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# Toll-like receptor 3 stimulation promotes Ro52/TRIM21 synthesis and nuclear redistribution in salivary gland epithelial cells, partially via type I interferon pathway

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## Summary

**Up-regulated expression of Ro52/tripartite motif-containing protein 21 (TRIM21), Ro60/TROVE domain family, member 2 (TROVE2) and lupus LA protein/Sjögren's syndrome antigen B (La/SSB) autoantigens has been described in the salivary gland epithelial cells (SGEC) of patients with Sjögren's syndrome (SS). SGECs, the key regulators of autoimmune SS responses, express high levels of surface functional Toll-like receptor (TLR)-3, whereas Ro52/TRIM21 negatively regulates TLR-3-mediated inflammation. Herein, we investigated the effect of TLR-3-signalling on the expression of Ro52/TRIM21, as well as Ro60/TROVE2 and La/SSB autoantigens, by SGECs. The effect of TLR-3 or TLR-4 stimulation on autoantigen expression was evaluated by polyI:C or lipopolysaccharide (LPS) treatment, respectively, of SGEC lines (10 from SS patients, 12 from non-SS controls) or HeLa cells, followed by analysis of mRNA and protein expression. PolyI:C, but not LPS, resulted in a two-step induction of Ro52/TRIM21 mRNA expression by SGECs, a 12-fold increment at 6 h followed by a 2·5-fold increment at 24–48 h, whereas it induced a late two-fold up-regulation of Ro60/TROVE2 and La/SSB mRNAs at 48 h. Although protein expression levels were not affected significantly, the late up-regulation of Ro52/TRIM21 mRNA was accompanied by protein redistribution, from nucleolar-like pattern to multiple coarse dots spanning throughout the nucleus. These late phenomena were mediated significantly by interferon (IFN)-β production, as attested by cognate secretion and specific inhibition experiments and associated with IFN regulatory factor (IRF)3 degradation. TLR-3-signalling had similar effects on SGECs obtained from SS patients and controls, whereas it did not affect the expression of these autoantigens in HeLa cells. TLR-3 signalling regulates the expression of autoantigens by SGECs, implicating innate immunity pathways in their over-expression in inflamed tissues and possibly in their exposure to the immune system.**

**Keywords:** Ro52/TRIM21 autoantigen, salivary gland epithelial cells, Sjögren's syndrome, TLR-3, type I interferons

## Introduction

Sjögren's syndrome (SS) is a chronic systemic autoimmune disease characterized by the presence of autoantibodies against the protein component of the human intracellular ribonucleoprotein–RNA complexes and more specifically the proteins Ro52/tripartite motif-containing protein 21 (TRIM21), Ro60/TROVE domain family, member 2 (TROVE2) and lupus LA protein/Sjögren's syndrome antigen B (La/SSB).

The mechanisms mediating the exposure of intracellular autoantigens to the immune system and the production of sufficient amounts of autoantibodies are largely unknown. Critical overview of the published data supports that autoimmune humoral responses are antigen-driven, because: (i) most of autoantibodies detected are of the immunoglobulin

  

(Ig)G class, suggesting that an antigen-dependent T cell help should be provided [1], (ii) the autoimmune responses are mainly polyclonal, targeting multiple epitopes within the autoantigen [1] and (iii) immunization of experimental animals with fragments of the autoantigens results in intra- and intermolecular spreading of the immune response, similar to that observed after immunization with foreign antigens [2,3]. The affected exocrine glands appear to serve as a major site of autoantibody formation, because (i) saliva of SS patients contains high levels of IgG and anti-Ro/SSA and anti-La/SSB autoantibodies, (ii) ectopic germinal centres in the salivary glands are seen in approximately 20% of patients and contain autoreactive B cells and (iii) many of the infiltrating plasma cells contain intracytoplasmic immunoglobulins with anti-Ro/SSA activity [4,5]. In this venue, the epithelial cell appears to play a central role. Indeed, the main characteristic of the immunopathological lesion of SS is the activation of epithelial cells; salivary gland epithelial cells (SGEC) are potent regulators of local innate and adaptive immune responses, as they bear the functional armamentarium of an antigen-presenting cell, including major histocompatibility complex (MHC) class II molecules and translocation of nuclear autoantigens into cytoplasm and cellular membrane. Also, they express a plethora of functional immunoregulatory molecules, including co-stimulatory molecules, cytokines, chemokines and almost all members of the Toll-like receptor (TLR) family. TLR-3 expression deserves particular attention, because: (i) its constitutive expression by SGECs is high and, in contrast to the cytoplasmic localization that is described in resting immunocytes, it is localized on the cell-surface, suggesting an activation of this pathway in SGECs, (ii) it is functional, as TLR-3 stimulation on SGECs by polyI:C results in the up-regulation of several immune-modulatory molecules, as well as anoikis-induced cell death [6–8] and (iii) its natural ligand is dsRNA, that can be delivered locally either from viruses or damaged cells [9].

Ro52/TRIM21 has been implicated in the negative regulation of TLR-3-mediated inflammation by promoting the ubiquitination and subsequent proteasomal degradation of interferon regulatory factors (IRFs) 3, 5, 7 and 8 [10,11]. Conversely, interferons (IFNs), which are produced upon TLR-3 ligation, have been shown to up-regulate Ro52/TRIM21 expression [12,13]. Although the regulatory role of Ro52/TRIM21 in TLR-3 signalling is well established, the reciprocal interaction has never been studied.

Herein, we investigated the effect of TLR-3 signalling on the expression of Ro52/TRIM21, and the other autoantigens of the Ro/La ribonucleoprotein hY-RNA complex Ro60/TROVE2 and La/SSB, in non-neoplastic long-term cultured SGECs, as well as in neoplastic epithelial HeLa cells that served as a control cell-line. TLR-3, but not TLR-4, signalling was found to result in a two-step induction of Ro52/TRIM21 mRNA expression by SGECs, while Ro60/TROVE2 and La/SSB mRNAs were up-regulated to a lesser extent. The second-step increment of Ro52/TRIM21 mRNA expression was accompanied by protein redistribution at the nucleus of the living cells. The late mRNA induction and protein redistribution were mediated largely by IFN-β production and associated with degradation of IRF3. In contrast to SGECs, TLR-3 signalling in HeLa did not affect the expression and localization of autoantigens, as well as IFN production, suggesting a differential regulation of signalling pathways in non-neoplastic and neoplastic epithelial cells.

## Materials and methods

### Reagents

Mouse monoclonal antibodies (mAbs) against human Ro60/TROVE2 (clone 1F2) and La/SSB (clone 1D6-H5) were purchased from AbD-Serotec (Oxford, UK) against IRF3 (clone 12A4A35), IRF5 (clone 11F4A09), IRF7 (clone 12G9A36), IRF8 (clone 7G11A45) and β-actin (clone 2F1-1) from Biolegend (San Diego, CA, USA), whereas to IRF9 (clone 1C10) were purchased from Abnova (Taipei, Taiwan). Phycoerythrin (PE)-conjugated monoclonal antibodies against TLR-3 (clone TLR-104) and TLR-4 (clone HTA125) were from Biolegend, whereas isotype controls were from BD Biosciences (Franklin Lakes, NJ, USA). Rabbit polyclonal antibodies against human Ro52/TRIM21, IFN-β (for use in paraffin-embedded tissues) and histone-H4 were from Novus-Biologicals (Cambridge, UK), Merck Millipore (Darmstadt, Germany) and Biolegend, respectively. The monoclonal and polyclonal isotype-matched antibody controls were from Dako (Glostrup, Denmark). Neutralizing mAbs against human IFN-α [clone IFN-α mouse monoclonal antibody (MMHA-2)] and IFN-α/β-receptor chain2 [clone human IFN-α/β R2 mAb (MMHAR-2)] were from PBL Interferon Source (Piscataway, NJ, USA), those against human IFN-β (clone 76703) and IFN-γ (clone 25718) from R&D Systems (Minneapolis, MN, USA) and isotype controls were from BD Biosciences.

Secondary antibodies used in confocal microscopy included goat anti-mouse IgG, DyLight™ 549-conjugated (Thermo Scientific, Rockford, IL, USA), Alexa Fluor® 488 F(ab′)2 fragment of goat anti-rabbit IgG and Alexa Fluor® 546 goat anti-mouse IgG (H + L) (Life Technologies, Carlsbad, CA, USA). Secondary antibodies conjugated with alkaline phosphatase, such as Pierce goat anti-mouse IgG (Thermo Scientific) and goat anti-rabbit Igs (Dako) were applied in immunoblotting.

Commercially available enzyme-linked immunosorbent assay (ELISA) kits were used for the assessment of cytokine production. Human IFN-α (sensitivity: 13 pg/ml), IFN-β (sensitivity: 1 IU/ml) and IFN-γ (sensitivity: 4 pg/ml) ELISA kits were from Life Technologies, interleukin (IL)-7 (sensitivity: 4 pg/ml) from R&D Systems, whereas IL-2

(sensitivity: 4 pg/ml), IL-4 (sensitivity: 0·6 pg/ml) and IL-17 (sensitivity: 2·58 pg/ml) were from Biolegend.

The analogue of TLR-3 ligand polyinosinic:cytidylic acid (polyI:C) and the TLR-4 ligand lipopolysaccharide (LPS; *Escherichia coli*) were purchased from Sigma (St Louis, MO, USA).

### Cell lines

Non-neoplastic, long-term cultured SGEC lines were established by standard explant outgrowth technique [14] from a lobule of minor salivary gland (MSG) biopsy obtained from individuals undergoing diagnostic evaluation for Sjögren's syndrome (SS). Twenty-two SGEC lines were included in the study. Ten were obtained from SS patients according to the American–European SS classification criteria [15] and 12 from non-SS controls (individuals who complained of sicca symptoms and did not fulfil the above-mentioned criteria, had a negative biopsy and did not exhibit serological features consistent with SS). Six of 10 SS patients were positive for anti-Ro52/TRIM21, four for anti-Ro60/TROVE2 and three for anti-La/SSB autoantibodies. All samples were gathered and used after informed consent. The study was approved by the Ethics Committee of the School of Medicine, National University of Athens, Greece (protocol no. 5107). The purity and epithelial origin of cultured SGEC lines was verified routinely by morphology, the uniform expression of epithelial-specific markers and the absence of markers indicative of lymphoid/monocytoid cells [14].

The neoplastic HeLa cells were cultivated in Dulbecco's modified Eagle's medium (DMEM) medium (Life Technologies) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin (all from Life Technologies).

The lack of endotoxin contamination was investigated by routinely testing all culture reagents by the chromogenic *Limulus* amoebocyte lysate assay (Sigma, St Louis, MO, USA).

### Stimulation of TLRs on epithelial cells

SGECs or HeLa were cultured to confluence in collagen-treated six-well plates or 16-well chamber slides (Nalge Nunc International, Rochester, NY, USA), as described previously [14]. Subsequently, cells were exposed to medium alone or medium containing polyI:C, (5 μg/ml, TLR-3 ligand) or LPS (1 μg/ml, TLR-4 ligand) for various time-points (6, 12, 24, 48 and 72 h). In preliminary experiments, the effect of treatment with suboptimal (0·5 μg/ml) or optimal (5 μg/ml) concentrations of polyI:C [6] was evaluated. Furthermore, in subsequent experiments the effect of TLR signalling was also studied at 2 and 4 h. HeLa cells served as a control epithelial cell line. The effect of TLR signalling on the expression of Ro52/TRIM21, Ro60/TROVE2 and La/SSB molecules at the mRNA level was examined by real-time quantitative polymerase chain reaction (PCR) and at the protein level by confocal microscopy and immunoblotting analyses.

### Reverse transcription–quantitative PCR (RT–qPCR)

DNase-treated RNA was isolated using the mirVana™ PARIS™ kit (Ambion, Applied Biosystems, USA) and the Ambion® TURBO DNA-free™ kit (Ambion). cDNA was prepared from 0·25-μg RNA by the high-capacity RNA to DNA kit (Applied Biosystems). Ro52/TRIM21, Ro60/TROVE2 and La/SSB mRNAs were analysed by quantitative real-time PCR using commercially available primers specific for each molecule (TaqMan® Gene Expression Assays; Applied Biosystems). Human HPRT1 (TaqMan® Gene Expression Assays) served as the reference gene. All samples were run in duplicate. The relative quantification of PCR products was performed by the $2^{-\Delta\Delta CT}$ method [16] using HeLa cells as the calibrator. The PCR conditions were the same for all genes and consisted of an initial denaturation step at 95°C for 10 min, followed by 50 cycles of 95°C for 15 s and 60°C for 1 min.

### Immunoblotting analysis

The expression of Ro52/TRIM21, Ro60/TROVE2 and La/SSB proteins, as well as IRF3, IRF5, IRF7, IRF8 and IRF9 by SGECs or HeLa cells was evaluated by standard sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of nuclear or cytoplasmic extracts followed by immunoblotting with specific antibodies [17]. Briefly, nuclear or cytoplasmic extracts of resting, polyI:C or LPS-treated SGECs were prepared by the NE-PER Nuclear and Cytoplasmic Extraction Reagent (Thermo Scientific), electrophorized in 10% polyacrylamide gels and transferred to polvinylidene difluoride (PVDF) membranes (Millipore). Immunoblotting was performed with specific or isotype control antibodies in 1% skimmed milk in Tris-buffered saline (TBS)/0·1% Tween-20 for 2 h, followed by an 1-h incubation with the appropriate alkaline phosphatase-conjugated secondary antibodies. Signals were visualized by enhanced chemiluminescence using the CDP-Star substrate (Roche, Basel, Switzerland).

### Confocal microscopy

The expression and localization of Ro52/TRIM21, Ro60/TROVE2 and La/SSB proteins in SGECs grown in 16-well chamber slides (Nalge Nunc) were assessed by confocal microscopy, as described previously [7]. Briefly, cells were fixed with methanol for 10 min followed by acetone for 2 min at −20°C. Non-specific antibody binding was blocked by incubation with 1·5% non-immune fetal bovine serum (FBS). Incubation with antibodies against human Ro52/TRIM21, Ro60/TROVE2 and La/SSB proteins or

isotype-matched control antibodies was performed overnight at 4°C in a humidified chamber and was followed by 30 min incubation with appropriate fluorescence-conjugated secondary antibodies at room temperature. Images were acquired by an Olympus FV1000 confocal laser scanning microscope.

### Immunohistochemistry

The *in-situ* expression of Ro52/TRIM21 and IFN-β was investigated in formalin-fixed paraffin-embedded minor salivary gland (MSG) biopsy tissue sections (5 μm). Immunohistochemical detection was performed by a standard immunoperoxidase technique using the EnVision system (Dako) [18]. Antigen retrieval was performed by microwaving in 10 mM-Tris/1 mM–ethylenediamine tetraacetic acid (EDTA) (pH 9·0). Non-immune FBS (10%) and 0·5% $H_2O_2$ in methanol were used to block non-specific antibody binding and endogenous peroxidase activity, respectively. Permeabilization by 0·1% Triton-X-100 in blocking buffer was used for IFN-β staining. Negative control staining was performed by replacing primary with irrelevant isotype-matched antibodies. Biopsy sections were counterstained with haematoxylin, dehydrated and mounted in Di-n-butylPhthalate in Xylene (DPX) (BDH Chemicals, Poole, UK). The *in-situ* expression of Ro52/TRIM21 in MSG biopsy sections was also investigated by confocal microscopy, where staining with primary antibodies was followed by incubation with Alexa Fluor®488 F(ab′)2 fragment of goat anti-rabbit IgG antibody.

### Flow cytometry

Flow cytometric analysis of surface TLR-3, TLR-4 and IFN-α/β R2 expression by SGECs or HeLa cells was performed by PE-conjugated antibodies against TLRs, MMHAR-2 antibody against IFN-α/β-receptor followed by incubation with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse secondary antibody (BD Biosciences) or appropriate isotype controls and standard techniques in a fluorescence activated cell sorter (FACS) Calibur flow cytometer, as described previously [19].

### Assessment of IFN production

TLR-3 signalling has been associated strongly with the induction of IFN expression. The production of IFNs as a response to TLR stimulation was investigated in resting, polyI:C or LPS-treated SGECs or HeLa cells at both mRNA and protein levels. The expression of IFN-α, IFN-β and IFN-γ mRNAs was examined by quantitative real-time PCR using commercially available specific TaqMan® Gene Expression Assays (Applied Biosystems). The secretion of respective IFNs was investigated in cell-free culture supernatants of resting, polyI:C or LPS-treated epithelial cells by specific commercial ELISAs, according to the manufacturer's instructions. Supernatants were collected at the end of the cultivation period and kept at −80°C until further testing.

### IFN blockade

The implication of IFNs in the TLR-3-mediated modulation of Ro52/TRIM21, Ro60/TROVE2 and La/SSB expression was evaluated by the addition of specific neutralizing antibodies against IFN-α (5 μg/ml), IFN-β (10 μg/ml), IFN-γ (5 μg/ml) or IFN-α/β-receptor (5 μg/ml) 30 min before treatment with polyI:C.

### Statistical analyses

Statistical analyses were performed by the non-parametric Mann–Whitney *U*-test, using GraphPad Prism version 4·0 software (GraphPad Software, San Diego, CA, USA). Only statistically significant differences are reported.

## Results

### Prolonged TLR-3 stimulation leads a significant proportion of epithelial cells to apoptotic cell death via anoikis

In line with previous data [6], prolonged polyI:C stimulation resulted in detachment-induced apoptotic cell death or anoikis of SGECs. This was evident from 24 h of treatment and reached approximately 30–40% cell loss at 72 h of treatment. On the contrary, LPS treatment did not result in detectable loss of cell anchorage and subsequent apoptotic death.

### PolyI:C treatment induces a two-step increment of Ro52/TRIM21 mRNA expression in SGECs, but not in HeLa cells

Stimulation of TLR-3 for several time-points (6, 12, 24, 48, 72 h) resulted in a significant up-regulation of Ro52/TRIM21 mRNA expression, which was readily evident from 6 h [mean fold increase compared to untreated cells ± standard error (s.e.): 12·07 ± 3·12, $P < 0·0001$] (Fig. 1a). This expression remained mainly unchanged until 12–24 h and was followed by a second-step increment that peaked at 48 h (mean fold increase compared to 12 h ± s.e.: 2·46 ± 0·27, $P < 0·0001$). Subsequent investigation of Ro52/TRIM21 mRNA expression by SGECs at earlier time-points of polyI:C treatment (2 and 4 h) revealed that the first increment of Ro52/TRIM21 mRNA levels occurs at 4 h (similar levels with 6 h) and remains stable until 12–24 h (data not shown). Stimulation with polyI:C was not found to readily affect Ro60/TROVE2 and La/SSB mRNA levels. Their expression was slightly but statistically significantly

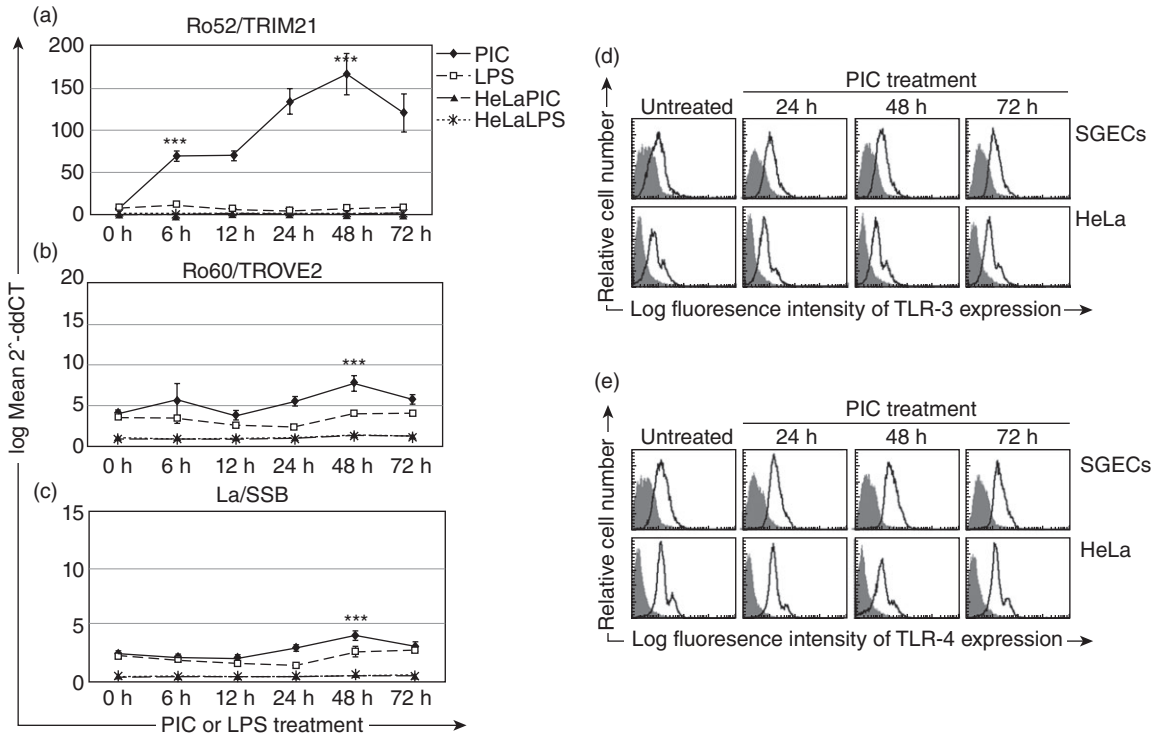


**Fig. 1.** (a–c) Mean fold increase of mRNA expression following polyI:C (PIC) or lipopolysaccharide (LPS) treatment of salivary gland epithelial cells (SGECs) or HeLa cells. In SGECs, tripartite motif-containing protein 21 (TRIM21) mRNA (a) induction was evident from 6 h of polyI:C treatment ($P < 0{\cdot}0001$), remained stable until 12–24 h and raised 2·5-fold at 48 h (compared to 12 h, $P < 0{\cdot}0001$) (bold continuous line). LPS did not affect Ro52/TRIM21 mRNA expression (discontinuous line). Neither PolyI:C (thin continuous line) nor LPS (dotted line) affected Ro52/TRIM21 mRNA expression by HeLa cells. Treatment of SGECs with polyI:C was found to result in a slight, but statistically significant ($P = 0{\cdot}0002$), induction of Ro60/TROVE domain family, member 2 (TROVE2) mRNA (b) at 48 h (compared to untreated, bold continuous line), whereas LPS had no effect (discontinuous line). Ro60/TROVE2 mRNA expression in HeLa cells was not affected by polyI:C (thin continuous line) or LPS (dotted line) treatment. Similarly, treatment of SGECs with polyI:C caused a slight, but statistically significant ($P = 0{\cdot}0014$), lupus LA protein/Sjögren syndrome antigen B (La/SSB) mRNA (c) induction at 48 h (compared to untreated, bold continuous line), whereas LPS had no effect (discontinuous line). In HeLa cells, neither polyI:C (thin continuous line) nor LPS (dotted line) affected La/SSB mRNA expression. Standard errors are representative of all 22 SGEC lines and three different experiments on HeLa cells, whereas statistical significance is indicated by asterisks (***$P < 0{\cdot}001$). Please note the different scale of the *y*-axes of the graphs. (d,e) Flow cytometric analysis of the surface Toll-like receptor (TLR)-3 (d) and TLR-4 (e) expression by untreated or polyI:C-treated Hela cells revealed significant constitutive expression of these receptors in both SGEC and HeLa cells, which is not affected significantly by TLR-3 stimulation.

increased upon 48 h of treatment (mean fold increase compared to untreated cells ± s.e.: 2·00 ± 0·23, $P = 0{\cdot}0002$ and 1·70 ± 0·12, $P = 0{\cdot}0014$ for Ro60/TROVE2 and La/SSB mRNAs, respectively) (Fig. 1b,c). The expression levels of Ro52/TRIM21, Ro60/TROVE2 and La/SSB mRNAs, as well as their pattern of induction by polyI:C treatment, was not found to differ between SGEC lines obtained from SS patients and non-SS controls and did not correlate with the presence of autoantibodies against any of these proteins (data not shown). Conversely, stimulation of SGECs with LPS, which is the TLR-4 ligand, had no effect on the expression levels of Ro52/TRIM21, Ro60/TROVE2 and La/SSB mRNAs. In contrast to SGECs, the expression of Ro52/TRIM21, Ro60/TROVE2 or La/SSB mRNAs by HeLa neoplastic cells was not affected by either polyI:C or LPS treatment (Fig. 1). This differential response of HeLa cells to polyI:C stimulation cannot be attributed to impaired TLR expression, as flow cytometric analysis supports that HeLa cells present significant surface TLR-3 and TLR-4 expression comparable to that of SGECs (Fig. 1d,e). Furthermore, the levels of surface TLR-3 and TLR-4 expression by SGECs or HeLa cells were not found to be affected by stimulation with polyI:C (Fig. 1d,e), suggesting that the observed phenomena are not induced by the up-regulated expression of surface TLR receptors.

### PolyI:C treatment induces the redistribution of Ro52/TRIM21 protein at the nucleus of SGECs, but not HeLa cells

PolyI:C treatment has not been found to affect the levels of Ro52/TRIM21, Ro60/TROVE2 and La/SSB proteins in SGECs, as attested by immunoblotting and confocal microscopy analysis (Fig. 2). However, polyI:C stimulation

resulted in late (48 and 72 h) nuclear redistribution of Ro52/TRIM21 protein in SGECs. Confocal microscopy revealed low cytoplasmic and strong nuclear staining of Ro52/TRIM21 protein in resting SGECs. This nuclear Ro52/TRIM21 staining was localized in one to three nuclear dots, resembling nucleolar staining. This pattern of expression remained unchanged during the first 24 h of polyI:C treatment, whereas it changed in late treatments, after 48 h. At 48 and 72 h, the nucleolar-like pattern of Ro52/TRIM21 expression changed to multiple coarse dots spanning throughout the nucleus (Fig. 2b). Treatment with LPS had no effect on Ro52/TRIM21, Ro60/TROVE2 and La/SSB protein expression or distribution (Fig. 2b). In accordance with mRNA results, SGEC lines obtained from SS patients and non-SS controls responded similarly. Suboptimal (0·5 μg/ml polyI:C) and optimal (5 μg/ml polyI:C) stimulation of TLR-3 [6] had similar effects on Ro52/TRIM21, Ro60/TROVE2 and La/SSB mRNA and protein expression (data not shown).

Neither polyI:C nor LPS stimulation had an effect on Ro52/TRIM21, Ro60/TROVE2 and La/SSB protein expression or distribution in HeLa cells. However, the constitutive expression pattern of Ro52/TRIM21 was different from that of SGECs. Ro52/TRIM21 protein was localized at nucleolar-like structures, but resided mainly at the cytoplasm and was not affected by TLR signalling (data not shown and Fig. 3b).

### Late polyI:C-induced up-regulation of Ro52/TRIM21 mRNA expression and protein redistribution in SGECs is mediated mainly by IFN-β production

The fact that the second-step increment of Ro52/TRIM21 mRNA and its nuclear redistribution in SGECs upon polyI:C treatment are late phenomena suggests that they are mediated by a factor produced downstream of TLR-3 signalling. Taking into consideration that these phenomena were not evident upon LPS treatment, which leads mainly to the production of TNF-α, and that IFNs, the major cytokines induced by TLR-3 stimulation, are potent inducers of Ro52/TRIM21 expression, we investigated the participation of IFNs in the polyI:C-induced Ro52/TRIM21 mRNA induction and protein redistribution. As expected, exogenous administration of types I and II IFNs, such as IFN-α, IFN-β and IFN-γ, readily resulted in significant up-regulation of Ro52/TRIM21 mRNA levels, which was evident from 6 h of treatment and remained relatively stable thereafter (mean fold induction of 6 h *versus* untreated SGECs ± s.e.: 3·0 ± 0·05, 7·7 ± 1·1 and 7·0 ± 0·9 for IFN-α, IFN-β and IFN-γ, respectively). Similarly, treatment of HeLa cells with IFN-α, IFN-β and IFN-γ for 6 h led to a respective 12·6 ± 0·05, 10·7 ± 1·2 and 5·8 ± 1·15-fold induction of the constitutive Ro52/TRIM21 mRNA expression, which remained unchanged thereafter. IFN treatment had no significant effect in Ro60/TROVE2 and La/SSB mRNA expression. Furthermore, the exogenous administration of IFN-α, IFN-β or IFN-γ in SGECs, but not in HeLa cells, caused the above-described nuclear redistribution of Ro52/TRIM21 from a nucleolar-like pattern to multiple coarse dots, an effect that was evident from 24 h of treatment (Fig. 3).

Subsequently, we investigated the IFN mRNA and protein expression by resting and polyI:C-stimulated SGECs. Expression of IFN-β mRNA, but not IFN-α or IFN-γ, was induced in polyI:C-treated SGECs. In fact, IFN-β mRNA was up-regulated robustly at 6 h of polyI:C, but not LPS treatment (mean fold induction ± s.e.: 1972 ± 797) and declined thereafter (Fig. 4a). In line with mRNA induction, secretion of IFN-β protein was detected in culture supernatants of polyI:C-treated SGECs. IFN-β secretion was evident from 6 h of treatment and peaked at 12 h (Fig. 4b), suggesting that it can be implicated in the second-step mRNA increment and the nuclear redistribution of Ro52/TRIM21 protein in SGECs. Production of IFN-α was not detected, whereas the stable, low amounts of IFN-γ that were found in the culture supernatants of resting or polyI:C-treated SGECs were due to the culture medium used, as attested by the lack of mRNA detection and the identification of similar levels of IFN-γ in unused culture medium (Fig. 4b). Neither polyI:C nor LPS treatment were found to induce IFN mRNA expression and secretion (data not shown) in HeLa cells. The secretion of IL-2, IL-4, IL-6, IL-7 and IL-17 by resting, polyI:C or LPS-treated epithelial cells was also investigated. As expected, resting or treated SGECs and HeLa were found to constitutively secrete significant amounts of IL-6, whereas they did not express IL-2, IL-4 and IL-17. In line with previous data [20], polyI:C was found to induce IL-7 secretion from 12 h in both SGECs and HeLa cells, with the peak observed at 72 h (20-fold and 10-fold, respectively). Similarly to polyI:C stimulation, LPS resulted in a 10-fold up-regulation of IL-7 secretion by HeLa cells, but was found unable to stimulate its production by SGECs.

The role of IFNs in the induction of Ro52/TRIM21 mRNA expression and protein redistribution in SGECs was verified further by blocking each IFN (IFN-α, IFN-β or IFN-γ) or the common type I IFN receptor (IFN-αβ-receptor) with specific neutralizing antibodies or isotype controls. From these, antibodies against IFN-β or the common IFN-αβ-receptor were found to significantly inhibit the polyI:C-driven mRNA increment (57·62% and 44·11% inhibition at 48 h of treatment, respectively) or nuclear redistribution of Ro52/TRIM21 in SGECs (Fig. 5). In fact, the neutralizing antibodies to IFN-β and the common IFN-αβ-receptor blocked 66·2 and 86·5%, respectively, of the second-step increment of polyI:C-induced Ro52/TRIM21 mRNA expression (12–48 h) (Fig. 5b). IFN blockade was not found to affect the low up-regulation of Ro60/TROVE2 and La/SSB mRNA at 48 h. Interestingly, polyI:C treatment was not found to affect the expression of

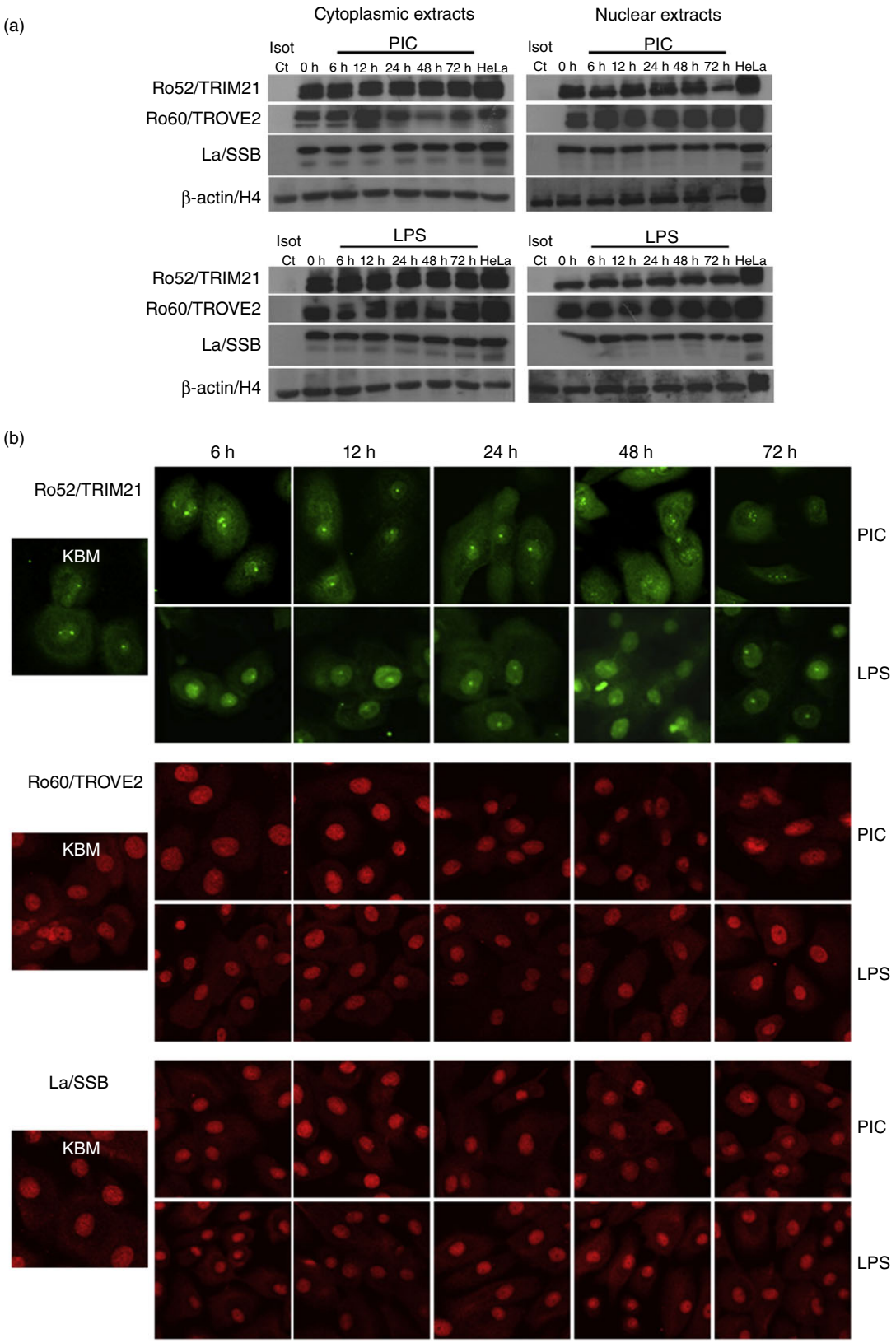
(a)
Cytoplasmic extracts
Nuclear extracts
Isot
PIC
Ct 0 h 6 h 12 h 24 h 48 h 72 h HeLa
Ro52/TRIM21
Ro60/TROVE2
La/SSB
β-actin/H4
LPS
(b)
6 h
12 h
24 h
48 h
72 h
Ro52/TRIM21
Ro60/TROVE2
La/SSB
KBM
PIC
LPS

**Fig. 2.** PolyI:C treatment does not affect the levels of tripartite motif-containing protein 21 (TRIM21), Ro60/TROVE domain family, member 2 (TROVE2) or lupus LA protein/Sjögren syndrome antigen B (La/SSB) protein expression, but induces a late redistribution of Ro52/TRIM21 in the nucleus of salivary gland epithelial cells (SGECs). (a) Immunoblotting analysis did not reveal any notable changes in protein expression levels of Ro52/TRIM21, Ro60/TROVE2 and La/SSB in either cytoplasmic (left side) or nuclear (right side) extracts of SGECs upon polyI:C (PIC; upper panel) or lipopolysaccharide (LPS) (lower panel) treatment. β-actin and histone-H4 were used as loading controls for cytoplasmic and nuclear extracts, respectively. A representative example of seven SGEC lines is shown. (b) Confocal microscopy analysis revealed that polyI:C treatment induced a nuclear redistribution of Ro52/TRIM21 protein in SGECs. In untreated cells, Ro52/TRIM21 is localized at the cytoplasm and one or two nuclear dots, resembling nucleolar staining. This pattern of nuclear expression remained stable until 24 h, whereas at 48 and 72 h it was redistributed to multiple coarse dots spanning the nucleus. LPS treatment had no effect on Ro52/TRIM21, Ro60/TROVE2 and La/SSB protein expression or distribution. A representative example of 13 SGEC lines (eight from SS and five from non-SS controls) is shown.

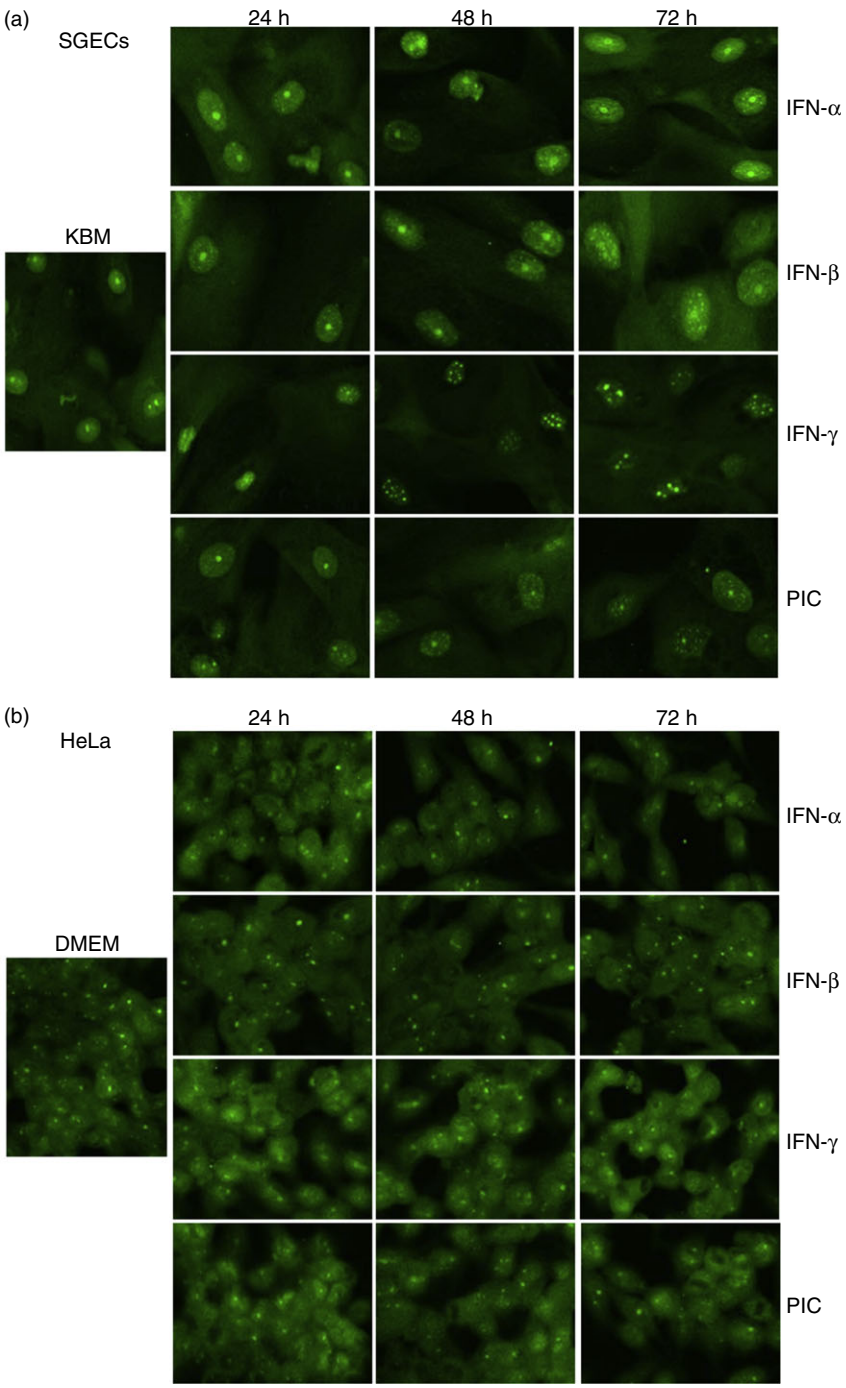


**Fig. 3.** Treatment with interferon (IFN)-α, IFN-β or IFN-γ induces the nuclear redistribution of tripartite motif-containing protein 21 (TRIM21) protein in salivary gland epithelial cells (SGECs), but not in HeLa cells. (a) IFN treatment of SGECs led to the nuclear redistribution of Ro52/TRIM21 protein from nucleolar-like pattern to multiple coarse dots spanning throughout the nucleus from 24 h. Representative example of three SGEC lines is shown. (b) Treatment with IFN-α, IFN-β or IFN-γ did not significantly alter the expression of Ro52/TRIM21 protein in HeLa cells. Figures are representative of three different experiments.

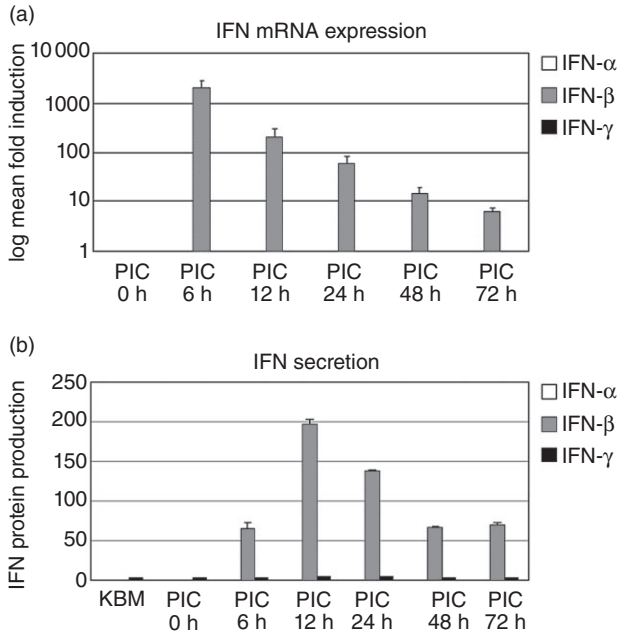


**Fig. 4.** Production of interferons (IFN) by salivary gland epithelial cells (SGECs). (a) Histogram indicating the mRNA production of IFN-α, IFN-β and IFN-γ by resting or polyI:C (PIC)-treated SGECs. Treatment with polyI:C readily induced the expression of IFN-β, but not IFN-α or IFN-γ, mRNA in SGECs at 6 h, and this expression declined thereafter. (b) Histogram indicating the secretion of IFNs by resting or polyI:C-treated SGECs. Treatment with polyI:C induced IFN-β secretion from 6 h, with a peak at 12 h. Minute stable amounts of IFN-γ were detected in resting (0 h), polyI:C-treated SGECs and unused cultured medium (KBM), whereas IFN-α was not detected. Standard errors correspond to results from three SGEC lines.

the common IFN-α/β-receptor on SGECs or HeLa cells, suggesting that the elevated expression of the receptor does not participate in the observed IFN-β-induced redistribution of Ro52/TRIM21 (data not shown).

Ro52/TRIM21 is an E3 ubiquitin-protein ligase that has been implicated in the regulation of IFN expression by mediating the ubiquitination and subsequent proteasomal degradation of several IRFs, including IRF3, IRF5, IRF7, IRF8 and IRF9 [21]. Thus, we subsequently investigated the expression of these IRFs in SGECs and HeLa cells, as well as the effect of TLR3 stimulation in their expression. Treatment with polyI:C was found to induce IRF3 degradation in SGECs at 48 and 72 h and to up-regulate IRF7, IRF8 and IRF9 expression from 24 h, whereas it down-regulated IRF5 expression without any evident degradation (Fig. 6). On the contrary, polyI:C treatment had no notable effect in the expression of the examined IRFs in HeLa cells (Fig. 6).

### *In-situ* expression of Ro52/TRIM21 and IFN-β at minor salivary gland (MSG) tissues

Strong cytoplasmic and nuclear Ro52/TRIM21 expression was detected in ductal and acinar epithelial cells at the MSG tissues of SS patients and non-SS controls, as well as in the infiltrating mononuclear cells of SS tissues (Fig. 7). Similarly, epithelial IFN-β staining was detected in the MSG tissues of both SS patients and non-SS controls. However, IFN-β expression was significantly stronger in SS tissues (Fig. 7). The distribution and protein expression of Ro52/TRIM21 at MSG tissues was also investigated by confocal microscopy. This method confirmed the nuclear expression of Ro52/TRIM21 protein in ductal epithelial cells and revealed differences between SS patients and non-SS controls. Thus, the vast majority of ductal epithelial cells at the MSG tissues of SS patients was found to present a coarse speckled nuclear staining, compared to a more homogeneous nuclear pattern at approximately half of the ductal cells of non-SS controls (Fig. 7).

## Discussion

The present study has clearly shown that TLR-3 signalling in non-neoplastic human salivary gland epithelial cells (SGEC) leads to a robust expression of the autoantigen Ro52/TRIM21 and a slight, but statistically significant, up-regulation of the other autoantigens of the Ro/La hYRNA ribonucleoprotein complex. A two-step up-regulation of Ro52/TRIM21 mRNA was stimulated by TLR-3, but not TLR-4, signalling in SGECs, in two phases; one at 4–6 h and the other at 12–48 h, which was mediated largely by the type I IFN pathway. Interestingly, the late Ro52/TRIM21 mRNA increment in SGECs was accompanied by a nuclear redistribution of the Ro52/TRIM21 protein from nucleolar-like pattern to multiple nuclear dots spanning throughout the nucleus visible from 48 h. Both late phenomena were partially, but significantly, inhibited by IFN-β blockade, whereas TLR-3 signalling in SGECs was associated with secretion of IFN-β, but not IFN-α or IFN-γ. Ro52/TRIM21 protein, with its capacity to act as an E3 ubiquitin-protein ligase [22,23] that mediates the ubiquitination and subsequent proteasomal degradation of IRFs (IRFs 3, 5, 7, 8 and 9), has been implicated in the negative regulation of TLR-3, TLR-7 and TLR-9 signalling and subsequent type I IFN production [10,11]. Conversely, proinflammatory stimuli, such as exposure to IFN-α or nitric oxide has been described to induce the expression and nuclear translocation of Ro52/TRIM21 [13,24] suggesting that, upon danger signals, Ro52/TRIM21 may ubiquitinate nuclear substrates. However, the significance of the nuclear localization and redistribution of Ro52/TRIM21 protein in non-neoplastic SGECs, including its substrate or interacting nuclear elements, needs to be elucidated. Interestingly, the late phenomenon of TLR-3 stimulation in SGEC was found to be associated with degradation of IRF3, possibly suggesting a functional role of Ro52/TRIM21 nuclear redistribution. In neoplastic epithelial HeLa cells, the response to TLR-3 or IFN stimuli and the pattern of expression of Ro52/TRIM21 molecules has been found to differ from that

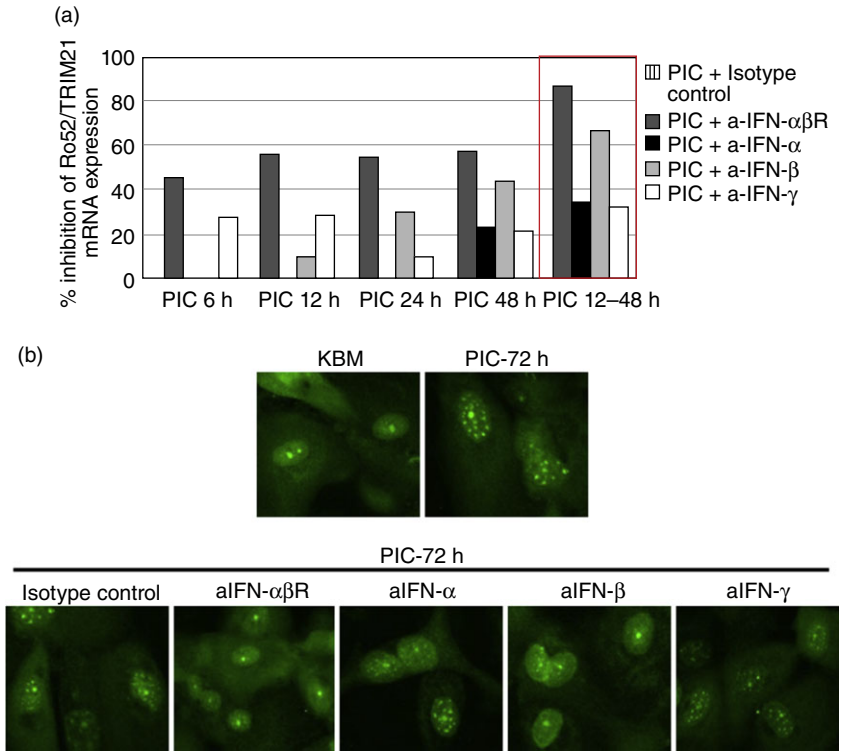


**Fig. 5.** Blockade of interferons (IFNs) by specific neutralizing antibodies revealed that the polyI:C-induced up-regulation of tripartite motif-containing protein 21 (TRIM21) mRNA and protein redistribution is mediated significantly by IFN-β. (a) Histogram showing the inhibition of Ro52/TRIM21 mRNA expression in resting or polyI:C (PIC)-treated salivary gland epithelial cells (SGECs) by specific neutralizing antibodies against IFN-α, IFN-β, IFN-γ, the common IFN-αβ-receptor (IFN-αβR) or isotype control. Antibodies against IFN-β or the common IFN-αβ-receptor significantly inhibit the polyI:C-driven mRNA increment, an effect that is more evident at the late increment (12–48 h; red box). (b) Blocking of polyI:C-induced redistribution of Ro52/TRIM21 protein by neutralizing antibodies against IFN-α, IFN-β, IFN-γ or IFN-αβ-receptor. Antibodies against IFN-β and IFN-αβ-receptor, but not against IFN-α or IFN-γ, block the polyI:C-driven nuclear redistribution of Ro52/TRIM21 in SGECs. Figures of blocking experiments at SGECs treated with polyI:C for 72 h are shown. These are representative of three distinct experiments.

in SGECs. Taking into consideration that this differential response cannot be attributed to impaired TLR-3 expression in HeLa cells, it possibly indicates that distinct pathways are operating in neoplastic and non-neoplastic cells.

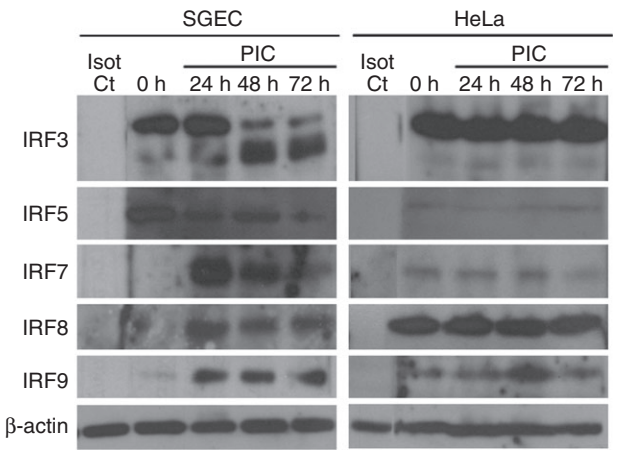


**Fig. 6.** Effect of polyI:C treatment in the expression of interferon regulatory factors (IRFs) by salivary gland epithelial cells (SGECs) and HeLa cells. Late polyI:C treatment resulted in the degradation of IRF3 in SGECs, but not in HeLa cells. Furthermore, stimulation of SGECs with polyI:C led to reduction of IRF5 expression, whereas it induced IRF7, IRF8 and IRF9 expression from 24 h. In line with mRNA and protein findings, IRF expression in HeLa cells was not affected by polyI:C treatment. β-actin was used as loading control. A representative example of four SGEC lines (two from SS and two from non-SS controls) is shown.

The exact aetiopathogenic pathways underlying SS remain unclear. Although an infectious agent has long been suspected as the aetiological agent of SS, no study so far has been conclusive. Epithelial cells are considered to be important players in the pathogenesis of the disorder [8]. Indeed, clinical observations demonstrated that organ involvement beyond the exocrine glands is due mainly to massive lymphocytic invasion, surrounding and infiltrating the epithelial structures [25]. Careful examination of salivary glands reveals activated T and B lymphocytes surrounding the salivary ducts. Epithelial cells are also activated, as they express regulatory and inflammatory cytokines and chemokines, as well as co-stimulatory and accessory molecules capable of inducing autoimmune responses. Furthermore, studies on long-term-cultured non-neoplastic SGECs have shown that they are fully equipped with molecules necessary for antigen presentation, transmittance of activation signals to T cells, recruitment of immune cells and perpetuation of immune responses. Moreover, SGECs have been reported to constitutively express high levels of several TLRs. Among them, the constitutive expression of TLR-3 was found to be remarkably increased in cultured SGECs, compared to various other types of cells, including macrophages and lymphocytes [7]. A major role of TLR-3 in the pathogenesis of autoimmune sialadenitis has also been suggested from studies conducted in mice. In fact, the administration of polyI:C in mice prone to develop a SS-like disease results in a transient, but significant, loss of function of their salivary glands that is accompanied by an up-regulation of various type I IFN-responsive genes *in situ*

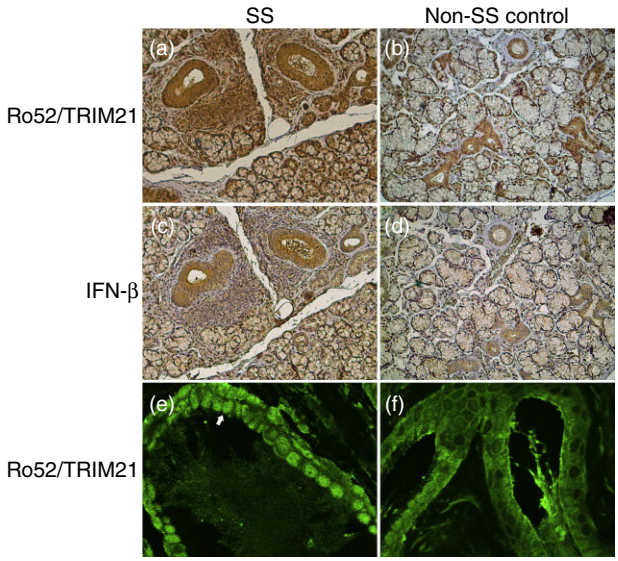


**Fig. 7.** Representative imunohistochemical (a–d) and confocal microscopy (e,f) detection of tripartite motif-containing protein 21 (TRIM21) (a,b,e,f) and interferon (IFN)-β (c,d) expression at the minor salivary glands (MSG). Ro52/TRIM21 was found to be expressed strongly in the ductal and acinar epithelial cells of Sjögren's syndrome (SS) patients (a) and controls (b), as well as in the infiltrating mononuclear cells of SS patients (a). The IFN-β staining was significantly more intense at the ductal epithelial cells of SS patients (c) compared to non-SS controls (d). Confocal microscopy revealed that the majority of the ductal epithelial cells at the MSGs of SS patients present a coarse nuclear Ro52/TRIM21 staining pattern (arrow) (e). In contrast, approximately half the ductal epithelial cells at the MSGs of non-SS controls display nuclear Ro52/TRIM21 expression, which is more homogeneous than that of SS patients (f).

[26]. Subsequent studies from the same group have reported that polyI:C injection to New Zealand black/white (NZB/W)-$F_1$ mice led to up-regulation of various chemokines, followed by an early recruitment of dendritic and natural killer (NK) cells and later of B cells at the submandibular glands [27]. Finally, experiments conducted on several strains of knock-out mice treated with polyI:C showed that a combined action of type I IFNs and IL-6 contributes towards salivary gland hypofunction [28].

As described in the opening lines of this report, the major site for autoantibody formation is the affected epithelial tissue. Several studies have demonstrated increased rates of apoptosis at the ductal and acinar glandular epithelia of SS patients [29–32]. Apoptotic cell death of SS epithelia represents a potential mechanism whereby nuclear antigens, such as Ro52/TRIM21, Ro60/TROVE2 and La/SSB autoantigens, are presented to the immune system in an immunogenic fashion [33]. During early apoptosis, La/SSB has been shown to redistribute diffusely to the cytoplasm. In later phases, Ro52/TRIM21, Ro60/TROVE2 and La/SSB autoantigens have been shown to be located mainly on the surface of apoptotic blebs and bodies [34]. Similarly, apoptosis is a procedure that could introduce autoantigens for presentation to autoreactive T cells. The role of apoptosis of epithelial cells in the induction of SS has been shown recently *in vivo* in an experimental mouse model. In this model, silencing of IκB-ζ expression in lacrimal epithelial cells led to increased apoptosis of the epithelial cells within the lacrimal glands and the development of an SS-like inflammatory lesion associated with high titres of serum anti-Ro/SSA and anti-La/SSB antibodies. Administration of caspase inhibitors inhibited the process, eventually proving the significant role of apoptosis in the disease induction [35].

Although the nuclear redistribution of Ro52/TRIM21 protein can be induced by both type I and type II IFNs, our data show that the autocrine mediator in SGECs is IFN-β. However, the elevated *in-situ* nuclear expression of Ro52/TRIM21 in the ductal epithelial cells at the MSG tissues of SS patients might also be regulated by the other IFNs. Types I and II IFNs have been strongly implicated in the pathophysiology of SS. More specifically, apart from the initial description of increased levels of circulating type I IFNs in patients with SS and systemic lupus erythematosus (SLE) [36], accumulated data presented over the last few years suggest that the activated type I IFN pathway plays a major role in the pathogenesis of SS, as type-I IFN-inducible genes are over-expressed in MSGs and peripheral lymphocytes of SS patients (reviewed in [37]). Moreover, the detection of a high number of IFN-α-producing plasmacytoid dendritic cells in the MSGs of SS patients compared to control tissues suggests their preferential homing within the target tissue [38–40]. In line with this, recent findings derived from the affected salivary glands in SS demonstrated that the epithelial cells display both types I and II IFN signatures, whereas the surrounding lymphocytes display type II [41]. In a broader view, these findings suggest that the major cytokines of the innate immunity (type I IFNs), which can mediate apoptotic cell death upon the appropriate stimulation, co-exist and probably co-operate with the immunoregulatory cytokine IFN-γ, the major inducer of specific adaptive immune responses within the pathological lesions [41]. The exact triggering of IFN production in the tissues of SS patients is not yet known; endogenous ligands might be implicated. In previous studies, it was shown that apoptotic bodies in complex with antibodies can serve as type I IFN inducers [42]. Other studies revealed that ribonucleoprotein complexes containing small RNA molecules (e.g. hY1-RNA), which are prominent targets of the autoimmune responses in SS, can also trigger IFN production via TLR-3 or TLR-7 ligation of their RNA part [43,44]. Also, IFN-γ is considered to boost signalling via TLR-3 and subsequent type I IFN responses [45] which, in turn, augment the expression of autoantigens, apoptosis and consequently the release of apoptotic bodies fully loaded with autoantigens that will further trigger TLR and IFN responses, thus creating a vicious cycle and providing

another mechanism for the co-operation of the two types of IFNs in regulating the augmentation and maintenance of TLR-mediated immune responses [41].

In summary, TLR-3 stimulation in SGECs causes an immediate and an indirect, IFN-β-dependent up-regulation of Ro52/TRIM21. This mechanism can create large quantities of the intracellular autoantigens that become visible to the immune system through the induction of epithelial cell apoptosis and release of the autoantigen in the apoptotic blebs. Thus, our findings implicate TLR-3, as the molecule at the crossroads of innate and adaptive immunity, as its ligation provides not only signals for IFN production, but also active synthesis of the intracellular autoantigen that can eventually prime adaptive immune responses.

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## Disclosure

Authors have no financial or personal conflicts of interest to declare.

## References

1 Routsias JG, Vlachoyiannopoulos PG, Tzioufas AG. Autoantibodies to intracellular autoantigens and their B-cell epitopes: molecular probes to study the autoimmune response. Crit Rev Clin Lab Sci 2006; **43**:203–48.

2 Routsias JG, Kyriakidis N, Latreille M, Tzioufas AG. RNA recognition motif (RRM) of La/SSB: the bridge for interparticle spreading of autoimmune response to U1-RNP. Mol Med 2010; **16**:19–26.

3 Yiannaki E, Vlachoyiannopoulos PG, Manoussakis MN *et al*. Study of antibody and T cell responses in rabbits immunized with synthetic human B cell epitope analogues of La (SSB) autoantigen. Clin Exp Immunol 2000; **121**:551–6.

4 Tengner P, Halse AK, Haga HJ, Jonsson R, Wahren-Herlenius M. Detection of anti-Ro/SSA and anti-La/SSB autoantibody-producing cells in salivary glands from patients with Sjogren's syndrome. Arthritis Rheum 1998; **41**:2238–48.

5 Tzioufas AG, Hantoumi I, Polihronis M, Xanthou G, Moutsopoulos HM. Autoantibodies to La/SSB in patients with primary Sjogren's syndrome (pSS) are associated with upregulation of La/SSB mRNA in minor salivary gland biopsies (MSGs). J Autoimmun 1999; **13**:429–34.

6 Manoussakis MN, Spachidou MP, Maratheftis CI. Salivary epithelial cells from Sjogren's syndrome patients are highly sensitive to anoikis induced by TLR-3 ligation. J Autoimmun 2010; **35**:212–8.

7 Spachidou MP, Bourazopoulou E, Maratheftis CI *et al*. Expression of functional Toll-like receptors by salivary gland epithelial cells: increased mRNA expression in cells derived from patients with primary Sjogren's syndrome. Clin Exp Immunol 2007; **147**:497–503.

8 Tzioufas AG, Kapsogeorgou EK, Moutsopoulos HM. Pathogenesis of Sjogren's syndrome: what we know and what we should learn. J Autoimmun 2012; **39**:4–8.

9 Alexopoulou L, Holt AC, Medzhitov R, Flavell RA. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. Nature 2001; **413**:732–8.

10 Espinosa A, Dardalhon V, Brauner S *et al*. Loss of the lupus autoantigen Ro52/Trim21 induces tissue inflammation and systemic autoimmunity by disregulating the IL-23-Th17 pathway. J Exp Med 2009; **206**:1661–71.

11 Yang K, Shi HX, Liu XY *et al*. TRIM21 is essential to sustain IFN regulatory factor 3 activation during antiviral response. J Immunol 2009; **182**:3782–92.

12 Rhodes DA, Ihrke G, Reinicke AT *et al*. The 52 000 MW Ro/SS-A autoantigen in Sjogren's syndrome/systemic lupus erythematosus (Ro52) is an interferon-gamma inducible tripartite motif protein associated with membrane proximal structures. Immunology 2002; **106**:246–56.

13 Strandberg L, Ambrosi A, Espinosa A *et al*. Interferon-alpha induces up-regulation and nuclear translocation of the Ro52 autoantigen as detected by a panel of novel Ro52-specific monoclonal antibodies. J Clin Immunol 2008; **28**:220–31.

14 Dimitriou ID, Kapsogeorgou EK, Abu-Helu RF, Moutsopoulos HM, Manoussakis MN. Establishment of a convenient system for the long-term culture and study of non-neoplastic human salivary gland epithelial cells. Eur J Oral Sci 2002; **110**:21–30.

15 Vitali C, Bombardieri S, Jonsson R *et al*. Classification criteria for Sjogren's syndrome: a revised version of the European criteria proposed by the American–European Consensus Group. Ann Rheum Dis 2002; **61**:554–8.

16 Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2[-delta delta C(T)] method. Methods 2001; **25**:402–8.

17 Kapsogeorgou EK, Abu-Helu RF, Moutsopoulos HM, Manoussakis MN. Salivary gland epithelial cell exosomes: a source of autoantigenic ribonucleoproteins. Arthritis Rheum 2005; **52**:1517–21.

18 Christodoulou MI, Kapsogeorgou EK, Moutsopoulos HM. Characteristics of the minor salivary gland infiltrates in Sjogren's syndrome. J Autoimmun 2010; **34**:400–7.

19 Kapsogeorgou EK, Moutsopoulos HM, Manoussakis MN. Functional expression of a costimulatory B7.2 (CD86) protein on human salivary gland epithelial cells that interacts with the CD28 receptor, but has reduced binding to CTLA4. J Immunol 2001; **166**:3107–13.

20 Jin JO, Shinohara Y, Yu Q. Innate immune signaling induces interleukin-7 production from salivary gland cells and accelerates the development of primary Sjogren's syndrome in a mouse model. PLOS ONE 2013; **8**:e77605.

21 Oke V, Wahren-Herlenius M. The immunobiology of Ro52 (TRIM21) in autoimmunity: a critical review. J Autoimmun 2012; **39**:77–82.

22 Espinosa A, Zhou W, Ek M *et al*. The Sjogren's syndrome-associated autoantigen Ro52 is an E3 ligase that regulates proliferation and cell death. J Immunol 2006; **176**:6277–85.
23 Wada K, Kamitani T. Autoantigen Ro52 is an E3 ubiquitin ligase. Biochem Biophys Res Commun 2006; **339**:415–21.
24 Espinosa A, Oke V, Elfving A, Nyberg F, Covacu R, Wahren-Herlenius M. The autoantigen Ro52 is an E3 ligase resident in the cytoplasm but enters the nucleus upon cellular exposure to nitric oxide. Exp Cell Res 2008; **314**:3605–13.
25 Moutsopoulos HM. Sjogren's syndrome: autoimmune epithelitis. Clin Immunol Immunopathol 1994; **72**:162–5.
26 Deshmukh US, Nandula SR, Thimmalapura PR, Scindia YM, Bagavant H. Activation of innate immune responses through Toll-like receptor 3 causes a rapid loss of salivary gland function. J Oral Pathol Med 2009; **38**:42–7.
27 Nandula SR, Scindia YM, Dey P, Bagavant H, Deshmukh US. Activation of innate immunity accelerates sialoadenitis in a mouse model for Sjogren's syndrome-like disease. Oral Dis 2011; **17**:801–7.
28 Nandula SR, Dey P, Corbin KL, Nunemaker CS, Bagavant H, Deshmukh US. Salivary gland hypofunction induced by activation of innate immunity is dependent on type I interferon signaling. J Oral Pathol Med 2013; **42**:66–72.
29 Jimenez F, Aiba-Masago S, Al Hashimi I *et al*. Activated caspase 3 and cleaved poly(ADP-ribose) polymerase in salivary epithelium suggest a pathogenetic mechanism for Sjogren's syndrome. Rheumatology (Oxf) 2002; **41**:338–42.
30 Kong L, Ogawa N, McGuff HS *et al*. Bcl-2 family expression in salivary glands from patients with primary Sjogren's syndrome: involvement of Bax in salivary gland destruction. Clin Immunol Immunopathol 1998; **88**:133–41.
31 Kong L, Ogawa N, Nakabayashi T *et al*. Fas and Fas ligand expression in the salivary glands of patients with primary Sjogren's syndrome. Arthritis Rheum 1997; **40**:87–97.
32 Manganelli P, Quaini F, Andreoli AM *et al*. Quantitative analysis of apoptosis and bcl-2 in Sjogren's syndrome. J Rheumatol 1997; **24**:1552–7.
33 Rosen A, Casciola-Rosen L, Ahearn J. Novel packages of viral and self-antigens are generated during apoptosis. J Exp Med 1995; **181**:1557–61.
34 Ohlsson M, Jonsson R, Brokstad KA. Subcellular redistribution and surface exposure of the Ro52, Ro60 and La48 autoantigens during apoptosis in human ductal epithelial cells: a possible mechanism in the pathogenesis of Sjogren's syndrome. Scand J Immunol 2002; **56**:456–69.
35 Okuma A, Hoshino K, Ohba T *et al*. Enhanced apoptosis by disruption of the STAT3-IkappaB-zeta signaling pathway in epithelial cells induces Sjogren's syndrome-like autoimmune disease. Immunity 2013; **38**:450–60.
36 Hooks JJ, Moutsopoulos HM, Geis SA, Stahl NI, Decker JL, Notkins AL. Immune interferon in the circulation of patients with autoimmune disease. N Engl J Med 1979; **301**:5–8.
37 Mavragani CP, Crow MK. Activation of the type I interferon pathway in primary Sjogren's syndrome. J Autoimmun 2010; **35**:225–31.
38 Bave U, Nordmark G, Lovgren T *et al*. Activation of the type I interferon system in primary Sjogren's syndrome: a possible etiopathogenic mechanism. Arthritis Rheum 2005; **52**:1185–95.
39 Gottenberg JE, Cagnard N, Lucchesi C *et al*. Activation of IFN pathways and plasmacytoid dendritic cell recruitment in target organs of primary Sjogren's syndrome. Proc Natl Acad Sci USA 2006; **103**:2770–5.
40 Vogelsang P, Brun JG, Oijordsbakken G, Skarstein K, Jonsson R, Appel S. Levels of plasmacytoid dendritic cells and type-2 myeloid dendritic cells are reduced in peripheral blood of patients with primary Sjogren's syndrome. Ann Rheum Dis 2010; **69**:1235–8.
41 Hall JC, Casciola-Rosen L, Berger AE *et al*. Precise probes of type II interferon activity define the origin of interferon signatures in target tissues in rheumatic diseases. Proc Natl Acad Sci USA 2012; **109**:17609–14.
42 Bave U, Alm GV, Ronnblom L. The combination of apoptotic U937 cells and lupus IgG is a potent IFN-alpha inducer. J Immunol 2000; **165**:3519–26.
43 Kelly KM, Zhuang H, Nacionales DC *et al*. 'Endogenous adjuvant' activity of the RNA components of lupus autoantigens Sm/RNP and Ro 60. Arthritis Rheum 2006; **54**:1557–67.
44 Laurie GW, Glass JD, Ogle RA, Stone CM, Sluss JR, Chen L. 'BM180': a novel basement membrane protein with a role in stimulus-secretion coupling by lacrimal acinar cells. Am J Physiol 1996; **270** (6 Pt 1):C1743–50.
45 Negishi H, Osawa T, Ogami K *et al*. A critical link between Toll-like receptor 3 and type II interferon signaling pathways in antiviral innate immunity. Proc Natl Acad Sci USA 2008; **105**:20446–51.