Salivary mucins induce a Toll-like receptor 4-mediated pro-inflammatory response in human submandibular salivary cells: are mucins involved in Sjögren’s syndrome?

María-José Barrera, Sergio Aguilera, Enno Veerman, Andrew F. G. Quest, David Díaz-Jiménez, Ulises Urzúa, Juan Cortés, Sergio González, Isabel Castro, Claudio Molina, Verónica Bahamondes, Cecilia Leyton, Marcela A. Hermoso and María-Julieta González

Abstract

Objectives. A hallmark characteristic of SS patients is the ectopic presence of the mucins MUC5B and MUC7 in the extracellular matrix of salivary glands that have lost apical–basolateral acinar-cell polarity. This study aims to determine whether exogenous salivary mucins induce gene expression of pro-inflammatory cytokines, as well as to evaluate whether the Toll-like receptor-4 (TLR4) pathway is involved in this response.

Methods. Differentiated human submandibular gland (HSG) cells were stimulated with mucins or oligosaccharide residues at different concentrations and for different periods of time. The expression of pro-inflammatory cytokines and their receptors was determined by semi-quantitative real time PCR (sqPCR). TLR4-mediated responses induced by mucin were evaluated with the Toll-IL-1 receptor domain containing adaptor protein (TIRAP) inhibitory peptide or using anti-hTLR4 blocking antibody. TLR4-receptor expression was also determined in SS patients, controls and HSG cells.

Results. Mucins induced a significant increase in CXCL8, TNF-α, IFN-α, IFN-β, IL-6 and IL-1β, but not B cell activating factor (BAFF). Cytokine induction was mediated by TLR4, as shown using TIRAP or using anti-hTLR4 antibody. Sugar residues present in MUC5B, such as sulpho-Lewis (SO3-3Galj1-3GlcNAc), also induced cytokines. Unexpectedly, mucins induced MUC5B, but not MUC7 expression.

Conclusion. Salivary mucins were recognized by TLR4 in epithelial cells initiating a pro-inflammatory response that could attract inflammatory cells to amplify and perpetuate inflammation and thereby contribute to the development of a chronic state characteristic of SS. The ectopic localization of MUC5B and MUC7 in the salivary gland extracellular matrix from SS patients and the current results reveal the importance of salivary epithelial cells in innate immunity, as well as in SS pathogenesis.

Key words: Sjögren’s syndrome, salivary mucins, human salivary cells, TLR4, inflammation, cytokines.
Salivary mucins induce a pro-inflammatory response

### Rheumatology key messages

- Mucins and sulpho-Lewis sugar residues are inducers of inflammatory responses in human salivary cells.
- Mucins induce pro-inflammatory cytokines mediated by TLR4 in human salivary cells.
- Salivary mucins act as DAMPs activating the innate immune response in SS.

### Introduction

Primary SS is a systemic disorder characterized by lymphocytic infiltration and functional impairment of salivary and lachrymal glands. While these represent the major affected sites, the inflammatory process can potentially spread to other organs [1]. Although the underlying cause of SS pathogenesis is not fully understood, several characteristics are well established, including loss of apicobasal polarity in salivary acinar cells [2–6]. Consistent with these observations, one working hypothesis attributes a significant role of the salivary gland epithelium itself in the initiation and perpetuation of local autoimmune responses. According to this idea, molecular changes in the epithelial cells result in recruitment, homing, activation, proliferation and differentiation of inflammatory cells [2, 7]. The loss of apicobasal polarity in salivary acinar cells in SS patients is associated with redistribution of the molecular machinery involved in exocytosis of secretory granules [3–6, 8]. Proteins involved in membrane fusion [soluble NSF attachment protein receptor (SNARE) proteins] relocate from the apical to the basolateral region of acinar cells [5], and redistribution of mature secretory granules in the cytoplasm is observed [5, 6]. In addition, exocytic fusion complexes formed by SNAREs, generally present in the apical plasma membrane, and secretory granules are found in the basolateral plasma membrane of salivary acinar cells from SS patients. Concomitantly with these changes, mucins, such as MUC5B and MUC7, are ectopically secreted into the extracellular matrix (ECM) [5].

Under physiological conditions, salivary mucins are secreted only towards the mouth and are efficient mucosal moisturizers that prevent desiccation [9, 10]. Mucins also form complexes with other proteins to bind bacteria and facilitate their clearance from the oral cavity [9]. However, the roles that ECM mucins play in the genesis of diseases are unknown.

Mucins are highly glycosylated glycoproteins, which are synthesized and N-glycosylated in the endoplasmic reticulum, O-glycosylated in the Golgi apparatus and then packaged into secretory granules [11, 12]. Mature secretory granules are localized in the apical region of polarized secretory cells, and their contents are released to the acini lumen, from where they access the oral cavity through ducts [11, 12]. Three types of mucin are predominantly expressed in salivary glands: (i) MUC1, a membrane-associated mucin [13], (ii) MUC7, a secreted monomeric mucin of low molecular mass (~200 kDa) [14] and (iii) MUC5B, a secreted gel-forming oligomeric mucin of high molecular mass (>1 MDa) [12, 14, 15]. The latter, MUC5B, is rich in sulpho-Lewis (SO$_3$-3Gal[1-3GlcNAc] residues [16]. Interestingly, in SS patients, decreased content and number of sulpho-Lewis-positive acini correlates with symptoms of mouth dryness, irrespective of whether labial salivary glands (LSGs) display normal or low unstimulated salivary flow [17].

We postulate that mucins present in the ECM may trigger an inflammatory response by acting as ligands activating potential receptors of epithelial or immune cells. A key question arising in this scenario concerns the type of receptor that could mediate such a response.

Toll-like receptors (TLRs) are a class of pattern recognition receptors that play a key role in innate immunity and trigger a specific immune response [18]. These receptors are stimulated by a variety of structural signatures found in pathogens, referred to as pathogen-associated molecular patterns, and TLR activation induces pro-inflammatory cytokines [18]. TLRs expressed on the cell surface, such as TLR1, 2, 4, 5, 6 and 10, recognize outer cell wall components of bacteria and fungi, whereas TLRs expressed in the intracellular compartments (TLR3, 7, 8 and 9) are involved in the recognition of nucleic acid components [19]. In addition, TLRs may also be activated by damage-associated molecular patterns (DAMPs) produced by the cell or the ECM, endogenous molecules released, activated or secreted by host cells and tissues undergoing stress, damage and non-physiological cell death [19]. Interestingly, TLR4 recognizes DAMPs that contain oligosaccharides and are present in the ECM, such as fibronectin, hyaluronic acid (HA) and heparan sulphate [19–21]. Termeer et al. [22] demonstrated that hyaluronan oligosaccharides activate dendritic cells via TLR4. Furthermore, the polysaccharide portion has been shown to play a role in Salmonella lipopolysaccharide-induced activation through human TLR4 [23]. After ligand binding, the cytoplasmic Toll/IL-1 receptor (TIR) domain of the TLRs associates with the TIR domain of adaptor proteins MyD88, TIRAP, TRIF and TRAM [24, 25]. Moreover and depending on the ligand, TLR4 activation may occur in a manner dependent on or independent of MyD88. In the first case, MyD88 and TIRAP protein adapters are recruited to the TIR domain and induce a signalling pathway leading to expression of pro-inflammatory cytokines and chemokines. Alternatively, the MyD88-independent pathway engages TRAM and TRIF as protein adapters to induce type-I interferon [24].

Salivary acinar cells express TLR4 and, for SS patients, a significant increase in TLR4 expression compared with controls is observed [26, 27]. Moreover, in the salivary glands of SS patients, chronic inflammation is apparent, although the mechanisms that trigger these processes are not known. In this study, we tested the hypothesis that salivary mucins are involved in the expression of pro-inflammatory cytokines, and explored the molecular sensor involved in this response. Furthermore, we examined whether mucin oligosaccharides might act as DAMPs...
that are recognized by TLR4, and activate the innate immune response.

Materials and methods

Patients with primary SS and controls

A total of 10 individuals participated in the present study. Six individuals were diagnosed as patients with primary SS according to the American/European consensus group criteria [28], and four subjects (age- and gender-matched) were controls. The controls were selected from individuals who did not fulfill the primary SS classification criteria. All control subjects were negative for RF, antinuclear, Ro and La antibodies. In LSG biopsies from controls, only mild, non-specific, chronic sialadenitis was observed (Chisholm and Mason, grade 1). A detailed description of demographic, serological and histological characteristics of the SS patients and controls is provided in supplementary Table S1, available at *Rheumatology* Online. Written consent was obtained from each subject according to the Declaration of Helsinki. The study was approved by the Ethical Committee of the Faculty of Medicine, University of Chile.

Biopsies

LSGs were obtained as previously described [29]. Following surgery, samples were immediately frozen in liquid nitrogen and stored at −80°C to extract RNA.

Cell culture

The human submandibular gland (HSG) cell line, originally established from an irradiated HSG [30], was kindly provided by Professor Bruce Baum (NIDCR, NIH, Bethesda, MD, USA). HSG cells were derived from intercalated ductal cells, which differentiate into acinar and myoepithelial cells [30]. Cells were cultured at 37°C, 5% CO₂, in DMEM: nutrient mixture F-12 (DMEM/F-12; Invitrogen, Carlsbad, CA, USA) supplemented with 5% characterized fetal bovine serum (FBS; Hyclone, Logan, UT, USA), 10 000 U/ml penicillin and 10 mg/ml streptomycin (Biological Industries, Israel). Cells were harvested using 0.25% trypsin/EDTA (Biological Industries, Israel). Cells were harvested using 0.25% trypsin/EDTA (Biological Industries, Israel) and suspended in culture media containing 5% FBS. Cells (5 × 10⁵ cells/well) were seeded in six-well plates. After 24 h, they were serum deprived for 24 h to induce differentiation into functional cells. The efficacy of this strategy has been corroborated in other cell types [31–33] as well as in our laboratory (Mónica Brito, unpublished data).

Mucin treatments

Differentiated HSG cells were incubated with or without mucin obtained from bovine submaxillary glands [BSM, lipopolysaccharide (LPS) free] (Sigma, St Louis, MO, USA) at different concentrations (0.001, 0.01, 0.1, 0.2 and 0.4 µM) for 2, 8 and 24 h. In addition, cells were also stimulated for 8 or 24 h with LPS (0.01, 0.1 and 0.2 µM) from *Escherichia coli* serotype O111:B4 (Sigma), MUC5B (MUC5B is from human source, isolated from human whole saliva [34], laboratory Dr Enno Veerman) (0.005 and 0.015 µM) and sulpho-Lewis (SO₂₃-Gal[1-3GlcNAc] (a synthetic carbohydrate antigen, which was obtained from Syntosome GmbH, Munich, Germany) [16] at concentrations of 21.6 and 54 µM. Cells were processed to extract RNA as described below.

IL-6 ELISA

HSG cells were incubated in the presence or absence of 0.4 µM BSM for 24 h. IL-6 concentrations were measured in culture supernatants using an ELISA kit for human IL-6 (DuoSet; R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s instructions. All samples were analysed in triplicate and expressed in picograms per millilitre. The assay is stable over time with a detection limit of 9.38 pg/ml, according to the manufacturer’s instructions.

Functional assay of TLR4

Cells were pre-treated for 2 h with medium containing or not containing TBX2 2 µg/ml (GKMAWFRGTLKPK KRPNSPESTQLQRTATPGGAVS), an inhibitory peptide of TIRAP, and then stimulated with LPS or mucin for 8 h. In the inhibition assays, cells were incubated in the presence or absence of anti-hTLR4-IgG 3 µg/ml mAb (mabg-htr4, clone W7C11; InvivoGen, San Diego, CA, USA) or IgG1 isotype control mAb (mabg1-ctrl, TBES; InvivoGen, San Diego, CA, USA) for 1 h at 37°C and then stimulated with LPS or mucin for 8 h.

Semi-quantitative real time-PCR

Relative levels of pro-inflammatory cytokines (TNF-α, IFN-α, IFN-β, IL-6, IL-1β and BAFF), CXCL8, TLR4, RelA (NF-κB-p65), MUC5B and MUC7 mRNA were determined by semi-quantitative real time PCR (sqPCR). RNA extraction, yield and purity were evaluated essentially as described [35]. Primer sequences for the genes studied were designed with the AmplifX 1.4 software (see supplementary Table S2, available at *Rheumatology* Online). For sqPCR reactions, the Brilliant II SYBR Green QPCR Master Mix kit (Stratagene, La Jolla, CA, USA) was employed. Template cDNA was obtained by reverse transcription of 1 µg of total RNA with SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). PCR amplification was realized as described [5]. PCR products were resolved by 2% agarose gel electrophoresis. The relative expression ratio of a target gene was expressed in comparison with h18S, as previously described [6] using the relative expression software tool (REST) program (V2.0.7). The mRNA expression ratio for the gene of interest versus h18S, both determined by sqPCR, is shown in box graphs. Ratios >1.0 indicated upregulation, and ratios <1.0 were indicative of downregulation.

Additionally, we determined cytokine receptor mRNA expression for TNF-α (TNFRSF1A), IFN-α (IFNAR1), IFN-β (IFNAR1), IFN-γ (IFNGR1), IL-6 (IL6R) and IL-1 β (IL1R1) in non-stimulated HSG cells (see primer sequences in supplementary Table S2, available at *Rheumatology* Online). The NF-κB immunofluorescence assay is available as supplementary data, available at *Rheumatology* Online.
Statistical analysis
All experiments were done in triplicate. Data presented in bar graphs are the mean (S.D.) of results obtained. Significant differences in mRNA levels between control and treated samples were assessed by comparing group means using the Pairwise Fixed Reallocation Randomization Test. IL-6 concentration mean values obtained by ELISA were compared using the Mann–Whitney test. \( P < 0.05 \) was considered significant.

Results
Mucins induced expression of pro-inflammatory components in HSG cells
Differentiated HSG cells were exposed to increasing concentrations (0–0.4 μM) of BSM for 24 h. sqPCR results showed that these cells expressed CXCL8, TNF-α, IFN-α, IFN-β, IL-6, IL-1β and BAFF. With the exception of BAFF, a significant increase in all cytokine-coding mRNAs was observed after 24 h of stimulation with 0.4 μM BSM (Fig. 1A). For CXCL8, TNF-α, IL-6 and IL-1β a significant increase in expression was already detectable after stimulation with 0.2 μM BSM (Fig. 1A). On the other hand, HSG cells expressed neither IFN-γ nor the anti-inflammatory cytokine IL-10 under basal conditions or following stimulation (data not shown). To determine the time-course of pro-inflammatory cytokine induction, HSG cells were exposed for 0, 2, 8 and 24 h to 0.4 μM BSM (Fig. 1B). For IL-6 and TNF-α induction, a similar pattern was observed. Expression of TNF-α mRNA increased significantly after only 2 h, while for IL-6, stimulation for 8 h was required. For both cytokines, mRNA levels remained elevated for up to 24 h (Fig. 1B). Considering that our main objective was to determine whether mucins induce the activation of pro-inflammatory cytokine genes, we evaluated their mRNA levels. However, only for IL-6 did we compare the mRNA and protein levels of IL-6, given the relevance of this cytokine in SS pathogenesis. For IL-6 quantified in the supernatants of HSG cells treated with mucins using ELISA, a significant increase was observed (Fig. 1C).

Additionally, we evaluated the expression of receptor mRNAs for some of the cytokines mentioned. Non-stimulated HSG cells were found to express the receptors for TNF-α, IL-6, IFN-α, IFN-β, IL-1β and IFN-γ (see supplementary Fig. S1, available at Rheumatology Online).

Mucin oligosaccharide residues induced expression of pro-inflammatory cytokines
MUC5B is a polymeric mucin synthesized in salivary acinar cells, and the most abundant mucin in saliva [34]. Sialylated and sulphated oligosaccharides account for 80% of its molecular mass and are responsible for the binding of water, a property that contributes to oral mucosa hydration [36]. Due to the importance of MUC5B in oral biology and its aberrant ECM location in LSGs of SS patients [5], we additionally sought to evaluate whether HSG cells induced a pro-inflammatory response upon exposure to purified human MUC5B or its sulpho-Lewis residues (SO₃-3Galβ1-3GlcNAc). The latter is a highly abundant oligosaccharide on MUC5B [37]. The results show that HSG cells responded to 0.005 μM MUC5B with a significant increase in TNF-α and IL-1β mRNA levels (\( P = 0.025 \), \( P = 0.001 \), respectively). Treatment with 0.015 μM MUC5B also induced a significant increase in IL-6 and TNF-α mRNA levels (\( P = 0.03 \), \( P = 0.02 \), respectively), while 54 μM sulpho-Lewis induced
HSG cells were cultured in the presence or absence of MUC5B (0.005 and 0.015 μM), sulpho-Lewis (Sulfo-Le, 21.6 and 54 μM) or BSM (0.4 μM) for 24 h. The bar graph shows the mRNA expression ratio of pro-inflammatory cytokines standardized to h18S. *P < 0.05 vs control culture (absence of BSM, Sulfo-Le or MUC5B in culture).

Mucins induced MUC5B expression by a NF-κB-independent mechanism

As lipoprotein P6 induces mucin transcription via TLR [19], we sought to determine whether addition of mucins to HSG cells induced expression of two relevant salivary mucins, MUC7 and MUC5B. HSG cells were stimulated with 0.4 μM BSM for 0, 2, 8 or 24 h. Interestingly, we observed notable differences in the patterns of mucin expression. For MUC5B a significant increase in expression was detectable after only 2 h of stimulation, and this was maintained for 24 h. In contrast, no significant increase in MUC7 expression was observed from 8 h onwards (Fig. 6A). In parallel, we also determined whether stimulation with mucins induced the expression of TLR4 and the NF-κB-p65 subunit. No significant changes in the mRNA levels of either protein were observed in HSG cells treated with mucins compared with untreated cells (Fig. 6A). Moreover, we also evaluated the activation of NF-κB-p65 in HSG cells by assessing NF-κB-p65 nuclear translocation. Immunofluorescence staining of paraformaldehyde-fixed HSG cells stimulated for 1 h with BSM (0.4 μM) or LPS (0.2 μM) revealed that NF-κB-p65 localized only to the cytoplasm. On the other hand, stimulation...
with 10 ng/ml TNF-α, as positive control, induced the nuclear translocation of NF-κB-p65 (Fig. 6B).

**Discussion**

In this study, we revealed for the first time that exogenous mucins induced the expression of pro-inflammatory cytokines in HSG cells. Moreover, evidence was obtained that oligosaccharides present in MUC5B, such as sulpho-Lewis (SO_3-Galβ1-3GlcNAc) were involved. Using two experimental strategies, we show that this response was mediated by TLR4, given that the TIRAP inhibitory peptide (TBX2) blocked the downstream receptor response, as did an antibody against TLR4. The TBX2 effect might be mediated by impaired recruitment of downstream effectors, while anti-TLR4 antibodies preclude the interaction of TLR4 with oligosaccharide ligands. Low et al. [38] showed that TBX2 selectively inhibits induction of pro-inflammatory cytokines after LPS stimulation of RAW 264.7 cells, as well as mouse and human primary macrophages. In addition to these suggested mechanisms, however, we cannot rule out the possibility that galectins and TIM family receptors might also play a role [40].

Muroi and Tanamoto [23] reported that LPS oligosaccharides of *Salmonella enterica* triggered an inflammatory response via TLR4 in THP-1 and HEK 293 cells, both of which expressed the human complex CD14/TLR4/MD-2. In RA, proteolytic products of HA have been detected [41]. HA is an important glycosaminoglycan that organizes the central core of aggrecan, the most abundant proteoglycan present in the cartilage matrix of joints [42]. Proteolytic products of HA generated at sites of inflammation were recognized by TLR4 and triggered a potent pro-inflammatory response [22]. The present results show...
Fig. 6 Mucins induced MUC5B expression by a NF-κB-independent mechanism

(A) HSG cells were cultured in the presence or absence of BSM (0.4 μM) for 2, 8 or 24 h. The bar graph shows mRNA expression ratios of TLR4, RelA (p65 subunit of NF-κB), MUC7 and MUC5B relative to h18S. *P < 0.05 vs control culture (absence of BSM in culture). (B) The distribution of NF-(B-p65 (green) was detected in non-stimulated HSG cells (a) or cells stimulated with LPS (0.2 μM) (b), BSM (0.4 μM) (c) or TNF-α (10 ng/ml) (d) for 1 h. Nuclei (red). Bars: 5 μm.
that oligosaccharides derived from human MUC5B can also initiate an inflammatory response.

Our results agree with previous studies in HSG cells, in which stimulation with LPS induced IL-6 expression through activation of TLR4 [26]. These authors also observed higher levels of TLR4 and MyD88 in LSGs from SS patients when compared with control subjects [26]. TLR4- and MyD88-specific immunoreactivity was observed both in infiltrating mononuclear cells and in acinar and ductal epithelial cells [26]. The present study confirmed that TLR4 expression in SS patients is significantly increased in comparison with that observed in control individuals (see supplementary Fig. S2, available at Rheumatology Online). The inhibition in the expression of cytokines observed in response to TLR ligands (TIRAP inhibitor peptide) treatment suggests that cytokine induction by mucins occurs through a MyD88-dependent pathway, given that MyD88 and TIRAP protein adapter recruitment to the TIR domain and downstream signalling events are required for induction of the expression of pro-inflammatory cytokines and chemokines.

We also evaluated whether the NF-κB pathway is involved in induction of pro-inflammatory cytokines following the binding of mucin to TLR4. To this end, expression of the p65 subunit of NF-κB at various time points after stimulation was determined by sqPCR. No significant changes in mRNA levels of this subunit were observed in mucin-treated HSG cells compared with untreated cells. Our immunofluorescence studies revealed that recognition of mucins and LPS via the TLR4 receptor did not induce NF-κB-p65 activation in HSG cells. These results are in agreement with those obtained by Kawakami et al. [26], who similarly did not observe nuclear translocation of NF-κB-p65 after treatment with TLR ligands.

Another novel finding of our study is that BSM induced MUC5B, but not MUC7 expression. Moreover, LPS induces expression of mucins, such as MUC5B and MUC5AC in certain airway epithelial cells [43, 44]. The MUC5B and MUC5AC genes are located in a cluster on chromosome 11p15.5 and have in common several regulatory elements identified in the 5′ and 3′ flanking regions of both genes (i.e. sites for c-Myc, N-Myc, Sp1 and NF-κB, AP-1, CREB, HNF-1, HNF-3, TGT3, GKL, TTF1, GRE) [45, 46]. MUC5AC is induced in response to LPS through a signalling pathway involving p38MAPK [47]. Given the similarity in gene regulation of MUC5AC and MUC5B, we suggest that the induction of MUC5B observed in HSG cells by mucin stimulation may also occur through the MAPK pathway. It has been reported that MUC5B might be upregulated by an autocrine–paracrine loop involving IL-6-dependent extracellular signal-regulated kinase (ERK)1/2 activation, which further enhances mucin-induced MUC5B expression [48].

The decrease in MUC7 levels suggests the existence of differential regulatory mechanisms for each mucin. Previous studies have shown that NCI-H292 cells expressing TLR4 do not increase MUC7 expression when stimulated with LPS (Pseudomonas aeruginosa). Instead, increased MUC5B expression was observed [49]. In addition, in A549 lung carcinoma cells expressing TLR4, MUC7 transcription did not increase in response to LPS [49]. The proximal promoter region of the MUC7 gene contains two binding sites for AP-1 and one for NF-κB. AP1 is thought to control constitutive MUC7 expression, while NF-κB is important in TNF-α-activated MUC7 transcription [50]. Such differences may explain why MUC7 and MUC5B responses to mucin stimulation are so distinct.

In summary, the alterations in cell polarity lead to loss of the innate epithelial barrier function, triggering a series of changes that result in the release of mucins to the ECM. Human salivary MUC5B, BSM and sulpho-Lewis are recognized by epithelial cell TLR4 and initiate a pro-inflammatory response. These signals, produced initially by epithelial cells, could attract inflammatory cells, which perpetuate inflammation and the development of chronic disease. These findings highlight the importance of salivary gland epithelial cell organization in controlling innate immunity, and in the aetiopathogenesis of SS.

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Supplementary data

Supplementary data are available at Rheumatology Online.

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