Dysregulated plasmacytoid dendritic cells in primary Sjögren's syndrome: normal functionality but decreased quantities

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Abstract

Objective

Primary Sjögren's syndrome (pSS) is an autoimmune disorder characterised by type I interferons (IFNs) signature, and plasmacytoid dendritic cell (pDC) is the primary producer of type I IFNs. However, the role of pDCs in the pathogenesis of pSS remains unclear. Our study aims to explore the dysregulation of pDCs in pSS, as well as the underlying mechanisms.

Methods

In the present study, we included a total of 104 patients with pSS (64 were untreated and 40 were treated with hydroxychloroquine) and 64 healthy controls. We examined the frequency, activation markers, cytokines secretion, and infiltration into affected tissue of pDCs derived from pSS. Clinical correlation analyses and co-culture systems of pDCs and B cells were conducted to explore the pathogenesis of pDC reduction in pSS.

Results

The frequency of pDC was significantly reduced in the peripheral blood of pSS. pDCs derived from pSS exhibited higher expression levels of Toll-like receptor 7 in the resting state. The IFN-α production by pDCs from pSS patients is similar to that of matched HC in vitro, regardless of whether the patients' IFN signature is negative or positive. Local invasion of pDCs into affected glands was detected but not common in pSS. In pSS patients, the proportion of circulating pDCs negatively correlated with serum IgG, IgA, and anti-SSA autoantibodies. pDCs promote proliferation, activation, differentiation, and antibody production of B cells. Conversely, excessive IgG promoted pDC apoptosis via neonatal Fc receptor (FcRn) and caused the decline of pDCs in pSS.

Conclusion

Our data enhances the understanding of pDC functionality in pSS and the mechanisms of their abnormal reduction in peripheral blood. We first report that excess IgG induced pDC apoptosis via FcRn and promoted the reduction of peripheral pDCs in patients with pSS.

Key words

primary Sjögren's syndrome, plasmacytoid dendritic cell, type I interferon, hypergammaglobulinaemia, neonatal Fc receptor

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Introduction

Primary Sjögren's syndrome (pSS) is a typical autoimmune disorder characterised by focal lymphocytic infiltrations, damage, and dysfunction in salivary and lacrimal glands (1). Lung, kidney and nervous system involvement could also occur in severe cases (2). pSS is acknowledged as a multifactorial disease which can be triggered by environmental, infectious, hormonal, and genetic factors (3). The activation of Type I interferon (IFN) as a key factor in the development of pSS is widely validated (3). Type I IFN signatures in peripheral blood mononuclear cells (PBMCs), B cells, monocytes, neutrophils, as well as the affected tissues were identified (4-9). Moreover, IFNα inducible proteins: MDA-5, IFIT-3 (10), and B cell-activating factor (BAFF) (11) were found to be highly expressed in salivary glands. Type I IFNs could enhance T cell and B cell responses and promote the production of autoantibodies (10, 12), implicating their essential roles in the pathogenesis of pSS.

Acting as a primary producer of type I IFNs and serving as a crucial link between innate and adaptive immunity, pDCs make non-negligible contributions to the development of autoimmune disease (13, 14). In patients with systemic lupus erythematosus (SLE), pDCs can be activated through various pathways such as immune complexes, neutrophil extracellular traps, and mitochondrial DNA(15-17). Activated pDCs subsequently trigger immune responses and promote the production of autoantibodies (18). Studies in systemic sclerosis (SSc) (19), Psoriasis (20), and autoimmune diabetes (21) have shown that infiltrating pDCs can exacerbate local inflammation in target tissue by releasing IFN-α and proinflammatory factors. Currently, studies on the role of pDCs in pSS are limited. Here, we conducted this study into the quantity, phenotype, and functions of pDCs to gain a deeper understanding of their roles in the pathogenesis and the associated type I IFN signatures in pSS. Through these investigations, we aim to uncover the dysregulation of pDCs in the pathological processes of pSS and provide new insights for treating this disease.

Methods

Patients and methods

All newly-onset pSS and SLE patients were enrolled in Peking Union Medical College Hospital (PUMCH). They met the 2016 ACR-EULAR classification (22) and the 1997 classification criteria of the American College of Rheumatology (23), respectively. Labial tissues were obtained from patients who underwent labial gland biopsy at the Department of Stomatology in PUMCH. All patients signed informed consent for their residual tissues after the pathological examination. The study was approved by the Ethics Committee of the Peking Union Medical College Hospital (no: K2525) and was conducted in accordance with the Helsinki Declaration. Informed consents of all the patients were obtained.

PBMC isolation and in vitro stimulation

Human PBMCs were isolated with Ficoll-Paque density (DAKEWE, China) as previously described (7). For the plasma stimulation, PBMCs from healthy controls (HCs) were seeded into 24-well plates at a density of 1×10^6/ml and maintained in RPMI 1640 (Gibco, A10491, USA) with 20% mixed plasma from ten pSS or HCs in the presence of 100 ng/ml IL-3 for 4 hours.

For IgG stimulation, freshly isolated PBMCs from HCs were first incubated with 100 ng/ml IL-3 for 2 hours, then stimulated with 0.5-2 g/L IgG (SP001, Solarbio). IL-3 is used to assist the survival of pDCs *in vitro* (24, 25).

In vitro purification and stimulation of pDC

pDCs were purified using CD304 microbeads (130-090-532, Miltenyi Biotec) and maintained in RPMI 1640 supplemented with 10% foetal bovine serum (FBS) (Gibco, USA), 100 U/ml penicillin and 100 μg/ml streptomycin (15140122, ThermoFisher). For cytokines detection, PBMCs were stimulated with 5 μg/ml R848 (tlrl-r848, InvivoGen) or 10 μg/ml ODN-2216 (tlrl-2216, InvivoGen) in the presence of 100 ng/ml IL-3 (Peprotech, 200-03). Freshly isolated PBMCs from pSS or

HC were cultured in the presence of IL-3, R848 or ODN-2216 was added at the 6th hour, and GolgiPlug was added at the 9th hour for IFN-α detection. Freshly isolated PBMCs from pSS or HC were cultured in the presence of IL-3 and GolgiPlug, stimulated by R848 or ODN2216 for 12 hours for IL-6 and TNF-α detection. Secretion of cytokines by pDCs was examined through intracellular staining at the 12th hour. R848 is a ligand for Toll-like receptor 7/8 (TLR7/8) and ODN-2216 is a ligand for TLR9.

IFN signature

12 key genes (IFITM1, IRF7, IFI27, IFI44L, IFIT1, IFIT2, MX1, IRF6, IRF1, IFI44, LY6E, IFIT3) of the type I interferon pathway to be included in our IFN signature model (6, 26) (Supplementary Table S1). Polymerase chain reaction (PCR) was performed to assess the relative expression levels of every gene and the IFN scores for each patient and compared these with HCs, categorising the pSS patients into two groups: one group was IFN-positive, and the other was IFN-negative. In our model, we first calculated the relative expression levels of the 12 genes in PBMC for each individual, ultimately using an IFN score >23.7 as the cut-off value. This cut-off value showed that 56.1% of the pSS group had a type I IFN signature and only 5.2% of the HC group. The proportion of IFN-positive patients among pSS patients and HC in our model is very close to that reported in the literature reference (6), indicating the validity of our model.

pDC and B cell coculture

B cells were purified using B cell isolation kit II (130-091-151, Miltenyi Biotec) following the manufacturer's protocol. B cells were activated with 5 μg/mL anti-IgM (314502, BioLegend), 500 ng/mL sCD40L (310-02, Peprotech), 100 ng/ml IL-4 (200-04, Peprotech), and 50 ng/ml IL-21 (200-21, Peprotech). For the experiments on the promotion of B cell differentiation by pDCs, purified $CD19^+$ B cells (5×10^5/ml) and pDC from allogeneic HCs were added at the ratio of 10:1 in a round-bottom 96-well cell culture plate. On day 7, the cell culture supernatant was collected, and B cell subpopulations were simultaneously detected by flow cytometry.

For the induction of pDC apoptosis by supernatant from B cell culture, B cells from patients with pSS and HCs matched for age and gender were cultured separately using the aforementioned protocol for 5 days in a 96-well plate. The B cell density was adjusted to 1×10^5 cells in 200 μl of 1640 RPMI per well. On day 5, pDCs from another HC were purified using CD304 magnetic beads, and 1.5×10^4 pDCs were added to each well of the B cell culture plate. After a 4-hour incubation, the viability of pDCs was assessed by flow cytometry.

Flow cytometry

Cells were harvested and resuspended at the concentration of 5×10^6 cells/ml. Fluorochrome-conjugated monoclonal antibodies for cell surface staining were as follows: anti-human CD303-APC, CD303-PE/Cyanine7, CD123-PerCP/Cyanine5.5, CD123-FITC, Lineage-FITC, HLA-DR-APC/Cyanine7, CD80-PE, CD83-FITC, CD86-PE, CD19-APC/Cyanine7, CD74-APC, CD27-PerCP/Cyanine5.5, IgD- PE/Cyanine7, CCR2- APC/Cyanine7, CCR4-PE, CCR5-PE, CCR7-PE, CCR10-PE, CD38-APC, CD138-PE, CD24-FITC.

The Intracellular Fixation & Permeabilization Buffer Set (eBioscience) was used for intracellular staining following the manufacturer's protocol. Fluorochrome-conjugated monoclonal antibodies for intracellular staining are as follows: anti-human Ki-67-PE/Cyanine7, TLR7-PE, TLR8-APC, TLR9-PE, IFNα-PE, TNF-α-PE, IL-6- PE/Cyanine7.

Apoptosis assay

Cell apoptosis was detected by PE Annexin V Apoptosis Detection Kit I (559763, BD Pharmingen). Briefly, cells were washed with 1ml 1×Annexin V binding buffer after regular surface staining, then incubated with PE-conjugated Annexin V and 7-AAD for 15 minutes at room temperature, all the samples were analysed by BD Accuri C6 flow cytometer (Becton Dickinson, USA).

FcRn blocking

For the plasma stimulation, PBMCs from HCs were seeded into 12-well

Table I. Summary of clinical feature of patients with pSS and matched healthy controls in this study.

Clinical features	Untreated pSS patients (n=64)	pSS patients treated with HCQ (n=40)	Healthy controls (n=64)
Female, n (%)	61 (95.3%)	40 (100.0%)	61 (95.3%)
Age	47.6 ± 12.37	44.0 ± 11.32	46.42 ± 11.45
Disease duration, years (median [IQR])	2 (0.73-5.0)	3 (0.9-9.0)	-
Fever (n, %)	2 (3.0%)	1 (2.5%)	0
Joint pain (n, %)	17 (26.6%)	10 (25.0%)	0
Ocular symptoms (n, %)	30 (46.9%)	29 (72.5%)	0
Oral symptoms (n, %)	44 (68.8%)	33 (82.5%)	0
Serological examinations (median, IQR)			
IgG (g/L)	17.78 (14.80-22.19)	17.69 (14.40-20.72)	N/A
IgA (g/L)	3.06 (2.41-3.95)	3.10 (2.41-3.99)	N/A
IgM (g/L)	1.06 (0.81-1.51)	0.99 (0.79-1.50)	N/A
RF (IU/ml)	65.0 (20.0-153.0)	80.00 (38.0-150.0)	N/A
ESR (mm/h)	15.5 (8.25-36.75)	16.0 (9.0-26.0)	N/A
CRP (mg/L)	0.78 (0.46-1.28)	0.58 (0.32-1.15)	N/A
Anti-SSA positive (n, %)	50 (78.1%)	21 (52.5%)	0
Anti-SSB positive (n, %)	24 (37.5%)	11 (27.5%)	0
Anti-Ro52 positive (n, %)	39 (46.9%)	19 (47.5%)	0
ESSDAI	4.5 (2.0-9.0)	3.0 (2.0-5.0)	-

Normally distributed data are presented as mean ± SD, whereas non-normally distributed data are presented as median (IQR).
RF: rheumatoid factor; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; ESSDAI: EULAR Sjögren's syndrome disease activity index; N/A: not applicable.

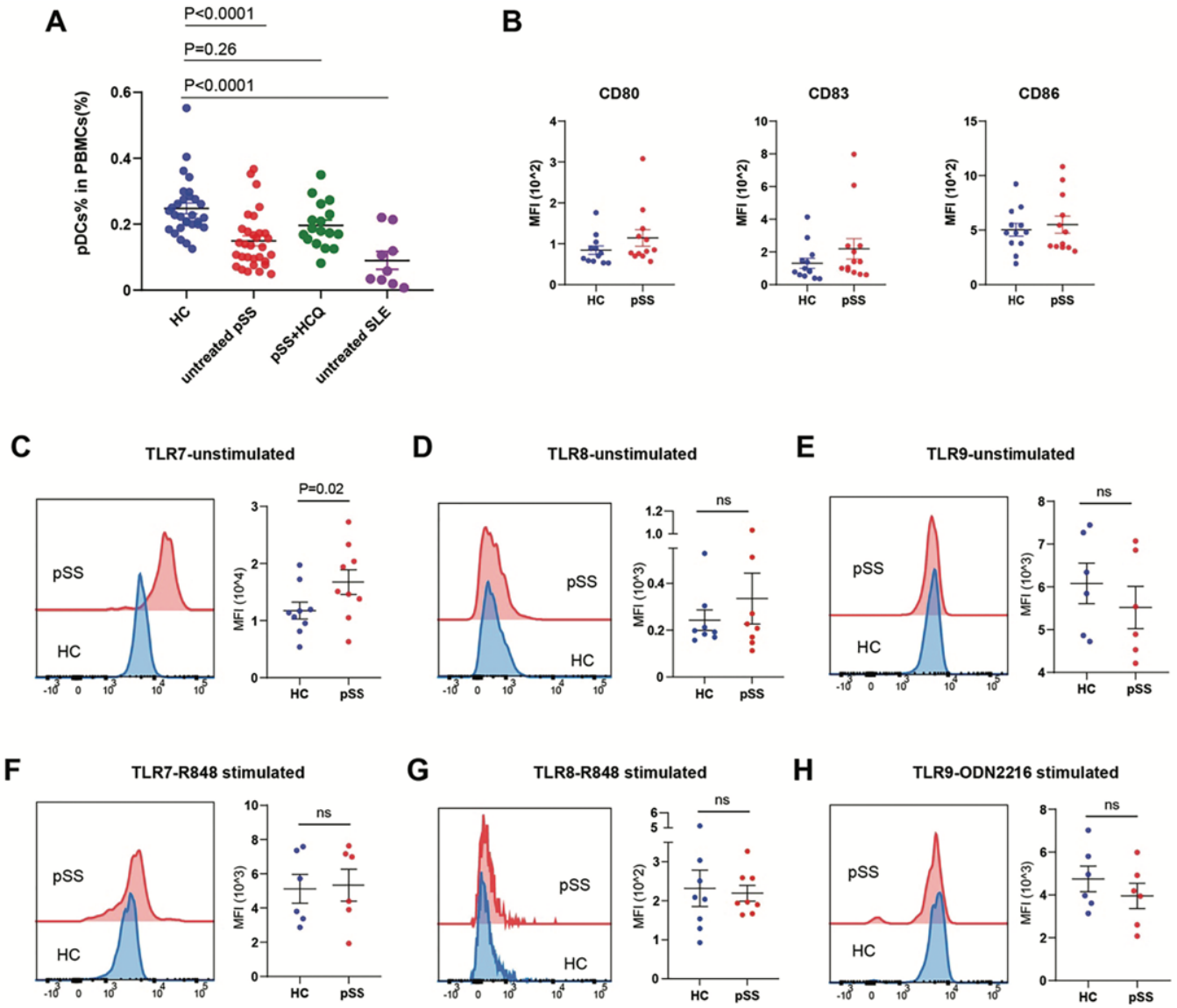

Fig. 1. Frequency and phenotype analyses of peripheral pDCs in pSS patients.
A: The percentage of peripheral pDCs in untreated patients with pSS or SLE, pSS patients treated with HCQ and matched HC (HC: n=29; untreated pSS: n=31; pSS treated with HCQ: n=17; untreated SLE: n=9). **B:** Comparison of activation markers (Mean Fluorescence Intensity, MFI) on the surface of pDCs between pSS (n=12) and HCs (n=12). Comparison of TLR7 (n=8), TLR8 (n=8), and TLR9 (n=6) expression levels between pDCs from pSS and HC without stimulation **(C-E)** and activated by TLR7/8 agonist **(F, G)** or TLR9 agonist **(H)** for 6 hours with 100ng/ml IL-3. Data follow normal distributions and are shown as mean ± SEM, one-way ANOVA was performed for comparisons among multiple groups, and unpaired two-tailed Student's t-test was applied for comparisons between two groups. Data were obtained from independent experiments, ns. means no significance.

plates at a density of 1×10^6/ml and maintained in RPMI 1640 (Gibco, A10491, USA), 100 U/ml penicillin, 100 μg/ml streptomycin (15140122, ThermoFisher), 100 ng/ml IL-3 and Batoclimab (MedChemExpress, HY-P99009) for 2 hours. Plasma from ten pSS patients was added at 2nd hour (The final concentration in the culture medium is 20%), pDC apoptosis was detected at 4th hour according to the methods mentioned above.

For IgG stimulation, PBMCs from HCs were seeded into 12-well plates at a density of 1×10^6/ml and maintained in RPMI 1640 supplemented with 10% foetal bovine serum (FBS) (Gibco, USA), 100 U/ml penicillin, 100 μg/ml streptomycin (15140122, ThermoFisher), 100 ng/ml IL-3 and Batoclimab (MedChemExpress, HY-P99009) for 2 hours. IgG (SP001, Solarbio) were added with indicated concentrations at 2nd hour, pDC apoptosis was detected at 4th hour according to the methods mentioned above.

Immunofluorescence

Freshly obtained labial tissue was embedded in O.C.T. (Tissue-Tek) compound and rapidly frozen in liquid nitrogen and then preserved at -80°C, 8μm frozen sections were prepared for further analysis. Sections were immersed in 95% ethanol for 15 min, blocked in normal goat serum for 3h at room temperature, followed by incubating with 25 μg/ml mouse anti-human BDCA-2 (CD303) monoclonal antibody (MAB62991, R&D), 1:50 diluted

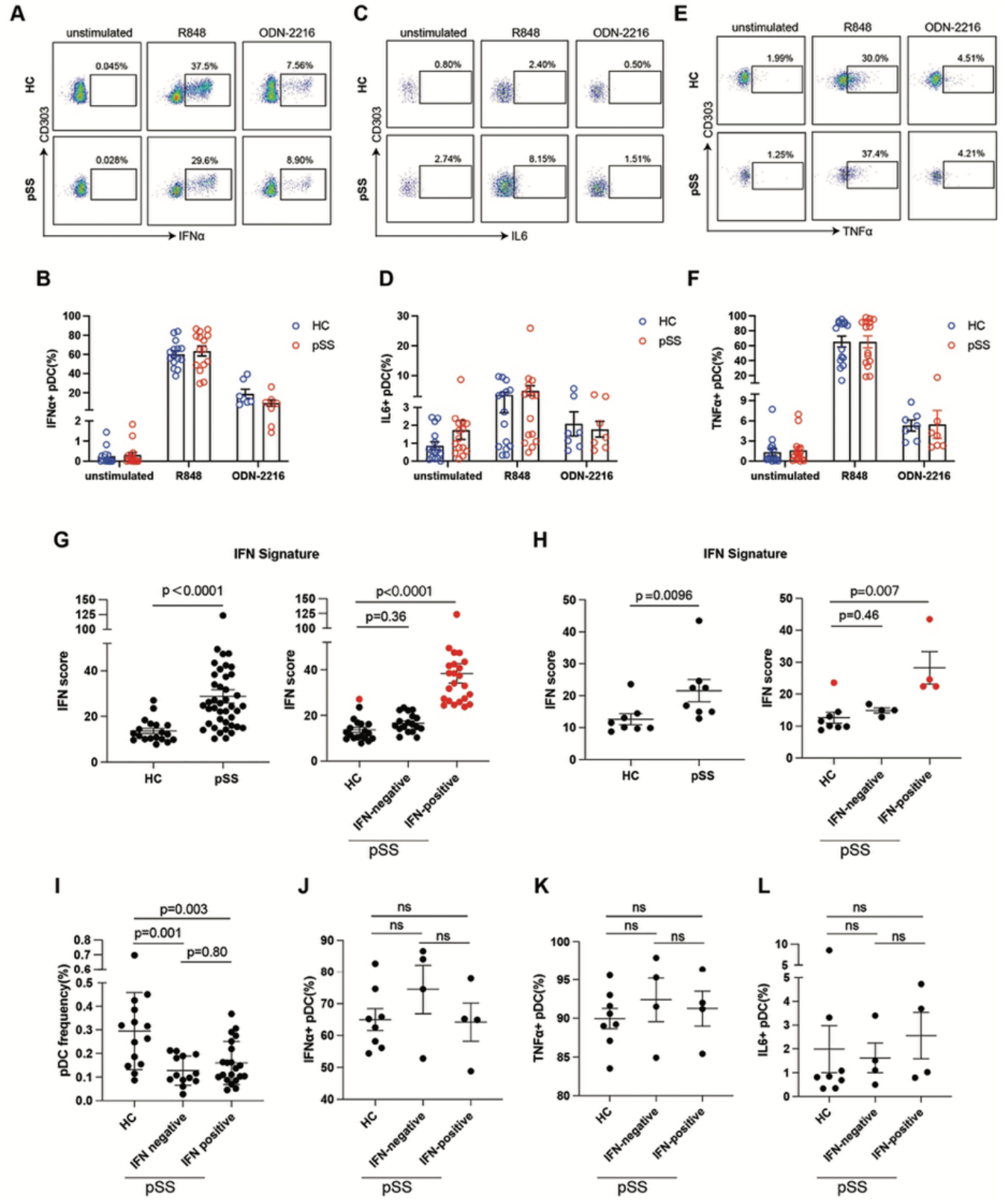

Fig. 2. Inflammatory cytokine secretion by pDCs and comparison of pDC status based on IFN signature.
Representative flow cytometry images and statistical graphs of IFN-α (**A, B**), IL-6 (**C, D**) and TNF-α (**E, F**) secretion by pDCs; Unstimulated and R848-stimulated states: HC (n=15) and pSS (n=15); ODN-2216-stimulated state: HC (n=7) and pSS (n=7). **G, H:** Comparison of IFN score between HC and pSS, as well as pSS subgroups (IFN positive or IFN negative). **I:** Comparison of peripheral pDCs percentage among HC and pSS subgroups (HC, n=19; pSS, n=41). Comparison of IFN-α (**J**), TNF-α (**K**) and IL-6 (**L**) secretion by pDCs among HC and pSS subgroups (HC, n=8; pSS, n=8). Data did not follow normal distributions, the Mann-Whitney test was used for comparisons between two groups and the Kruskal-Wallis test was used for comparisons among multiple groups. Data were obtained from independent experiments, ns. means no significance.

mouse anti-human IFN-α monoclonal antibody (sc-373757, Santa Cruz Biotechnology, Inc.) or 1:500 diluted rabbit anti-human EpCAM monoclonal antibody (also known as CD326, ab223582, Abcam) at 4°C overnight. CoraLite594-conjugated goat anti-mouse secondary antibody (proteintech, China) or CoraLite488-conjugated goat anti-rabbit secondary antibody (proteintech, China) was incubated at a dilution of 1:500 for 1h at room temperature. Additionally, the slides were mounted by an antifading mounting medium with DAPI (S2110, Solarbio) and scanned by Pannoramic MIDI (3DHISTECH, Hungary).

ELISA

The levels of IgG, IgM, and IgA in culture supernatants were measured with Human IgG/IgM/IgA Precoated ELISA Kit (1128162, 1128182, 1128172 DAKEWE, China) respectively. The level of IFN-α in plasma from pSS patients and matched HCs was determined by Human IFN-α Precoated ELISA Kit (1110012, DAKEWE, China) following the manufacturer's instructions.

Statistical analysis

All the data were analysed using IBM SPSS statistics (v. 25.0, IBM, Armonk, NY, USA) and GraphPad Prism 8 (GraphPad Software, Inc., La Jolla, CA, USA) software. We first conducted a normality test on the continuous variables (When n≥50, the Kolmogorov-Smirnov test is used; when n<50, the Shapiro-Wilk test is used). For data that followed normal distributions, comparisons among multiple groups were performed using one-way ANOVA (with Bonferroni-adjusted *p*-values), and comparisons between two groups were conducted using the t-test. For data that did not follow a normal distribution, comparisons among multiple groups were made using the Kruskal-Wallis test (with Dunn's multiple adjusted *p*-values), while comparisons between two groups were performed using the Mann-Whitney test. Spearman correlation was applied for correlation analysis. Statistical significance was defined as a two-sided *p*-value <0.05.

Results

The frequency and phenotype analyses of peripheral pDCs in patients with pSS

We first analysed the frequency of pDCs in PBMCs from treatment-naïve pSS (n=31), hydroxychloroquine (HCQ)-treated pSS (n=17), SLE (n=9), and HCs (n=29) by flow cytometry. The demographic characteristics of all the pSS patients and HCs were demonstrated in Table I. The gating strategy of pDCs is shown in Supplementary Fig. S1A. Consistent with previous studies (27, 28), the percentage of pDCs was significantly lower in PBMCs from treatment-naïve SLE or pSS patients compared to HCs (Suppl. Table S2). Interestingly, the reduction of pDCs rebounded in pSS patients after HCQ therapy (Fig 1A). However, we did not observe hyperactivated surface markers of pDCs in treatment-naive pSS, as evidenced by the comparable expression levels of the activation markers CD80, CD83, and CD86 to those in HCs (Fig 1B).

Detection of TLR7/8/9 expressions showed that unstimulated pDCs (freshly isolated) from pSS patients exhibited higher TLR7 expressions compared with HCs (Fig 1C), while the expressions of TLR8 and TLR9 were similar to HC (Fig 1D-E). However, the difference in TLR7 expression vanished after TLR7/8 ligand (R848) stimulation (Fig 1F), indicating a unique activation pattern in patients with pSS. The expressions of TLR8 and TLR9 in pDCs were comparable between pSS and HC after specific ligand stimulation (Fig 1G-H).

Inflammatory cytokine secretion by pDCs and comparison of pDC status based on IFN signature

Given the abnormal reduction of peripheral pDC in pSS, we next investigated the function of pDCs. To determine whether pDCs from pSS patients display the enhanced capacity of IFN-α secretion, we stimulated freshly isolated PBMCs with TLR7/TLR8 ligand (R848) or TLR9 ligand (ODN-2216) and determined representative cytokines production by flow cytometry. Under similar stimulating conditions, there was no difference in the IFN-α secretion of pDCs between HC and pSS patients (Fig. 2A-B). The same pattern was observed in the IL-6 and TNF-α production (Fig. 2C-F).

Considering the interference of disease heterogeneity in pSS, we then classified pSS patients into two groups according to their IFN signature (6, 26, 29). The IFN score in pSS is significantly higher than that in HC (Fig. 2G-H, Suppl. Fig. S2A-L). Further subgroup analysis of pSS patients yields an IFN-negative group with IFN scores similar to that of HC, and an IFN-positive group with IFN scores significantly higher than that of HC (Fig. 2G-H). Interestingly, independent of the IFN signature, pSS patients exhibit a significant decrease in peripheral pDCs compared to HC, while the pDC quantity does not differ between pSS subgroups (Fig. 2I). Furthermore, the levels of IFN-α, TNF-α, and IL-6 secreted by pDCs after stimulation showed no significant differences among the three groups: HC, pSS (IFN-negative), and pSS (IFN-positive) (Fig. 2J-L).

Evaluation of pDC-infiltration into affected tissues in patients with pSS

Although pDCs derived from pSS patients did not secrete more IFN-α under the same culture conditions *in vitro*, the involvement of exocrine glands is a characteristic of pSS. If pDCs infiltrate the affected tissues in large numbers, they may cause excessive local type I IFN signalling, which could indirectly lead to a decrease in peripheral pDC. We subsequently examined the infiltration of pDCs in labial glands using immunofluorescence to investigate their impact on the tissue type I IFN signature in patients with pSS. We detected the labial glands of nine pSS patients whose labial gland biopsy indicated typical focal lymphocytic infiltration. Among these cases, only two displayed a small amount of pDC infiltration, while the remaining seven cases showed no evidence of pDC infiltration (Fig. 3A). Recent studies highlighted the role of salivary epithelial cells in producing type I IFNs (30-32), consistently, our immunofluorescent results indicated that epithelial cells are responsible for the production of tissular IFN-α in pa-

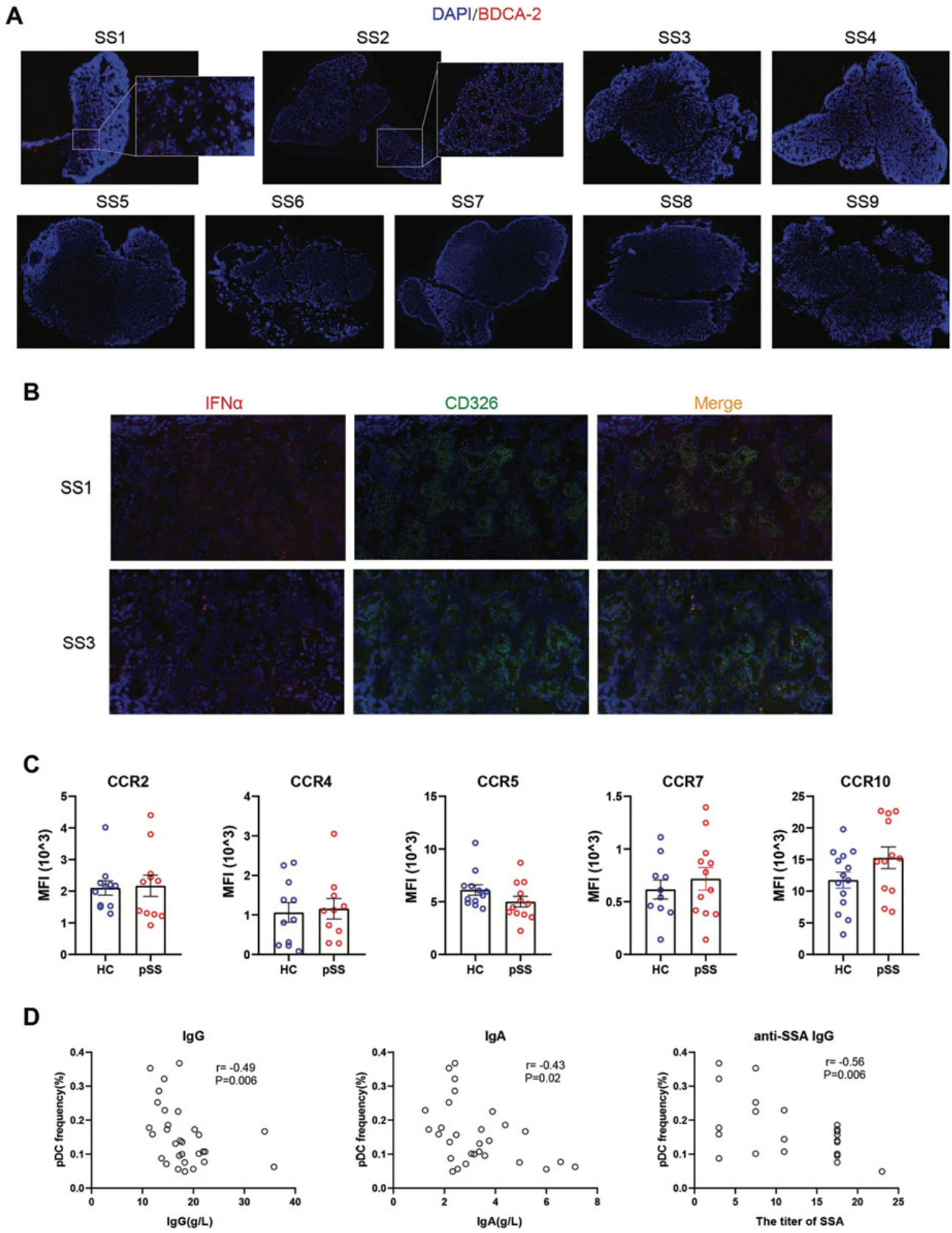

Fig 3. Infiltrations of pDCs in the labial gland and the clinical correlation analyses.
A: Immunofluorescent results of 9 pSS patients who had labial pathological reports with typical focal lymphocytic infiltrations. **B:** Immunofluorescent colocalisation of epithelial cells (CD326) and IFN-α in salivary glands of pSS patients (n=2). **C:** Comparison of surface chemotactic receptors (MFI) in pDCs between pSS and HCs, CCR2 (HC: n=11; pSS: n=11), CCR4 (HC: n=11; pSS: n=10), CCR5 (HC: n=12; pSS: n=12), CCR7 (HC: n=10; pSS: n=12), CCR10 (HC: n=14; pSS: n=12). **D:** Spearman correlation analysis of pDC percentage in PBMC and serum IgG (n=30), IgA (n=29), anti-SSA IgG (n=23).

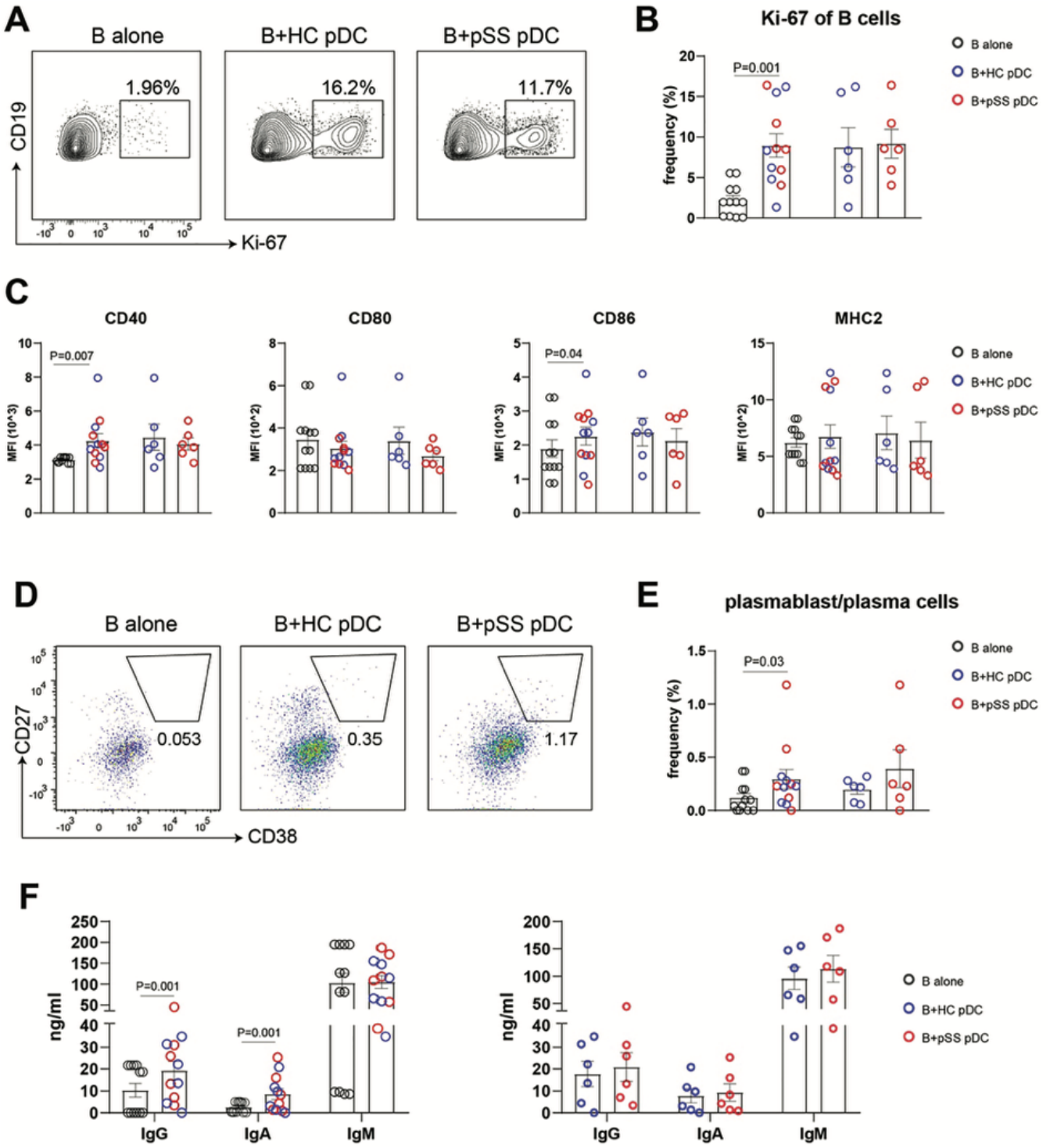

Fig 4. Co-culture of unstimulated pDCs and B cells. Purified CD19+ B cells from allogeneic healthy donors cultured alone or with pDCs (without extra activation) from pSS and HC in the presence of 100ng/ml IL-3 for 7 days, the ratio of pDC to B cell was 1:10.
A: Representative flow cytometric graph of ki-67 in B cells cultured alone or cultured with pDCs. **B:** The effect of pDCs on B cell proliferation (n=12) and the effect comparison between HC (n=6) and pSS (n=6) derived pDC on B cells. **C:** Expression of activation markers on B cells cultured alone or cultured with pDCs (HC: n=6, pSS: n=6). **D:** Representative flow cytometric graph depicting the proportion of plasma cells/plasmablasts within the B cell population. **E:** The impact of pDCs on the proportion of plasma cells/plasmablasts within the B cell population and the effects comparison between HC (n=6) and pSS (n=6) derived pDCs. **F:** Secretion of IgG, IgA and IgM by B cells cultured alone or cultured with pDCs (n=12). Data follow normal distributions and are shown as mean ± SEM. Paired and unpaired two-tailed Student's t-test was performed. Data were obtained from independent experiments.

tients with or without pDC infiltration (Fig. 3B). Additionally, we assessed the expression of key chemokine receptors, including CCR2, CCR4, CCR5, CCR7, and CCR10, on the surface of pDCs and found no significant differences between patients with pSS and HCs (Fig. 3C, Suppl. Fig. S3A). Together, these data suggest that the significant reduc-

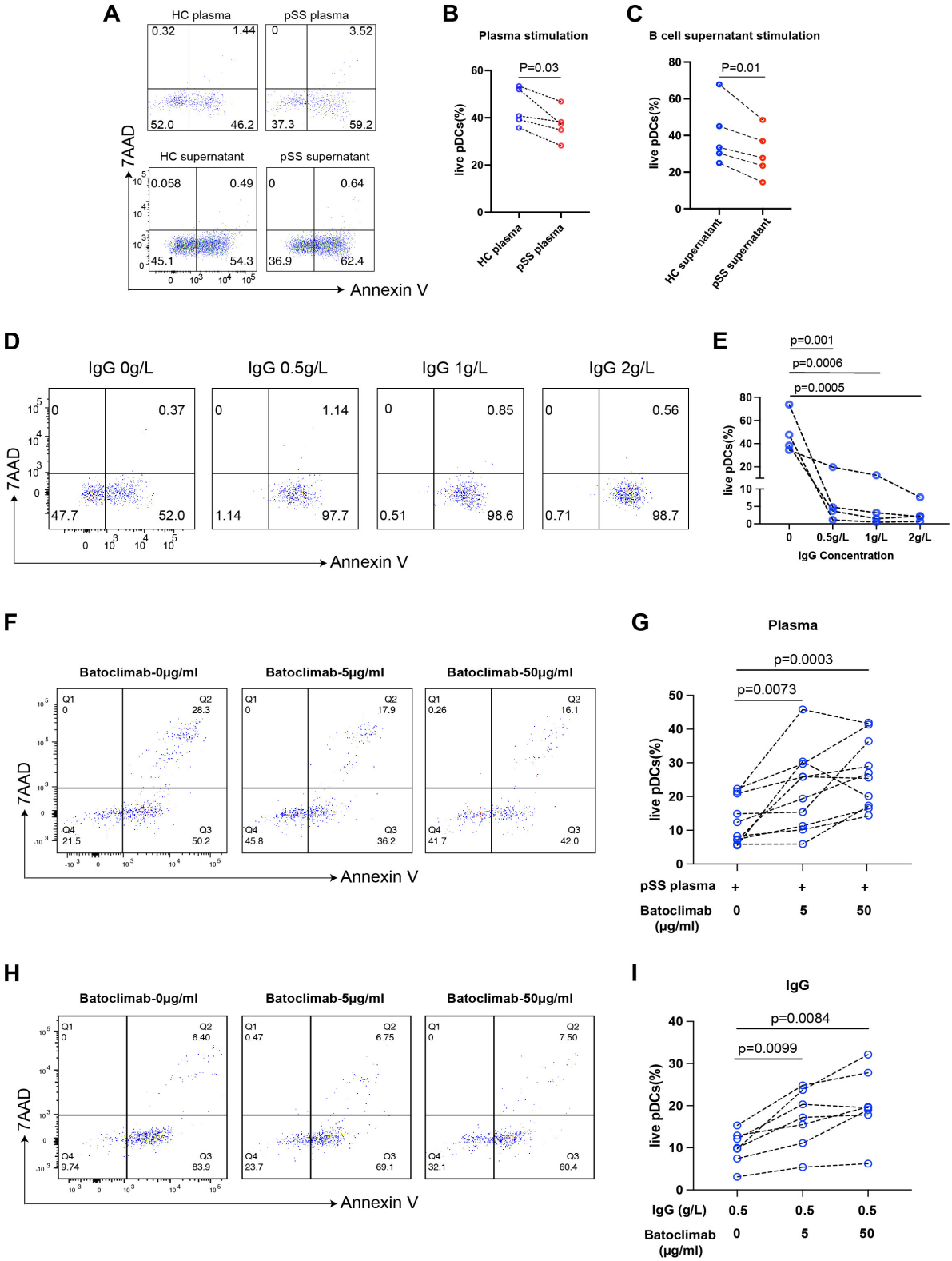

Fig. 5. IgG-induced pDC apoptosis via FcRn is a key factor in reduced peripheral pDCs in pSS patients.
A: Representative flow cytometric graph and summary graphs of pDCs viability. Statistical graph of pDC apoptosis detection, stimulated by HC or pSS-derived plasma (n=5) **(B)**, as well as by B cell culture supernatant (n=5) **(C)**. Representative flow cytometric graph **(D)** and Statistical graph **(E)** of apoptosis detection, pDCs were stimulated by increasing concentration of IgG for 2 hours with 100ng/ml IL-3 (n=4). Representative flow cytometric graph **(F)** and Statistical graph **(G)** of apoptosis detection, pDCs were stimulated by pSS plasma with or without Batoclimab (n=7). Representative flow cytometric graph **(H)** and Statistical graph **(I)** of apoptosis detection, pDCs were stimulated by IgG with or without Batoclimab (n=10). Data followed normal distributions, the Paired two-tailed Student's t-test was performed for comparisons between two groups**,** and One-way ANOVA was performed for comparisons among multiple groups. Data were obtained from independent experiments.

tion of pDCs in peripheral blood cannot be explained by excessive inflammatory activation or extensive infiltration into affected tissues.

pDCs correlated with the clinical phenotype of pSS and promoted B cell antibody production

We then conducted correlation analyses between the percentage of peripheral pDCs and clinical parameters in pSS patients to explore the relationship between pDCs and clinical phenotype, as well as motivating factors for the decrease of peripheral pDC. The results demonstrated that the percentage of pDCs was negatively correlated with serum IgG, IgA, and anti-SSA autoantibody levels (Fig. 3D, Suppl. Fig. S3B-J). Therefore, we established the pDC and B cells co-culture system to explore the potential role of pDC in promoting B cell responses and antibody production in patients with pSS. When co-cultured with resting or TLR7-activated pDCs, the proliferation and activation of B cells were significantly elevated, and the proportion of plasmablast/plasma cells in B cells was also significantly increased (Fig. 4A-E, Suppl. Fig. S4A-E). Consistently, we explored the effects of pDCs on antibody production and found that even resting pDCs could effectively promote IgG and IgA production by B cells (Fig. 4F). Furthermore, no difference was found in the effects of HC or pSS-derived pDCs on naive or memory B cells (Fig. S5A-D). However, there was no difference in the effects of HC or pSS-derived pDCs on B cells.

The promotion of pDC apoptosis by IgG via FcRn is a critical reason for the reduction of peripheral pDCs in pSS patients

Finally, we sought to explore the potential mechanism of reduced pDC percentage in the peripheral blood of pSS patients. Incubation of plasma from pSS patients could significantly reduce pDCs viability than that from HCs (Fig. 5A, B). The survival rate of pDCs stimulated by plasma from pSS patients receiving HCQ treatment is higher than that of plasma from treatment-naive pSS patients (Suppl. Fig. S6A, B). Consistently, we found that compared to the B-cell culture supernatant from HC, B-cell culture supernatant from patients with pSS significantly promotes apoptosis in pDCs (Fig. 5A-C). Considering the observed promotion of pDC apoptosis by plasma or B-cell culture supernatant of pSS, along with the negative correlation between pDC percentage and plasma IgG (Fig. 3D), we infer that plasma-derived IgG produced by overactivated B cells in pSS may serve as the key inducers of pDC apoptosis. As we expected, IgG dramatically increased the apoptotic rates of pDCs in a dose-dependent manner (Fig. 5D-E), and we found that IgA can also promote the apoptosis of pDCs (Suppl. Fig. S7). The viability of freshly isolated pDCs showed no significance between pSS and HCs (Fig. S8A), thereby excluding apoptosis caused by defects of pDCs in patients with pSS. Although pSS had higher serum IFN-α levels (Fig. S8B), they had no significant effects on the apoptosis of pDC (Fig. S8C-G).

We further explored the pathway through which IgG mediate pDC apoptosis. Since Fc receptors are important receptors for IgG, and IgG can bind to Fc receptors through Fc regions (33), we attempted to block the Fc receptors and then stimulated pDCs with pSS plasma and IgG separately to assess pDC survival. The results showed that blocking Fc receptors could not rescue pDCs from apoptosis induced by pSS plasma and IgG (Suppl. Fig. S9A-D). In addition to Fc receptors, FcRn can also bind IgG, and its important role in autoimmune diseases has gradually gained attention in recent years (34, 35). Our results showed that blocking FcRn by Batoclimab can rescue pDCs from apoptosis induced by pSS plasma (Fig. 5F-G) and IgG (Fig. 5H-I). This suggests that the promotion of pDC apoptosis by IgG via FcRn is the important reason for the reduction of pDCs in the peripheral blood of pSS patients. These results indicate that excessive IgG in the serum produced by overactivated B cells of pSS patients contributes to the reduction of pDCs in the peripheral blood via FcRn (Fig. 6).

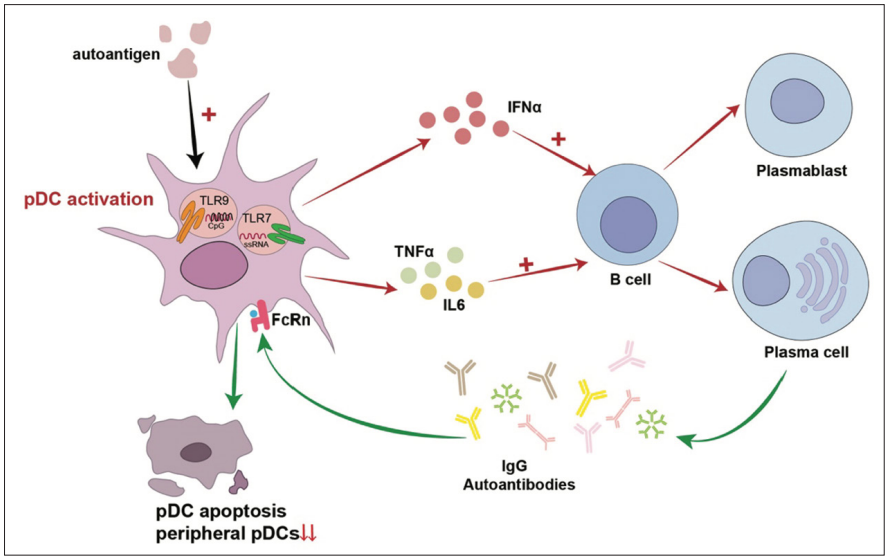

Fig. 6. The interplay between pDCs and B cells in pSS patients.
pDCs promote the proliferation, activation, and differentiation into plasmablast/plasma cells of B cells. Conversely, hyperactive B cells in pSS induce the apoptosis of pDC through excess IgG via FcRn.

Discussion

The critical role of type I IFN signalling in the pathogenesis of pSS has been widely addressed (3). Although acting as the primary producer of IFN-α, the role of pDCs in the pathogenesis of pSS remains inclusive. In the present study, we started from the abnormal reduction of pDCs in pSS patients and explored the functions of pDCs, ultimately proposing the mechanisms behind the decrease of peripheral pDCs in pSS. In particular, we first report that excess IgG induced pDC apoptosis via FcRn and promoted the reduction of peripheral pDCs in patients with pSS.

Consistent with previous studies, we have detected a peripheral reduction of pDCs in pSS (27, 28). Besides, earlier research has reported that the quantity of pDCs correlates with anti-Ro/SSA and anti-La/SSB antibodies, but not with serum IFN-α levels, CRP, ESR, or other clinical parameters (27). We first proposed a negative feedback loop, in which, pDCs promote antibody production by B cells, and in turn, excessive immunoglobulin induces pDC apoptosis in pSS. Hyperactivated B cells, characteristic autoantibodies (SSA, SSB), and hypergammaglobulinaemia are hallmarks of pSS (36). Additionally, we gave a good explanation that excess IgG produced by hyperactivated B cells should be responsible for the apoptosis and reduction of pSS pDCs in peripheral blood via FcRn. FcRn is named for its earliest discovery of being able to transport IgG from the maternal circulation to the foetal capillaries in the placental villi (37). Subsequent studies gradually revealed the widely expression of FcRn in immune cells, with high expression levels in dendritic cells (DCs), and plays an important role in maintaining the stability of circulating IgG levels (35). FcRn binds to IgG and prevents their degradation by lysosomes, thereby extending the half-life of IgG and increasing its concentration and functionality in the body (35). FcRn can transport the bound IgG from within the cell to the cell surface, thus allowing IgG to enter the extracellular space again and exert its immunoregulatory effects (38, 39). FcRn blockade (batoclimab, efgartigimod, nipocalimab, etc.) can reduce circulating total IgG levels in HCs (35). In several diseases mediated by autoantibodies (immune thrombocytopenic purpura, myasthenia gravis and pemphigus), clinical trials of FcRn blockers have shown certain efficacy in reducing the levels of circulating pathogenic antibodies and decreasing disease activity (34). Our findings suggest the role of IgG and FcRn in mediating the abnormal reduction of pDCs in pSS, providing clues for future research targeting FcRn in pSS. In addition, the specific mechanisms through which IgA mediates the apoptosis of pDCs remain to be determined. Our findings, however, demonstrate that Batoclimab treatment significantly reduced pSS plasma-induced pDC apoptosis, and the increase of plasma IgG level is more common among pSS patients. These results support that pDC apoptosis mediated by IgG through FcRn receptor may be the key factor contributing to the reduction of pDC. Given the low abundance of pDCs in peripheral blood and their further decrease in patients with pSS (only 1-4×10^4 pDCs can be isolated from 10ml of whole blood), it is hard to conduct in-depth molecular studies on pDCs derived from pSS, looking forward to future technical advancements to help elucidate the further mechanism.

Moreover, we also found that in pSS patients receiving HCQ treatment, the frequency of peripheral pDCs was significantly higher than that of untreated pSS patients. The survival rate of pDCs incubated in the plasma of pSS patients treated with HCQ was also notably higher than that of untreated pSS patients. HCQ can directly alter the pH of endosomes, thereby inhibiting the activation of TLR7 and TLR9 by ligands (40). It can also bind directly to nucleic acids, blocking the interaction between Toll-like receptors and their ligands at the cellular level, which inhibits TLR9 signalling and directly suppresses pDC activation (41, 42). Additionally, HCQ effectively inhibits B cell activation (43), with studies reporting significant reductions in serum IgG(44) levels in pSS patients after treatment. This suggests that the decrease in serum IgG levels and the increase in pDC survival rates following HCQ treatment may also contribute to the recovery of the percentage of pDCs in the peripheral blood of pSS patients, but further experimental validation is needed.

Nonetheless, pDC is noted for its unique ability to produce type I IFNs, our data suggest that it might not be the only source of hyperactive type I IFN signalling in pSS. Recent studies in pSS have emphasised the critical role of abnormally activated epithelial cells (32, 45), especially the active IFN signalling in salivary gland epithelial cells (SEGCs) (32). SEGCs from pSS were found to be sensitive to the stimulation of TLR agonists and produced type I IFNs as a response to the stimulation (30, 31, 46). Type I IFNs released by SEGCs could further promote the secretion of BAFF in an NF-κB dependent way (11, 30). These data and our results collectively confirmed that SEGCs are not purely innocent victims but can also be the trigger of type I IFN signalling in pSS. Taken together, locally infiltrated pDCs could exacerbate the activation of local type I IFN signalling together with SEGCs, thereby promoting tissue damage.

In SSc, pDCs directionally migrated towards target organs and secreted IFN-α and CXCL4, thereby accelerating tissue fibrosis (19, 47). TLR7 is more predominantly expressed in B cells, monocytes, and pDCs, while TLR8 shows greater expression in monocytes, neutrophils, and myeloid dendritic cells (mDCs) (48, 49). It is worth noting that in SSc, an increase of TLR8 expression in peripheral pDCs was detected, and TLR8 upregulation is closely associated with pDC-mediated fibrosis (19). Nonetheless, our data showed no such abnormal TLR8 elevation in pSS (both before and after ligand stimulation), suggesting that pDCs in these two diseases may exhibit different TLR8 expression patterns and disease characteristics. TLR8 has also been reported to regulate functions on TLR7 signalling and play a role in autoimmune diseases (50). However, to date, studies on TLR8 in pDC and its role in autoimmune disease may be inadequate. Reduction of pDCs in the peripheral blood as well as the concomitant infiltration and activation in related tissue were reported in SLE (51, 52). A recent randomised controlled trial in SLE clarified that litifilimab (anti-BDCA2 antibody, BDCA2 is an exclusive marker of pDC) is effective in disease remission (53), which indicated the feasibility of pDC purge strategy in SLE treatment. Whereas newly published research revealed that SLE-derived pDC had senescence and inert phenotype, active keratinocytes should be responsible for the IFN signalling rather than pDC (28). In general, pDCs contributed to the pathogenesis of autoimmune diseases through

type I IFNs or interplay with other immune cells (13).

A previous study based on transcriptional analysis proposed that pDCs from patients with pSS secreted more type I IFNs after TLR7 stimulation (54). However, the study by Antonios *et al*. revealed that pDCs from patients with SLE or pSS did not show stronger secretion ability for inflammatory cytokines, especially IFN-α (28), which is consistent with our findings. These divergent results may be caused by different detection methods, patient heterogeneity, and low abundance of circulating pDCs. It is worth mentioning that we also classified pSS patients based on their IFN signature and further compared the secretion levels of inflammatory factors among patient subgroups. Considering the heterogeneity of disease in pSS, it is valuable to classify pSS patients into two groups according to their IFN signature, since the two groups of patients have been reported to have different disease characteristics (6, 26, 29). However, despite the IFN signature, pDC quantity does not differ between pSS subgroups, as well as the secretion of IFN-α, TNF-α, and IL-6. This suggests that the abnormal decrease of pDCs in pSS patients may be independent of the IFN signature, which is consistent with our results that IFN-α is not the main cause of pDC apoptosis.

In conclusion, we analysed the aberrant phenotype and dysregulation of pDCs in pSS. Significantly, we disclosed the interaction between dysregulated pDCs and hyperactivated B cells in pSS. We innovatively demonstrated that excessive IgG contribute to the apoptosis and decline of pDCs via FcRn in pSS. Our research provides novel insights into the immune disorder of pSS and offers new perspectives on future disease intervention targets and treatment strategies.

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