve CD4$^+$ T cells of pSS patients and HCs directly *ex vivo*; (**C**) frequencies of fraction diluted as well as the division index of naïve CD4$^+$ T cells following stimulation with 30 ng/ml IL-7 *in vitro*; and (**D**) surface expression (MFI) of IL-7R of naïve CD4$^+$ T cells of pSS patients and HCs. *$P$ <0.05.

FIG. 3 Naïve CD4$^+$ T cells of pSS patients have a longer replicative history




Graphs show (**A**) telomere lengths of naïve and memory CD4$^+$ T cells with age in pSS patients and HCs; (**B**) correlations of telomere lengths of naïve CD4$^+$ T cells with age of pSS patients and HCs; and (**C**) expression of senescence-associated $\beta$-galactosidase of naïve CD4$^+$ T cells of pSS patients and HCs. *$P$ <0.05.

significantly shorter telomeres compared with those without (naïve: 6.1 [5.3–6.8] *vs* 6.4 [5.6–7.4], $P = 0.012$; memory: 5.6 [5–6.8] *vs* 6.4 [5.5–7.1], $P = 0.001$). In HCs, telomere length correlated significantly with age, whereas in pSS patients, no significant correlation could be observed (Fig. 3B). Although it was previously reported that female sex and smoking can influence telomere length [23], we did not observe such an effect in our cohort.

Next, we wanted to test the hypothesis that reduced telomere length is associated with expression of senescence-associated β-galactosidase (SABG), a common biomarker for cellular senescence. As shown in Fig. 3C, we observed an increase in SABG in naïve T cells of pSS patients compared with HCs. Taken together; these findings suggest a naïve T-cell phenotype reminiscent of senescent immune cells.

## Insufficient telomerase activity in pSS

In healthy individuals, critical shortening of telomeres is prevented by the induction of telomerase enzyme. To investigate whether telomerase insufficiency contributes to shortening of telomeres in pSS patients, we determined telomerase by TRAP-assay. Young pSS patients showed an increase in telomerase activity, whereas pSS patients older than 45 years showed similar results compared with HCs (Fig. 4A). Correlating telomerase activity with age, we observed that naïve CD4$^+$ T cells from HCs older than 45 years had a 6-fold higher telomerase activity than younger individuals. In contrast, telomerase activity in pSS patients was not associated with age ($corr_{coeff} = -0.033$; $P = 0.829$; Fig. 4B). Similar observations were made in the other T-cell subsets. Correlating telomerase activity with telomere length, we noted an increase of telomerase activity along with shortening of telomeres in HCs, whereas this association was not found in pSS patients (Fig. 4C).

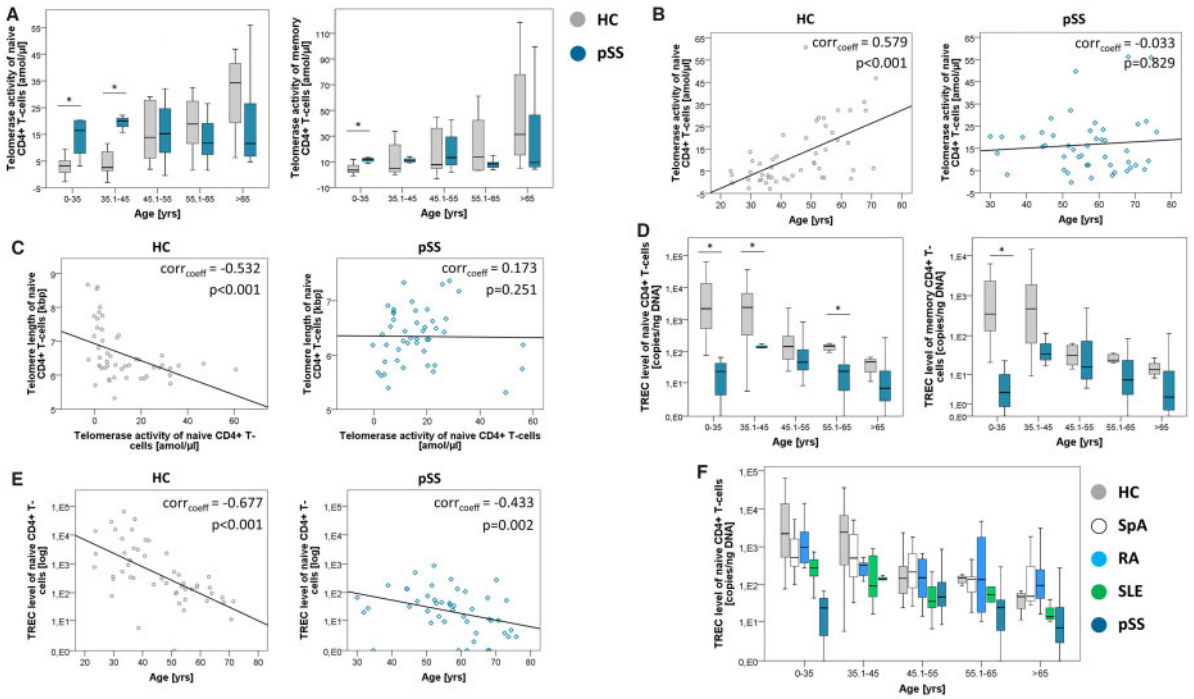
## Increased replicative history of naïve CD4$^+$ T cells in pSS

Shortened telomeres and increased telomerase activity may indicate increased cell divisions in early age. TRECs that are small stable DNA episomes formed during T-cell receptor rearrangement are diluted by each post-thymic division and can be utilized to assess the replicative history of T cells [15].

We found a striking premature decline of TRECs in naïve CD4$^+$ T cells especially in young pSS patients as compared with HCs (Fig. 4D). TREC analysis of memory CD4$^+$ T cells yielded similar results with a smaller number of TRECs overall. TREC levels of naïve CD4$^+$ T cells decline with age [24]. We confirmed this observation in our cohort of healthy subjects. In contrast, we could not observe a similar decrease in pSS patients (Fig. 4E) suggesting enhanced cell divisions at an early age and reduced proliferation rates in established disease. Low TREC levels of naïve CD4$^+$ T cells correlated with their abundance ($r = 0.402$, $P < 0.001$, Supplementary Fig. S2, available at *Rheumatology* online). These findings suggest that naïve pSS CD4$^+$ T cells have undergone extensive replication that is linked to the inability to sustain normal cell numbers.

Immuno-senescence of naïve T cells has been observed in other autoimmune diseases not typically associated with lymphopenia such as RA or ankylosing spondylitis [15, 24]. Therefore, we compared naïve CD4$^+$ T-cell TREC levels of RA, ankylosing spondylitis, SLE and pSS (Fig. 4F). We found that naïve CD4$^+$ T cells in pSS patients had the lowest amount of TRECs

FIG. 4 Lack of correlation of telomerase activity with telomere length in in pSS patients




Graphs show (**A**) telomerase activity of naïve and memory CD4$^+$ T cells with age in pSS patients and HCs; (**B**) correlations of telomerase activity of naïve CD4$^+$ T cells with age of pSS patients and HCs; (**C**) correlations of telomerase activity with telomere lengths of naïve CD4$^+$ T cells of pSS patients and HCs; (**D**) TREC levels of naïve and memory CD4$^+$ T cells with age in pSS patients and HCs; (**E**) correlations of TREC levels of naïve CD4$^+$ T cells with age of pSS patients and HCs; and (**F**) TREC levels of naïve CD4$^+$ T cells with age in RA, SpA, SLE and pSS patients as well as HCs. *$P$ <0.05.

next to those in SLE, another disease known to frequently cause lymphopenia.

Taken together these results indicate extensive replicative history of naïve CD4$^+$ T cells that may lead to a senescent phenotype unable to sustain homeostasis. Our data suggests a close association of these findings to the occurrence of lymphopenia in pSS.

## Discussion

In this study, we identified fundamental alterations in naïve T-cell homeostasis, including reduced homeostatic proliferation, a history of extensive replication and signs of cellular senescence as a potential cause for lymphopenia in pSS patients.

In healthy individuals, the number of naïve T lymphocytes in the periphery is almost constant over time [25]. This homeostasis requires a strict control in order to maintain cell numbers with advancing age. Naïve T-cell homeostasis, therefore, is dependent on several interconnected events including thymic output, antigen-dependent and antigen-independent proliferation, maturation into memory T cells, accumulation in lymphoid or inflamed tissues, increased apoptosis, virus infection and disease treatment.

In general, the life span of naïve T cells is longer compared with other cell populations [26–28], and their survival depends on TCR-MHC interactions and cytokines, primarily IL-7 [29, 30]. Here, we show that naïve CD4$^+$ T cells of pSS patients have a reduced frequency of proliferating Ki67$^+$ cells compared with HC. Moreover, we observed a reduced proliferative response of naïve CD4$^+$ T cells from pSS patients to stimulation with IL-7 *in vitro*, suggesting that homeostatic T-cell proliferation is disturbed in patients with disease. This finding can potentially be explained by a diminished expression of IL-7R (CD127) found on naïve pSS CD4$^+$ T cells. Interestingly, signalling down-stream of the IL-7R was not altered *in vitro*. Recent thymic emigrants were shown to express low levels of IL-7R too, but they were more responsive to cytokine stimulation than mature naïve T cells *in vitro*. *In vivo*, however, mature naïve T cells had a greater proliferative response in the presence of IL-7, demonstrating a discrepancy between *in vivo* and *in vitro* data [31]. Expression of IL-7R decreases with age [22]. Furthermore, prolonged inflammation such as chronic viral infection reduced expression of IL-7R on T cells is associated with T-cell senescence and lymphopenia [32]. We observed similar alterations in naïve CD4$^+$ T cells of pSS patients: (i) decreased ability to proliferate; (ii) evidence of an

extensive replicative history; (iii) accumulation of senescence-associated β-galactosidase; (iv) shortened telomeres; and (v) insufficient telomerase activity in naïve $CD4^+$ T cells of pSS patients to compensate for the aforementioned deficiencies. Attrition of telomeres in aging cells occurs as a consequence of cellular replication and DNA damage. Like stem cells, naïve T cells express telomerase as telomeres shorten [21]. In pSS, we show that naïve $CD4^+$ T cells already have shortened telomeres in young patients resulting in early telomerase activity. This seems to be a consequence of the extensive replicative history of these cells as indicated by low TRECs. As we observed this phenomenon already in young pSS patients, the triggering event must have had occurred very early in the disease or even before its onset. Dilution of TRECs also occurs in viral infection [33] which has been linked to both T-cell senescence and pSS [34]. Interestingly, we observed that CMV seropositivity correlated with the occurrence of terminally differentiated $CD4^+$ T cells, but neither with TREC levels nor with telomere length of naive $CD4^+$ T cells (data not shown). Nevertheless, our findings could reflect a pronounced anti-viral reaction preceding or even triggering pSS.

To some extent, increased replicative history and a premature aging phenotype of naïve T cells has been reported for various autoimmune diseases including RA and ankylosing spondylitis [15, 24]. Therefore, it is conceivable that part of the disturbed T-cell homeostasis is a consequence of persistent inflammation. In pSS, these changes are more profound, resulting in peripheral blood lymphopenia commonly not found in RA or ankylosing spondylitis.

In addition to the lack of homeostatic proliferation, naïve $CD4^+$ T-cell lymphopenia could be a result of cell death, memory cell generation, naive T-cell homing to lymphoid tissues, or thymic failure [35]. Increased loss of naïve $CD4^+$ T cells by enhanced apoptosis has been described in the context of RA and chronic infection [36, 37]. In pSS, a higher rate of T-cell apoptosis *in vitro* and *ex vivo* has been reported in the past [38, 39]. However, this occurred mainly in $CD95^+$ T cells and not in naïve ($CD95^-$) T cells, which is in line with our observations.

Loss of circulating naïve $CD4^+$ T cells due to increased migration of cells to other compartments such as the salivary glands or the lymphatic tissues is another potential reason for lymphopenia. Large-scale egression of circulating naïve $CD4^+$ T cells into the salivary glands seems unlikely because previous studies revealed mostly antigen-experienced T cells in pSS salivary glands [40, 41]. Furthermore, loss of circulating naïve $CD4^+$ T cells with age was accompanied by equal reductions in lymph nodes and spleen in healthy individuals [42, 43]. These data suggest an equilibrium between the compartments. However, we cannot rule out that enhanced recruitment to the lymph nodes and spleen occurs in pSS.

Our study has some limitations: first, due to the cross-sectional design of the study only a snapshot from peripheral naïve T-cell homeostasis was observed. Therefore, time-, age- and disease course-dependent changes cannot be elucidated unequivocally. Given the fact that disease onset is probably even before time of diagnosis and that disease progression takes place over years, it is difficult to prove that the disturbed naïve T-cell homeostasis observed is the cause of lymphopenia rather than a subsequent bystander effect.

Second, isolation of naïve $CD4^+$ T cells was based on expression of surface markers CD45RA and CD45RO. Although only a minor fraction of the analysed $CD4^+$ cells were terminally differentiated effector T cells, those might also re-express CD45RA and therefore potentially contribute to the effects observed in naïve T cells.

In conclusion, this work describes fundamental alterations in naïve T-cell homeostasis of pSS patients and therefore potentially identifies the cause of lymphopenia, a significant risk factor in the disease pathogenesis.

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## Supplementary data

Supplementary data are available at *Rheumatology* online.

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