Invariant NKT cells are expanded in peripheral blood but are undetectable in salivary glands of patients with primary Sjögren's syndrome

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Abstract

Objective

Invariant NKT (iNKT) cells play a role in regulating the function of autoreactive B cells before their entry into germinal centres. Absence and/or reduction of iNKT cells have been demonstrated in patients with systemic lupus erythematosus (SLE) together with an increase of autoreactive B cell activity. Primary Sjögren's syndrome (pSS) is a systemic autoimmune disease in which lymphocyte infiltration and organisation in lymphoid structures of inflamed salivary glands occurs. The aim of the study was to investigate the percentage and function of iNKT in the salivary glands and peripheral blood of patients with pSS.

Methods

Minor salivary gland biopsies were obtained from patients with pSS and with non-specific chronic sialoadenitis (nSS). Flow cytometry analysis of CD1d/α-GalactosylCeramide (α-GalCer) tetramer positive cells, producing IFN-γ and IL-17, and quantitative gene expression analysis by TaqMan real-time PCR for Vα24 were performed on salivary glands biopsies and peripheral blood samples obtained from patients and controls. Flow cytometry and immunofluorescence analysis for autoreactive B lymphocytes and ELISA for anti-SSA antibodies (Ab) detection were also performed.

Results

An increase of iNKT was detected ex vivo in peripheral blood of pSS patients; after α-GalCer stimulation this subset produce IL-17 and IFN-iNKT were undetectable in the salivary glands of pSS patients and anti-SSA specific B cells were found in target tissue. Invariant NKT cells were able to inhibit autoantibody production by B cells obtained from salivary glands of pSS.

Conclusion

Impaired iNKT migration to inflamed sites might induce the activation of autoreactive B cells specific for SSA-antigen in salivary glands of pSS patients.

Key words

iNKT cells, Sjögren's syndrome, autoantibodies, B cells

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Introduction

Primary Sjögren's syndrome (pSS) is an autoimmune epithelitis characterised by chronic lymphocyte infiltration, organisation in lymphoid structures and auto-Ab production, leading to architectural destruction of the exocrine glands and resulting in the classical clinical signs and symptoms of mouth and eye dryness (1). The pathogenesis of pSS seems to be multifactorial and both innate and adaptive immunity play an important role leading to the organisation of ectopic lymphoid structures responsible of local B cell expansion and auto-antibody (autoAb) production (2). Many studies have underlined the involvement of autoreactive B lymphocytes in the pathogenesis of autoimmune disorders and demonstrated that B cell activation increases in the absence or reduction of a cell subset that bridges innate and adaptive immunity, named natural killer T (NKT) cells (3, 4).

These lymphocytes are mainly characterised by the expression of a unique (invariant) T cell receptor (TCR), Vα24Jα18Vβ11, capable of recognising glycolipids such as alpha-galactosylceramide (α-GalCer), associated with the non-polymorphic CD1d molecule (5, 6). Upon α-GalCer/CD1d recognition, iNKT cells expand and produce large amounts of cytokines and chemokines both *in vitro* and *in vivo* (7, 8). Invariant NKT cells include $CD8^+$, $CD4^+$ and double negative $CD4^-/CD8^-$ subsets all producing different cytokines, thus potentially playing both protective and harmful roles in the progression of autoimmune diseases (3, 4, 9).

Activated iNKT cells have been reported to enhance Ab responses against T-dependent and T-independent antigens of pathogens (10) and to inhibit auto-Ab-producing B cells, playing a supportive and regulatory role on B cell functions. Invariant NKT cells appear in fact to inhibit autoAb production in a contact and CD1d-dependent manner (11) and to regulate the function of autoreactive B cells before their entry into germinal centres (12).

According to our knowledge, no specific evidence of the role of iNKT in pSS has been described. The aim of this study was to investigate the frequency and function of iNKT cells in the salivary glands and in the peripheral blood of patients with pSS.

In this study we demonstrate that:

1. iNKT cells were undetectable in the salivary glands of pSS patients, but are significantly expanded in the peripheral blood ex vivo and produced IL-17 and IFN-γ after α-GalCer stimulation;
2. iNKT cells from pSS patients express at low levels the chemokines receptors CXCR3, CCR6 and CCR5 compared to iNKT from nSS subjects;
3. a significant increase of autoreactive B lymphocytes specific for SSA antigen occur in the inflamed salivary glands;
4. activated iNKT cells *in vitro* inhibit anti-SSA Ab production by B cells obtained from pSS. These results seem to suggest that iNKT absence in salivary glands may results in B cells activation and anti-SSA production.

Material and methods

Patients

For this study, twenty patients meeting the American-European Consensus Group criteria for pSS (20) were enrolled. Sixteen patients classified as having non-specific chronic sialoadenitis (nSS) showing various degrees of mononuclear cell infiltration in the absence of focal organisation (these patients do not fulfill the American-European Consensus Group criteria for SS) were also included as a control group. All patients and controls underwent to minor salivary gland biopsy for histological diagnosis and peripheral blood were also collected at the time of enrolment. The presence of other underlying autoimmune diseases or hepatitis C virus infection was carefully excluded. All pSS patients selected were positive for antinuclear antibodies, anti-SSA/Ro, anti-SSB/La. Patients and controls also underwent serological evaluation for rheumatoid factor, levels of C-reactive protein and erythrocyte sedimentation rate. Unstimulated whole sialometry and the Schirmer Test were also performed. The baseline characteristics of patients and controls are shown in Table I.

Flow cytometric analysis of iNKT in salivary gland mononuclear cells (SGMCs) and peripheral blood mononuclear cells (PBMCs) after αGalCer stimulation

Peripheral blood from pSS patients and control subjects were collected in heparinised tubes. PBMCs were obtained after centrifugation on Ficoll-Hypaque (Pharmacia, Uppsala, Sweden).

Salivary glands were digested as previously described (14). The medium used throughout was RPMI 1640 (Invitrogen Life Technologies) supplemented with 5% heat-inactivated pooled human AB^+ serum, 2 mM l-glutamine, 20 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, 5×10^{-5} M 2-ME. The cells were counted using Trypan Blue dye exclusion. The lymphocytes were cultured at 5 x 10^5/well in u-bottomed 96 well plates (Nunc, Copenhagen Denmark).

PBMCs or SGMCs were incubated for 5 days with (20 ng/ml) at 37°C 5% CO_2. After incubation, cells were washed in complete medium and incubated with PE-labelled CD1d/αGal-Cer tetramers or PE-labelled CD1d-vehicle (Proimmune, Oxford, UK), washed, and analysed by flow cytometry on a FACSCalibur using the CellQuest Pro software. Cells were also stained with FITC-labelled- anti- CXCR3, CCR6 and CCR5 mAb (Beckton Dickinson, US). To assess the cytokine content of $tetramer^+$ iNKT cells, cells were fixed and permeabilised (Cytofix/Cytoper, Beckton Dickinson, US) and then stained with allophycocyanin-labelled anti-IFN-γ mAb (Beckton Dickinson, US) and PerCP-labelled anti-IL-17 mAb (Beckton Dickinson, US) in incubation buffer (PBS containing 1% FCS and 0.1% sodium azide) for 30 min at 4°C. Cells were then washed twice in PBS with 1% FCS and analysed by flow cytometry. Viable lymphocytes were gated by forward and side scatter, and analysis was performed on 100,000 acquired events for each sample. Mean of percentages obtained from patients and controls was statistically compared.

RT-PCR for iNKT receptor Vα24-Jα18

RNA was extracted from PBMCs and SGMCs isolated from pSS patients and nSS, before and after 5 days of *in vitro* treatment with α-Gal-Cer, using the commercially available illustra RNAspin Mini Isolation Kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK), according to manufacturer's instructions. Total RNA was reverse transcribed to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Samples were stored at –20°C until use. For quantitative TaqMan RT-PCR, master mix and TaqMan gene expression assays for *GAPDH* (glyceraldehyde 3-phosphate dehydrogenase, Hs99999905_m1) control and iNKT receptor Vα24-Jα18 (Forward primer: 5'-CCTCCCAGCTCAGCGATTC-3'; Reverse primer: 5'-TATAGCCTCCC-CAGGGTTGA-3'; Probe: FAM-5'-C-CTCCTACATCTGTGTGGTGAGC-GACA-3'-TAMTph), were obtained from Applied Biosystems. Samples were run in duplicate using the Step-One Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). Relative changes in gene expression between paired patients before and after treatment, were determined using the $\Delta\Delta C_t$ method. Levels of the target transcript were normalised to a GAPDH endogenous control, constantly expressed in both groups (ΔC_t). For $\Delta\Delta C_t$ values, additional subtractions were performed between untreated and treated samples ΔC_t values. Final values were expressed as **f**old **o**f **i**nduction (FOI).

Detection of iNKT and autoreactive B lymphocytes by immunofluorescence

Salivary gland and tonsil (obtained from healthy controls) tissue samples were immediately fixed with 4% formaldehyde and embedded in paraffin. Staining was performed on 5-μm-thick paraffin-embedded sections as previously described (14). After dewaxing and dehydration, sections were stained with TCR-Vα24 antibody (Immunotech SA, Marseilles, France). In order to study the general SSA-specific B cell pattern in the SG, double-staining experiments were carried out with CD19 in combination with SSA-Ag as described by Aqrawi *et al*. (15). Three sections for each sample were analysed by two different scientists or technician to avoid causalities of showed results. Briefly, tissues were blocked with normal bovine serum and then incubated with SSA antigen (SSA-Ag) (20 μg/ml) and stained with the primary antibody against SSA (rabbit anti-human SSA at a concentration of 1:100) and anti-CD19 (mouse anti-human CD19 at a concentration of 1:100). Purified rabbit IgG control antibody was used instead of the primary antibody as a control. The secondary antibodies (goat anti-rabbit Ig, rabbit anti-mouse Ig), were added and incubated for 30 min. Nuclei were counter stained with Toto3. The images were acquired with confocal microscope (Nikon).

Co-culture of B cells and iNKT

PBMCs obtained from 5 patients were magnetically separated for Vα24-Jα18 (anti-human Vα24-Jα18 -PE eBioscience, San Diego, CA), using anti PE-microbeads (Miltenyi Biotec, Germany) to isolate iNKT. The negative cellular fraction was next enriched for $CD19^+$ cells (Beckton Dickinson, US) using anti PE-microbeads (Miltenyi Biotec, Germany) and stimulated with SSA antigen. Next, the B lymphocytes were cultured alone or with activated iNKT (as indicated above) (iNKT:B cells ratio 1:1 and 1:10) in RPMI medium supplemented with 10% FCS in U-bottom 96-well plates and incubated at 37°C, 5% CO2 for 48 hours. After incubation, supernatants were collected to test anti-SSA production by ELISA.

Anti-SSA ELISA

A sandwich ELISA system was developed using the anti-SSA mAbs as the capturing Ab. The ELISA kit was a kind gift by Dr. Lakshmanan Suresh (Immco Diagnostics, Buffalo, NY). Supernatants derived from co-culture of iNKT and B lymphocytes were collected and stored at –80°C. All samples were titrated two-fold in the same solution and assayed in triplicate on the ELISA plate. The ELISA test was performed according to manufacturer instructions. The ELISA system could detect anti-SSA with a detection limit around 25 pg/ml.

Statistics

Statistical analysis of quantitative

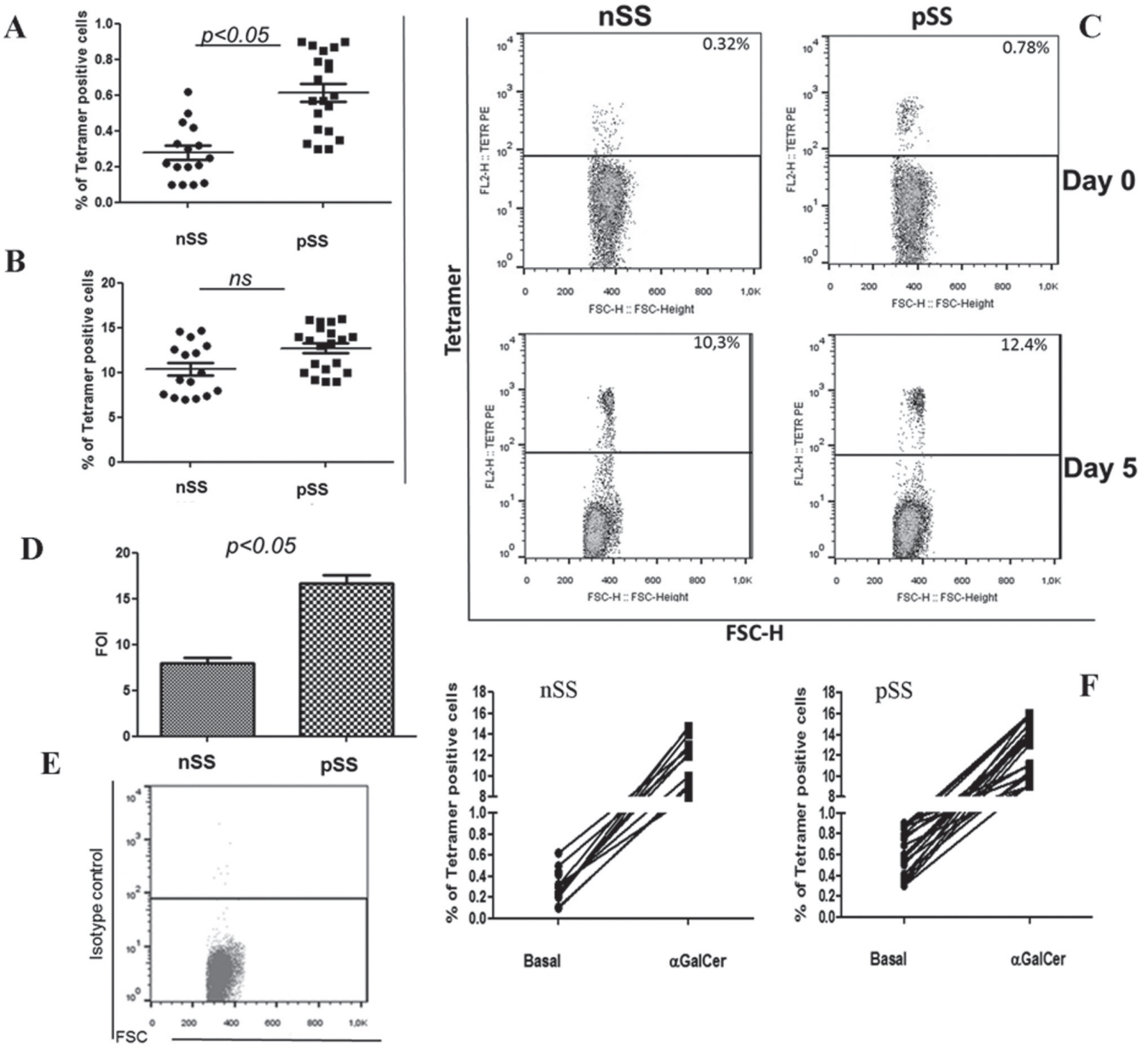


Fig. 1. iNKT percentage from PBMCs of pSS and nSS before and after αGalCer stimulation (the experiment was performed two times for each subject enrolled in the study). **A-B:** Mean percentages of iNKT cells before (**A**) and after (**B**) αGalCer stimulation of PBMCs of patients and controls. **C:** Dot plot analysis of iNKTcells, stained with PE-labelled CD1d-αGalCer tetramer, before and after stimulation with αGalCer in one representative patient and control. **D:** Relative expression of Vα24 mRNA among PBMC of patients with pSS and nSS. Data are normalised for GAPDH and plotted as fold of induction. Results represent the mean±SEM. **E** Dot plot analysis showing isotype control. **F:** Dot plot displaying the coupled values of iNKT percentages before and after stimulation with αGalCer.

variables was performed using the Mann-Whitney rank-sum test. Pearson's correlation analysis was utilised to quantify the expression associations between the genes of interest. Data are expressed as mean ± SEM. *p*-values <0.05 were considered significant.

Results

As shown in Figure 1A and C, the mean of percentages of peripheral blood iNKT cells were significantly higher in pSS patients than in nSS before αGal-Cer culture (Fig. 1F). This difference was not statistically significant after *in vitro* stimulation with αGal-cer (Fig. 1B-C-F). The mean expansion index (*i.e.*) of iNKT, calculated before and after *in vitro* culture, was higher in controls (*i.e.* 43.7) than in patients (*i.e.* 23.1). These data were confirmed by RT-PCR, showing an increase of almost three-fold of Vα24 gene expression (Fig. 1D) in pSS when compared to nSS.

Then we tried to better characterise iNKT cells in pSS patients by analysing their cytokine production. An increased percentage of IL-17$^+$ or IFN-γ$^+$ iNKT was observed in pSS compared to nSS (Fig. 2A-B), after *in vitro* activation with αGal-Cer. The surface expression of CXCR3, CCR5 and CCR6 was down regulated in iNKT of pSS when compared to nSS (Fig. 2C-E). Despite the expansion of iNKT in peripheral blood of pSS patients, these

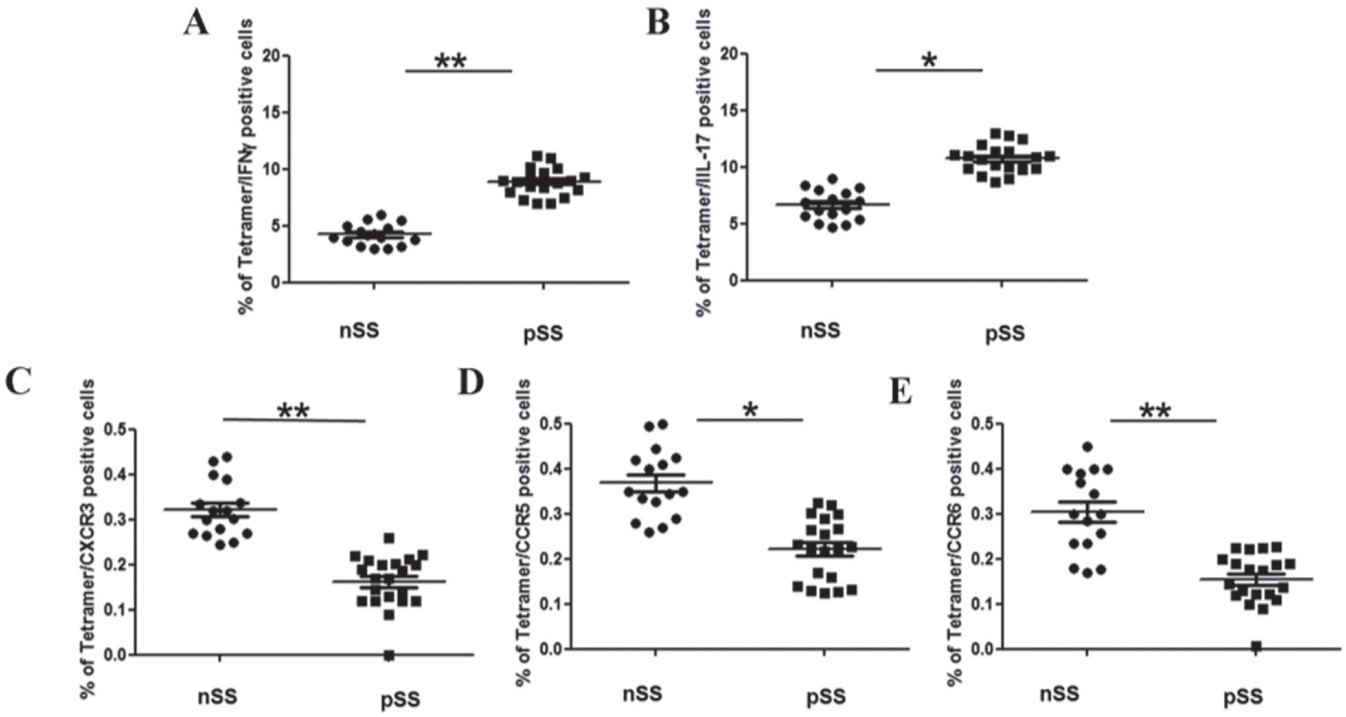


Fig. 2. Expression of IFN-γ and IL-17, and chemokines receptors among iNKT from peripheral blood of pSS patients and control after stimulations (the experiment was performed two times for each patient or control enrolled in the study). **A-B** Mean percentage of IFN-γ and IL-17 producing iNKT cells after αGalCer stimulation in patients and controls. **C-E** Mean percentages of CXCR3, CCR5 and CCR6 expressing iNKT respectively. Results represent the mean±SEM.

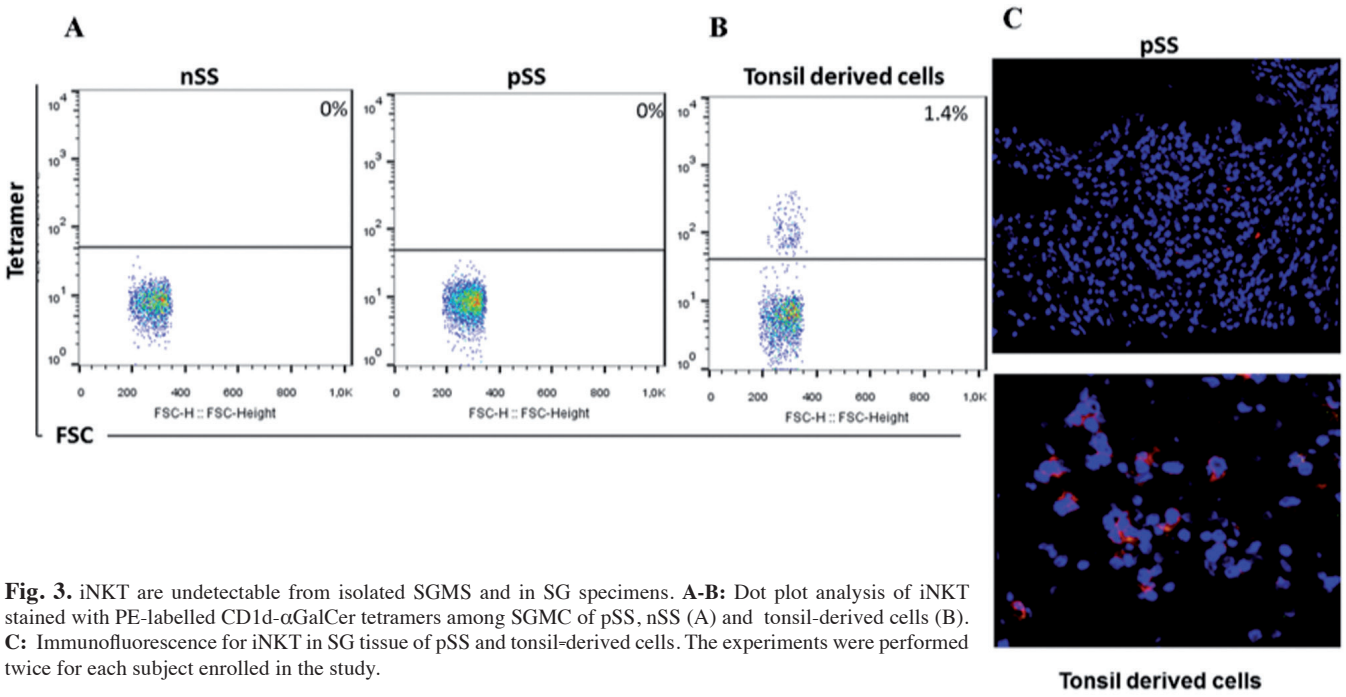


Fig. 3. iNKT are undetectable from isolated SGMS and in SG specimens. **A-B:** Dot plot analysis of iNKT stained with PE-labelled CD1d-αGalCer tetramers among SGMC of pSS, nSS (A) and tonsil-derived cells (B). **C:** Immunofluorescence for iNKT in SG tissue of pSS and tonsil-derived cells. The experiments were performed twice for each subject enrolled in the study.

cells were not detectable among SGMCs stimulated with αGal-Cer of both patients and controls by flow cytometry (Fig. 3A-B) and IF assays (Fig. 3C). As a positive control for IF staining of iNKT, tonsil-derived cells were shown in Figure 3C. Vα24 gene expression was undetectable in biopsy and in salivary gland mononuclear cells cultured with αGal-cer of either pSS and nSS (data not shown).

Considering the important role of iNKT cells in the control of B cell autoreactivity and given the absence of iNKT in SGMCs we next evaluated the presence of auto-reactive B cells in the salivary gland specimens of pSS and nSS patients. Interestingly we found SSA

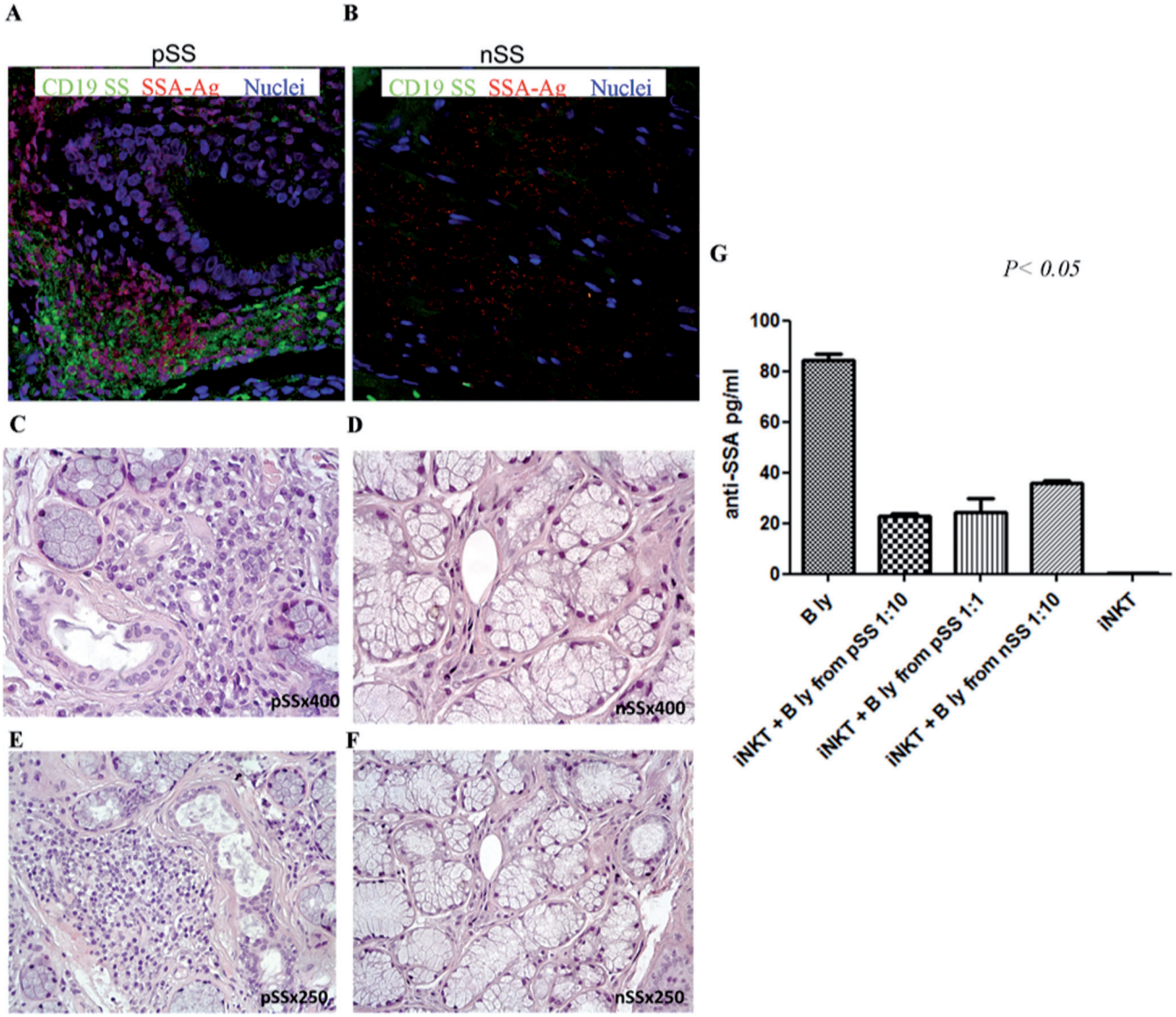


Fig. 4. iNKT inhibit anti-SSA secretion from autoreactive B cells. **A-B** Immunofluorescence for CD19 (green) and SSA-Ag (red) in SG tissue of pSS (A) and nSS (B); nuclei were contrasted with Toto3 iodide. The experiments were performed two times for each subject enrolled in the study. **C-F** Sequential slide stained with haematoxilin and eosin respectively in pSS and nSS. **G** Anti-SSA were detected by supernatants obtained from co-cultures of B lymphocytes derived from salivary glands of pSS and nSS patients with iNKT derived from patients and controls at different ratios. Experiments were repeated three times. Data are reported as antiSSA (pg/ml) detected.

antigen-specific autoreactive CD19$^+$ B cells in the SGMC of pSS patients only (Fig. 4A-B). The absences of iNKT in the salivary glands of pSS patients among infiltrating cells (Fig. 4C-F), could contribute to the lack of regulation of autoreactive B cells. In this regard, we evaluated the production of anti-SSA autoAb by salivary gland derived B lymphocytes after co-culture with different ratios of αGal-Cer-activated iNKT cells. Interestingly, we found a statistically significant reduction of anti-SSA auto-Ab when B cells from patients were co-cultured with iNKT cells from patients but not when the cells were obtained from controls (Fig. 4G). The number of iNKT in the cultures was directly related with the inhibitory function displayed by these cells on autoreactive B cells.

Discussion

The presence of high percentages of autoreactive B cells and the contemporary absence of iNKT cells characterised the salivary glands of patients with pSS, conversely, high percentages of iNKT cells were observed in the peripheral blood of pSS patients. iNKT cells obtained from peripheral blood, after *in vitro* stimulation with αGalCer, expressed both IFN-γ and IL-17 and displayed low levels of the chemokine receptors CXCR3, CCR6 and CCR5 on the cell surface in pSS patients respect to controls. These findings seem to suggest that despite an activate phenotype, iNKT from pSS patients may have a possible low capacity to migrate towards inflammatory sites in response to relevant inflammatory chemokines. Furthermore, circulating iNKT were able to inhibit autoantibody production when *in vitro* co-cultured with B lym-

phocytes obtained from the salivary glands of pSS patients.

Moreover iNKT, cultured with αGalCer, inhibit autoreactive B cells in a contact- and CD1d-dependent manner but are also able to activate non autoreactive B cells and/or activate autoreactive B cells via cytokines (11, 12). Low percentage of iNKT cells has been demonstrated in other autoimmune diseases such as systemic lupus erythematosus (SLE) and correlated with disease activity, suggesting a role in the control of autoreactive responses and disease pathogenesis (4, 9, 16, 17). In contrast with these observations and our present study, iNKT cell activation has been observed in the salivary gland of patients with pSS by Awada *et al*. (18). In their study, however, iNKT cells were identified as $CD3^+$ $CD16^+$ $CD56^+$ cells, clearly not sufficient to identify iNKT cells (18). Conversely, in our study the glycolipid αGal-Cer was used to specifically stimulate iNKT cells (10, 19) and extremely specific probe capable to bind selectively the invariant TCR of NKT cells (the CD1d/ αGalCer tetramer) was used to proper identify these cells.

The immune system is able to sequestrate autoreactive B lymphocytes in the MZ and to organise these cells in ectopic GC-like structures as aggregates (20, 21). In this regard, the absence of iNKT cells in the salivary gland tissue might be responsible, at least in part, for the lack of control of B lymphocyte activation *in situ*, potentially leading to autoreactive B cells organisation in structures similar to germinal centre (GC). This capability of iNKTs to suppress autoAb production, without causing global suppression of B cells, may have important implications for the development of iNKT-based therapy for autoimmune diseases.

More studies are clearly required to better characterise the exact role of iNKT in pSS, including their complete profile of cytokine/chemokine production, their migratory pattern and the mechanisms underlying their regulatory activities to design possible future iNKT cell-based immunotherapeutic strategies. Nevertheless, our results may suggest that in pSS expanded and activated circulating iNKT cells could be detected as a marker of the disease activity.

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