Original Article

Activation of Toll-like receptor 7 signaling in labial salivary glands of primary Sjögren's syndrome patients

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Running title: TLR7 in SS

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Abbreviations

AECG, American-European Consensus Group; ANA, anti-nuclear antibody; ANOVA, analysis of variance; APC, allophycocyanin; BAFF, B-cell activating factor; cGAS, cyclic GMP-AMP synthase; ELISA, enzyme-linked immunosorbent assay; ER, endoplasmic reticulum; ESSDAI, European League Against Rheumatism Sjögren's Syndrome Disease Activity Index; FITC, fluorescein isothiocyanate; HRP, horseradish peroxidase; IFITM3, IFN-induced transmembrane protein 3; IFI27, IFN-α-inducible protein 27; IFN, interferon; IgG, immunoglobulin G; IHC, immunohistochemistry; IL, interleukin; IQR, interquartile range; IRF7, interferon regulatory factor 7; ISGF3G, IFN-stimulated transcription factor 3; LSG, labial salivary gland; MDA5, melanoma differentiation associated gene-5; MFI, mean fluorescence intensity; MHC, major histocompatibility complex; MNCs, mononuclear cells; MyD88, myeloid differentiation primary response gene 88; M3R, M3 muscarinic acetylcholine receptor; NA, not assessed; NFκB, nuclear factor kappa B; PBMC, peripheral blood mononuclear cell; pDC, plasmacytoid dendritic cell; PE,

phycoerythrin; pSS, primary Sjögren's syndrome; PVDF, polyvinylidene fluoride; RF, rheumatoid factor; RIG-I, retinoic acid-inducible gene-I; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SGECs, salivary gland epithelial cells; ssRNA, single-strand RNA; STING, stimulator of interferon genes; TBS-T, Tris-buffered saline containing 0.1% Tween 20; TLRs, Toll-like receptors; TRAF6, tumor necrosis factor receptor-associated factor 6; TRITC, tetramethyl rhodamine isothiocyanate.

SUMMARY

Objectives: To determine the expressions of Toll-like receptors (TLRs) 7–9 and type I interferon (IFN) signal in labial salivary glands (LSGs) and cultured salivary gland epithelial cells (SGECs) from primary Sjögren's syndrome (pSS) patients.

Methods: We performed an immunohistochemistry analysis of LSGs from 11 patients with pSS as defined by American-European Consensus Group classification criteria and five healthy subjects. The pSS patients' SGECs were analyzed by immunofluorescence and western blotting. IFN-α expression was examined by immunosorbent assay and flow cytometry.

Results: Mononuclear cells (MNCs) from pSS patients' LSGs showed TLR7-dominant expression. B cells, plasma cells, and plasmacytoid dendritic cells (pDCs) co-expressed with TLR7. MyD88, TRAF6, and IRF7 co-expressed with the pDC marker CD303 in LSGs. Ducts from pSS patients dominantly expressed TLR7, and TLR7 in the ducts co-expressed with MyD88, TRAF6 and IRF7. Type I IFNs including IFN-α and IFN-β were detected in MNCs and ducts in pSS patients' LSGs. Increased TRAF6 expression and the nuclear translocation of IRF7 in SGECs were detected by immunofluorescence following loxoribine (a TLR7 ligand) stimulation despite IFN-β pretreatment. Western blotting showed increased TRAF6 expression in SGECs following IFN-β and loxoribine stimulation. Although no increase in IFN-α was detected in supernatant from stimulated SGECs, the IFN-α in supernatant from stimulated peripheral blood pDCs from pSS patients was

significantly increased.

Conclusion: Our findings suggest that TLR7 is dominantly expressed in both MNCs and ducts with downstream signals for type I IFNs, indicating that TLR7-dominant innate immunity is related to the development of sialadenitis in pSS.

(250 words)

Introduction

Primary Sjögren's syndrome (pSS) is a systemic autoimmune disease characterized by periductal lymphocytic infiltration of the salivary and lacrimal glands, which results in reduced secretory functions and oral and ocular dryness [1]. Although the pathogenesis of pSS is not yet established, innate immune responses including type I interferon (IFN) activity were shown to be associated with the pathogenesis of pSS [2, 3], as were the conventional acquired immunity responses including major histocompatibility class II-mediated antigen presentation [4]. Toll-like receptors (TLRs), especially TLRs 7–9, are important innate immune receptors that recognize a range of RNA and DNA molecules from viruses and self-antigens, and lead to the production of type I IFN via downstream molecules such as myeloid differentiation primary response gene 88 (MyD88), tumor necrosis factor receptor-associated factor 6 (TRAF6), and interferon regulatory factor 7 (IRF7) signaling [5]. The expressions of TLR7 and TLR9 were high in the peripheral blood mononuclear cells (PBMCs) of pSS patients [6]. However, little is known regarding the expression pattern and the roles of TLR7–9 in the salivary glands. In this study, we analyzed the expressions of TLR7–9 in labial salivary glands (LSGs) of pSS patients. Based on the TLR7 dominant expression observed, we also analyzed TLR7 downstream signals and function in an *in vitro* examination.

Materials and Methods

Patients

For the immunohistochemical analysis, we retrospectively analyzed materials from 11 patients with pSS and five control subjects who visited Nagasaki University Hospital during the period from 2008 to 2013. The patients' pSS classification was based on the 2002 American-European Consensus Group (AECG) Sjögren's Syndrome (SS) classification criteria [7]. The control subjects had sicca symptoms but did not fulfill the AECG SS classification criteria (non-SS sicca control subjects). LSG biopsies specimens were obtained from all participants for our assessment of the pathological findings of pSS.

For the determination of focus scores (i.e., the number of foci per 4 mm^2) in LSGs, the number of foci in a section from LSGs was counted and the surface area of the section was measured by a hybrid cell count system mounted on a microscope (BZ-X700; Keyence, Osaka, Japan) [8]. The clinical and serological characteristics of the pSS patients and control subjects are summarized in Table 1. All patients gave their informed consent to be subjected to the protocol, which was approved by the Institutional Ethics Committee of Nagasaki University Hospital (approval no. 09102822-3).

Culture of salivary gland epithelial cells (SGECs)

We performed the culturing of SGECs as described [9]. Briefly, the LSG tissues were cut with fine

needles and scalpels and placed in six-well plates coated with type-I collagen (Iwaki, Tokyo) with culture medium consisting of defined keratinocyte-SFM culture medium (Invitrogen Life Technologies, Carlsbad, CA), 0.4 μg/ml hydrocortisone (Sigma-Aldrich, St. Louis, MO), 25 μg/ml bovine pituitary extract (Kurabo, Osaka, Japan), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco, Grand Island, NY). When an outgrowth of SGECs was observed, the cells were transferred into 100-mm^2 plates coated with type-I collagen (Iwaki) after the cells reached confluence. After the SGECs reached confluence on the 100-mm^2 plates, the cells were cultured onto 60-mm^2 plates coated with type-I collagen (Iwaki) for western blotting and an enzyme-linked immunosorbent assay (ELISA).

When the SGECs reached confluence, the cells were treated with 1 mM loxoribine (InvivoGen; San Diego, CA), a TLR7 ligand, for 10 min, 60 min, or 6 hr, and/or 1000 U/ml IFN-β (Betaferon®; Bayer Pharma, Berlin, Germany) for 12 hr. For immunofluorescence, SGECs were distributed onto 12-mm^2 cover slips coated with a Type I collagen, Cellmatrix (Nitta Gelatin, Osaka, Japan) in 24-well plates (Corning, New York, NY) after the SGECs reached confluence on the 100-mm^2 plates. Subsequently, the SGECs were treated with 1 mM loxoribine for various lengths of time between 0 min and 60 min and/or 1000 U/ml IFN-β for 12 hr.

Isolation and culture of peripheral blood mononuclear cells (PBMCs)

Heparinized whole blood (7 ml) was obtained from each pSS patient and control subjects (patients other than those described in Table 1). PBMCs were isolated from whole blood by centrifugation using Ficoll-Paque™ Plus (GE Healthcare Bio-Sciences, Uppsala, Sweden); the cells were subsequently seeded on six-well plates (Iwaki) and cultured in RPMI 1640 medium (Wako, Osaka, Japan) supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Paisley, Scotland), 0.25 μg/ml amphotericin B (Sigma-Aldrich), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco). The cells were then treated with 1 mM loxoribine for 6 hr and/or 1000 U/ml IFN-β for 12 hr for the ELISA and flow cytometry.

Immunohistochemistry (IHC)

We performed IHC to examine the expressions of TLR7–9 and TLR7 downstream molecules in LSGs. A cancer tissue array including four types of cancer — colon, breast, lung, and prostate — and normal tissue (US Biomax, Derwood, MD) was used as the positive control for TLR7–9 expression. The primary antibodies used were rabbit anti-TLR7 polyclonal (working dilution 1:100; Enzo Life Sciences, Farmingdale, NY), rabbit anti-TLR8 polyclonal (working dilution 1:100, Sigma-Aldrich), rabbit anti-TLR9 polyclonal (working dilution 1:100; Bioss Antibodies, Woburn, MA), mouse anti-IFN-α monoclonal (working dilution 1:100, Santa Cruz), and rabbit anti-IFN-β polyclonal antibody (working dilution 1:100, Abcam, Cambridge, MA).

In summary, paraffin-embedded sections from LSGs and the tissue array were incubated with 3% H_2O_2 solution for the inhibition of endogenous peroxidase activity after microwave epitope retrieval, and blocked with 5% normal horse serum. They were incubated with each primary antibody diluted with 5% normal horse serum for 60 min at room temperature (RT), followed by peroxidase-conjugated secondary antibody (Histofine Simple Stain, Nichirei Biosciences, Tokyo) for 30 min at RT. After incubation, they were reacted with 3, 3'-diaminobenzidine solution for 10 min and counterstained with hematoxylin solution. Images were taken using a digital microscope color camera (DFC295; Leica Microsystems, Tokyo).

Immunofluorescence

We performed an immunofluorescence examination to determine the localization of TLR7, TLR8, and TLR7 downstream molecules in mononuclear cells (MNCs) of LSGs *in vivo*, and the expression of TLR7 downstream molecules in SGECs *in vitro*. The primary antibodies used were rabbit anti-TLR7 polyclonal (working dilution 1:100), mouse anti-TLR7 monoclonal (working dilution 1:100; Thermo Fisher Scientific, Rockford, IL), rabbit anti-TLR8 polyclonal (working dilution 1:100), rabbit anti-MyD88 polyclonal (working dilution 1:100, Santa Cruz), rabbit anti-TRAF6 monoclonal (working dilution 1:100; Abcam), rabbit anti-IRF7 polyclonal (working dilution 1:100; Abnova, Taipei City, Taiwan), mouse anti-CD4 monoclonal (working dilution

1:100; Agilent Technologies, Santa Clara, CA), mouse anti-CD8 monoclonal (working dilution 1:100, Agilent Technologies), mouse anti-CD20 monoclonal (working dilution 1:1, Agilent Technologies), mouse anti-CD68 monoclonal (working dilution 1:100, Agilent Technologies), mouse anti-VS38c monoclonal (working dilution 1:100, Agilent Technologies), and mouse anti-CD303 antibody (working dilution 1:100; Dendritics, Lyon, France) *in vivo*, and rabbit anti-TRAF6 monoclonal antibody (working dilution 1:100) and rabbit anti-IRF7 polyclonal antibody (working dilution 1:100) *in vitro*. The procedure until incubation with primary antibodies was basically the same as that of the IHC *in vivo*.

After incubation with primary antibodies, the sections from LSGs were reacted with secondary antibodies including donkey anti-mouse IgG conjugated with fluorescein isothiocyanate (FITC) antibody (Jackson ImmunoResearch Laboratories, West Grove, PA), donkey anti-rabbit IgG conjugated with tetramethyl rhodamine isothiocyanate (TRITC) antibody (Jackson ImmunoResearch), and Hoechst dye 33258 (Sigma-Aldrich) for 45 min at RT in the dark. The sections were then mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA).

In vitro, SGECs on 12-mm^2 cover slips were incubated in 4% paraformaldehyde for 10 min at 4°C and then immersed in methanol for 10 min at −20°C after loxoribine and/or IFN-β stimulation. The cells were blocked with 5% normal horse serum and incubated in each primary

antibody for 60 min at RT, followed by secondary antibodies including rabbit anti-IgG conjugated with TRITC and Hoechst dye 33258 for 45 min at RT in the dark. The cells were subsequently mounted in Vectashield mounting medium. Images were captured by a microscope (BZ-X700). The mean fluorescence intensity (MFI) of cells in a given area was calculated by the hybrid cell count system that was mounted on the BZ-X700 microscope.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining

We performed TUNEL staining to examine apoptosis in SGECs, as described [10]. Briefly, we used a Mebstain Apoptosis Kit Direct (MBL, Nagoya, Japan). After the SGECs were treated with loxoribine for 6 hr and/or IFN-β for 12 hr, or 50 ng/ml recombinant tumor necrosis factor-related apoptosis inducing ligand (TRAIL) (R&D Systems Minneapolis, MN) for 3 hr as a positive control, the cells were incubated with 50 μl of terminal deoxynucleotidyl transeferase (TdT) solution at 37°C for 1 hr after incubation with TdT buffer for 10 min. The cells were subsequently mounted in Vectashield mounting medium. Images were captured by a microscope (BZ-X700).

Western blotting

We performed western blotting to detect the protein of TLR7, its downstream molecules, and other molecules (B-cell activating factor [BAFF], Ro52, and major histocompatibility [MHC] class I)

after stimulation with loxoribine and/or IFN-β. Western blotting was performed as described [9]. After SGECs were lysed and the protein concentrations were measured, identical amounts of protein were subjected to 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were transferred to a polyvinylidene fluoride (PVDF) filter, and the filter was blocked using 5% nonfat dried milk in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) for 60 min, and incubated with rabbit anti-TLR7 polyclonal antibody (working dilution 1:250), rabbit anti-TRAF6 monoclonal antibody (working dilution 1:1000), mouse anti-BAFF monoclonal antibody (working dilution 1:1000; Novus Biologicals, Littleton, CO), rabbit anti-MHC class I polyclonal antibody (working dilution 1:1000; Proteintech, Rosemont, IL) or rabbit anti-Ro52 polyclonal antibody (working dilution 1:1000; Cloud-Clone Corp; Katy, TX) at 4°C overnight, or mouse anti-β actin monoclonal antibody (working dilution 1:5000, Santa Cruz) for 60 min at RT.

The filter was then washed with TBS-T and incubated with mouse or rabbit anti-IgG coupled with horseradish peroxidase (HRP) as secondary antibodies (working dilution 1:1000; MBL). After the incubation, we used an Amersham Hyperfilm enhanced chemiluminescence system (GE Healthcare UK, Little Chalfont, UK) for the detection of the bands. The density of each band was measured by Image J software developed at the U.S. National Institutes of Health for the determination of the pixel intensity.

ELISA

The levels of IFN-α in the culture supernatants of SGECs and PBMCs after stimulation with loxoribine and/or IFN-β were measured using the VeriKine™ Human IFN-α ELISA Kit (Product #41100, PBL Assay Science, Piscataway, NJ) following the manufacturer's instructions. Briefly, the cells were seeded on 96-well ELISA plates and incubated for 60 min at RT. They were incubated with detection antibody for 60 min at RT, followed by HRP-conjugated secondary antibody for 60 min at RT. After that, they were incubated with substrate solution for 15 min at RT, and stop solution was added. Subsequently, the absorbance at 450 nm was determined by using a microplate reader.

Flow cytometry

After the isolated PBMCs from three pSS patients were stimulated with loxoribine and/or IFN-β followed by treatment with 5 μg/ml of brefeldin A for 12 hr to save the IFN-α activity, the PBMCs were stained with phycoerythrin (PE)/Cy7-conjugated mouse anti-CD123 antibody (Biolegend, San Diego, CA) and allophycocyanin (APC)-conjugated mouse anti-CD303 antibody (Biolegend) for 30 min at 4°C. After permeabilization, the PBMCs were stained with FITC-conjugated mouse anti-IFN-α antibody (MACS Miltenyi Biotek, Bergisch Gladbach, Germany) and PE-conjugated

mouse anti-TLR7 antibody (ThermoFisher Scientific) at 4°C overnight, and they were then analyzed by flow cytometry using a BD FACS Verse (BD Biosciences, San Jose, CA) and FlowJo ver. 10 software (Tree Star, Ashland, OR). PE/Cy7-conjugated mouse IgG1, APC-conjugated mouse IgG1, FITC-conjugated mouse IgG2a, and PE-conjugated mouse IgG1 (Biolegend) were used as isotype controls.

Statistical analysis

We used the Mann-Whitney U-test or Fisher's exact test to compare the clinical and serological characteristics between the pSS and control groups, and a repeated-measures analysis of variance (ANOVA) to determine the differences in protein expressions detected by western blotting and the percentages of IFN-α+ cells measured by flow cytometry. Spearman's rank correlation coefficient was used to determine the correlation between the mean fluorescence intensity of IFN-α or TLR7 stains in the mononuclear cells of LSGs and the percentage of IFN-α+TLR7+ cells among the PBMCs.

All statistical analyses in this study were performed using JMP software, ver. 13 (SAS, Cary, NC). *P*-values<0.05 were considered significant.

Results

Subject characteristics

Table 1 summarizes the characteristics of the 11 patients and the five control subjects. All of the pSS patients were female. The group of pSS patients had significantly greater anti-SS-A/Ro antibody and anti-nuclear antibody positivity, and significantly higher serum IgG levels and LSG biopsy focus scores compared to the control group ($p<0.001$, $p<0.001$, $p=0.009$ and $p=0.0019$).

The dominant expression of TLR7 in the MNCs and ducts of the pSS patients' LSGs

The expression levels of TLR7 were stronger in infiltrating MNCs and ducts of LSGs from the pSS patients compared to those of the control subjects (Fig. 1A,D). In contrast, TLR9 was weakly expressed in the MNCs and ducts from both the pSS patients and controls (Fig. 1C,F). TLR8 was expressed in the MNCs of several pSS patients (Fig. 1B,E). The immunostaining results for TLR7–9 are summarized in Supplementary Table S1.

The dominant expression of TLR7 in plasmacytoid dendritic cells (pDCs), CD8+ T cells, B cells, and plasma cells of LSGs from the pSS patients

To clarify which cells expressed TLR7, we performed double immunofluorescence staining with TLR7 and several cell markers in LSGs from the pSS patients. The results showed that the TLR7-positive cells were co-localized mainly with plasmacytoid dendritic cells (pDCs; CD303-positive cells). CD8+ T cells (CD8-positive cells), B cells (CD20-positive cells), and

plasma cells (VS38c-positive cells) were also co-localized with TLR7-positive cells, next to the co-localization of pDCs, whereas other cell marker-positive cells were partly co-localized with TLR7-positive cells (Fig. 2). We also performed double immunofluorescence staining with TLR8 and several cell markers in LSGs from the pSS patients, and the results demonstrated that TLR8-positive cells were also co-localized mainly with pDCs (CD303-positive cells) (Suppl. Fig. S1).

The expression of TLR7 downstream molecules in pDCs from the pSS patients

We examined the results of the double immunofluorescence staining for TLR7 downstream molecules and CD303 in LSGs from the pSS patients, and we observed that TLR7 was expressed in pDCs. TLR7 downstream molecule (MyD88, TRAF6 and IRF7)-positive cells as well as TLR7-positive cells were co-localized mainly with pDCs (Fig. 3).

The expression of TLR7 and its downstream molecules in ducts from the pSS patients

We examined the results of the IHC staining with TLR7 downstream molecules in ducts of LSGs from the pSS patients. TLR7 was strongly expressed in ducts of LSGs from the pSS patients compared to the expressions of TLR8 and TLR9 (Fig. 4A). In the serial sections, immunofluorescence showed that the TLR7 downstream molecules MyD88, TRAF6, and IRF7

were also expressed in ducts of LSGs from the pSS patients (Fig. 4B). In addition, the expression levels of type I IFNs (IFN-α and IFN-β) were also stronger in the MNCs and ducts of LSGs from the pSS patients compared to those of the control subjects (Fig. 5A).

Activation of TLR7 signaling stimulated by TLR7 ligand in SGECs and PBMCs

We examined the expression of TLR7 signaling in SGECs after stimulation with TLR7 ligand, and we observed that TLR7 signaling was expressed in LSG ducts from the pSS patients. Our time-course experiments using immunofluorescence staining showed that TRAF6 expression was most strongly detected, and IRF7 expression was most aggregated around the nuclei, suggesting that IRF7 was translocated into the nucleus after stimulation with loxoribine for 10 min as well as by loxoribine for 10 min and IFN-β for 12 hr (Fig. 5B). In the western blotting, the expressions of TLR7 and TRAF6 were not changed by stimulation with loxoribine for 10–60 min and/or IFN-β (data not shown). However, the TRAF6 expression was significantly increased by stimulation with IFN-β coupled with loxoribine for 6 hr. In contrast, the TLR7 expression was not changed by stimulation with IFN-β and/or loxoribine for 6 hr (Fig. 5C). IFN-α was not detected in the supernatants of SGECs in the presence of IFN-β and/or loxoribine by ELISA.

In contrast, the ELISAs showed that the levels of IFN-α were increased after stimulation with loxoribine and IFN-β in the supernatants of PBMCs from the pSS patients (Fig. 6A).

Additionally, the flow cytometry revealed a significant increase in IFN-α after stimulation with loxoribine and IFN-β in TLR7+CD123/303+ pDCs from the pSS patients (Fig. 6B). ELISAs also showed a significant increase in IFN-α in supernatants from the control subjects following stimulation with loxoribine and IFN-β (data not shown).

We also examined the correlation between the MFI of IFN-α or TLR7 stains in mononuclear cells of LSGs revealed by immunofluorescence and the percentage of IFN-α+TLR7+ plasmacytoid dendritic cells among PBMCs after stimulation with loxoribine and IFN-β shown by flow cytometry from the same pSS patients. The results showed a tendency toward a positive correlation between IFN-α or TLR7+ mononuclear cells of LSGs and IFN-α+TLR7+ plasmacytoid dendritic cells among PBMCs (Fig. 6C).

Apoptosis and activation of BAFF, Ro52, and MHC class I stimulated by TLR7 ligand in SGECs

We examined the induction of apoptosis and the activation of BAFF, Ro52, and MHC class I in SGECs after stimulation with a TLR7 ligand. TUNEL staining showed that apoptosis was not induced by stimulation with loxoribine and/or IFN-β (Suppl. Fig. S2A). Although neither apoptosis nor BAFF expression was induced by loxoribine and/or INF-β, both Ro52 and MHC class I were significantly up-regulated with these stimulations in the western blotting (Suppl. Fig. S2B–D).

Discussion

The findings revealed by our present analyses indicate that TLR7 and its downstream signaling are expressed strongly in ducts and MNCs (especially pDCs) of labial salivary glands from pSS patients *in vivo*, and we observed that TLR7 downstream molecules are expressed in SGECs from pSS patients after TLR7 ligand stimulation *in vitro*. A key contributor to the development of pSS is thought to be an adaptive immune response in which self-reactive T cells recognize autoantigens such as M3 muscarinic acetylcholine receptor (M3R) [11] and alpha-fodrin [12]. The involvement of innate immune activity was recently reported to be related to the pathogenesis of many autoimmune diseases [13]. TLR7–9 are representative pattern recognition receptors that recognize the nucleic acid sequences of viruses and self-antigens, and these activities produce cytokines such as type I IFN or induce the activation of the nuclear factor kappa B (NFκB) pathway [5].

Previous studies identified the overexpression of type I IFN-inducible genes such as IFN-α-inducible protein 27 (IFI27), IFN-induced transmembrane protein 3 (IFITM3), IFN-stimulated transcription factor 3 (ISGF3G), and IRF1 in the salivary glands and peripheral blood from pSS patients, suggesting that the type I IFN pathway is a key player in the pathogenesis of pSS [2, 3, 14]. Our earlier investigation demonstrated the expression and function of TLR2–4 in patients with SS [15], and in the present study we focused on TLR7–9 from the view point of receptors of nucleic acid sequences of viruses and/or self-antigens.

Several studies showed that TLR7–9 are expressed in PBMCs from SS patients [6, 16, 17] and one study suggested that apoptosis induced TLR7 and -9 expressions in pDCs [18]. However, the expression of TLR7–9 in salivary glands has not been well investigated. Zheng et al. reported that TLR7 and TLR9 were dominantly expressed in lymphocytes of the parotid glands from pSS patients compared to those of control subjects [6]. In contrast, Maria et al. reported that TLR7 was expressed by few cells within the lymphocytic foci [16]. Our present findings demonstrate that TLR7 was more strongly expressed than TLR9 in mononuclear cells of labial salivary glands. Because the expression of TLR7–9 was confirmed by positive control sections, the dominant expression of TLR7 in mononuclear cells was evident in our severely infiltrated samples from the pSS patients.

Fukui et al. reported that the multitransmembrane endoplasmic reticulum (ER)-resident protein Unc93B1 dominantly up-regulated TLR7 ligand-induced activity due to a down-regulation of TLR9 ligand-induced activity [19]. It was reported that single-strand RNA (ssRNA) as a TLR7 ligand was recognized by TLR7 but not TLR8 and activated the transcription of TNFα in synovial tissues from rheumatoid arthritis patients, suggesting that the activation of TLR7 was more closely associated with inflammation compared to TLR8 activation [20]. Similarly, TLR7-mediated activation might dominantly occur in pDCs of labial salivary glands from pSS patients. Alveolar macrophages and dendritic cells produced interleukin (IL)-33 induced by TLR7 activation in the

respiratory tract of respiratory syncytial virus ssRNA-infected mice [21]. These findings strongly suggest the dominance of TLR7 with respect to the provocation of inflammation in LSGs.

Our present data demonstrated the strong expression of TLR7 in the ducts of labial salivary glands from pSS patients. We also evaluated the downstream signaling of TLR7 expressed in the ducts of labial salivary glands and TLR7-mediated signal transduction in our *in vitro* experiments. Our findings suggest that the ducts as well as mononuclear cells are involved in TLR7-mediated inflammation. Because TLR7 was expressed mainly in mononuclear cells (including pDCs and B cells), we speculate that the TLR7 signaling activation in the ducts is associated with mononuclear cells that expressed TLR7. However, the expression of TLR7 signaling in SGECs after stimulation with a TLR7 ligand in our *in vitro* analysis revealed that TLR7 signaling was activated in epithelial cells independently.

Another study showed that TLR7 was expressed in HSY cells (human salivary gland cells), whereas the levels of IL-6 and IL-8 in cultured supernatants did not increase after stimulation with ssPoly U as a TLR7 ligand [22]. Spanchidou et al. reported that immunoregulatory molecules including intercellular adhesion molecule-1, CD40, and MHC class I were up-regulated after stimulation with TLR2–4 ligands in SGECs from pSS patients, suggesting that epithelial cells affected the role of the innate immune response [23]. Additionally, an analysis of a murine model of SS revealed that epithelial cell apoptosis was essential for the development of inflammation [24].

These data support the concept that epithelial cells have an important role in inflammation, including the activation of innate immunity.

Because it was reported that IFN-β pretreatment with loxoribine led to an increased production of IFN-α upon loxoribine stimulation in PBMCs from patients with multiple sclerosis [25], we analyzed SGECs and PBMCs stimulated with IFN-β in addition to loxoribine. However, the level of IFN-α in the cultured supernatant of SGECs was not detected by an ELISA. In contrast, the expression of IFN-α in TLR7+CD123/303+ pDCs in cultured PBMCs from our pSS patients was significantly increased after stimulation with IFN-β and loxoribine. In light of these findings, we suspected that the production of IFN-α might depend on a synergistic action of IFN-β and TLR7 ligation. Eventually, we confirmed that compared with SGECs, the TLR7-positive pDCs among PBMCs had a crucial role with respect to the IFN-α production and secretion mechanism in pSS, considering that no IFN-α was secreted by stimulated SGECs regardless of the positive expression of IFN-α in the ducts of labial salivary glands. With regard to the secretion of soluble factors from SGECs in response to IFNs, there is a report [26] showing that the secretion of BAFF was upregulated by stimulation with IFNs such as IFN-γ and IFN-α. That report suggested that SGECs might have various functions to secrete some soluble factors in response to IFNs, although IFN-α itself was not secreted into the culture medium.

As noted above, the increased type I IFN activity observed in the salivary glands and peripheral bloods from pSS patients in previous studies. However, the relationship regarding TLR7 signaling between PBMCs and MNCs in salivary glands from pSS patients remains unclear. Our present results demonstrated only a tendency toward a positive correlation between IFN-α+ or TLR7+ cells of LSGs and IFN-α+TLR7+ cells of PBMCs that were obtained from the same pSS patients, suggesting that the TLR7 signal activation was not always correlated between peripheral blood and salivary glands.

Our present findings demonstrated TLR7 expression in CD8+ T cells in labial salivary glands from the pSS patients. In general, TLR7 was weakly expressed in CD8+ T cells. Another study indicated that TLR7 signaling enhanced the cross-priming of CD8+ T cells, suggesting that this signaling may have influenced the TLR7 expression itself [27].

An earlier investigation showed that apoptotic particles induced TLR7 and TLR9 expressions in pDCs *in vitro* [18]. Although our prior study demonstrated that TLR3 signal activation induced apoptosis in SGECs from individuals with pSS [9], it is not yet known whether stimulation of the TLR7 signaling pathway induces apoptosis. However, our present experiments demonstrated that TLR7 signal activation induced no apoptosis regardless of type I IFN activation in SGECs from the pSS patients.

We also investigated the expression of relevant molecules including Ro52 and MHC class I

(Suppl. Fig. S2). Since we observed a significant increase of Ro52 with loxoribine stimulation with IFN-β, we speculated that a TLR7-mediated augmentation of SS-related antigen exists. Because Higgs et al. reported that TLR7 stimulation promoted the association of Ro52 with IRF7 [28], TLR7 might have a pathogenic function with regard to the control of the expression of Ro52. In, addition, we observed that stimulation with loxoribine and IFN-β augmented MHC class I expression in SGECs. Although it has been shown that another TLR7 agonist, i.e., imiquimod, weakly up-regulated MHC class I in vaginal epithelial cells [29], our present findings might demonstrate a specific effect of TLR7 stimulation on SGECs regarding the association of endogenous antigen.

Several limitations of this study should be mentioned. First, we analyzed the expression of TLRs in mainly pDCs among mononuclear cells. In future analyses, we should analyze the expression of TLR7 in another cell type such as B cells. Because TLR7 has the potential to induce autoantibody production through B-cell activation, a TLR7-mediated autoantibody production system in SS should be investigated in future studies. Second, a recent study showed that retinoic acid-inducible gene-I (RIG-I) and melanoma differentiation associated gene-5 (MDA5) (as innate sensors associated with the production of type I IFNs other than TLRs) were up-regulated in pDCs and monocytes and highly expressed in mononuclear cells of LSGs from IFN-positive pSS patients [13]. Because it has been reported that the expressions of RIG-I and MDA5 are induced by TLR7

signal activation, we should analyze these functions in LSGs in future studies. Because the upregulation of cyclic GMP-AMP synthase (cGAS), a stimulator of interferon genes (STING) pathway, was shown in monocytes of patients with systemic lupus erythematosus [30], the pathogenic characteristics of these molecules in pSS should also be investigated. As a third limitation of the present study, we did not perform peripheral blood transcriptomics or gene expression analysis for the 11 pSS patients.

In conclusion, the results of our analyses showed that TLR7 signaling for type I IFNs is strongly expressed in ducts and mononuclear cells — especially the plasmacytoid dendritic cells of labial salivary glands — from pSS patients. Our findings suggest that TLR7-mediated downstream signals might be activated due to stimulation with ssRNA. In addition, ductal epithelial cells as non-immune cells as well as mononuclear cells expressed TLR7, indicating that it is possible that ductal epithelial cells per se act as receptors of adventive microbes and/or endogenous antigens. To investigate these speculations, it is desirable to determine whether or not the direct activation of microbes and/or self-antigens with ssRNA activates epithelial cells from patients with pSS. By elucidating these mechanisms, the role of TLR7 signaling activation in the pathogenesis of pSS will be clarified.

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Author's contributions

Study conception and design: H. Nakamura

Salivary gland biopsy: T. Shimizu, H. Nakamura, A. Takatani, Y. Horai, S. Kurushima, T. Michitsuji, Y. Nakashima

Acquisition of data: T. Shimizu, H. Nakamura

Analysis and interpretation of data: T. Shimizu, H. Nakamura, M. Umeda, A. Kawakami

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Disclosure

The authors declare that they have no conflicts of interest.

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Table 1. Background information of enrolled subjects

Variables	pSS (n=11)	Non-SS (n=5)	*p*-value
Age (yrs), median (IQR)	61 (57–65)	52 (40–68)	0.65[a]
Female, n (%)	11 (100%)	5 (100%)	1[b]
Xerostomia, n (%)	10 (90.9%)	3 (60%)	0.21[b]
Xerophthalmia, n (%)	6 (54.5%)	3 (60%)	1[b]
Schirmer test positivity, n (%)	4 (36.4%)	2 (40%)	0.84[b]
Saxon test positivity, n (%)	9 (81.8%)	3 (60%)	0.35[b]
Anti-SS-A/Ro antibody positivity, n (%)	11 (100%)	0 (0%)	<0.001[b]
Anti-SS-B/La antibody positivity, n (%)	4 (36.3%)	0 (0%)	0.12[b]
ANA positivity, n (%)	11 (100%)	0 (0%)	<0.001[b]
RF positivity, n (%)	7 (63.6%)	4 (80%)	0.51[b]
Serum IgG, mg/dl, median (IQR)	2140 (1550–2550)	1047 (656–1246)	0.009[a]
LSG biopsy, focus score	6.9 (4–19.2)	0	0.0019[a]
ESSDAI score, median (IQR)	6 (4–6)	n.a.	n.a.

Non-SS: These subjects were classified as non-SS sicca control subjects based on the AECG classification criteria. [a]Mann-Whitney U-test. [b]Fisher's exact test. *P*-values <0.05 were considered significant. ANA: anti-nuclear antibody, ESSDAI: European League Against Rheumatism Sjögren's Syndrome Disease Activity Index, IgG: immunoglobulin G, IQR: interquartile range, LSG: labial salivary gland, n.a.: not assessed, pSS: primary Sjögren's syndrome, RF: rheumatoid factor.

Supplementary Table S1. Staining extent of TLR7–9 in LSGs from pSS patients and non-SS sicca control subjects

		pSS											Non-SS				
	No.	1	2	3	4	5	6	7	8	9	10	11	1	2	3	4	5
TLR7	MNCs	++	++	++	++	++	++	++	++	++	++	NA	−	±	−	±	±
	Ducts	++	++	++	++	++	++	++	++	++	++	NA	++	++	−	−	−
TLR8	MNCs	+	±	+	−	+	±	±	++	++	+	NA	−	+	−	−	±
	Ducts	−	−	−	−	−	−	−	−	−	−	NA	−	±	−	−	−
TLR9	MNCs	−	−	±	−	NA	−	−	±	−	−	−	−	−	−	−	±
	Ducts	−	−	±	±	NA	+	+	±	±	±	+	−	−	−	±	±

The frequency of TLR7–9 positive area: ++ > 50%, +=11–50%, ±=1–10% and −=0% of MNCs in each focus, and ducts in each 4 mm^2 section.

Non-SS: These subjects were classified as non-SS sicca control subjects based on the AECG classification criteria.

LSG: labial salivary gland, MNC: mononuclear cell, NA.: not assessed, TLR: Toll-like receptor.

Figure legends

Fig. 1. Expressions of Toll-like receptors (TLRs) 7–9 in labial salivary glands (LSGs) from the primary Sjögren's syndrome (pSS) patients and non-SS sicca control subjects. Representative samples of LSGs stained with anti-TLR7 (**A**), anti-TLR8 (**B**), and anti-TLR9 antibodies (**C**) from pSS patients (n=10). Representative samples of LSGs from non-SS patients (n=5) and cancer tissue array (*right lower panels*) stained with anti-TLR7 (**D**), anti-TLR8 (**E**), and anti-TLR9 antibody (**F**). *Insets:* Representative staining in the cancer tissue array. Bar: 80 μm (LSGs), 200 μm (cancer tissue array). Non-SS: These subjects were classified as non-SS sicca control subjects based on the AECG classification criteria.

Fig. 2. Co-localization of TLR7 and mononuclear cells (MNCs) in LSGs from the pSS patients. Representative samples of LSGs stained with cell markers for T cells (anti-CD4 and anti-CD8 antibodies), B cells (anti-CD20 antibody), plasma cells (anti-VS38c antibody), macrophages (anti-CD68 antibody), or pDCs (anti-CD303 antibody) (*green*), and anti-TLR7 antibody (*red*) from pSS patients (n=3). Hoechst (*blue*) was used for counterstaining the nuclei. *Insets:* Representative staining for each panel. Bar: 40 μm.

Fig. 3. Expression of TLR7 and its downstream molecules in plasmacytoid dendritic cells (pDCs) of LSGs from the pSS patients. Representative samples of LSGs from pSS patients (n=4) stained with a cell marker for pDCs (anti-CD303 antibody) (*green*) and anti-TLR7 (**A**), anti-MyD88 (**B**), anti-TRAF6 (**C**), or anti-IRF7 antibody (**D**) (*red*). Hoechst (*blue*) was used for counterstaining the nuclei. *White arrowheads:* The expression of TLR7 or its downstream molecules in pDCs. Bar: 20 μm. IRF7: interferon regulatory factor 7, MyD88: myeloid differentiation primary response gene 88, TRAF6: tumor necrosis factor receptor associated factor 6.

Fig. 4. Expression of TLR7 and its downstream molecules in ducts of LSGs from the pSS patients. A: Representative samples showing the expressions of TLR7–9 in LSGs from pSS patients. *Insets:* Representative ductal expressions of TLR7–9. Bar: 80 μm. **B:** Representative samples of LSGs from pSS patients (n=5) stained with anti-TLR7 (*green*), anti-MyD88, anti-TRAF6, and anti-IRF7 antibody (*red*). Hoechst (*blue*) was used for counterstaining the nuclei. *White arrowheads:* The same ductal expression, using serial sections. Bar: 20 μm.

Fig. 5. Expression of type I IFNs in LSGs and TLR7 signal activation in salivary gland epithelial cells (SGECs). A: Representative samples showing the expressions of IFN-α and IFN-β

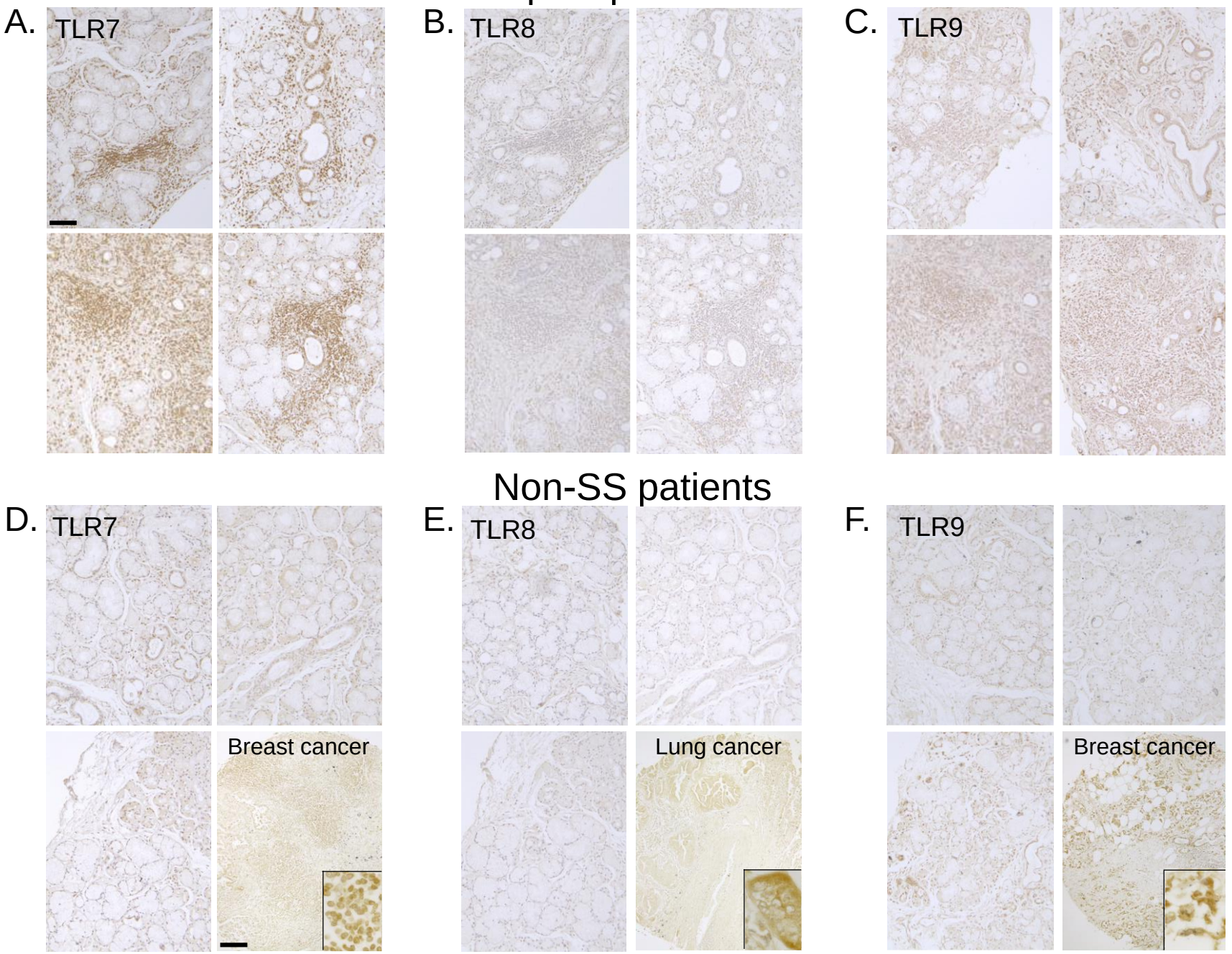
in LSGs from pSS patients (n=4) and non-SS patients (n=4). Bar: 20 μm. *Insets:* Representative expressions of IFN-α and IFN-β. **B:** Representative images showing the expressions of TRAF6 and IRF7 (*red*) in SGECs from pSS patients (n=3). Bar: 20 μm. **C:** TLR7 and TRAF6 signal in SGECs from pSS patients (n=5) stimulated with 1 mM loxoribine for 6 hr and/or 1000 U/ml IFN-β for 12 hr analyzed by western blotting. *p<0.05 by ANOVA. Error bars: std. dev. NS: not significant.

Fig. 6. TLR7 signal activation in SGECs and PBMCs from the pSS patients. A: The levels of IFN-α in SGECs and PBMCs from pSS patients (n=6) stimulated with 1 mM loxoribine for 6 hr and/or 1000 U/ml IFN-β for 12 hr measured by ELISA. **B:** The expressions of IFN-α and TLR7 in CD123+CD303+ pDCs from pSS patients (n=6) stimulated with 1 mM loxoribine for 6 hr and/or 1000 U/ml IFN-β for 12 hr, analyzed by flow cytometry. **p<0.01 by ANOVA. Error bars: std. dev. **C:** The correlation between the mean fluorescence intensity (MFI) of IFN-α or TLR7 stains in mononuclear cells of LSGs and the percentage of IFN-α+TLR7+ pDCs in flow cytometry from pSS patients (n=6). The MFI of immunostaining was captured and calculated with a hybrid cell count system. The correlations were determined using Spearman's rank correlation coefficient. *P*-values <0.05 were considered significant. ND: not detected, PBMC: peripheral blood mononuclear cell.

Supplementary Fig. S1. Co-localization of TLR8 and MNCs in LSGs from the pSS patients.

Representative samples of LSGs stained with cell markers for T cells (anti-CD4 and anti-CD8 antibodies), B cells (anti-CD20 antibody), plasma cells (anti-VS38c antibody), macrophages (anti-CD68 antibody), or pDCs (anti-CD303 antibody) (*green*), and anti-TLR8 antibody (*red*) from pSS patients (n=2). Hoechst (*blue*) was used for counterstaining the nuclei. *Insets:* Representative staining for each panel. Bar: 40 μm.

Supplementary Fig. S2. The apoptosis and expressions of BAFF, Ro52, and MHC class I in SGECs from the pSS patients. A: Representative images showing the TUNEL staining (*green*) in SGECs from pSS patients (n=3). Bar: 20 μm. Hoechst (*blue*) was used for counterstaining of the nuclei. **B:** BAFF, **C:** Ro52, **D:** MHC class I signal in SGECs from pSS patients (n=5) in which the SGECs were stimulated with 1 mM loxoribine for 6 hr and/or 1000 U/ml IFN-β for 12 hr, analyzed by western blotting. $^{**}p<0.01$ by ANOVA. Error bars: std. dev. BAFF: B-cell activating factor, MHC: major histocompatibility complex.


pSS patients
A.
TLR7
B.
TLR8
C.
TLR9
Non-SS patients
D.
TLR7
Breast cancer
E.
TLR8
Lung cancer
F.
TLR9
Breast cancer

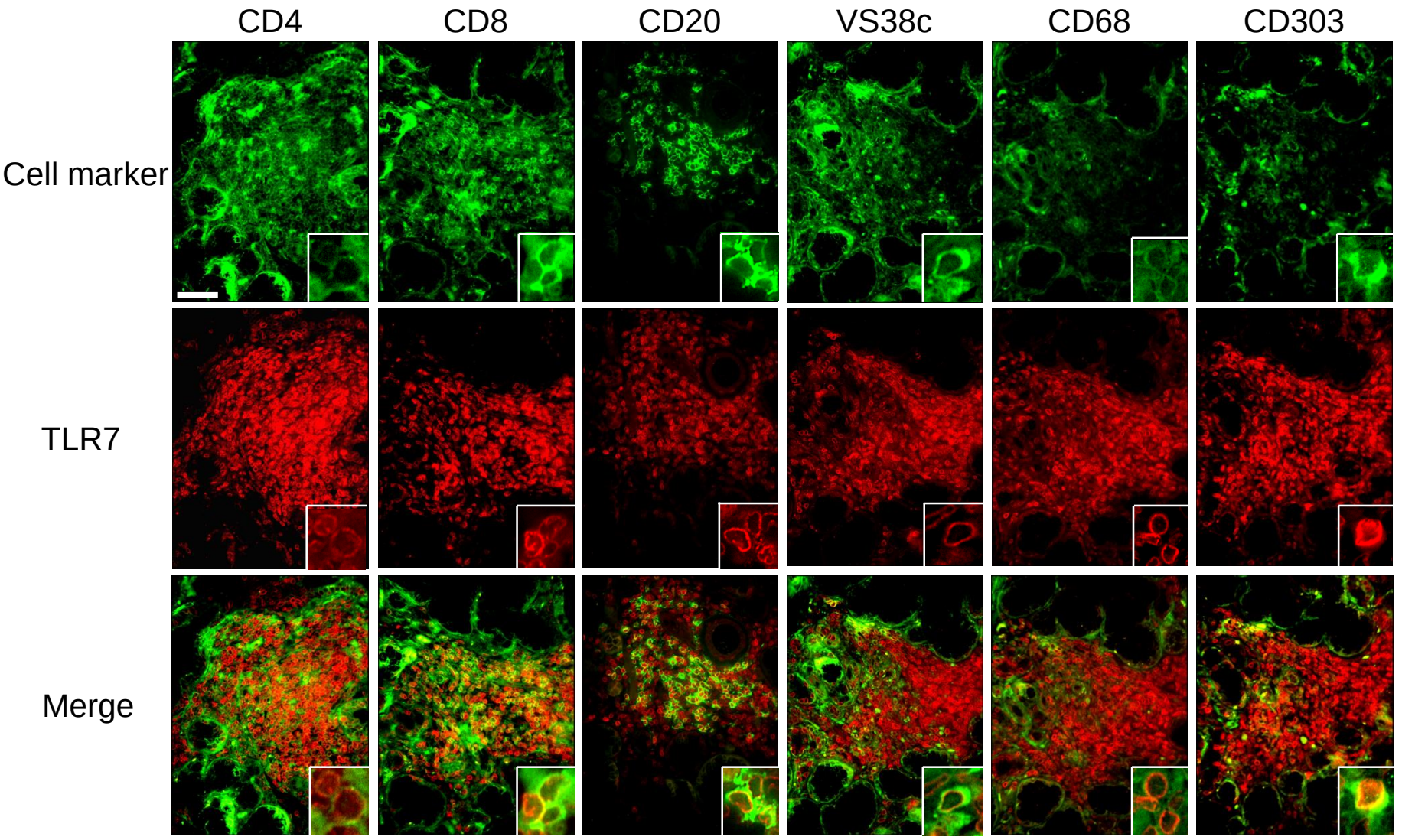
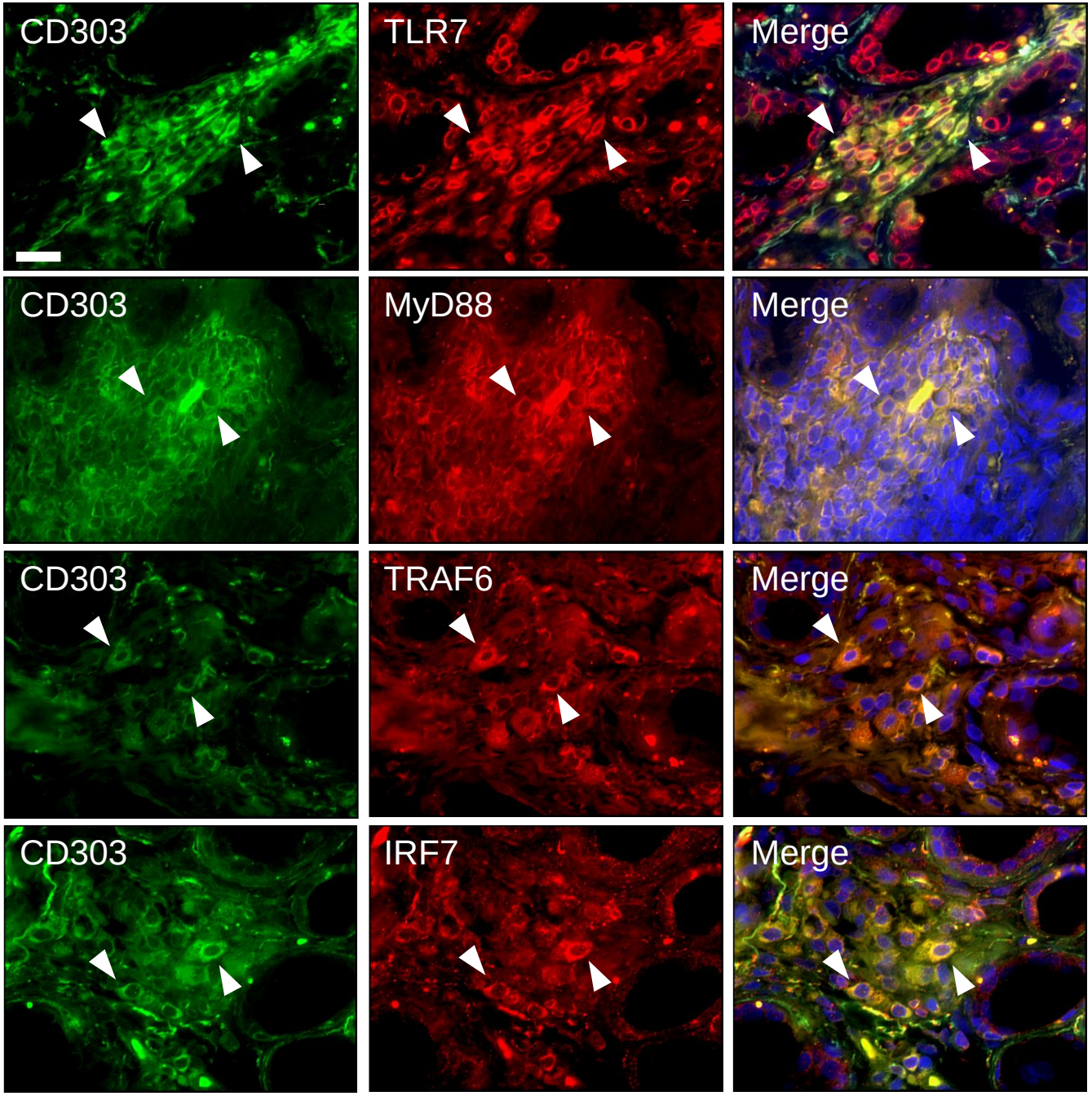
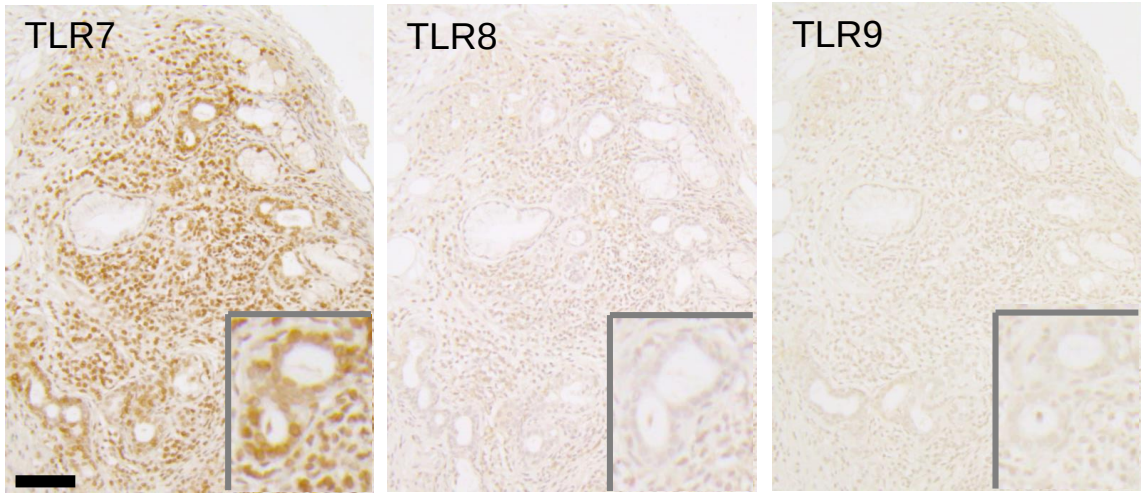

CD4
CD8
CD20
VS38c
CD68
CD303
Cell marker
TLR7
Merge

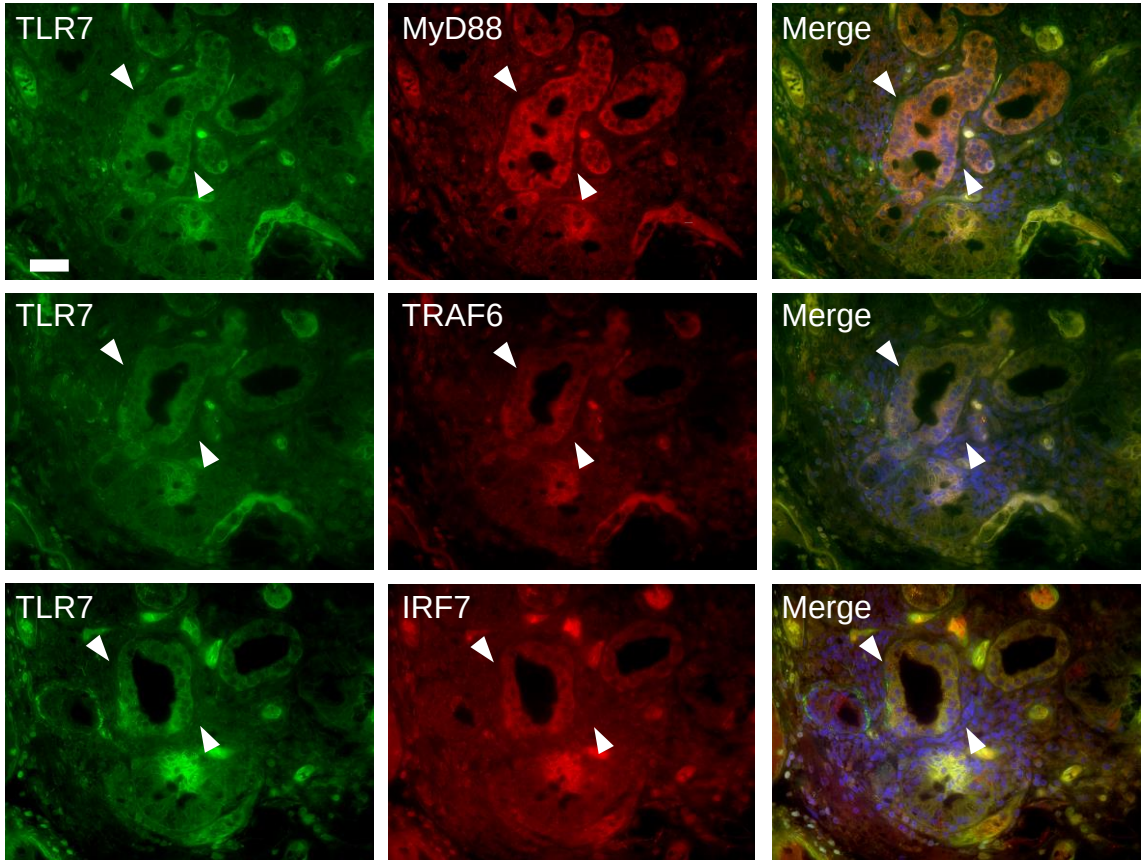
Fig. 3


CD303
TLR7
Merge
CD303
MyD88
Merge
CD303
TRAF6
Merge
CD303
IRF7
Merge

A.


TLR7
TLR8
TLR9

B.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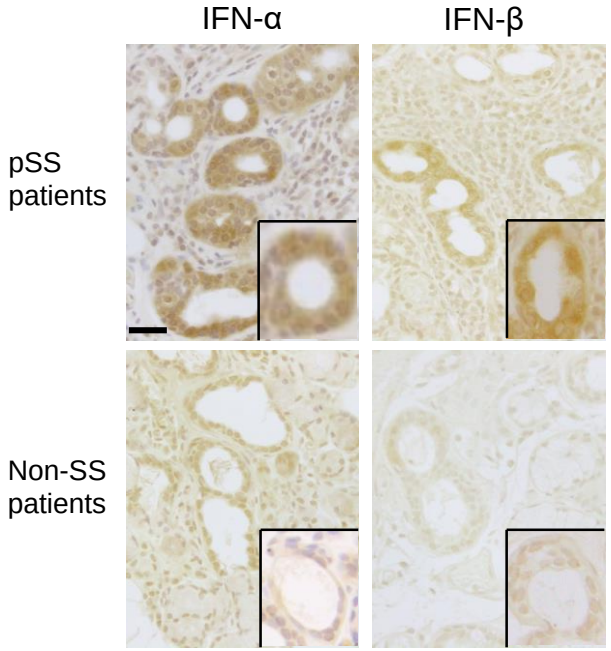
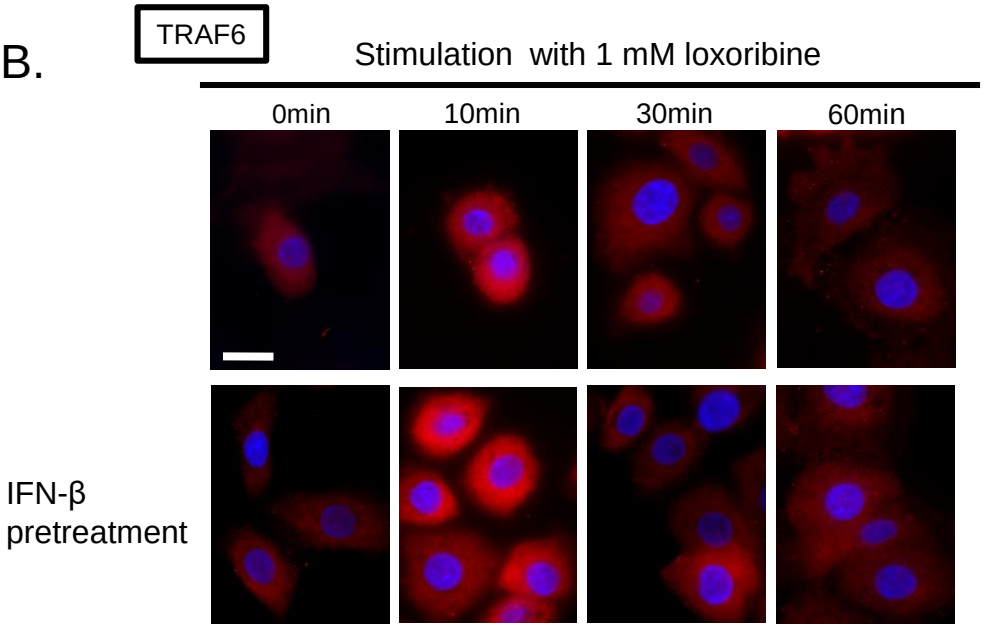
MyD88
Merge
TLR7
TRAF6
Merge
TLR7
IRF7
Merge

Fig. 5

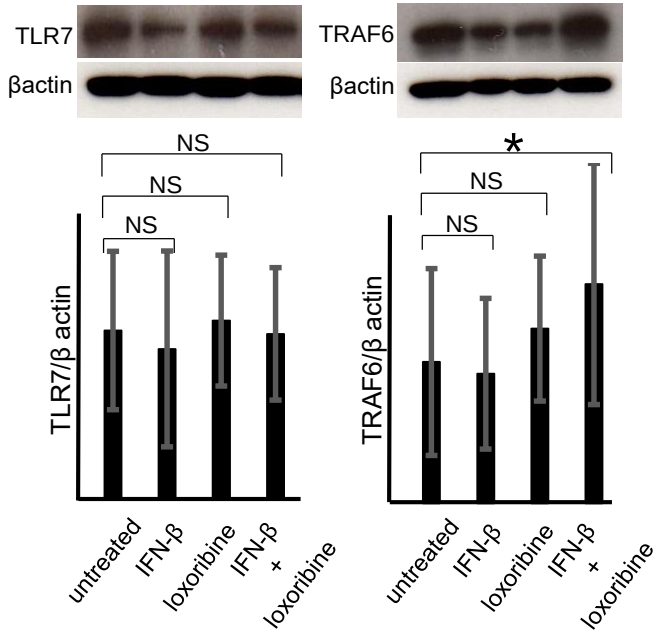
A.


IFN-α
IFN-β
pSS patients
Non-SS patients

B.


TRAF6
Stimulation with 1 mM loxoribine
0min
10min
30min
60min
IFN-β pretreatment

C.


TLR7
βactin
TRAF6
βactin
NS
NS
NS
*
NS
NS
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TRAF6/β actin
untreated
IFN-β
loxoribine
IFN-β + loxoribine

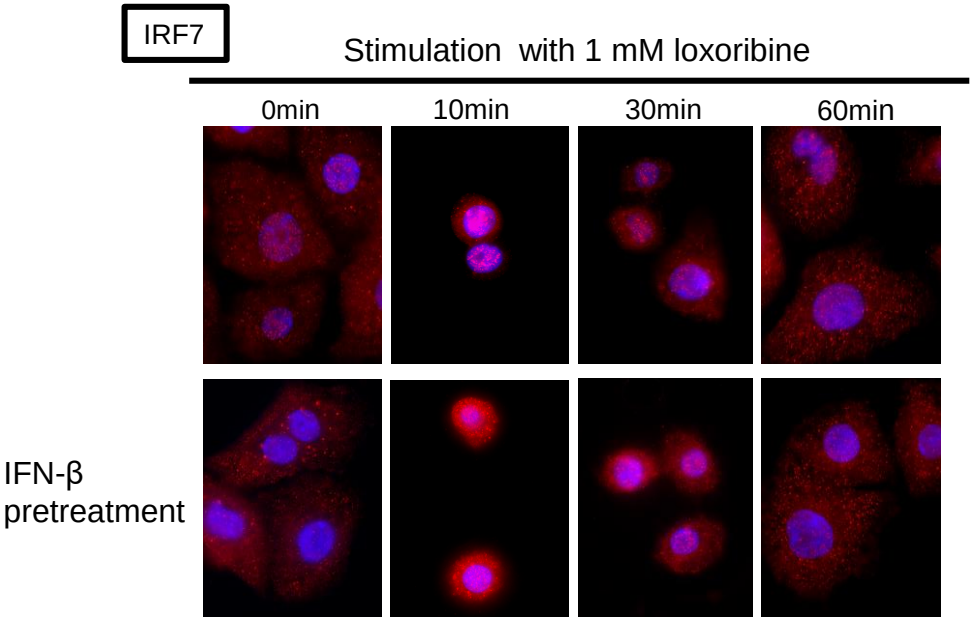
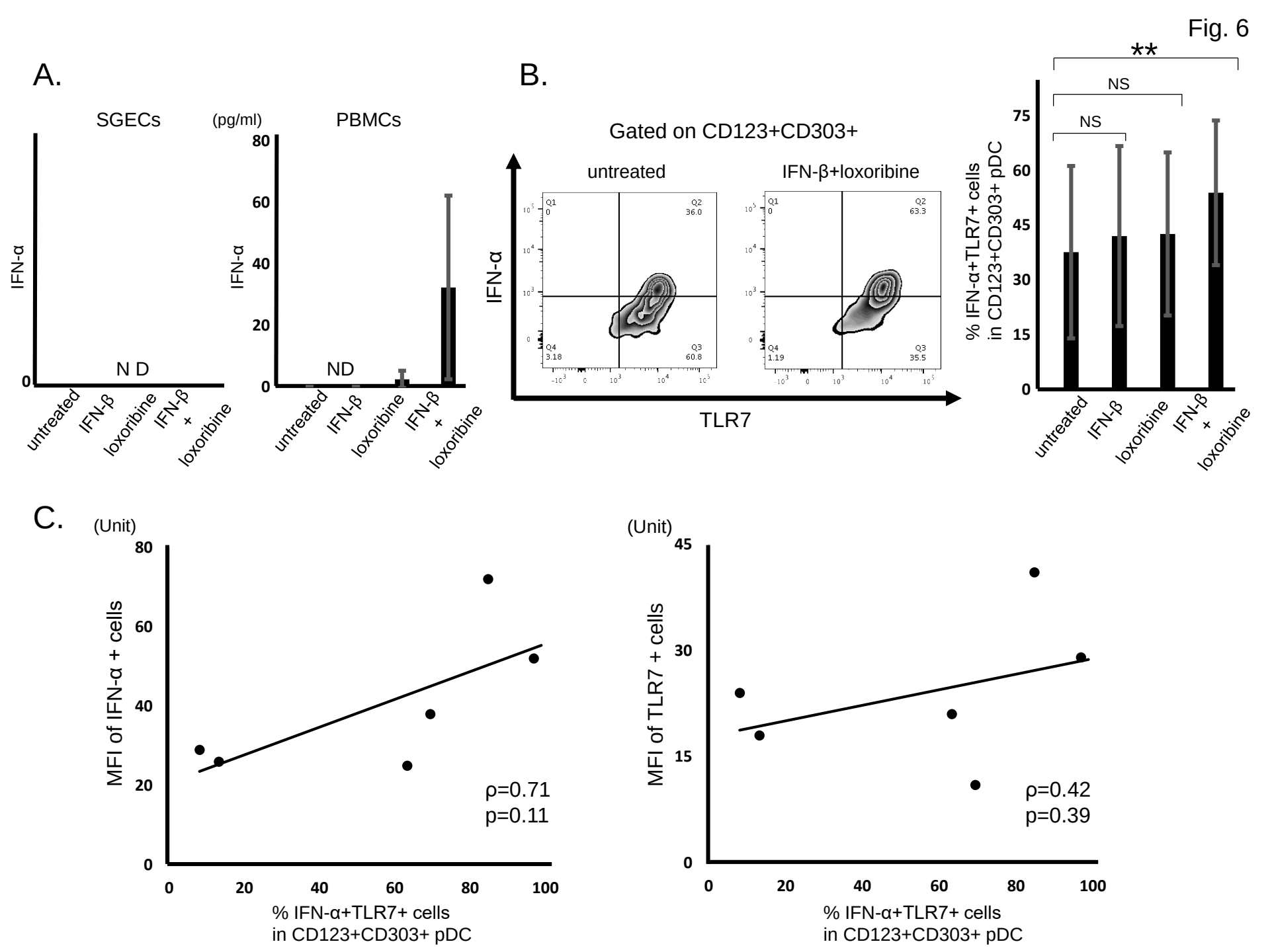
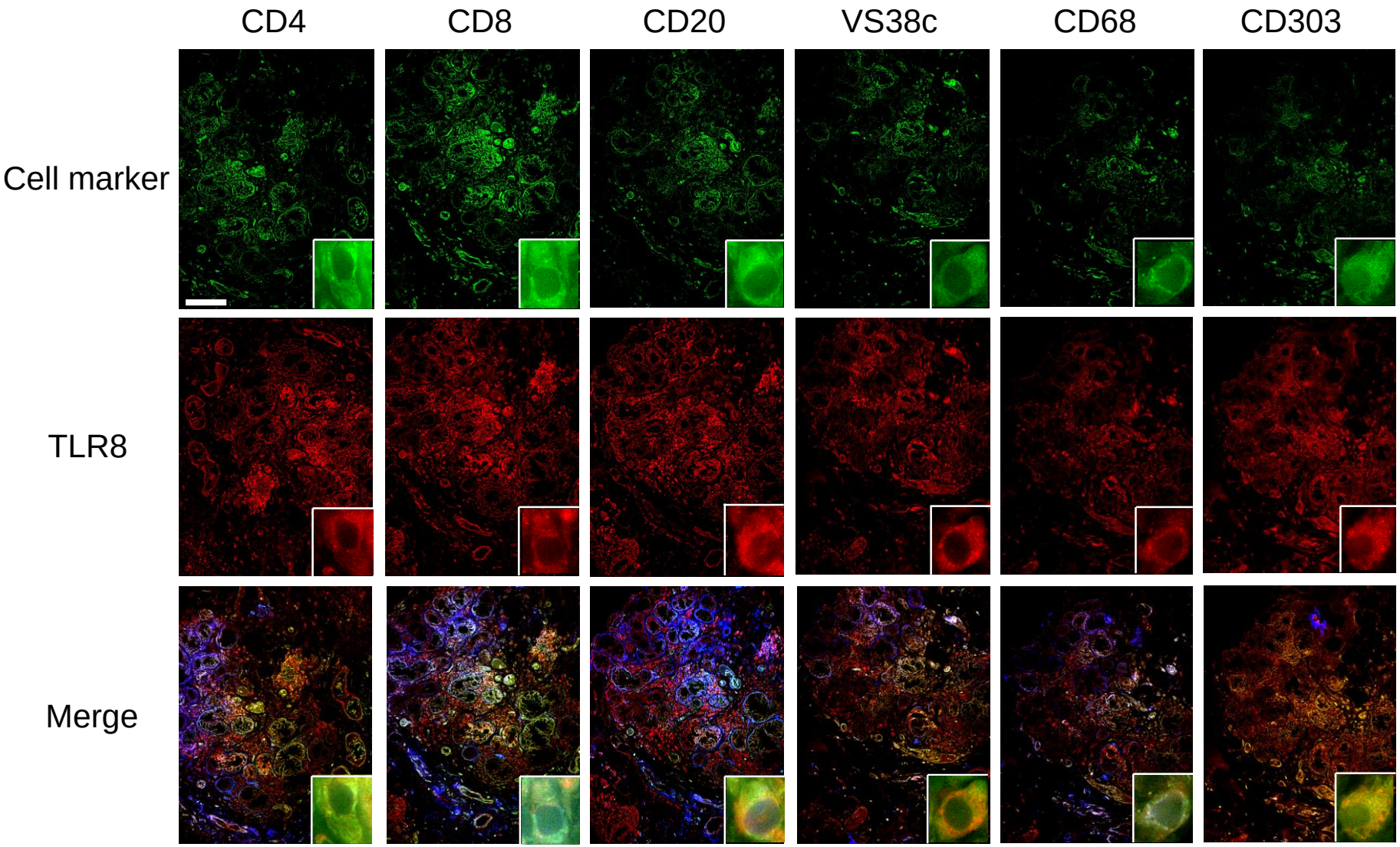
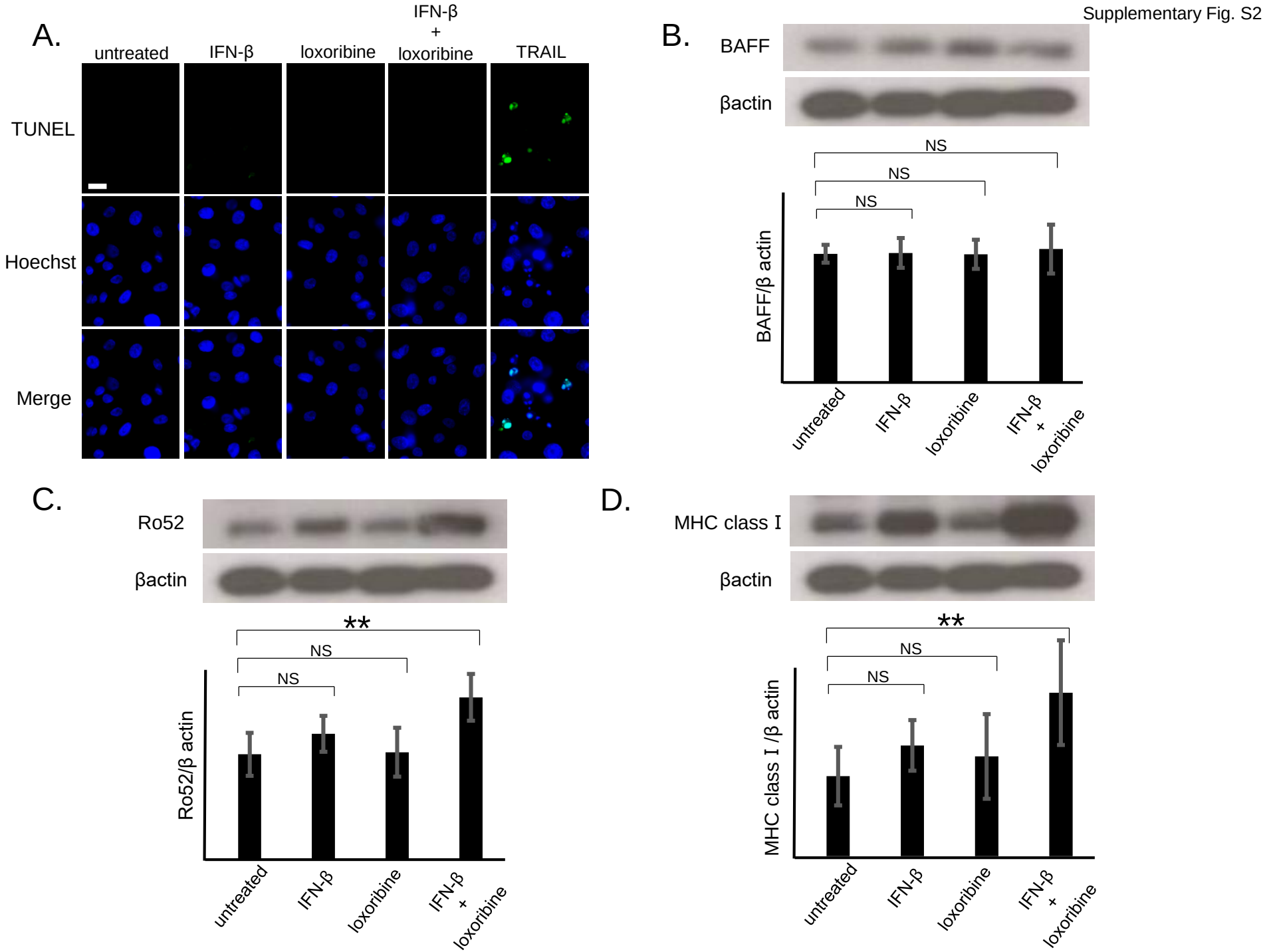

IRF7
Stimulation with 1 mM loxoribine
0min
10min
30min
60min
IFN-β pretreatment

Fig. 6


A.
SGECs
(pg/ml)
PBMCs
IFN-α
N D
ND
untreated
IFN-β
loxoribine
IFN-β + loxoribine
B.
Gated on CD123+CD303+
untreated
IFN-β+loxoribine
Q1 0
Q2 36.0
Q4 3.18
Q3 60.8
Q2 63.3
Q4 1.19
Q3 35.5
TLR7
**
NS
% IFN-α+TLR7+ cells in CD123+CD303+ pDC
C.
(Unit)
MFI of IFN-α + cells
ρ=0.71
p=0.11
% IFN-α+TLR7+ cells in CD123+CD303+ pDC
MFI of TLR7 + cells
ρ=0.42
p=0.39


CD4
CD8
CD20
VS38c
CD68
CD303
Cell marker
TLR8
Merge


A.
untreated
IFN-β
loxoribine
IFN-β + loxoribine
TRAIL
TUNEL
Hoechst
Merge
B.
BAFF
βactin
NS
NS
NS
BAFF/β actin
untreated
IFN-β
loxoribine
IFN-β + loxoribine
C.
Ro52
βactin
**
NS
NS
Ro52/β actin
untreated
IFN-β
loxoribine
IFN-β + loxoribine
D.
MHC class I
βactin
**
NS
NS
MHC class I /β actin
untreated
IFN-β
loxoribine
IFN-β + loxoribine