

The redundant role of plasmacytoid dendritic cells in Primary Sjögren's syndrome

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Abstract

Objective: The aim of our present study is to investigate the role of plasmacytoid dendritic cells (pDCs) in the pathogenesis and type I interferon (IFN) signatures in Primary Sjögren's Syndrome (pSS) patients. **Methods:** In the present study, we compared the percentage, activation markers, and representative cytokines secretion of pDCs derived from treatment-naïve pSS and matched healthy controls (HCs) by flow cytometry. We performed pDC/B co-culture system to explore the contribution of pDC to B cell functions in pSS. **Results:** The percentage of pDC was significantly reduced in the peripheral blood of pSS. The activation markers (CD80, CD83, and CD86) expressions, chemokine receptors, and representative cytokines production (IFN- α , IL-6, and TNF- α) of pDC were similar between pSS and HCs. Only a few pDCs infiltration were detected in the labial gland. The percentage of pDCs was negatively correlated with serum IgG, IgA, and anti-SSA autoantibody levels and resting pDCs were able to efficiently promote B cells proliferation, activation, differentiation, and antibody production *in vitro*. However, there was no difference between HC and pSS-derived pDCs. Finally, we found that incubation of plasma from pSS patients could significantly induce pDCs apoptosis than that from HCs and both IgG and IgA dramatically increased the apoptotic rates of pDCs. **Conclusion:** Our data have deciphered the redundant role of pDC in the type I signature and disease development in pSS. Also, we demonstrated the decreased percentage of pDC in pSS patients might result from apoptosis induced by the excess of immunoglobulin (IgG and IgA).

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Statements and Declarations

Competing interests:

The authors have declared no conflicts of interest.

Ethics approval and consent to participate:

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 consent of all the patients were obtained.

Consent for publication:

The manuscript is approved by all authors for publication, and written informed consent to publish were obtained.

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Methods: In the present study, we compared the percentage, activation markers, and representative cytokines secretion of pDCs derived from treatment-naïve pSS and matched healthy controls (HCs) by flow cytometry. We performed pDC/B co-culture system to explore the contribution of pDC to B cell functions in pSS.

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Conclusion: Our data have deciphered the redundant role of pDC in the type I signature and disease development in pSS. Also, we demonstrated the decreased percentage of pDC in pSS patients might result from apoptosis induced by the excess of immunoglobulin (IgG and IgA).

Key words: Primary Sjögren's syndrome, Plasmacytoid dendritic cell, Type I interferon, B cell, Hypergammaglobulinemia

Introduction

Primary sjögren's syndrome (pSS) is a typical autoimmune disorder characterized 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]. Previous studies have demonstrated the type I IFN signatures in peripheral blood mononuclear cells (PBMCs), B cells, monocytes, neutrophils as well as the affected tissues

[3-10]. Moreover, IFN- α inducible proteins: MDA-5, IFIT-3 [11], and B cell-activating factor (BAFF) [12] were found highly expressed in salivary glands. Type I IFN could enhance T cell and B cell responses and promote the production of autoantibodies [11, 13], implicating their important roles in the pathogenesis of pSS.

Acting as a premier type I IFN producer and a key bridge between innate and adaptive immunity, pDCs also play a nonnegligible contribution to autoimmune disease development. pDCs in SLE patients can be activated through various pathways (immune complexes, neutrophil extracellular traps, mitochondrial DNA, *etc.*), which in turn trigger immune responses and promote the production of autoantibodies [14]. In studies of systemic sclerosis (SSc) [15], Psoriasis [16], rheumatoid arthritis [17] and autoimmune diabetes [18], pDCs could infiltrate into the target tissue and exacerbate local inflammation by releasing IFN- α and proinflammatory factors.

Currently, studies on the role of pDCs in pSS are limited. Previous studies have reported the reduced frequencies of pDCs in peripheral blood [19, 20] of pSS patients. A recent study performed a transcriptional analysis of circulating pDCs and identified the aberrant activation of pDC in pSS patients [21]. Here, we systematically study the percentage, phenotype, and functions of pDCs for a better understanding of their roles in the pathogenesis and type I IFN signatures in pSS patients.

Methods

Patients and ethics

All newly-onset pSS and SLE patients were enrolled in Peking Union Medical College Hospital (PUMCH) and 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. Patients all signed informed consent for their residual tissues after the pathological examination. This study was approved by the Ethics Committee of PUMCH (No: K2525).

PBMC Isolation and *In vitro* stimulation

Human PBMCs were isolated with Ficoll-Paque density (DAKEWE, China) as previously described[8]. For the plasma stimulation, PBMCs from 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 and IgA stimulation, freshly isolated PBMCs from HCs were first incubated with 100 mg/ml IL-3 for 2 hours, then stimulated with human IgG (SP001, Solarbio) and human IgA (SP016, Solarbio) with indicated concentrations. IL3 is used to assist the survival of pDCs in vitro [24, 25].

In vitro purification and stimulation of pDC

pDC was purified using CD304 microbeads (130-090-532, Miltenyi Biotec) and maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) (Gibco, USA), 100 U/ml penicillin and 100 μ g/ml streptomycin (15140122, ThermoFisher). For cytokines detection, PBMCs were stimulated with 5 μ g/ml R848 (tlrl-r848, InvivoGen) or 10 μ g/ml ODN-2216 (tlrl-2216, InvivoGen) in the presence of 100 ng/ml IL-3 (Peprotech, 200-03). R848 is a ligand for Toll-like receptor 7 (TLR7) and OND-2216 is a ligand for TLR9.

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 μ g/mL anti-IgM (314502, BioLegend), 500 ng/mL sCD40L (310-02, Peprotech), 100 ng/ml IL-4 (200-04, Peprotech), and 50 ng/ml IL21 (200-21, Peprotech). For pDCs and B cells coculture, purified CD19⁺ B cells (5×10^5 /ml) and pDC from allogeneic HCs were added at the ratio of 10:1. The supernatants were harvested at day 7.

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, CD123, Lineage, HLA-DR, CD80, CD83, CD86, CD19, CD40, CD74, CD27, IgD, CCR2, CCR4, CCR5, CCR7, CCR10, CD38, CD138, CD24.

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

Detailed information about conjugated fluorescence and manufacturers for antibodies is shown in Supplementary Table 1.

Apoptosis assay

Cell apoptosis was detected by PE Annexin V Apoptosis Detection Kit I (559763, BD Pharmingen). Briefly, cells were washed with 1ml $1 \times$ 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 analyzed by BD Accuri C6 flow cytometer (Becton Dickinson, USA).

Immunofluorescence

Freshly labial tissue was embedded in O.C.T. (Tissue-Tek) and $8 \mu\text{m}$ frozen sections were prepared. Sections were immersed in 95% ethanol for 15 min, blocked in normal goat serum for 3h at room temperature, followed by incubating with $25 \mu\text{g/ml}$ mouse anti-human BDCA-2 (CD303) monoclonal antibody (MAB62991, R&D), 1:50 diluted 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. Further, the slides were mounted by an antifading mounting medium with DAPI (S2110, Solarbio) and scanned by Panoramic 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 analyses were conducted by IBM SPSS statistics (Version 25.0, IBM, Armonk, NY, USA) and GraphPad Prism 8 (GraphPad Software, Inc., La Jolla, CA, USA) software. The data were first performed with the normality distribution. Student's t-test was used for variables in a normal distribution, otherwise, the Mann-Whitney test was used. Linear regression and Pearson's correlation were applied to explore the correlation analysis. A p -value < 0.05 was defined as statistically significant.

Results

The frequency and phenotype analysis of peripheral pDC in pSS patients

We first analyzed the frequency of pDC in PBMCs from treatment-naïve pSS ($n=31$), HCQ-treated pSS ($n=17$), SLE ($n=9$), and HCs ($n=29$) by flow cytometry. The demographic characteristics of all the included pSS patients were demonstrated in Supplementary Table 2 and SLE patients were demonstrated in Supplementary Table 3. The gating strategy of pDCs was shown in Figure S1a. In accordance with previous studies [20, 26], the percentage of pDCs was significantly lower in PBMCs from treatment naïve SLE and pSS patients compared with HCs. Interestingly, the reduction of pDCs rebounded in pSS patients after Hydroxychloroquine (HCQ) therapy (Fig 1a). However, we did not observe the hyperactivation status of pDCs in pSS as the activation markers CD80, CD83, and CD86 expressions were equal to HCs (Fig 1b, Fig S1c).

Detection of TLR7/9 expressions showed that unstimulated pDCs from pSS patients showed higher TLR7 but not TLR9 expressions compared with HCs (Fig 1c, d). However, the difference vanished after TLR7 (R848) or TLR9 ligand (ODN2216) stimulation (Fig 1e, f).

pDCs did not contribute to peripheral and affected tissue type I IFN signature in pSS patients

To determine whether pDC from pSS patients displays the enhanced capacity of IFN- α secretion, we stimulated freshly isolated PBMCs with TLR7 (R848) or TLR9 ligand (ODN-2216) and determined representative cytokines production by flow cytometry. However, upon stimulation, there was no difference in the IFN- α secretion of pDCs between HC and pSS patients (Fig 2a-b). The same was observed in the IL-6 and TNF- α production (Fig 2c-f).

We next detected the infiltration of pDCs in labial glands by immunofluorescence to explore whether they contribute to tissue type I IFN signature in pSS patients. Only one patient had a few pDCs infiltration, while the other three hardly had any pDCs in the labial gland (Fig 3a). Recent researches highlighted the role of salivary epithelial cells in producing type I IFNs [27-29], consistently, our immunofluorescent results indicated that epithelial cells are responsible for the production of tissular IFN- α in patients with or without pDC infiltration (Fig 3b). We also detected the main chemokine receptors, including CCR2, CCR4, CCR5, CCR7, and CCR10 on the pDC surface showed no difference between pSS patients and HCs (Fig 3c, Fig S2j).

pDCs promote B cell class-switching and antibody production

Correlation analysis between the frequency of peripheral pDCs and clinical parameters in pSS patients showed that the percentage of pDCs was negatively correlated with serum IgG, IgA, and anti-SSA autoantibody levels (Fig S2a-i, Fig 3d). We, therefore, established the pDC/B cells co-culture system to explore the potential role of pDC in promoting B cell responses and antibody production in pSS patients.

We found that resting or TLR-7-activated pDCs were able to efficiently promote the proliferation, activation, and differentiation into plasmablast/plasma cell of B cells (Fig 4a-e, Fig S3a-e). Also, we explored the effects of pDCs on antibody production and found that even resting pDCs could promote IgG, and IgA production by B cells (Fig 4f). However, there was no difference between HC and pSS-derived pDCs. Similarly, no difference was found in the effects of HC or pSS derived pDCs on naive or memory B cells (Fig S4).

pDCs are more sensitized to pSS plasma-induced apoptosis

Finally, we sought to explore the potential mechanism of reduced pDC frequency in the peripheral blood of pSS patients. Incubation of plasma from pSS patients could significantly reduce pDCs viability than that from HCs (Fig 5a). Considering that pSS is characterized by high autoantibodies in serum and pDC could promote antibody production as we demonstrated above, we inferred that plasma-derived IgG and IgA might be important inducers of pDC apoptosis. As we expected, both IgG and IgA dramatically increased the apoptotic rates of pDCs in a dose-dependent manner (Fig 5b-e). The viability of freshly isolated pDCs showed no significance between pSS and HCs (Fig S5a). Although pSS had higher serum IFN- α levels (Fig S5b), they had little effect on the pDC apoptosis (Fig S5c-e). These results indicate that hyper immunoglobulin in the serum of pSS patients contributes to the reduction of pDC in the peripheral blood (Fig 6).

Discussion

The critical role of the type I IFN pathway in the pathogenesis of pSS has been widely addressed [30, 31]. Acting as the premier producer of IFN- α , the role of pDC in the pathogenesis of pSS still remains inclusive. In the present study, we have deciphered the redundant role of pDC in the type I signature and disease development in pSS.

Consistent with previous studies, we have detected a peripheral reduction in pSS [20, 26]. We have demonstrated a negative feedback loop, in which, pDC promoted antibody production and in turn, high immunoglobulin could induce pDC apoptosis. It has been reported that IgG-complexed adenoviruses induce the apoptosis of human pDCs [32]. Hyperactivated B cells, characteristic autoantibodies (SSA, SSB), and

hypergammaglobulinemia are hallmarks of pSS [33]. Previous studies have found that pDCs could efficiently promote the differentiation of B cells into plasmablasts and plasma cells through type I IFNs and IL-6 [34, 35]. Moreover, pDCs could enhance the autoreactivity of B cells via type I IFNs in a T cell-independent manner [36]. Although our data showed that, pSS-derived pDC did not display a stronger capacity for promoting plasma cell differentiation and antibody production, we gave a good explanation that the excess immunoglobulin (IgG and IgA) produced by hyperactivated B cells might result in the apoptosis and reduction of pDC in peripheral blood of pSS. In addition, Hydroxychloroquine (HCQ) effectively inhibits B cell activation [37] and a recent systematic review revealed that serum IgA in patients with pSS decreased significantly after using HCQ [38], which is consistent with our results that pDC frequency rebounded after HCQ therapy.

Nonetheless, pDC is noted for its unique ability in producing type I IFNs, our data suggest that it might not be the culprit of hyperactive type I IFN signaling in pSS. Recent studies in pSS have emphasized the critical role of dysregulated epithelial cells [29, 39], especially the active IFN signaling in salivary gland epithelial cells (SEGCs) [29]. 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 [27, 28, 40]. Type I IFNs released by SEGCs could further promote the secretion of BAFF in an NF- κ B dependent way [12, 27]. These results imply that gland epithelial cells are not purely innocent victims, but can also be the trigger of type I IFN signaling in pSS.

In SSc, pDCs directionally migrated to target organs and secreted IFN- α and CXCL4, thereby accelerated tissue fibrosis [15, 41]. In another autoimmune disease with fibrosis signature, IgG4-related disease, active pDCs and relevant IFN- α signaling were also observed in related pancreas, pDC also enhanced the production of IgG4 by B cells [42]. Dual role of pDCs on IFN- α production and promoting activation of pathogenic T cells in psoriasis has been revealed in psoriasis [43]. Study in RA mouse model suggested that pDC aggravated joint inflammation and bone erosion via TLR7 dependent type I IFNs [17]. Reduction of pDC in the peripheral blood as well as the concomitant infiltration and activation in related tissue were reported in SLE [44, 45]. A recent randomized controlled trial in SLE clarified that Litifilimab (anti-BDCA2 antibody, BDCA2 is an exclusive marker of pDC) is effective in disease remission [46], 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 tupe I IFN signaling rather than pDC [26]. In general, pDCs contributed to the pathogenesis of autoimmune diseases through type I IFNs or interplay with other immune cells [47]. However, further studies are needed to elucidate the potential mechanism about pDC's contribution to SLE and pSS.

Our available evidence does not support an overactive phenotype (resting and activating status) of pDCs from pSS patients, particularly in the production of IFN- α . However, a previous transcriptional study reported that pDCs from patients with pSS secreted more type I IFNs after TLR7 stimulation [21]. Antonios et al.'s work that pDCs from patients with SLE or pSS did not show stronger secretion ability for inflammatory cytokines, especially IFN α [26], which is consistent with our findings. These divergent results may be caused by the difference of detection methods, further study will help to elucidate underlying mechanism.

In conclusion, we demonstrated the decreased percentage of pDC in pSS patients, which might result from the excess immunoglobulin (IgG and IgA) induced apoptosis. Moreover, we showed that pDC might not be the major contributor to the hyperactivation of type I interferon signaling in pSS patients. Our research provides a good addition to pSS pathogenesis and gives implications that targeting pDC might not be a good strategy for clinical pSS treatment.

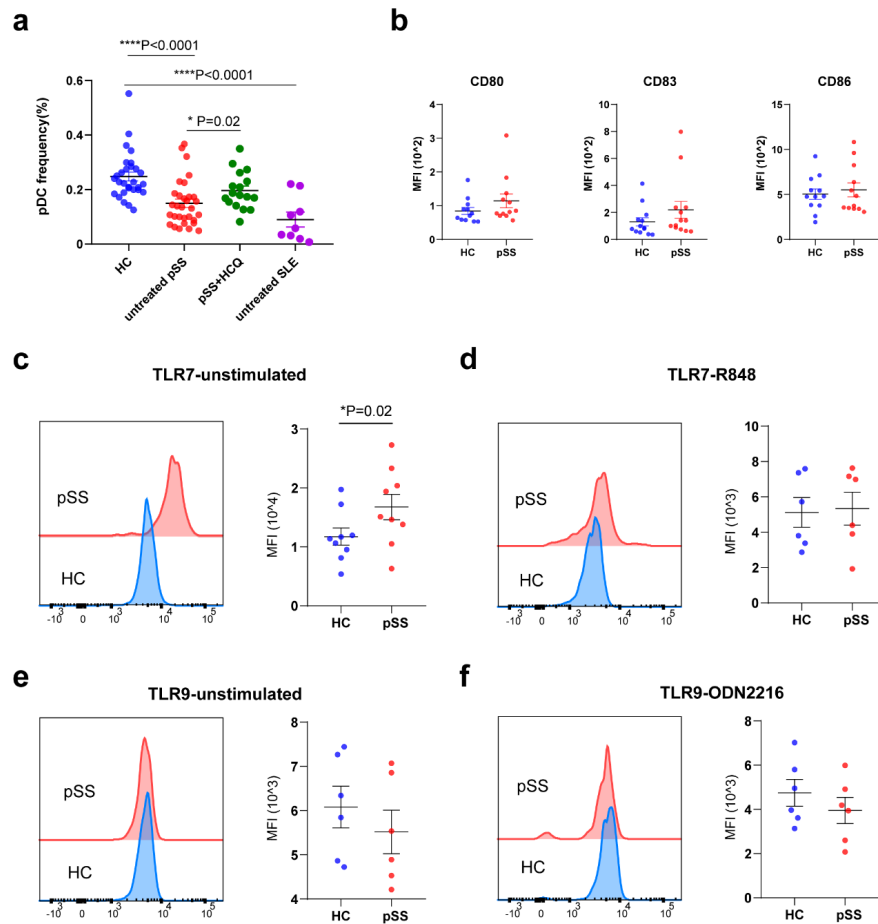


Fig 1. Frequency and phenotype analysis of peripheral pDCs in pSS patients.

a: The frequency 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) and TLR9 (n=6) expression levels between pDCs from pSS and HC without stimulation (**c, d**) and activated by TLR7 agonist (**e**) or TLR9 agonist (**f**) for 6 hours with 100ng/ml IL3 (n=6). Data are shown as mean $\pm$ SEM. Unpaired two-tailed Student's t-test was applied. *P-value < 0.05, **P-value < 0.01, ***P-value < 0.001, ****P-value < 0.0001.

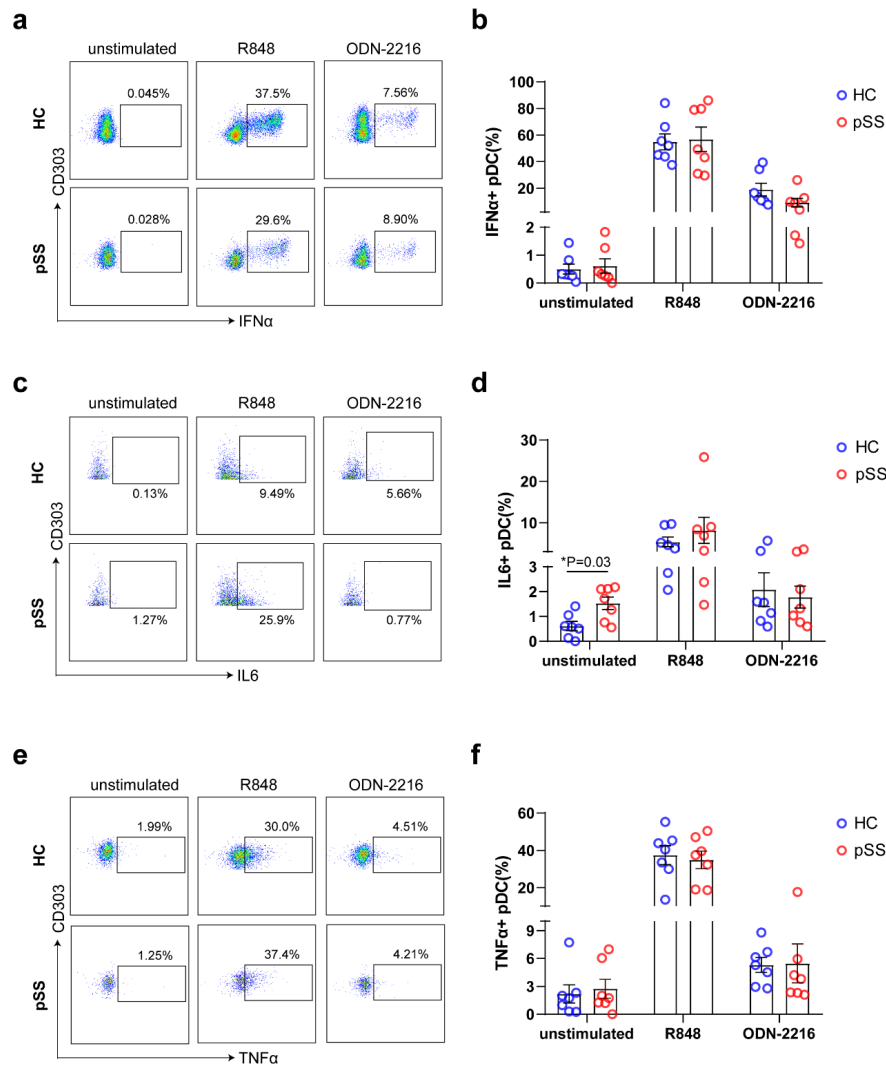


Fig 2. Detection of characteristic cytokines in pDCs with different activation states.

a: Freshly isolated PBMCs from pSS or HC were cultured in the presence of IL3, R848 (TLR7 agonist) or ODN2216 (TLR9 agonist) was added at the 6th hour, GolgiPlug was added at the 9th hour. secretion of IFN- α by pDCs was examined through intracellular staining at the 12th hour. pDCs were gated as CD303+CD123+HLA-DR+LIN- cells; **b:** Comparison of IFN- α secretion between HC (n=7) and pSS (n=7). **c:** Freshly isolated PBMCs from pSS or HC were cultured in the presence of IL3 and GolgiPlug, stimulated by R848 or ODN2216 for 12 hours, intracellular staining was used to detect the secretion of IL-6; **c:** Comparison of IL-6 secretion between HC (n=7) and pSS (n=7); Representative flow cytometric graphs (**d**) and statistical graph(**f**) for TNF- α secretion (detected by the same method as IL-6). Data are shown as mean $\pm$ SEM. Unpaired two-tailed Student's t-test was applied.

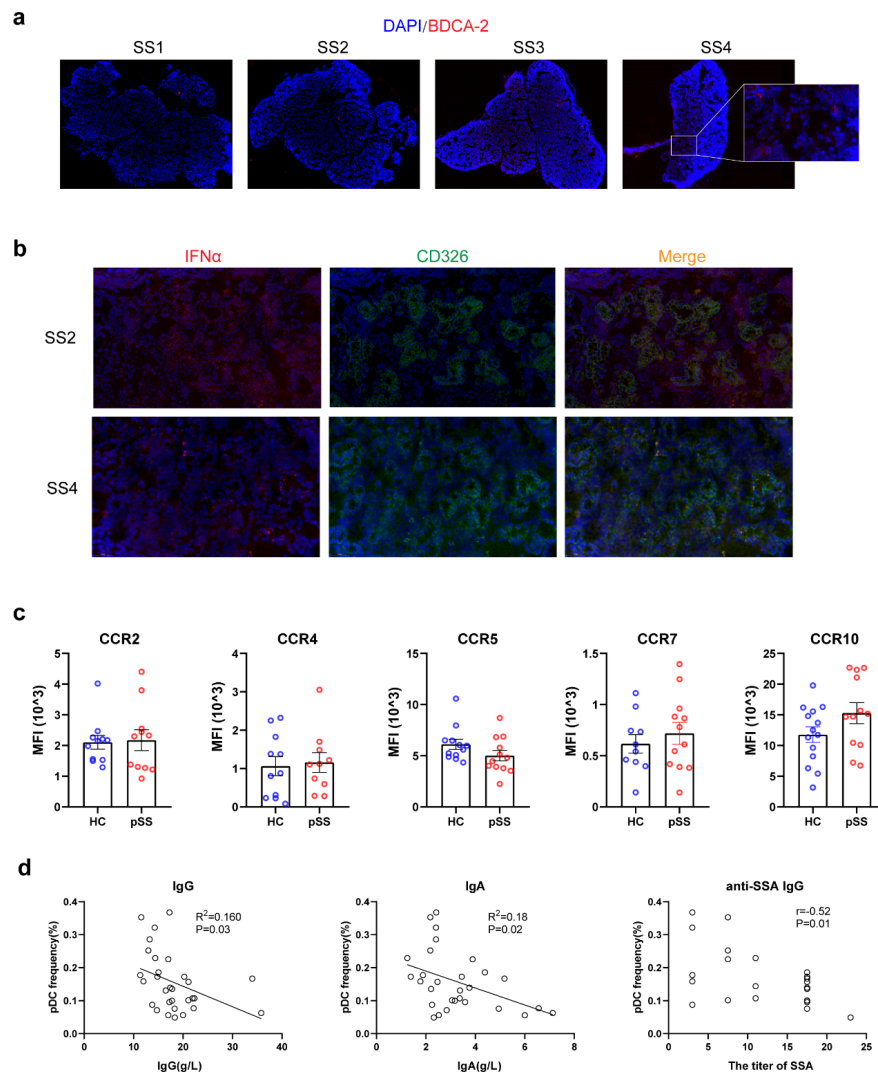


Fig 3. Few infiltrations of pDCs in labial gland and the clinical correlation analysis. **a:** Immunofluorescent results of 4 pSS patients who had labial pathological report with typical focal lymphocytic infiltrations. **b:** Immunofluorescent colocalization of epithelial cells (CD326) and IFN- α in 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:** Correlation analysis of pDC percentage in PBMC and serum IgG (n=30), IgA (n=29), anti-SSA IgG (n=23). Data are shown as mean $\pm$ SEM. Unpaired two-tailed Student's t-test, Linear regression and Pearson's correlation were performed for IgG and IgA, Spearman rank correlation were performed for anti-SSA IgG.

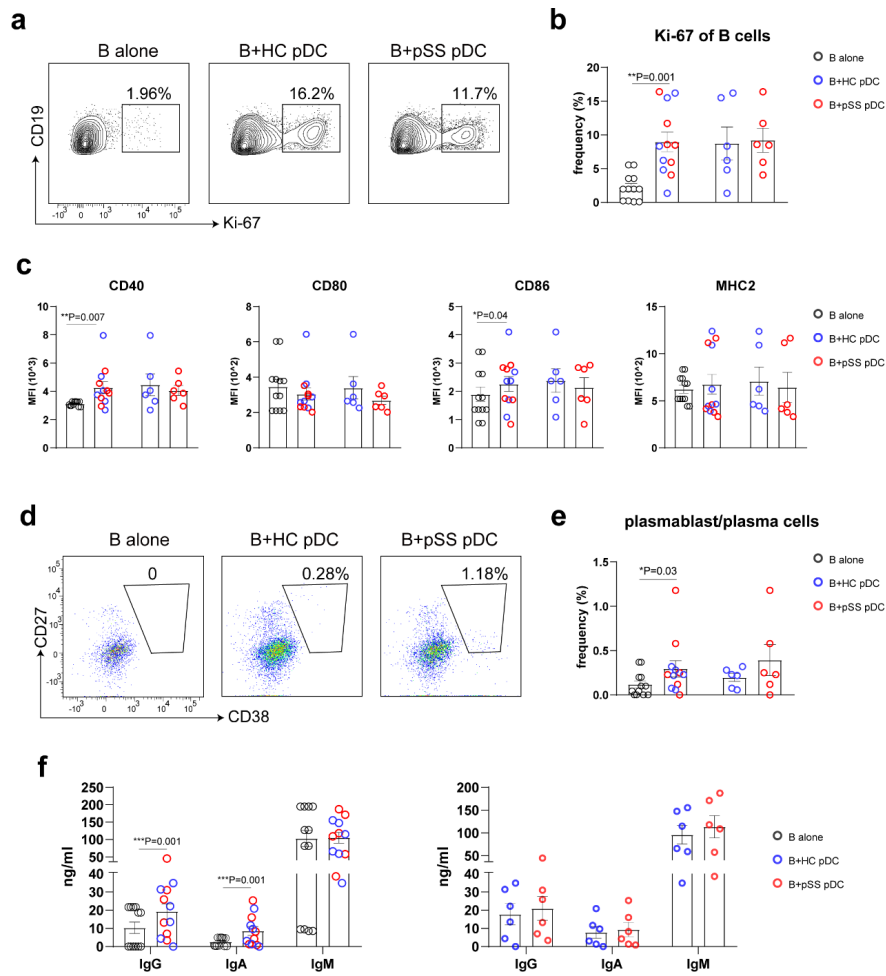


Fig 4. Co-culture of unstimulated pDCs and B cells. Purified CD19+ B cells from allogeneic healthy donors cultured alone or with pDCs (without extra activation) from pSS and HC in the presence of 100ng/ml IL3 for 7 days, the ratio of pDC to B cell was 1:10. **a:** Representative flow cytometric graph 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. **d:** Representative flow cytometric graph of B cells differentiation into plasma cells/plasmablasts. **e:** The effect of pDCs on B cell differentiation (n=12) and the effects comparison between HC (n=6) and pSS (n=6) derived pDCs on B cell differentiation. **f:** Secretion of IgG, IgA and IgM by B cells cultured alone or cultured with pDCs (n=12). Data are shown as mean $\pm$ SEM. Paired and unpaired two-tailed Student's t-test was performed. *P-value < 0.05, **P-value < 0.01, ***P-value < 0.001, **** P-value < 0.0001.

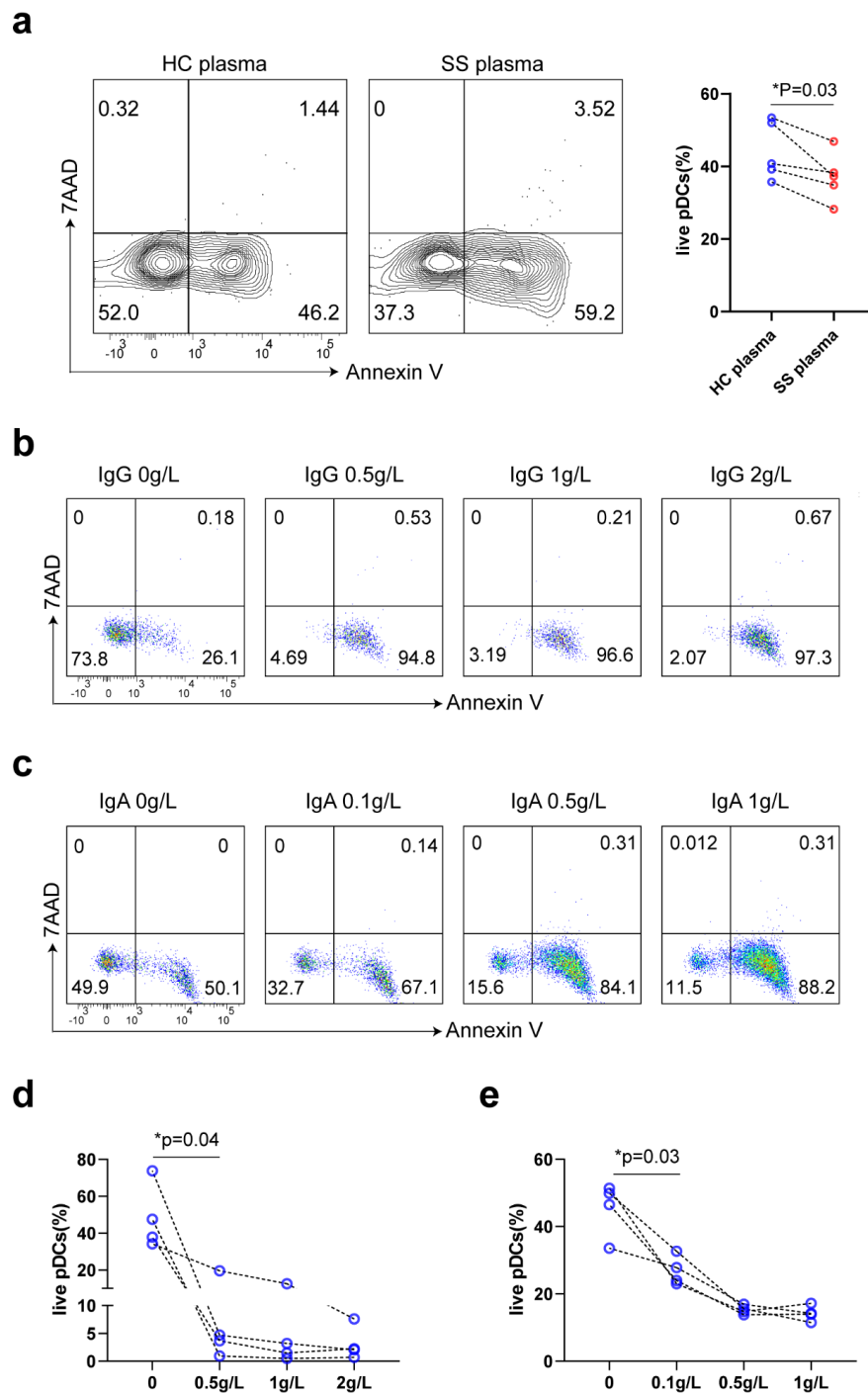


Fig 5. pSS plasma, IgG and IgA down-regulate the viability of pDCs.

a. Representative flow cytometric graph and summary graph of pDCs viability. pDCs were stimulated by HC or pSS derived plasma (n=5) for 4 hours with 100ng/ml IL3. Representative flow cytometric graph of apoptosis detection, pDCs were stimulated by increasing concentration of IgG (**b**) or IgA (**c**) for 2 hours with 100ng/ml IL3 (n=4). Statistical graph of pDCs viability when stimulated by different concentrations of human IgG (**d**) and IgA (**e**). Unpaired two-tailed Student's t-test was performed. *P-value < 0.05,

P-value < 0.01, *P-value <0.001, **** P-value <0.0001.

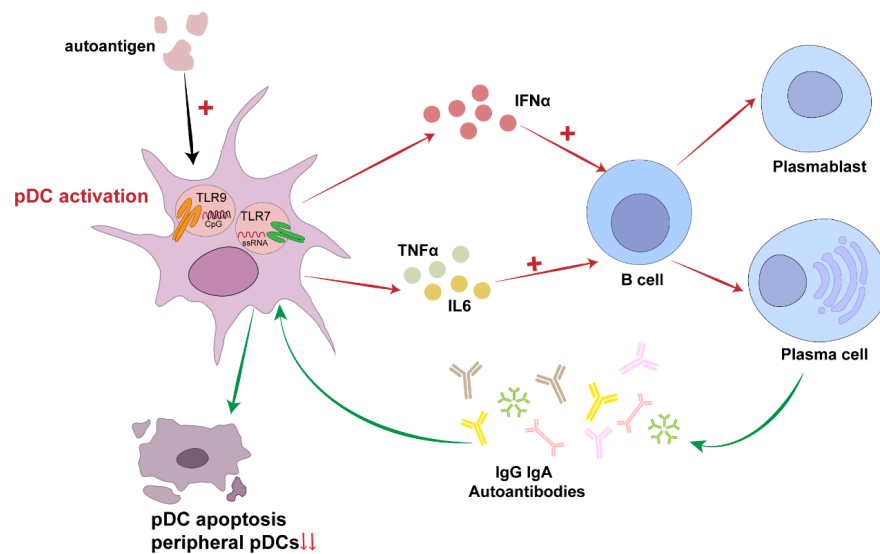


Fig 6. The interplay between pDC and B cell contributed to the pathogenesis of primary Sjögren's syndrome.

pDCs promote the proliferation, activation, and differentiation into plasmablast/plasma cell of B cells, hyperactive B cells in pSS induce the apoptosis of pDC via excess IgG, IgA and autoantibodies.

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