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Concise report

Increased expression of Fas on group 2 and 3 innate lymphoid cells is associated with an interferon signature in systemic lupus erythematosus and Sjögren's syndrome

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Abstract

Objective. The role of innate lymphoid cells (ILCs) in the pathophysiology of rheumatic diseases is emerging. Evidence from animal studies implicate type I IFN, produced by plasmacytoid dendritic cells, to be involved in regulating the survival of group 2 and group 3 ILCs (ILC2s and ILC3s) via the upregulation of Fas (CD95) expression. For the first time, we explored the frequency and phenotype of circulating ILCs in SLE and primary Sjögren's syndrome (pSS) in relationship to the IFN signature.

Methods. Frequencies and phenotypes of ILC subsets and plasmacytoid dendritic cells were assessed by flow cytometry in peripheral blood of patients with SLE ($n = 20$), pSS ($n = 20$) and healthy controls ($n = 17$). Patients were stratified by the presence or absence of an IFN signature as assessed by RT-qPCR on circulating mononuclear cells.

Results. ILC1 frequencies were increased in peripheral blood of patients with SLE as compared with healthy controls and correlate with disease activity in pSS patients. Overall, the frequencies of ILC2s or ILC3s did not differ between patients with SLE, pSS and healthy controls. However, patients with a high type I IFN signature expressed elevated levels of Fas on ILC2s and ILC3s, which coincided with decreased frequencies of these cells in blood.

Conclusion. The presence of a type I IFN signature is related to Fas expression and frequencies of circulating ILC2s and ILC3s in patients with SLE and pSS, potentially altering the homeostatic balance of ILCs.

Key words: systemic lupus erythematosus, Sjögren's syndrome, innate lymphoid cells, type I interferon, Fas

CLINICAL SCIENCE

Rheumatology key messages

- Group 1 innate Llymphoid cells: increased in lupus and associated with disease activity in Sjögren's syndrome.
- Interferon signature correlates with Fas on group 2/3 innate lymphoid cells in lupus and Sjögren's.

Introduction

Innate lymphoid cells (ILCs) are immune cells of lymphoid morphology that play an important role in epithelial homeostasis. ILCs lack the expression of T cell receptors and other 'lineage' markers characteristic for other haematopoietic cells, but do express CD45, and CD117 (c-Kit) and/or CD294 (CRTH2) in addition to high expression of IL-7 receptor (CD127) [1]. Besides their location at barrier sites, ILCs can be found in low numbers in blood. In response to environmental triggers, ILCs rapidly release cytokines. The expression of transcription factors and the production of cytokines distinguishes three subsets of ILCs that mirror the well-known subsets of helper T

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cells (Th). ILC1s express T-bet and produce IFN γ and TNF α, ILC2s express GATA-3 and produce interleukin (IL) 4, 5 and 13 and ILC3s express RORγt and produce IL-17 and 22, analogous to Th1, Th2 and Th17 cells, respectively. Due to their high expression of the IL-7 receptor, survival of ILCs, in particular ILC2s and ILC3s, is critically dependent on IL-7 [1, 2].

Recent studies have linked alterations in the frequency and function of ILCs to the pathophysiology of rheumatic diseases, in particular RA and spondyloarthropathies [2]. SLE and pSS are systemic autoimmune diseases with involvement of epithelial tissue including the salivary and lacrimal glands, skin and oral mucosa. Central to the pathogenesis of SLE and pSS is the increased activity of T and B cells leading to production of pathogenic autoantibodies. Increased activity of the IL-7 axis drives T and B cell hyperactivity in SLE and pSS and is related to major pathologic features such as lupus nephritis and dryness [3, 4].

In SLE and pSS, increased levels of type I IFNs (e.g. IFNα) are associated with disease activity, and these IFNs regulate multiple key immunological processes, including B cell hyperactivity. Mechanistically, immune complexes of RNA/DNA binding autoantibodies have been shown to activate plasmacytoid dendritic cells (pDC) to produce type I IFN (IFNα) in SLE and pSS, resulting in an overexpression of type I IFN-inducible genes, known as the IFN signature [5].

Evidence from animal studies that model HIV and asthma indicate a close relationship between IFNα-producing pDCs and the fate of group 2 and group 3 ILCs. pDC-derived IFNα induces the expression of Fas (first apoptosis signal receptor, CD95) and other proapoptotic molecules on ILC2s and ILC3s, rendering them susceptible to apoptosis [6–8]. In patients with HIV, ILC2s and ILC3s are decreased in frequency and characterized by a high expression of Fas [9]. However, no previous studies have investigated ILCs in patients with SLE and pSS in relation to the IFN signature. Here we explored the frequency and phenotype of circulating ILCs in SLE and pSS in relation to pDC activation and the subsequent IFN signature.

Methods

Patients

Peripheral blood mononuclear cells (PBMCs) from patients and healthy controls were isolated by ficoll density gradient centrifugation and cryopreserved in liquid nitrogen until further use. A total of 20 patients with SLE, 20 patients with pSS and 17 age- and sex-matched healthy controls were included in this study. SLE patients met the ACR classification criteria and patients with pSS met the American-European Consensus Group criteria . None of the included patients had clinical evidence of an ongoing infection. This study was approved by the ethical committee of the UMC Utrecht, in accordance with the Helsinki declaration. All patients and healthy controls signed informed consent prior to the donation of blood. Patient characteristics can be found in Supplementary Table S1, available at *Rheumatology* online.

Flow cytometry

ILCs were identified as previously described by our group [10]. A list of used antibodies can be found in Supplementary Table S2, available at *Rheumatology* online. 5–10 × 10e6 PBMC were stained and subsequently acquired on an LSR Fortessa (Becton Dickinson). Dead cells were excluded using the fixable viability dye eF506 (eBioscience). From the lymphocyte gate, ILCs were identified as CD45+Lin-CD127+. The expression of CD117 (c-Kit) and CD294 (CRTH2) were used to identify ILC1s (CD117-CD294-), ILC2s (CD294+) and ILC3s (CD117+CD294-) subsets. The gating strategies for Fas (CD95) were set based on the appropriate fluorescence minus one, using the identical panel of antibodies but leaving out the anti-Fas antibody. The level of Fas expression per cell subset was determined using the MFI.

A second flow cytometry panel was used to identify the frequency of pDCs among CD45+ cells (defined as CD123+BDCA2+BDCA4+ cells) and the expression of Siglec-1 (Sialic acid-binding immunoglobulin-type lectin-1, CD169, an IFN inducible protein [11]) on CD14+ monocytes.

qPCR

RNA was extracted from PBMCs using the Allprep universal kit (Qiagen) according to the manufacturer's instructions. Complementary DNA was synthesized from 200 ng RNA using iScript (BioRad). RT-qPCR was performed on a Quantstudio 12k Flex (Thermofisher). The expression of IFI44, IFI44L, MX1, Ly6E and IFIT3 normalized to the housekeeping gene GUSB were used to calculate an IFN scores. A list of primer sequences can be found in Supplementary Table S3, available at *Rheumatology* online.

Statistics

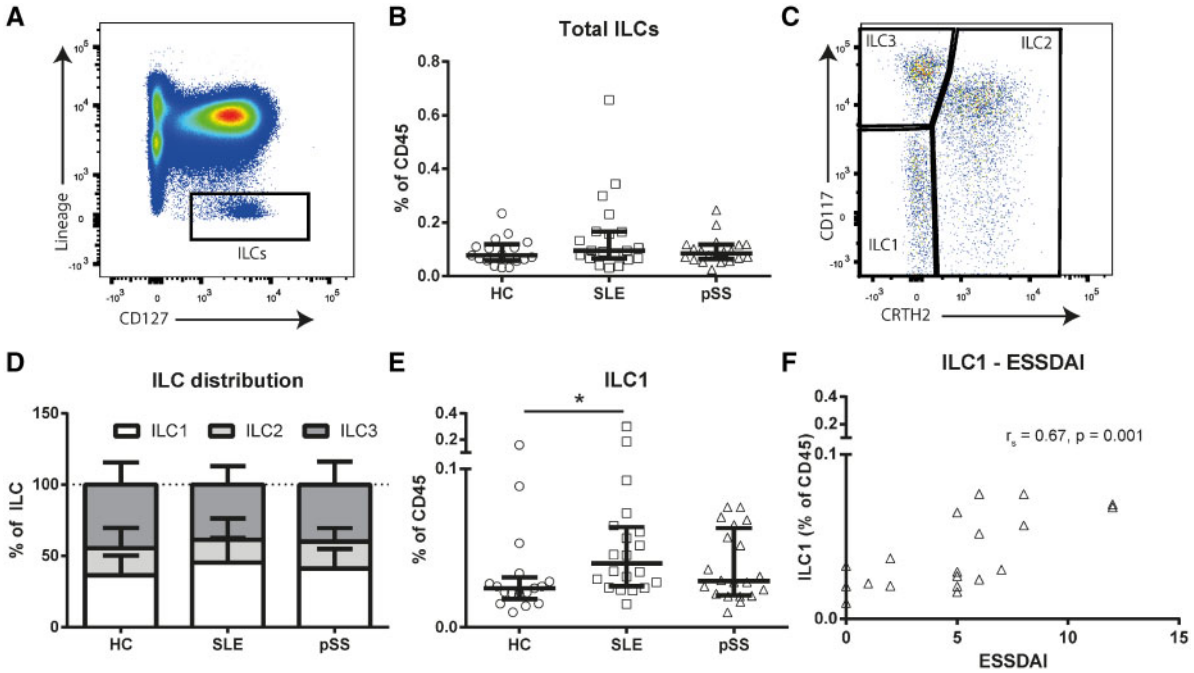
The type I IFN scores were calculated as previously described [12, 13]. An optimal cut-off to discern IFN-high and IFN-low patients' subgroups was identified at the maximum J-statistic of the Youden's index of the receiver operating characteristics curve as described [13]. Mann–Whitney U tests and Spearman rank correlations were used to test statistical significance. Because multiple groups were compared, Kruskal-Wallis test with *post-hoc* Dunn's correction was applied to correct for multiple testing. All tests were conducted two sided at an alpha level of 0.05. In Figs 1 and 2, HCs are depicted as circles, patients with SLE as squares and patients with pSS as triangles.

Results

ILC1s are increased in SLE and are associated with disease activity in pSS

We readily identified circulating ILCs among PBMCs by their high expression of CD127 and the absence of lineage markers (Fig. 1A). The total frequencies of ILCs did not differ among SLE and pSS patients as compared with HCs (Fig. 1B). Using the expression of CD117 and CD294, we discerned three populations of ILCs: CD117-CD294- ILC1s, CD294+ ILC2s and CD117+CD294- ILC3s

FIG. 1 Increased circulating ILC1 frequency in patients with SLE and pSS with high disease activity



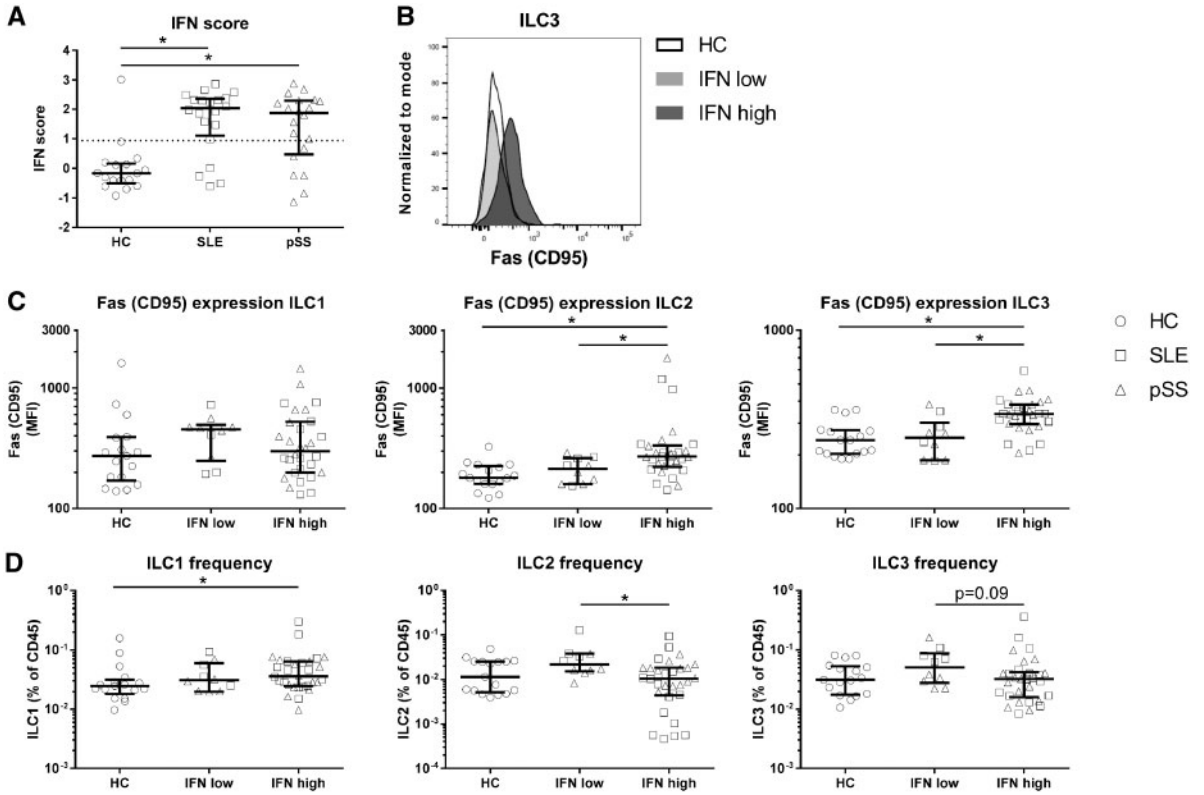
(**A**) Identification of ILCs in the peripheral blood of SLE and pSS patients based on lack of expression of lineage markers and expression of IL-7Rα (CD127). (**B**) Frequency of total ILCs in patients with SLE and pSS and HCs. (**C**) Identification of the three ILC subsets on the basis of the expression of CD117 and CD294. (**D**) ILC subset distributions in HC, SLE and pSS. (**E**) ILC1 frequencies in HC, SLE and pSS. (**F**) Correlation of ILC1 frequencies with disease activity in patients with pSS. SLE patients and pSS patients are combined for stratification based on IFN signature. HCs are depicted as circles, patients with SLE as squares and patients with pSS as triangles. SLE: $n = 20$, pSS: $n = 20$, HC: $n = 17$. HC: healthy controls; ILC: innate lymphoid cells.

(Fig. 1C). There were no changes in the proportions of ILC subsets among total ILCs among patients with SLE, pSS and HCs (Fig. 1D). With the exception of ILC1s being increased in patients with SLE as compared with HCs (Fig. 1E), no differences were found in the frequencies of ILCs subsets among patients with SLE, pSS and HCs (Supplementary Fig. S1, available at *Rheumatology* online). Within the pSS group, higher ILC1 frequencies were associated with higher disease activity as measured by ESSDAI ($r = 0.68$, $P = 0.001$, Fig. 1F) as well as serum IgG levels ($r = 0.53$, $P = 0.015$, Supplementary Fig. 2, available at *Rheumatology* online) and with presence of anti-La/SSB autoantibodies ($P = 0.01$, Supplementary Fig. S2, available at *Rheumatology* online). No correlations with SLE clinical features were found, including SLEDAI, although overall mostly patients with low disease activity were included.

The presence of a type I IFN signature is associated with elevated Fas expression on ILC2 and ILC3 subsets and decreased cell frequency

Elevated IFN scores were observed in patients with SLE and pSS ($P < 0.001$, Fig. 2A), which strongly correlated to monocyte Siglec-1 (CD169) expression ($r = 0.82$, $P < 0.001$, Supplementary Fig. S3, available at *Rheumatology* online), confirming the robust identification of the IFN signature. Setting a threshold using the HCs, 16/20 (80%) and 14/20 (70%) patients with SLE and pSS respectively were characterized by a high type I IFN signature (IFN-high, Fig. 2A). In patients with a high IFN signature, Fas expression was upregulated on both ILC2 and ILC3 subsets (Fig. 2B and C). In contrast, Fas expression on ILC1s was not related to the IFN signature (Fig. 2C). The increased expression of Fas on the ILC2 and ILC3 subsets in IFN-high patients was associated with a decrease in ILC2s ($P = 0.01$, Fig. 2D) and a similar trend in ILC3s ($P = 0.09$, Fig. 2D) as compared with IFN-low patients. ILC1 frequencies did not differ between IFN-high and IFN-low patients (Fig. 2D). Circulating pDCs were decreased in SLE and pSS and correlated with Fas expression on ILC2 and ILC3 subsets ($r = -0.39$, $P = 0.01$, and $r = -0.38$, $P = 0.02$ for ILC2 and ILC3 respectively, Supplementary Fig. S4, available at *Rheumatology* online). If correction for multiple testing is applied (Kruskal-Wallis with *post hoc* Dunn's correction), most comparisons mentioned above and in Fig. 2 remain statistically significant, except for the following comparisons. For Fas expression on ILC2 between IFN-low and IFN-high patients and ILC1 frequency between HC and IFN-high patients trends towards statistical significance are

FIG. 2 Elevated Fas-expression on ILC2s and ILC3s from IFN-high patients is associated with decreased ILC2/3 frequencies



(**A**) IFN scores in HCs and patients with SLE and pSS. (**B**) Representative FACS plot. (**C**) Expression of Fas (CD95) on ILC2s and ILC3s in HCs and patients with a low or high type I IFN signature. (**D**) Frequencies of ILC2s and ILC3s in HCs and patients with a low or high type I IFN signature. The gating strategies for Fas (CD95) were set based on the appropriate FMO, using the identical panel of antibodies but leaving out the Fas antibody. The level of Fas expression per cell subset was determined using the MFI. SLE patients and pSS patients are combined for stratification based on IFN signature. HCs are depicted as circles, patients with SLE as squares and patients with pSS as triangles. SLE: $n = 20$ ($n = 16$ IFN-high), pSS: $n = 20$ ($n = 14$ IFN-high). HC: healthy controls; ILC: innate lymphoid cells; Fas: CD95; FMO: fluorescence minus one; MFI: median fluorescence intensity.

found when correcting for multiple testing (P = 0.07 and P = 0.08, respectively).

Discussion

Here we evaluated for the first time the frequency and phenotype of circulating ILC subsets in patients with SLE and pSS. ILC1s were found elevated in blood of patients with SLE and were associated with disease activity in patients with pSS. When comparing SLE and pSS, no differences were observed in the frequencies of ILC subsets. However, high expression of Fas (CD95) on circulating ILC2 and ILC3 subsets was associated with an increased IFN signature and reduced frequencies of circulating ILC2s and ILC3s.

Increased Fas expression on ILC2s and ILC3s may render these cells more susceptible to apoptosis. These observations corroborate previous studies in mice that reported a link between type I IFN, pDC activation and apoptosis of circulating ILC2s and ILC3s [6–8]. We here report unprecedented data on a potential similar regulation in the blood of patients with systemic autoimmune diseases. The differences between ILC2s and ILC3s with upregulated Fas and ILC1s with unaffected Fas expression is not explained, but may be related to responsiveness to IFNs or other activating factors. Hence, Fas regulation may be associated with specific activation facilitating a delicate balance between effector functions including their homing to the sites of inflammation and apoptosis.

ILCs were only recently identified. In the pre-ILC era, a subset of ILC1-like NK cells were reported to be increased in patients with SLE compared with HC [1]. Here we report that circulating ILC1s, classified according to current phenotypical definitions, are increased in patients with SLE and in patients with pSS with higher disease activity.

How ILC1s may contribute to disease activity in pSS is unclear. IFN-γ is thought to play an important role in pSS [14], and because ILC1s can produce high levels of this cytokine, this may play a role in the disease process. In addition, they can secrete granzymes and perforins that can contribute to immunopathology at epithelial sites [15]. Why numbers of ILC1s are increased in the blood of pSS patients and SLE patients is left unexplained, but could be related to increased expansion and recirculation. This remains to be studied.

In mice models of RA, the ILC2 subset is crucial for the resolution of autoimmune inflammation by producing IL-9, which promotes regulatory T cell function and restores immune homeostasis. In humans, circulating ILC2s are reduced in active RA and normalize to normal levels in remission [16]. Whether reduced numbers in the blood represent systemic cell death (reducing regulatory ILC function) or increased migration of pro-inflammatory ILC2s from the site of inflammation remains to be established. In our cohort, the frequency of circulating ILC2s is reduced in patients with a high IFN signature. Presence of a high IFN signature is associated with disease activity in these patients [11, 13]. Hence, possibly the decrease in ILC2s in IFN-high SLE and pSS patients may contribute to ongoing inflammation via similar mechanisms as in RA, either decrease of the frequency of suppressive ILC2s or enhanced migration of pro-inflammatory ILC2s. The latter is supported by data from patients with SSc and pSS as described below [17–19]. In systemic sclerosis, another disease characterized by a type I IFN signature, ILC2s are elevated in the skin [17]. Migration of ILCs into target tissues might therefore represent another explanation for the reduced levels of circulating ILC2s and ILC3s in IFN-high patients. No studies so far have assessed ILCs in affected tissues of patients with SLE. In pSS, inflammatory ILC2s and IL-22 producing NKp44$^+$ cells, which might represent ILC3s, are found in the salivary glands of patients with pSS and correlate with the amount of lymphocytic infiltration [18, 19]. In addition, by secreting IL-4 and IL-22 ILC2s have been indicated to contribute to B cell hyperactivity and lymphocyte infiltration in (experimental) Sjögren's-like disease [20]. Lymphoid tissue inducer cells (LTi) cells are a subset of ILC3s and are present in lymphoid aggregates potentially contributing to lymphoid neogenesis [1]. Thus, reduced circulating frequencies of ILC2s and ILC3s may reflect their migration to inflammatory sites. Inconsistent with reduced numbers of ILC2s in systemic autoimmune diseases, Guggino *et al.* found elevated frequencies of inflammatory ILC2s in the circulation of pSS patients [19]. In the present study, elevated frequencies of total ILC2s were not found in pSS patients, and were reduced in IFN+ patients. The difference between the studies may be explained by patient heterogeneity, where a lower number of IFN+ patients may result in somewhat higher ILC2 counts. In addition, the difference between the studies may be explained by the use of different gating strategies, as in our study we used the gating strategy described by Vély *et al.* [21] and inflammatory ILC2s were not assessed.

Survival of ILCs, including LTi cells is dependent on IL-7, which is elevated in salivary glands of pSS patients [14]. The IL-7/IL-7R axis plays an important role in formation of ectopic lymphoid structures in non-lymphoid tissues, including the salivary gland, and this has been shown to require presence of LTi cells [22]. Altogether, this suggests a role for ILCs in development of lymphocytic infiltrates, including germinal centre-like structures, in the glands of pSS patients.

A limitation of our study is that the patients with SLE were treated with immunosuppressive drugs and had relatively low disease activity, potentially hindering the analysis on the relationship between ILCs and SLE disease activity. However, there was no difference in hydroxychloroquine use between SLE patients with or without an IFN signature (χ^2, P = 0.6), reassuring that the difference between IFN-low and -high patients is not merely a consequence of differences in medication use. Characteristics of IFN-high *vs* IFN-low patients are shown in Supplementary Table S4, available at *Rheumatology* online. Future studies should also evaluate whether immunosuppressive medication can impact on ILC phenotype and function. Similarly, whether ILC subtypes are altered in affected organs of patients with SLE is currently unknown and should be investigated.

The absolute numbers calculated using absolute lymphocyte counts and the percentage of ILCs from the lymphocyte gate, also indicated significant decreases in ILC2 and ILC3 numbers (Supplementary Fig. S5, available at *Rheumatology* online). These reduced absolute counts suggest that increased Fas expression is related to an absolute decrease of ILC numbers and not just to relative changes. A decrease in ILCs in IFN+ patients is associated with lymphopenia, which is typical of patients with SLE and pSS. The mechanism is not completely understood but in line with regulation of ILC frequencies may depend on increased apoptosis of circulating cells and their migration.

In this study we did not assess presence of ILCs in affected tissues from SLE and pSS patients. The presence of the various ILC subsets and their distribution in kidneys or skin from SLE patients and salivary glands from pSS patients needs to be further assessed in future studies. We hypothesize that ILCs are dysfunctional at the site of inflammation and in the circulation of SLE and pSS patients, potentially contributing to epithelial dysfunction in the affected organs. Studies in mice [23] and previous work in psoriasis patients with and without arthritis suggest that ILCs recirculate in low numbers during chronic inflammation [10]. Studying local ILCs will add to the understanding of the role of ILCs in SLE and pSS immunopathology.

Perturbations in the immune system are often shared between different autoimmune diseases, with the IFN signature as a clear example. It is becoming increasingly clear that both from an immunological as well as a clinical perspective, stratification of patients with SLE and pSS on a molecular level, as by the IFN signature, may aid in the identification of subgroups with different immunological

alterations and even response to therapy [12, 13, 24]. Here we report that alterations in the frequency and phenotype of circulating ILCs is related to the IFN signature in SLE and pSS. On the basis of our observations, we propose that future studies on ILCs in patients with autoimmune diseases, whether in blood or tissue, should take into account the presence or absence of the (local) IFN signature, as this may significantly impact their function and role in autoimmune diseases.

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Supplementary data

Supplementary data are available at *Rheumatology* online.

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