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Original article

Aberrant MUC1 accumulation in salivary glands of Sjögren's syndrome patients is reversed by TUDCA *in vitro*

Isabel Castro[1,*], Nicolás Albornoz[2,*], Sergio Aguilera[3], María-José Barrera[4], Sergio González[5], Matilde Núñez[2], Patricia Carvajal[2], Daniela Jara[2], Carolina Lagos[2], Claudio Molina[4], Ulises Urzúa[6], Marcela A. Hermoso[7] and María-Julieta González[2]

Abstract

Objectives. Xerostomia in SS patients has been associated with low quality and quantity of salivary mucins, which are fundamental for the hydration and protection of the oral mucosa. The aim of this study was to evaluate if cytokines induce aberrant mucin expression and whether tauroursodeoxycholic acid (TUDCA) is able to counteract such an anomaly.

Methods. Labial salivary glands from 16 SS patients and 15 control subjects, as well as 3D acini or human submandibular gland cells stimulated with TNF-α or IFN-γ and co-incubated with TUDCA, were analysed. mRNA and protein levels of Mucin 1 (MUC1) and MUC7 were determined by RT-qPCR and western blot, respectively. Co-immunoprecipitation and immunofluorescence assays for mucins and GRP78 [an endoplasmic reticulum (ER)-resident protein] were also performed. mRNA levels of RelA/p65 (nuclear factor-κB subunit), TNF-α, IL-1β, IL-6, SEL1L and EDEM1 were determined by RT-qPCR, and RelA/p65 localization was evaluated by immunofluorescence.

Results. MUC1 is overexpressed and accumulated in the ER of labial salivary gland from SS patients, while MUC7 accumulates throughout the cytoplasm of acinar cells; however, MUC1, but not MUC7, co-precipitated with GRP78. TUDCA diminished the overexpression and aberrant accumulation of MUC1 induced by TNF-α and IFN-γ, as well as the nuclear translocation of RelA/p65, together with the expression of inflammatory and ER stress markers in 3D acini.

Conclusion. Chronic inflammation alters the secretory process of MUC1, inducing ER stress and affecting the quality of saliva in SS patients. TUDCA showed anti-inflammatory properties decreasing aberrant MUC1 accumulation. Further studies are necessary to evaluate the potential therapeutic effect of TUDCA in restoring glandular homeostasis in SS patients.

Key words: Sjögren's syndrome, MUC1, endoplasmic reticulum stress, tauroursodeoxycholic acid, cytokines, NF-κB

CLINICAL SCIENCE

Rheumatology key messages

- MUC1 is overexpressed and accumulated in the endoplasmic reticulum of salivary glands from SS patients.
- Pro-inflammatory cytokines induced aberrant MUC1 accumulation and ER stress in human salivary gland cells.
- Tauroursodeoxycholic acid decreased MUC1 accumulation, and markers of inflammation and ER stress in human salivary gland cells.

Introduction

SS is a chronic autoimmune disease characterized by the inflammation of the exocrine glands [1], with patients demonstrating a persistent sensation of eye and mouth dryness due to the functional impairment of salivary and lachrymal glands [1]. Some SS patients complain of oral

[1]Departamento de Tecnologia Medica, [2]Programa de Biología Celular, Instituto de Ciencias Biomédicas, Facultad de Medicina, Universidad de Chile, [3]Departamento de Reumatología, Clínica INDISA, [4]Facultad de Odontología, Universidad San Sebastián, [5]Escuela de Odontología, Facultad de Ciencias, Universidad Mayor, [6]Departamento de Oncología Básico-Clínico y and [7]Programa de Inmunología, Instituto de Ciencias Biomédicas (ICBM), Facultad de Medicina, Universidad de Chile, Santiago, Chile

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*Isabel Castro and Nicolás Albornoz contributed equally to this study.

Correspondence to: María-Julieta González, Programa de Biología Celular y Molecular, Instituto de Ciencias Biomédicas (ICBM), Facultad de Medicina, Universidad de Chile, Independencia 1027, Postal code 8380453, Santiago, Chile. E-mail: jgonzale@med.uchile.cl

dryness even though they have a normal unstimulated salivary flow [2], indicating that this symptom is not just a consequence of the reduced volume of saliva [3]. The hydration of the oral mucosa is maintained by salivary mucins (MUCs), which retain water through sulphated and sialylated oligosaccharides [4]. These large glycoproteins are synthesized in the endoplasmic reticulum (ER), and are highly *O*-glycosylated in the Golgi apparatus, packaged into secretory granules before exocytosis towards the acinar lumen [5]. At this stage, some MUCs remain associated to the plasma membrane (transmembrane MUC1) while others constitute part of the saliva (MUC1/SEC, MUC7 and MUC5B). The structural complexity of MUCs together with the high demand for protein synthesis maintain healthy salivary gland (SG) epithelial cells under basal ER stress condition and controlled by the unfolded protein response (UPR) to restore proteostasis [6, 7]. SG from SS patients show alterations related to chronic ER stress such as ER cistern dilatation [8] and altered levels of UPR [Inositol-requiring enzyme 1α (IRE1α, X box-binding protein 1 (XBP1), glucose-regulated protein 78 (GRP78), activating transcription factor 6α (ATF6α), activating transcription factor 4)] [9, 10] combined with ER-associated degradation (ERAD) components [SEL1L adaptor subunit of ERAD E3 ubiquitin ligase (SEL1L) and ER degradation enhancing alpha-mannosidase like protein 1 (EDEM1), among others] [10]. Additionally, expression and localization changes of exocytic machinery components [11], cytoplasmic accumulation of secretory granules [8], and MUC7 and MUC5B presence in the extracellular matrix [11] indicate that the mucin secretory process is altered in salivary acinar cells from SS patients. However, the factors that underlie these changes are unknown.

As has been documented, SG from SS patients are characterized by a periductal focal lympho-plasmocytic infiltrate together with elevated levels of pro-inflammatory cytokines (TNF-α and IFN-γ, among others) which are produced by epithelial and/or inflammatory cells [12, 13]. The stimulation of 3D acini with TNF-α and/or IFN-γ affect the expression of proteins involved in the UPR, reproducing *in vitro* the ER stress condition observed in SG of SS patients [9, 10]. Moreover, pro-inflammatory cytokines induce MUC1 and MUC7 expression in several cell lines [14, 15], including some MUC1 variants in human submandibular gland (HSG) cells [16]. Interestingly, tauroursodeoxycholic acid (TUDCA), a chemical physiological chaperone, reduces protein misfolding and colitis in mice [17], and suppresses nuclear factor (NF)-κB signalling ameliorating colitis-associated tumorigenesis [18]. TUDCA is a taurine conjugate of ursodeoxycholic acid, a US Food and Drug Administration-approved hydrophilic bile acid for the treatment of primary biliary cholangitis [19]. It has been recently postulated that SS and primary biliary cholangitis are both immune-mediated epithelitis with similar pathogenic mechanisms; in this context it is possible to speculate that the small intrahepatic bile ducts could trigger chronic ER stress [20]. TUDCA has cytoprotective, anti-apoptotic and membrane-stabilizing properties [21], while alleviating ER stress and decreasing inflammatory-related protein expression [22, 23]. The effect of TUDCA has been studied in a wide range of cell types demonstrating the recovery of animal models of human diseases, such as acute and chronic non-liver diseases, obesity, stroke, acute myocardial infarction and spinal cord injury [24].

These antecedents suggest that in SG of SS patients, pro-inflammatory cytokines could induce the overexpression of MUCs, leading to ER synthesis machinery overload and inducing intracellular accumulation of MUCs and ER stress. We hypothesize that TUDCA restores epithelial cells homeostasis ameliorating aberrant MUCs expression.

Methods

Patients with primary SS and control subjects

The study group included 16 patients diagnosed with primary SS, based on the American-European Consensus Group Criteria [25]. The control group included 15 individuals who did not fulfil the primary SS classification criteria, did not suffer systemic diseases and in whom lip biopsy analysis was normal or revealed mild diffuse chronic sialadenitis. Major salivary gland function was determined by scintigraphy as previously described [26]. Written consent was obtained from subjects according to the Declaration of Helsinki. This study was approved by the Ethical Committee of the Facultad de Medicina, Universidad de Chile. A detailed description of demographic, serological and histological characteristics of the SS patients and control subjects is summarized in supplementary Table S1, available at *Rheumatology* online.

Biopsies

Labial salivary glands (LSG) biopsies from SS patients and control subjects were performed according to Daniels [27]. Following surgery, samples were immediately frozen in liquid nitrogen or fixed for morphological and immunofluorescence studies.

Cell culture

HSG cells were cultured as monolayer or 3D acini as previously described [10, 28]. HSG cells or 3D acini were incubated with or without 1 or 10 ng/ml human recombinant TNF-α or IFN-γ (BioLegend, CA, USA) or 0.05 μg/ml of tunicamycin (a canonical ER stress inducer) (Sigma, MO, USA) for 24 h. Alternatively, HSG cells or 3D acini were incubated with cytokines for 6 h and then co-incubated with TUDCA (sodium salt, #580549; Calbiochem, CA, USA) (150 or 250 μM) up to 24 h and subsequently lysed or fixed as described below.

RNA extraction and real time-PCR

RNA was extracted with yields and purity evaluated essentially as previously described [10]. One microgram of total RNA was reverse transcribed with oligo(dT), random primers and the Superscript II enzyme (Invitrogen, CA, USA). Specific primers for *MUC1* (20 splice variants), *MUC7*,

MUC1-SEC, *RelA/p65*, *TNF-α*, *IL-6*, *IL-1β*, *SEL1L*, *EDEM1* and *h18S* genes were designed with the AmplifiX 1.4 software (supplementary Table S2, available at *Rheumatology* online). Real time-PCRs were carried out using Brilliant II SYBR Green PCR Master Mix Kit and MxPro-MX 3000 P thermocycler (Agilent Technologies, CA, USA). Each specific transcript tested was expressed as the ratio to h18S, using the efficiency-calibrated model [29].

Western blotting and immunoprecipitation assays

LSG biopsies or HSG cells were homogenized using radioimmunoprecipitation assay buffer containing the Complete Protease Inhibitor Cocktail (Roche, Mannheim, Germany). Protein content was quantified using the Bradford method [30], and proteins were separated by SDS–PAGE under reducing conditions, then transferred into nitrocellulose membranes. Blots were then blocked with 5% milk and probed with specific primary antibodies (supplementary Table S3, available at *Rheumatology* online) and horseradish peroxidase-conjugated secondary antibodies. Next, target proteins were detected by chemiluminescence, quantified by densitometry and normalized to β-actin.

For immunoprecipitation assays, LSG or HSG cell extracts were prepared in a buffer containing 20 mM Tris–HCl pH 7.4, 150 mM NaCl, 1% NP-40 and protease inhibitors. Supernatants obtained after centrifugation were used for immunoprecipitation assays (500 μg of total protein per assay) with antibody-coated Protein-A/G-Sepharose beads.

Immunofluorescence

LSG were fixed in 1% paraformaldehyde or Bouin's solution for 6 h and embedded in paraffin. Additionally, HSG cells cultured on glass coverslips or 3D acini cultured on 8-well chamber slides were fixed in 2% paraformaldehyde or Bouin's solution and permeabilized with 0.1–1% Triton X-100. Next, LSG sections, HSG cells or 3D acini were blocked with 0.25% casein and sequentially incubated with specific primary and Alexa Fluor 488 or 546-conjugated secondary antibodies (supplementary Table S3, available at *Rheumatology* online). Hoechst 33342 was used for nuclear staining. As a negative control, rabbit or mouse IgG fractions were employed.

Quantitative analysis of the fluorescence staining intensity

Digital images were obtained in an Olympus FluoView FV10i confocal laser scanning microscope (Olympus, Tokyo, Japan). For each analysed protein, the pinhole diameter, laser intensity and sensitivity were maintained between LSG sections from SS patients and control subjects, as well as among different cell culture conditions. The images were deconvolved with the Huygens Pro 4.3.0 software and the co-localization analysis was performed with the Imaris Classic 7.2.3 software. The fluorescence intensity was determined using the ImageJ software (http://imagej.nih.gov/ij/).

Statistical analysis

The statistical significance of differences between groups was analysed with the GraphPad Software (San Diego, CA, USA), using the Mann–Whitney test with *P*-values <0.05 considered significant.

Results

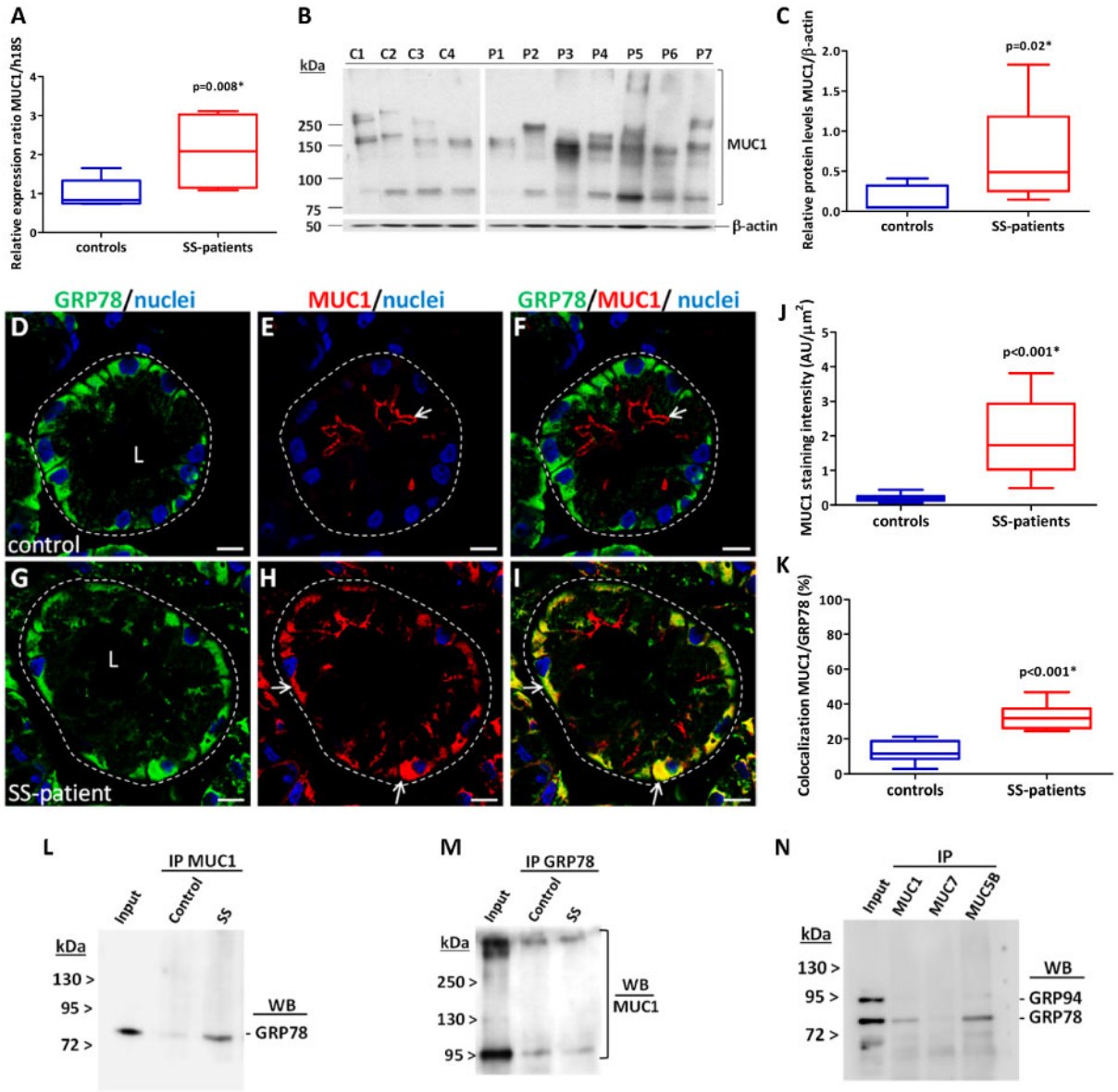
MUC1 overexpressed and accumulated in the ER of LSG from SS patients

MUC1 has numerous splicing variants encoding different membrane-anchored and soluble isoforms, ranging from 90 to 300 kDa. The extracellular MUC1 domain contains a variable number of tandem repeats of a sequence rich in serine and threonine, residues that covalently bond a variety of *O*-glycans. In this study we designed primers that recognize 20 splicing variants of MUC1 with results showing an increase of *MUC1* mRNA levels in LSG from 16 SS patients compared with 15 controls ($P = 0.008$) (Fig. 1A). Using the M8 antibody directed against the variable number of tandem repeats domain of MUC1, we also observed an increase of MUC1 protein levels in LSG from 16 SS patients compared with 15 controls ($P = 0.02$) (Fig. 1B and C). A cytoplasmic accumulation of MUC1, co-localizing with the ER marker GRP78, was also observed (Fig. 1G–I), while in controls MUC1 was localized in the apical surface of acinar cells (Fig. 1D–F). The staining intensity of MUC1 and the percentage of co-localization with GRP78 were higher in LSG from SS patients ($P < 0.0001$) (Fig. 1J and K). This result was also validated by MUC1 and GRP78 co-immunoprecipitation assays (Fig. 1L), which suggests its association. We have previously described a significant reduction of GRP78 protein levels in LSG from SS patients compared with controls [9], which could explain why SS patients samples did not show increased detection of MUC1 in Fig. 1M when antibodies against GRP78 were used as immunoprecipitants.

MUC7 accumulated in secretory granules of LSG from SS patients

MUC7 shows three splicing variants encoding a single protein of ∼200 kDa. In LSG from SS patients ($n = 16$), no change in *MUC7* mRNA levels was observed ($P = 0.24$) (Data not shown); however, an increase of MUC7 protein levels was detected compared with controls ($n = 15$) ($P = 0.03$) (Fig. 2A and B), accompanied by a decrease in the Sialyl-Lewis^a/MUC7 ratio (Fig. 2A–C). Consistent with the western blot results, MUC7 showed higher staining intensity and accumulation throughout the cytoplasm of salivary acinar cells from SS patients (Fig. 2G–I), with no co-localization with GRP78 (Fig. 2J and K). However, in controls, MUC7 demonstrated apical localization, where the secretory granules accumulate prior to exocytosis (Fig. 2D–F). In LSG from SS patients only MUC1 and MUC5B co-precipitated with GRP78, confirming the absence of MUC7 and GRP78 co-localization, but that GRP78 is associated with MUC1 and MUC5B (Fig. 1N).

FIG. 1 MUC1 is overexpressed and accumulated in the ER of LSG from SS patients



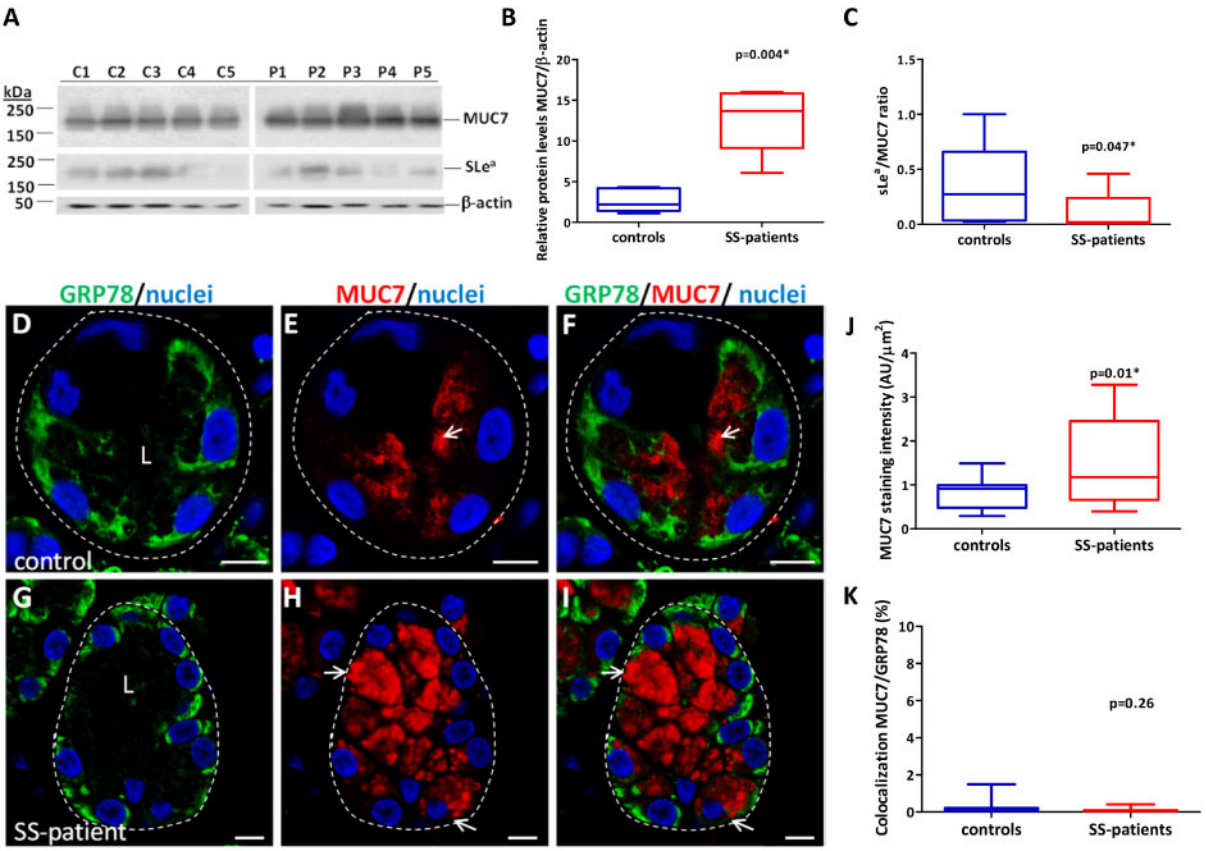
(**A**–**C**) Relative MUC1 transcript levels and representative WB of MUC1 (90–300 kDa) in LSG extracts from controls (C) ($n = 15$) and SS patients (P) ($n = 16$). (**D**–**K**) Representative acini showing MUC1 (red) and GRP78 (green). In SS patients MUC1 was accumulated in the basolateral region, co-localizing with GRP78 in the ER (arrows, G–I). Bars 10 μm. (**L**–**N**) MUC1, MUC7, MUC5B or GRP78 were immunoprecipitated from protein extracts of LSG from controls and SS patients and then analysed by WB with anti-GRP78 or anti-MUC1 antibodies. *P-values <0.05 were considered significant. ER: endoplasmic reticulum; IP: immunoprecipitation; LSG: labial salivary glands; WB: western blot.

TNF-α and IFN-γ induced MUC1 expression and accumulation combined with NF-κB activation in 3D acini

The incubation of 3D acini with TNF-α or IFN-γ increased mRNA and protein levels of MUC1 (Fig. 3A–C), with basolateral MUC1 accumulation co-localizing with GRP78 in epithelial cells (Fig. 3D and E). Interestingly, MUC1 accumulation in the ER was detected by MUC1 and GRP78 co-precipitation in 3D acini stimulated with TNF-α and IFN-γ (Fig. 3F and G).

The stimulation of 3D acini with cytokines induced an increase of *RelA/p65* mRNA levels (Fig. 4A). In addition, 24 h stimulation of 3D acini with TNF-α induced the expression of IL-1β and IL-6 transcripts (Fig. 4B) and stimulation with IFN-γ induced the expression of TNF-α transcripts (Fig. 4B). The exposure of 3D acini to inflammatory stimuli also induced nuclear translocation of the

FIG. 2 MUC7 is accumulated throughout the cytoplasm of acinar cells in LSG from SS patients



(**A**–**C**) Representative WB of MUC7 and Sialyl-Lewis^a moieties (~200 kDa) in LSG extracts from control subjects (C) ($n = 15$) and SS patients (P) ($n = 16$). (**D**–**I**) Representative acini showing MUC7 (red) in a supranuclear cytoplasmic localization (arrow) and GRP78 (green) in the basolateral region of epithelial cells in sections of LSG from controls (D–F). In SS patients MUC7 was accumulated throughout the cytoplasm of acinar cells, even in the basal region (arrow), without showing co-localization with GRP78 (G–**K**). Bars 10 μm. *P-values <0.05 were considered significant. LSG: labial salivary glands; WB: western blot.

NF-κB subunit RelA/p65 (Fig. 4C–E), and increased MUC1 detection in the basolateral region of epithelial cells (Fig. 4F–H). These results suggest that MUC1 overexpression induced by pro-inflammatory cytokines could be mediated by NF-κB activation in 3D acini.

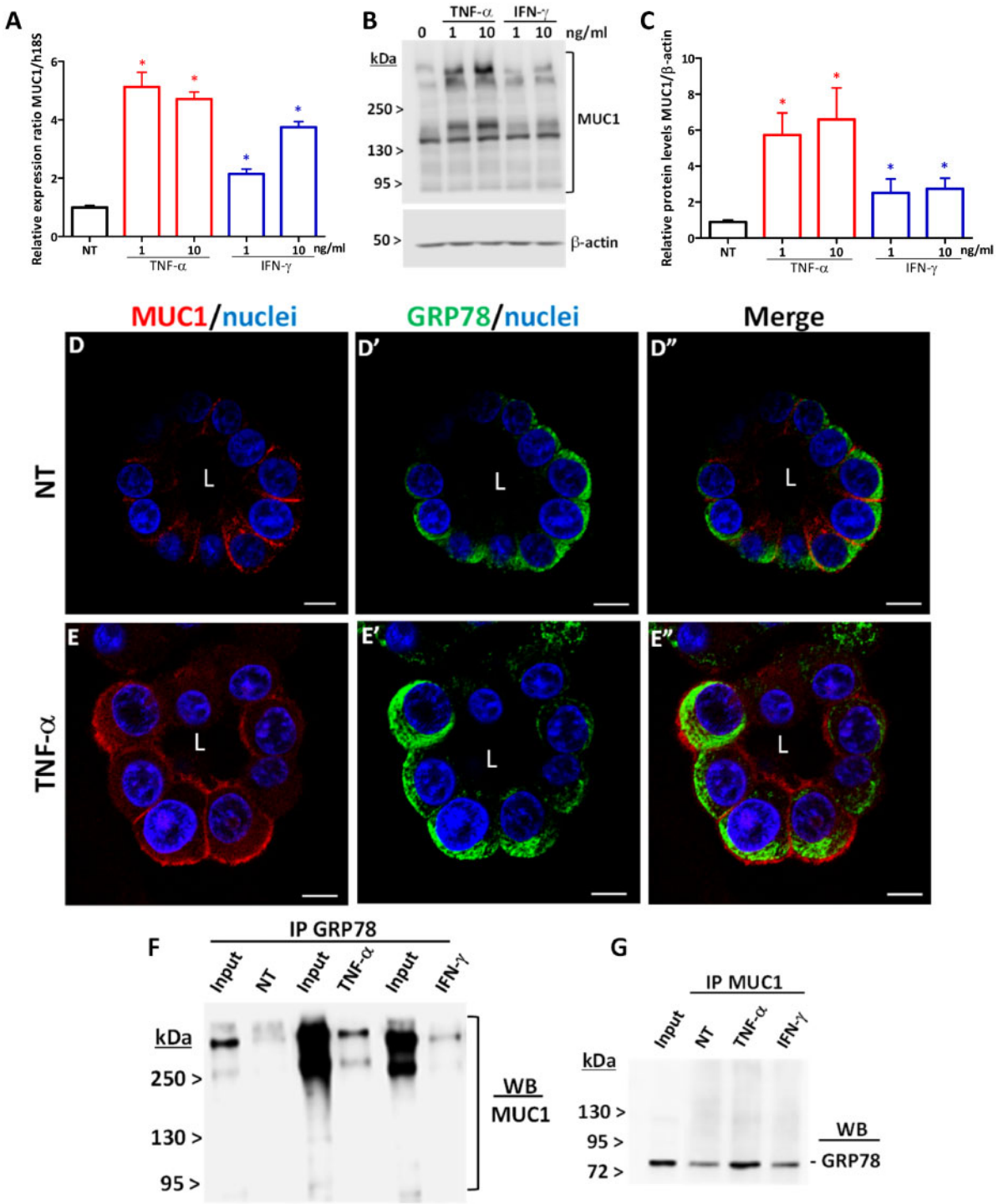
TUDCA decreased inflammatory and ER stress signals diminishing MUC1 accumulation

TUDCA is a highly hydrophilic taurine conjugated form of ursodeoxycholic acid [24], which alleviates ER stress and decreases expression of inflammatory-related proteins, such as inducible nitric oxide synthase, monocyte chemotactic protein 1 and NF-κB [22, 23]. Here, the incubation of 3D acini with TNF-α or IFN-γ induced an increase of *RelA/p65*, *TNF-α*, *IL-1β* and *IL-6* mRNA levels that was diminished by TUDCA (Fig. 5A–D), as well as TNF-α-induced RelA/p65 nuclear translocation (Fig. 5E–H), altogether suggesting an anti-inflammatory effect of TUDCA on TNF-α-stimulated HSG cells. Moreover, TUDCA decreased the mRNA levels, protein levels and staining intensity of SEL1L and EDEM1 (ERAD components induced by UPR) in response to TNF-α and IFN-γ stimulation (supplementary Figs 1 and 2, available at *Rheumatology* online). TUDCA also weakened the increase of MUC1 and MUC1-SEC mRNA levels (Fig. 6A and B) in 3D acini stimulated with TNF-α or IFN-γ. Furthermore, TNF-α increased cytoplasmic MUC1-SEC immunostaining that decreased by co-incubation with TUDCA (Fig. 6C–F), with the fluorescence intensity of MUC1-SEC induced by TNF-α similar to the staining in cells stimulated with the canonical ER stress inducer tunicamycin (Fig. 6C and G).

Discussion

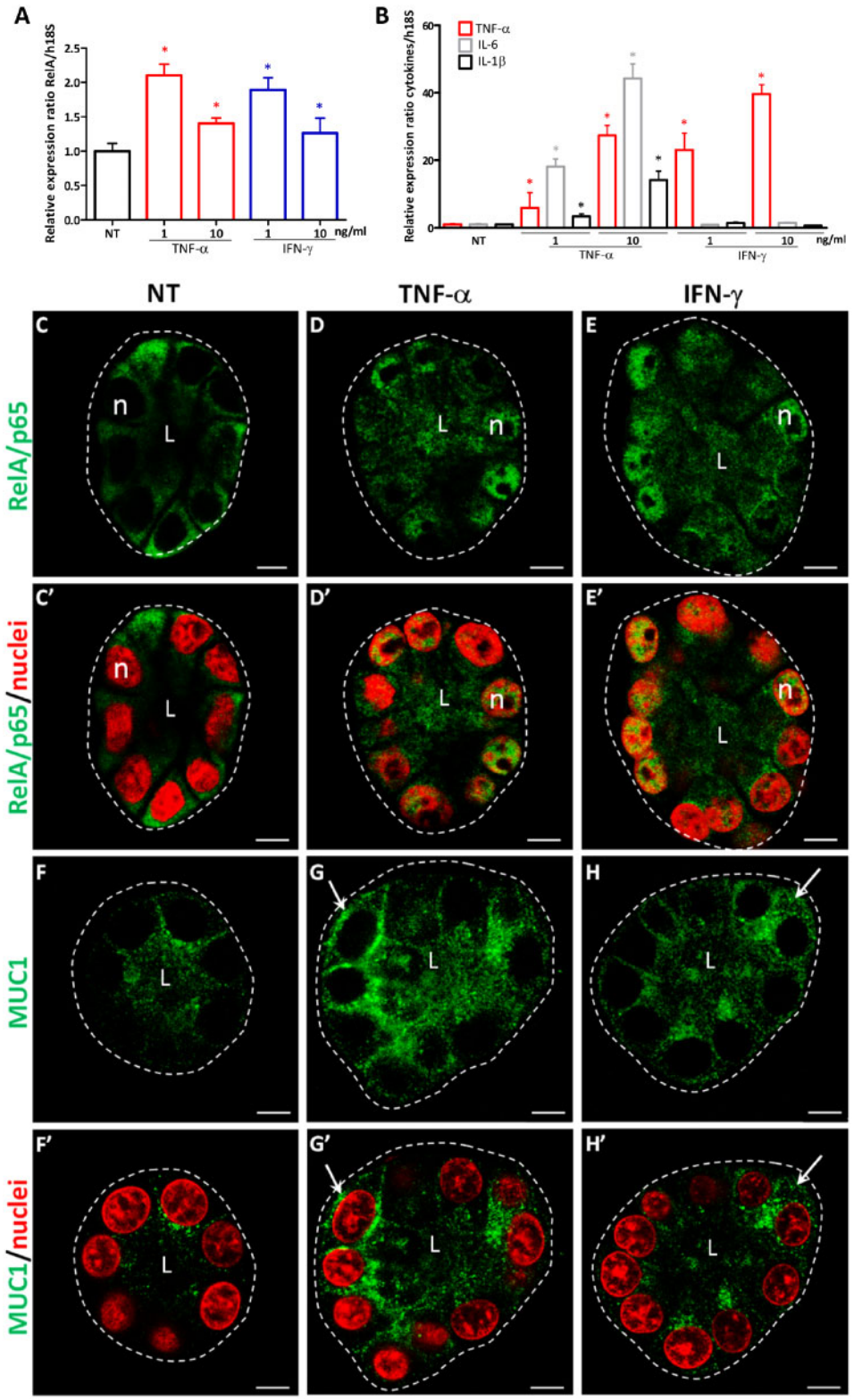
One serious problem that SS patients suffer daily is the dryness of their oral mucosa. We have previously demonstrated that this dryness is not just a consequence of the reduced volume of saliva [3], but that MUCs play a leading role in mucosa hydration and protection, with hydrophilic groups essentially retaining water molecules in the epithelial surface [31, 32]. Here, MUC1 was observed

FIG. 3 TNF-α and IFN-γ induce MUC1 expression and accumulation in 3D acini



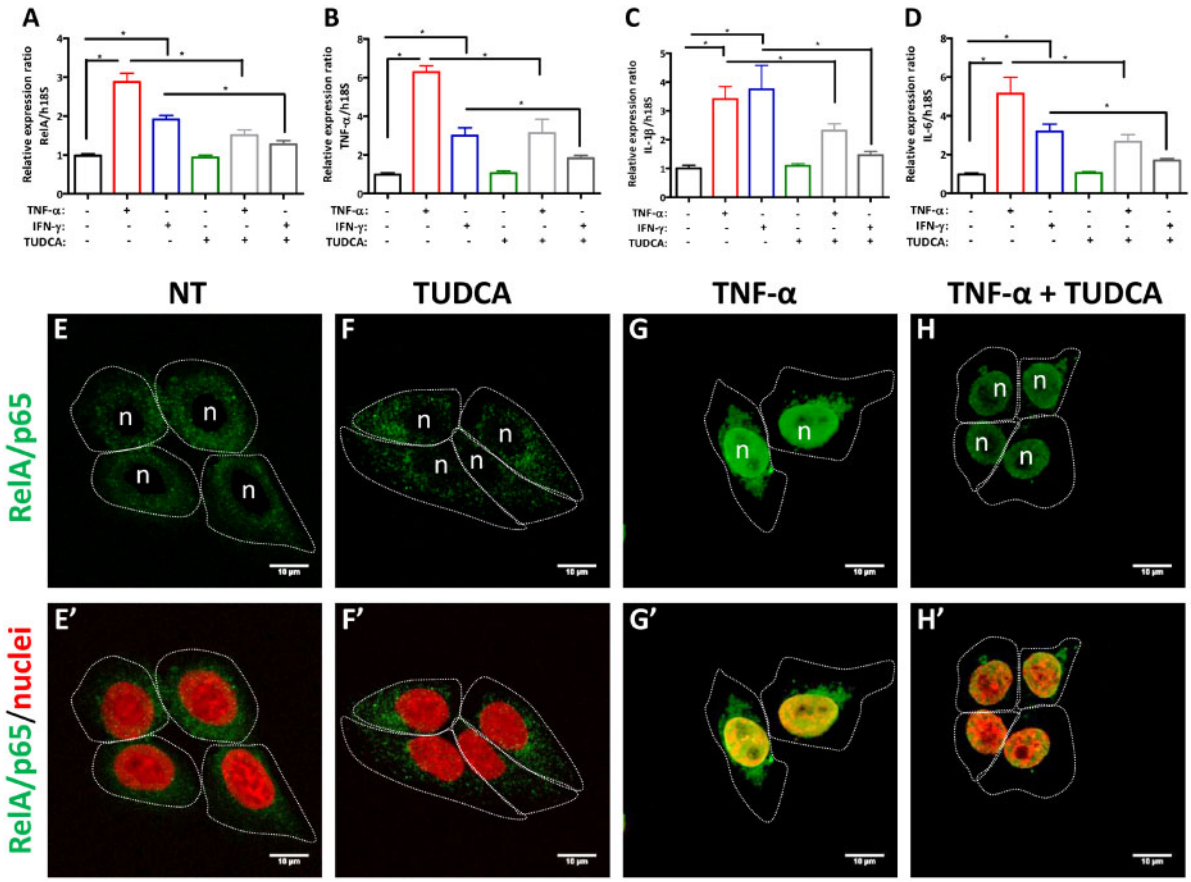
(**A**–**C**) Relative MUC1 transcript levels and representative MUC1 WB (90–300 kDa) in 3D acini stimulated with TNF-α or IFN-γ. Relative mRNA and protein levels were normalized to NT condition. (**D**) Representative NT acini showing MUC1 (red) in a lateral and supranuclear cytoplasmic localization and GRP78 (green) in the basolateral region. (**E**) Representative acini stimulated with 10 ng/ml TNF-α for 24 h showing MUC1 also in the basolateral region. Bars 10 μm, L: lumen. (**F**, **G**) GRP78 or MUC1 were immunoprecipitated from protein extracts of cytokine stimulated 3D acini and then analysed by WB. Data are representative of five independent experiments. *P-values <0.05 were considered significant. IP: immunoprecipitation; NT: non-treated; WB: western blot.

FIG. 4 TNF-α and IFN-γ induce the activation of NF-κB and inflammatory marker expression in 3D acini



(**A**, **B**) Relative transcript levels of RelA/p65, TNF-α, IL-6 and IL-1β in 3D acini stimulated with TNF-α or IFN-γ. Relative mRNA levels were normalized to NT condition. (**C**) Representative RelA/p65 (green) staining in epithelial cell cytoplasm of NT acini. (**D**, **E**) In 3D acini stimulated with 10 ng/ml TNF-α or IFN-γ a nuclear (n) RelA/p65 staining was detected. (**F**–**H**) Representative acini showing MUC1 (green) in non-treated (NT) or in 3D acini stimulated with 10 ng/ml TNF-α or IFN-γ. Bars 10 μm, L: lumen. Data are representative of five independent experiments. **P*-values <0.05 were considered significant. NF-κb: nuclear factor-κB; NT: non-treated.

FIG. 5 TUDCA reduces pro-inflammatory markers and NF-κB activation in HSG cells stimulated with TNF-α or IFN-γ



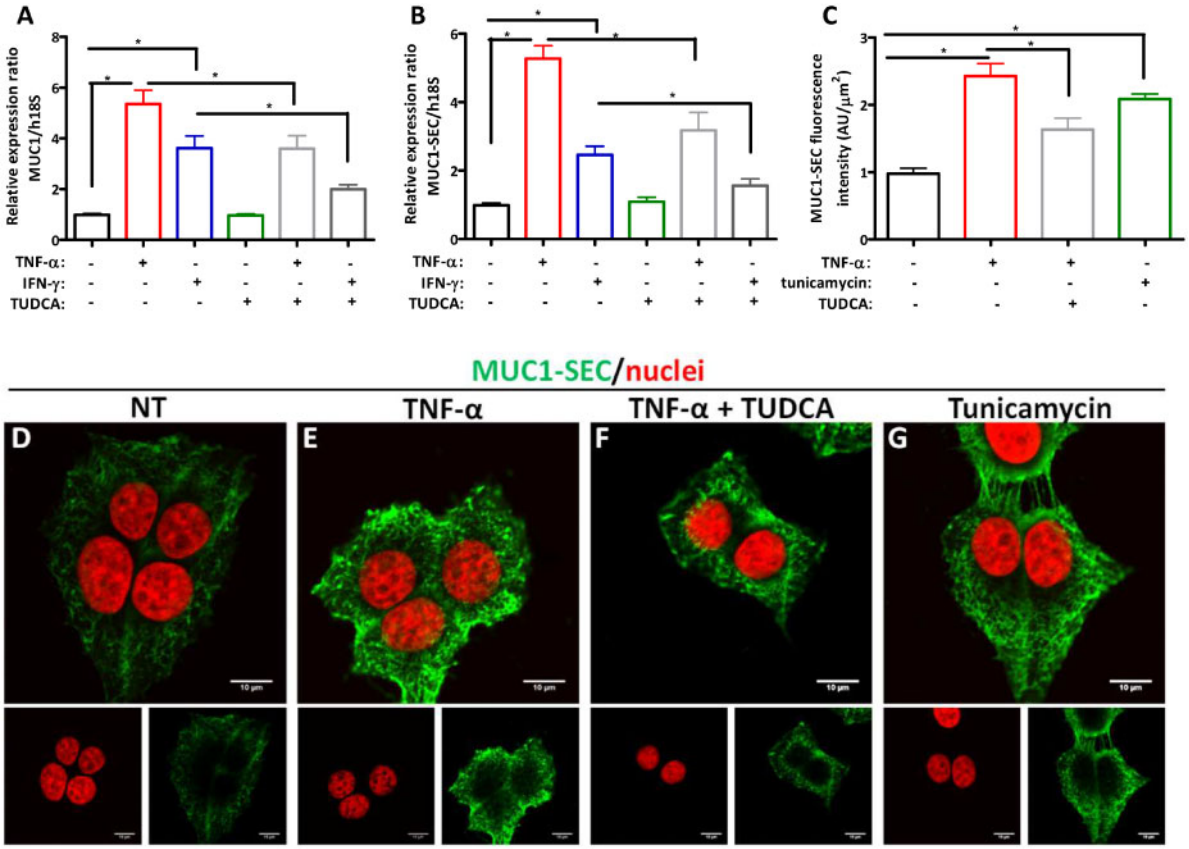
(**A**–**D**) Relative transcript levels of RelA/p65, TNF-α, IL-1β and IL-6 in 3D acini stimulated with 10 ng/ml TNF-α or IFN-γ and co-incubated with 250 μM TUDCA. (**E**, **F**) Representative RelA/p65 (green) staining in the cytoplasm of non-treated (NT, E) or 250 μM TUDCA-stimulated (F) HSG cells. (**G**, **H**) In HSG cells stimulated with 10 ng/ml TNF-α, an intense nuclear (n) staining of RelA/p65 was detected, which was decreased by co-incubation with TUDCA. Bars 10 μm. Data are representative of five independent experiments. *P-values <0.05 were considered significant. HSG: human submandibular gland; NF-κb: nuclear factor-κB; TUDCA: tauroursodeoxycholic acid.

accumulated in the ER of LSG from SS patients, co-localizing and co-precipitating with GRP78, a chaperone that binds to unfolded proteins in the ER lumen. Previous evidence from our laboratory has revealed changes indicative of ER stress and altered UPR in LSG from SS patients, such as ER cistern dilation [8], decreased IRE1α/XBP1 pathway activation and GRP78 levels [9], and ATF6α pathway activation, promoting an increase in ERAD [10]. Under physiological conditions, a variable percentage of proteins fold inadequately during ER synthesis, with this percentage being proportional to the amount and complexity of synthesized proteins [33]. MUC1 is an example of a complex protein because of its extracellular domain comprising a variable number of tandem repeat regions of 20 amino acids that can be repeated from 20 to 125 times, each repeat including five potential *O*-glycosylation sites [34]. MUC1 can interact with outside (MUC7 and MUC5B) [31] and inside cell proteins through their extracellular and cytoplasmic domains, respectively [34]. Given its structural complexity when exposed to cellular stress, MUC1 could become misfolded and inadequately associated with other proteins, which can occur when ER-Ca^{2+} levels decrease or with the increase of reactive oxygen species levels, among other causes [35].

In the present study, cytokine-induced MUC1 overexpression and accumulation in 3D acini were observed under similar conditions to those that activated the ATF6α pathway of the UPR and ERAD [10]. These results suggest that in LSG of SS patients, the inflammatory environment increased the synthesis of salivary MUCs, promoting ER stress, explaining MUC1 accumulation observed in this compartment. In murine fibrosarcoma L929 cells, TNF-α induces reactive oxygen species accumulation, activating the UPR and ER cistern dilation [36]. Moreover, in β pancreatic cells, the incubation with TNF-α, IFN-γ and IL-1β activates the UPR, evidenced by the increase in PERK and eIF2α phosphorylation [37].

No changes were found in MUC7 transcript levels, although protein levels increased and sialylation rate decreased in LSG of SS patients. Reduced MUC7

FIG. 6 TUDCA reduces MUC1 expression and accumulation in HSG cells stimulated with TNF-α or IFN-γ



(**A**, **B**) Relative MUC1 and MUC1-SEC transcript levels. (**C**, **F**) Representative MUC1-SEC (green) staining in the cytoplasm of non-treated (NT) HSG cells (**D**). In HSG cells stimulated with 10 ng/ml TNF-α, an intense cytoplasmic MUC1 staining was detected (**E**), which was decreased by co-incubation with TUDCA (F). (**G**) HSG cells were stimulated with 0.05 μg/ml tunicamycin (positive control of ER stress), showing an intense MUC1-SEC staining. At the bottom, images (D)–(G) are shown as separated channels. Bars 10 μm. Data are representative of five independent experiments. **P*-values <0.05 were considered significant. ER: endoplasmic reticulum; HSG: human submandibular gland; TUDCA: tauroursodeoxycholic acid.

sialylation confirmed previous studies showing alterations in the Golgi glycosylation machinery [3, 38, 39] and changes in MUC7 sialic acid content in residual saliva of patients with xerostomia [40]. In LSG of SS patients MUC7 showed an intense staining distributed throughout the cytoplasm of acinar cells, without co-localizing with GRP78 in the ER. However, MUC7 co-localizes with Rab3D in the cytoplasm near the acinar lumen in LSG of control subjects, while in SS patients, both proteins co-localize in the basolateral region [41], suggesting that MUC7 accumulates in mature secretion granules in the basolateral region. We believe that this is probably due to pro-inflammatory cytokine-induced loss of baso-apical directionality of the secretory process [42], concurrent with loss of acinar cell polarity [8], soluble N-ethylmaleimide-sensitive factor attachment protein receptor complexes in the basolateral plasma membrane [11, 43], and exocytosis of MUC7 and MUC5B toward the extracellular matrix in LSG from SS patients [28]. Interestingly, ectopically secreted salivary MUCs act as damage-associated molecular patterns recognized by Toll-like receptor 4, inducing an inflammatory loop through transcription of TNF-α, IFN-α, IFN-β, IL-6, IL-1β, CXCL8 and MUC5B in HSG cells [28], supporting the existence of a cross-regulation between cytokines and MUCs that would perpetuate inflammation in LSGs from SS patients [28, 44].

Stimulation of 3D acini with TNF-α or IFN-γ increased MUC1 transcript and protein levels and its relocation to the basolateral region of epithelial cells reproducing our findings in LSGs of SS patients. Furthermore, the *MUC1* gene promoter sequence presents response elements for cytokine-activated transcription factors [14, 45], with four STAT response elements being located near the TATA box. In breast cancer cell lines, IFN-γ and IL-6 activate STAT1 and STAT3, respectively, interacting with STAT elements in the −503 and −485 positions of the MUC1 promoter inducing its overexpression [46]. In addition, MUC1 expression increases in airway cells after infection with *Pseudomonas aeruginosa*, dependent on TNF

receptor 1 receptor activation, since receptor silencing inhibits MUC1 overexpression [47]. Additionally, TNF-α activates NF-κB interaction with response elements in the *MUC1* promoter inducing its transcription [14].

Given that LSG from SS patients showed an overexpression and accumulation of MUC1, we hypothesize whether these changes are due to NF-κB activation. With this in mind, we determined and demonstrated that TNF-α or IFN-γ induced nuclear translocation of RelA/p65 in 3D acini, while TNF-α stimulated expression of RelA/p65, IL-6 and IL-1β transcripts, whereas IFN-γ induced RelA/p65 and TNF-α transcripts. Thus, pro-inflammatory cytokines induced nuclear RelA/p65 translocation in 3D acini, suggesting that MUC1 overexpression could be mediated by NF-κB activation.

Interestingly, TUDCA has recently being reported that to inhibit NF-κB signalling and DNA-binding activity, as well as IL-8 and IL-1α expression and suppressed IκBα phosphorylation and degradation in TNF-α-stimulated human colon cancer (HCT 116) cells [18].

Here, we confirmed an anti-inflammatory effect of TUDCA evidenced by decreased translocation of RelA/p65 and mRNA cytokine levels in HSG cells stimulated with TNF-α. A cytokine-induced increase of SEL1L and EDEM1 was subsequently reverted by TUDCA, corroborating the cytokine role as local ER stressors inducing ERAD hyperactivation [10], and the modulator role of TUDCA as a chemical chaperone on ER stress [48]. The current study also shows that TUDCA decreased cytokine-induced aberrant MUC1 accumulation in salivary gland epithelial cells. Previously, the effect of TUDCA has been studied in a wide range of cell types and human animal models of diseases associated with apoptosis, inflammation and ER stress [24]. In a mouse model of allergic asthma, TUDCA decreased the expression of MUC5AC, ATF6α, GRP78, C/EBP homologous protein and proinflammatory markers (IL-6, IL-13, IL-17) [49]. The administration of TUDCA led to an enrichment of ursodeoxycholic acid, which replaces hydrophobic bile acids, thereby attenuating cholestasis and hepatocellular injury in patients with primary biliary cholangitis [50].

All our results taken together suggest that the anti-inflammatory properties of TUDCA ameliorate MUC1 overexpression and aberrant accumulation, probably by modulating N-FκB activation. Further *in vivo* studies are necessary to assess the potential therapeutic effect of TUDCA in restoring glandular function in SS patients.

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Supplementary data

Supplementary data are available at *Rheumatology* online.

References

1 Mavragani CP, Moutsopoulos HM. Sjogren's syndrome. Annu Rev Pathol 2014;9:273–85.

2 Eliasson L, Birkhed D, Carlén A. Feeling of dry mouth in relation to whole and minor gland saliva secretion rate. Arch Oral Biol 2009;54:263–7.

3 Castro I, Sepúlveda D, Cortes J *et al*. Oral dryness in Sjogren's syndrome patients. Not just a question of water. Autoimmun Rev 2013;12:567–74.

4 Chaudhury NM, Shirlaw P, Pramanik R, Carpenter GH, Proctor GB. Changes in saliva rheological properties and mucin glycosylation in dry mouth. J Dent Res 2015;94:1660–7.

5 Thornton DJ, Rousseau K, McGuckin MA. Structure and function of the polymeric mucins in airways mucus. Annu Rev Physiol 2008;70:459–86.

6 Barrera MJ, Aguilera S, Castro I *et al*. Endoplasmic reticulum stress in autoimmune diseases: can altered protein quality control and/or unfolded protein response contribute to autoimmunity? A critical review on Sjögren's syndrome. Autoimmun Rev 2018;17:796–808.

7 Michael DG, Pranzatelli TJF, Warner BM, Yin H, Chiorini JA. Integrated epigenetic mapping of human and mouse salivary gene regulation. J Dent Res 2019;98:209–17.

8 Goicovich E, Molina C, Pérez P *et al*. Enhanced degradation of proteins of the basal lamina and stroma by matrix metalloproteinases from the salivary glands of Sjögren's syndrome patients: correlation with reduced structural integrity of acini and ducts. Arthritis Rheum 2003;48:2573–84.

9 Sepúlveda D, Barrera MJ, Castro I *et al*. Impaired IRE1α/XBP-1 pathway associated to DNA methylation might contribute to salivary gland dysfunction in Sjögren's syndrome patients. Rheumatology (Oxford) 2018;57:1021–32.

10 Barrera MJ, Aguilera S, Castro I *et al*. Pro-inflammatory cytokines enhance ERAD and ATF6α pathway activity in

salivary glands of Sjögren's syndrome patients. J Autoimmun 2016;75:68–81.

11 Barrera MJ, Sánchez M, Aguilera S *et al*. Aberrant localization of fusion receptors involved in regulated exocytosis in salivary glands of Sjögren's syndrome patients is linked to ectopic mucin secretion. J Autoimmun 2012;39:83–92.

12 Fox PC, Brennan M, Di Sun P. Cytokine expression in human labial minor salivary gland epithelial cells in health and disease. Arch Oral Biol 1999;44:S49–52.

13 Roescher N, Tak PP, Illei GG. Cytokines in Sjögren's syndrome. Oral Dis 2009;15:519–26.

14 Andrianifahanana M, Moniaux N, Batra SK. Regulation of mucin expression: mechanistic aspects and implications for cancer and inflammatory diseases. Biochim Biophys Acta 2006;1765:189–222.

15 Li S, Intini G, Bobek LA. Modulation of MUC7 mucin expression by exogenous factors in airway cells in vitro and in vivo. Am J Respir Cell Mol Biol 2006;35:95–102.

16 Sung HH, Castro I, González S *et al*. MUC1/SEC and MUC1/Y overexpression is associated with inflammation in Sjögren's syndrome. Oral Dis 2015;21:730–8.

17 Cao SS, Zimmermann EM, Chuang BM *et al*. The unfolded protein response and chemical chaperones reduce protein misfolding and colitis in mice. Gastroenterology 2013;144:989–1000.e6.

18 Kim YH, Kim JH, Kim BG *et al*. Tauroursodeoxycholic acid attenuates colitis-associated colon cancer by inhibiting nuclear factor kappaB signaling. J Gastroenterol Hepatol 2019;34:544–51.

19 Lleo A, Marzorati S, Anaya JM, Gershwin ME. Primary biliary cholangitis: a comprehensive overview. Hepatol Int 2017;11:485–99.

20 Selmi C, Gershwin ME. Chronic autoimmune epithelitis in Sjögren's syndrome and primary biliary cholangitis: a comprehensive review. Rheumatol Ther 2017;4:263–79.

21 Gronbeck KR, Rodrigues CM, Mahmoudi J *et al*. Application of tauroursodeoxycholic acid for treatment of neurological and non-neurological diseases: is there a potential for treating traumatic brain injury? Neurocrit Care 2016;25:153–66.

22 Yanguas-Casás N, Barreda-Manso MA, Nieto-Sampedro M, Romero-Ramírez L. Tauroursodeoxycholic acid reduces glial cell activation in an animal model of acute neuroinflammation. J Neuroinflammation 2014;11:50.

23 Yanguas-Casás N, Barreda-Manso MA, Pérez-Rial S, Nieto-Sampedro M, Romero-Ramírez L. TGFβ contributes to the anti-inflammatory effects of tauroursodeoxycholic acid on an animal model of acute neuroinflammation. Mol Neurobiol 2017;54:6737–49.

24 Vang S, Longley K, Steer CJ, Low WC. The unexpected uses of urso- and tauroursodeoxycholic acid in the treatment of non-liver diseases. Glob Adv Health Med 2014;3:58–69.

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

26 Schall GL, Anderson LG, Wolf RO *et al*. Xerostomia in Sjögren's syndrome. Evaluation by sequential salivary scintigraphy. JAMA 1971;216:2109–16.

27 Daniels TE. Labial salivary gland biopsy in Sjögren's syndrome. Assessment as a diagnostic criterion in 362 suspected cases. Arthritis Rheum 1984;27:147–56.

28 Barrera MJ, Aguilera S, Veerman E *et al*. Salivary mucins induce a Toll-like receptor 4-mediated pro-inflammatory response in human submandibular salivary cells: are mucins involved in Sjögren's syndrome? Rheumatology (Oxford) 2015;54:1518–27.

29 Yuan JS, Reed A, Chen F, Stewart CN Jr. Statistical analysis of real-time PCR data. BMC Bioinformatics 2006;7:85.

30 Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54.

31 Offner GD, Troxler RF. Heterogeneity of high-molecular-weight human salivary mucins. Adv Dent Res 2000;14:69–75.

32 Hattrup CL, Gendler SJ. Structure and function of the cell surface (tethered) mucins. Annu Rev Physiol 2008;70:431–57.

33 Hasnain SZ, Tauro S, Das I *et al*. IL-10 promotes production of intestinal mucus by suppressing protein misfolding and endoplasmic reticulum stress in goblet cells. Gastroenterology 2013;144:357–68.

34 Martínez-Sáez N, Peregrina JM, Corzana F. Principles of mucin structure: implications for the rational design of cancer vaccines derived from MUC1-glycopeptides. Chem Soc Rev 2017;46:7154–75.

35 Chakrabarti A, Chen AW, Varner JD. A review of the mammalian unfolded protein response. Biotechnol Bioeng 2011;108:2777–93.

36 Xue X, Piao JH, Nakajima A *et al*. Tumor necrosis factor alpha (TNFalpha) induces the unfolded protein response (UPR) in a reactive oxygen species (ROS)-dependent fashion, and the UPR counteracts ROS accumulation by TNFalpha. J Biol Chem 2005;280:33917–25.

37 Chan JY, Biden TJ, Laybutt DR. Cross-talk between the unfolded protein response and nuclear factor-κB signalling pathways regulates cytokine-mediated beta cell death in MIN6 cells and isolated mouse islets. Diabetologia 2012;55:2999–3009.

38 Alliende C, Kwon YJ, Brito M *et al*. Reduced sulfation of muc5b is linked to xerostomia in patients with Sjögren syndrome. Ann Rheum Dis 2008;67:1480–7.

39 Castro I, Aguilera S, Brockhausen I *et al*. Decreased salivary sulphotransferase activity correlated with inflammation and autoimmunity parameters in Sjögren's syndrome patients. Rheumatology (Oxford) 2012;51:482–90.

40 Chaudhury NM, Proctor GB, Karlsson NG, Carpenter GH, Flowers SA. Reduced mucin-7 (Muc7) sialylation and altered saliva rheology in Sjögren's syndrome associated oral dryness. Mol Cell Proteomics 2016;15:1048–59.

41 Bahamondes V, Albornoz A, Aguilera S *et al*. Changes in Rab3D expression and distribution in the acini of Sjögren's syndrome patients are associated with loss of cell polarity and secretory dysfunction. Arthritis Rheum 2011;63:3126–35.

42 Ewert P, Aguilera S, Alliende C *et al*. Disruption of tight junction structure in salivary glands from Sjögren's syndrome patients is linked to proinflammatory cytokine exposure. Arthritis Rheum 2010;62:1280–9.

43 Cortés J, Hidalgo J, Aguilera S *et al*. Synaptotagmin-1 overexpression under inflammatory conditions affects secretion in salivary glands from Sjögren's syndrome patients. J Autoimmun 2019;97:88–99.

44 Tarang S, Kumar S, Batra SK. Mucins and toll-like receptors: kith and kin in infection and cancer. Cancer Lett 2012;321:110–9.

45 Jonckheere N, Van Seuningen I. The membrane-bound mucins: from cell signalling to transcriptional regulation and expression in epithelial cancers. Biochimie 2010;92:1–11.

46 Gaemers IC, Vos HL, Volders HH, van der Valk SW, Hilkens J. A stat-responsive element in the promoter of the episialin/MUC1 gene is involved in its overexpression in carcinoma cells. J Biol Chem 2001;276:6191–9.

47 Choi S, Park YS, Koga T, Treloar A, Kim KC. TNF-α is a key regulator of MUC1, an anti-inflammatory molecule, during airway Pseudomonas aeruginosa infection. Am J Respir Cell Mol Biol 2011;44:255–60.

48 Siddesha JM, Nakada EM, Mihavics BR *et al*. Effect of a chemical chaperone, tauroursodeoxycholic acid, on HDM-induced allergic airway disease. Am J Physiol Lung Cell Mol Physiol 2016;310:L1243–59.

49 Shekels LL, Lyftogt CT, Ho SB. Bile acid-induced alterations of mucin production in differentiated human colon cancer cell lines. Int J Biochem Cell Biol 1996;28:193–201.

50 Ma H, Zeng M, Han Y *et al*. A multicenter, randomized, double-blind trial comparing the efficacy and safety of TUDCA and UDCA in Chinese patients with primary biliary cholangitis. Medicine (Baltimore) 2016;95:e5391.