Immune response against the coiled coil domain of Sjögren's syndrome associated autoantigen Ro52 induces salivary gland dysfunction

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ABSTRACT

Objective. *The structural domains of Ro52, termed the RING, B-box, coiled coil (CC) and B30.2/SPRY are targets of anti-Ro52 in multiple autoimmune disorders. In Sjögren's syndrome patients, the presence of anti-Ro52 is associated with higher disease severity, and in mice, they induce salivary gland hypofunction. This study was undertaken to investigate whether immune responses against different domains of Ro52, influences salivary gland disease in mice.*

Methods. *Female NZM2758 mice were immunised with Ro52 domains expressed as recombinant fusion proteins with maltose binding protein (MBP) [MBP-RING-B-box, MBP-CC, MBP-CC(ΔC19), MBP-B30.2/SPRY]. Sera from immunised mice were studied for IgG antibodies to Ro52 by immunoprecipitation, and to salivary gland cells by immunofluorescence. Pilocarpine-induced saliva production was measured to evaluate salivary gland function. Submandibular glands were investigated by histopathology for inflammation and by immune-histochemistry for IgG deposition.*

Results. *Mice immunised with different Ro52-domains had comparable reactivity to Ro52 and to salivary gland cells. However, only mice immunised with the CC domain and its C-terminal truncated version CC(ΔC19) showed a significant drop in saliva production. None of the mice developed severe salivary gland inflammation. The salivary gland hypofunction significantly correlated with increased intra-lobar IgG deposits in the submandibular salivary glands.*

Conclusion. *Our data demonstrate that epitope specificity of anti-Ro52 antibodies plays a critical role in the induction of glandular dysfunction. Clearly, screening Sjögren's syndrome patients for relative levels of Ro52 domain specific antibodies will be more informative for associating anti-Ro52 with clinical measures of the disorder.*

Introduction

Anti-Ro52 antibodies are detected in patients with Sjögren's syndrome (SjS), systemic lupus erythematosus, autoimmune hepatitis, primary biliary cirrhosis and idiopathic inflammatory myopathy (1). In SjS, the frequency of anti-Ro52 has been reported to be as high as 70% and these autoantibodies are generally associated with higher severity of disease (2). Ro52, also known as TRIM21, consists of RING, B-box and coiled coil (CC) domains. Structurally, this RBCC motif is characteristic of the family of tripartite motif (TRIM) containing proteins (3). The C-terminal B30.2/SPRY domain puts Ro52 in the C-IV class of TRIM proteins. Functionally, Ro52 is an E3 ubiquitin ligase, with a distinct role for each domain (4). The RING domain is involved in the interaction with the E2 ubiquitin-conjugating enzyme (5). The CC and the B30.2/SPRY domains are necessary for the cytoplasmic localisation of Ro52 (6). In cytoplasm, Ro52 also functions as an intracellular Fc receptor (7). The B30.2/SPRY domain binds the Fc region of human IgG with an affinity higher than that between IgG and Fcγ receptors. The intracellular binding of Ro52 with an endocytosed IgG-virus complex has been shown to be important for proteasomal degradation of the viral particle, and for the subsequent activation of innate immunity (8).

All structural domains of Ro52 are targeted by autoantibodies. By using monoclonal and polyclonal antibodies generated against Ro52 and its peptides, as well as sera obtained from patients, multiple B cell epitopes have been mapped within these domains (9, 10). While the antibodies targeting the RING domain

neutralise Ro52 function *in vitro* (5), the antibodies targeting the CC domain are implicated in the induction of congenital heart block *in vivo* (11). In SjS patients, antibodies targeting all regions of Ro52 are detected at varying levels (12). However, whether antibody responses targeting a particular region on Ro52 are linked with distinct clinical features of the disorder is not known.

Previously, using an experimental mouse model system, we demonstrated the direct role of anti-Ro52 in causing salivary gland hypofunction (13). Thus, based on the findings discussed above, the present study tested the hypothesis that immune responses to different domains on Ro52 have distinct effects on the induction of salivary gland disease. To test this hypothesis, New Zealand mixed (NZM) 2758 female mice were immunised with recombinant Ro52 proteins representing different domains of Ro52. Our data show that immunisation with each of the Ro52 fragments generated robust antibody responses against the whole Ro52 protein. However, only mice immunised with the CC domains developed significant salivary gland hypofunction.

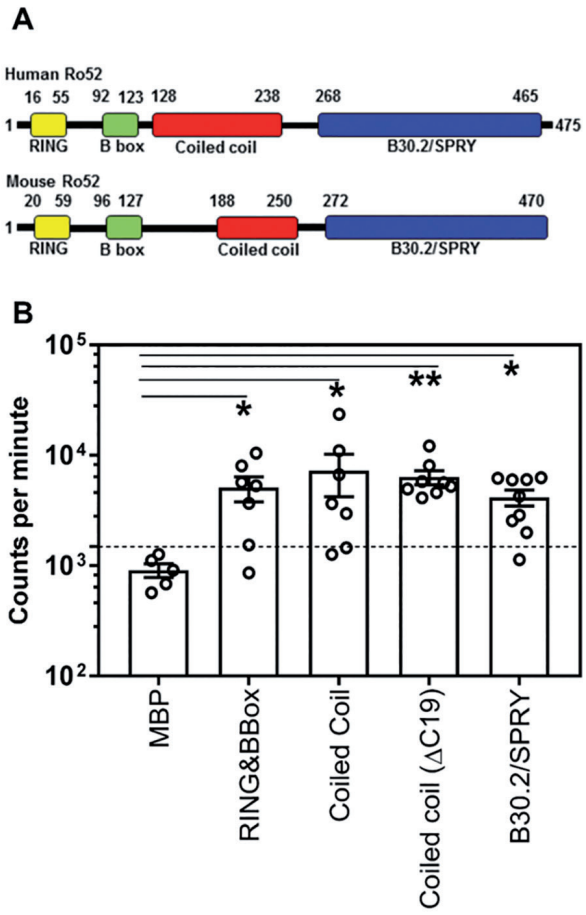

Fig. 1. Immunoprecipitating anti-Ro52 antibodies are generated in mice immunised with Ro52-domain recombinant proteins.
A. Schematic representation and comparison of human and mouse Ro52 domains. Amino acid boundaries are based on the Family and Domains information from www.uniprot.org.
B. Sera from mice immunised with different Ro52 domains were used to immunoprecipitate *in vitro* transcribed, translated and ^{35}S-Met labelled mouse Ro52. Data are represented as counts per minute and the dotted line represents mean CPM value + 2SD obtained in sera from MBP immunised mice. Statistical significance was determined by Kruskal-Wallis test and Dunn's multiple comparisons test. A $p<0.05$ at 95% confidence interval was considered significant.
$*p<0.05$, $**p<0.005$.
Similar results were obtained in an additional experiment.

Materials and methods

Mice

All experiments performed in this study were approved by the Oklahoma Medical Research Foundation's Institutional Animal Care and Use Committee. NZM2758 mice were bred and maintained in specific pathogen free colony and fed ad libitum in the Oklahoma Medical Research Foundation vivarium. Female mice (12–14 wk old) were immunised with purified recombinant proteins adsorbed on to alum adjuvant as described previously (13). Briefly, on day 0, mice were immunised with 50μg of recombinant proteins adsorbed on to alum in one foot pad, and subcutaneously at the base of the tail. On days 14 and 42, mice were injected intraperitoneally with 50μg of the respective recombinant proteins adsorbed on to alum. Pilocarpine-induced saliva was measured as described previously (14), with a few modifications. Mice were injected with pilocarpine and saliva was collected by placing a highly absorbent piece of sponge (Salimetrics, Carlsbad, CA, USA) in the animal's mouth for 15 minutes. The weight of saliva produced (mg) was calculated as the difference between the dry and wet sponge weights.

Recombinant proteins

The mouse Ro52 domains, RING-B-box, CC, CCΔC19 (CC lacking 19 C-terminal amino acids) and the B30.2/SPRY were amplified by PCR using full length mRo52 cDNA (NM_009277.2) as template. The amino acid boundaries are shown in Figure 1A and they were selected based on domain information obtained at http://www.uniprot.org. The PCR fragments were cloned into the pMAL-c5E vector (New England BioLabs, Ipswich, MA, USA) to generate maltose-binding protein (MBP)-Ro52 domain fusion proteins. All proteins were purified under native conditions using amylose columns by following the manufacturer's instructions. Empty vector was used for the production of MBP.

Antibody analysis

Reactivity to native mouse Ro52, mouse Ro60 and mouse SSB/La was analysed by a quantitative immunoprecipitation assay as described previously (13). *In vitro* transcribed, translated (Promega, Madison, WI, USA) and ^{35}S-Met (PerkinElmer, USA) labelled mRo52, mRo60, and mLa, respectively, were used as substrates. Protein-A Sepharose (G-Biosciences, St Louis, MO USA) was used for antibody binding. Results are presented as counts per minute (CPM) for each sample.

Autoantibodies in sera were detected by indirect immunofluorescence. SCA9-15 cells were grown on coverslips, fixed with 4% paraformaldehyde, and incubated with sera from immunised mice. Bound antibody was detected by FITC labelled anti-mouse IgG. For quantitative immunofluorescence, the format of in-cell-western (LiCOR Biosciences, Lincoln, NE, USA) was used by following the manufacturer's instructions. Briefly, SCA9-15 cells were grown overnight in clear bottom, black, 96

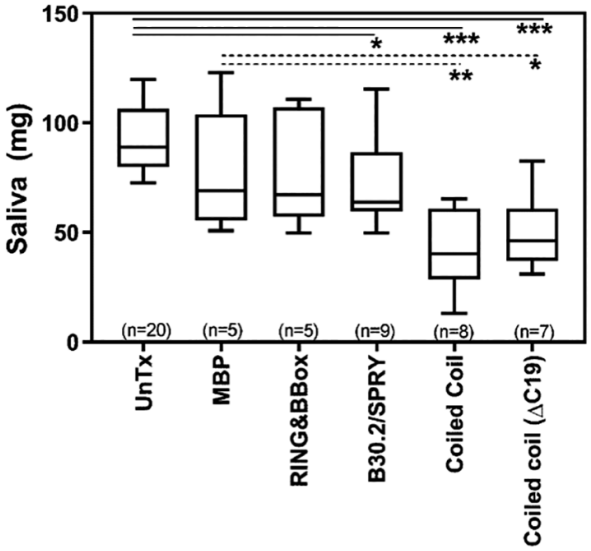

Fig. 2. Mice immunised with recombinant proteins containing the mRo52 CC domains show highest drop in saliva production.
Pilocarpine-induced saliva was measured, 7-8 weeks post-immunisation and data are represented as saliva weight (mg). Saliva from untreated NZM2758 mice from our cohort of mice was used as baseline. Statistical significance was determined by one-way ANOVA and Holm-Sidak's multiple comparisons test.
A $p<0.05$ was considered significant. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

well plates (5000 cells per well), fixed with 4% paraformaldehyde and permeabilised with 0.1% Triton X-100. Cells were incubated with Odyssey blocking buffer (LiCOR Biosciences), followed by an overnight incubation with diluted (1:100) mouse sera. IR Dye 800CW labelled goat anti-mouse IgG was used for the detection of bound antibody. To normalise for cell numbers, CellTag700 (LiCOR Biosciences) was added to the wells. The plates were scanned in the 700 and 800 channels in the Odyssey Imaging system and data analysed with ImageStudio software (LI-COR Biosciences). A ratio of fluorescence intensities at 800/700 channels was considered as the relative amount of antibody bound to SCA9-15 cells.

Salivary gland analyses
Submandibular salivary glands were harvested, collected in formalin, and processed for histopathology. Haematoxylin and eosin stained sections were evaluated for inflammatory infiltrates as previously described (13). IgG in salivary glands were detected in formalin-fixed, paraffin-embedded sections using rabbit anti-mouse IgG (Novus Biologicals, Littleton, CO, USA), followed by Signal Stain Boost IHC detection reagent (Cell Signaling technology, Danvers, MA, USA) and developed with ImmPACT DAB substrate (Vector Laboratories, Burlingame, CA, USA). The sections were counter stained with haematoxylin. Images of acini and ducts within a lobule were captured at 20x magnification and the extent of IgG deposited was scored on a scale of increasing severity from 0 to 5; with 0 representing no deposits and 5 representing extensive deposition in the gland all over the section. All slides were coded and then scored twice by an observer blinded to the experimental details.

Statistical analyses
All statistical analyses were carried out using Graph Pad Prism 7.0 software (GaphPad Software Inc, La Jolla, CA, USA). Each dataset was subjected to normality tests to determine subsequent use of parametric or non-parametric analyses. Multiple comparisons were carried out by non-parametric, Kruskal-Wallis one way ANOVA on ranks test followed by Dunn's post-test. Correlations were determined by Pearson's method. A p-value <0.05 at a 95% confidence interval was considered significant.

Results

Ro52 domain protein immunisation induces comparable levels of auto-antibodies
The structural/functional domains of mouse Ro52 (Fig. 1A) were generated as MBP fusion proteins and mice were immunised with the respective purified proteins. Sera were analysed for the presence of immunoprecipitating high affinity IgG anti-Ro52 antibodies (Figure 1B). Sera from mice immunised with MBP alone were used as controls. Samples giving a value over the control mice immunised with MBP (mean CPM+2SD) were considered positive. All Ro52 domain fusion proteins induced anti-Ro52 antibodies. The anti-Ro52 antibody levels and frequency of anti-Ro52 positivity between the different Ro52 domain immunised groups were not significantly different. Thus, regardless of the Ro52-domain used for immunisation, mice generated comparable levels of immunoprecipitating anti-Ro52 antibodies. Furthermore, when sera were evaluated for reactivity with a salivary gland cell line SCA9-15 by a quantitative indirect immunofluorescence assay, comparable levels of autoantibodies were observed in different groups of mice (Supplementary Fig. 1). The antibodies showed a dominant cytoplasmic staining (Supplementary Fig. 2). However, no discernable distinctions between staining patterns emerged between different groups of mice. Collectively, these data demonstrate that the Ro52 domains used in this study were equally immunogenic.
Intermolecular B cell epitope spreading to Ro60 and La/SSB proteins has been reported in mice immunised with whole Ro52, (15, 16). Thus, to determine whether epitope spreading had occurred in mice immunised with Ro52 fragments, the sera were analysed for the presence of anti-Ro60 and anti-La antibodies by immunoprecipitation assays. None of the immunised groups showed presence of these antibodies (Supplementary Fig. 3), indicating a lack of epitope spreading.

Salivary gland hypofunction is most prominent in mice immunised with CC proteins
To determine whether Ro52 domain immunisations affect salivary gland function, pilocarpine-induced saliva was measured (Fig. 2). The mean saliva amount from untreated NZM2758 mice in our cohort was used as baseline. Compared to saliva produced in the untreated mice (92.77±17.76 mg), a small but insignificant drop in saliva production was seen in the RING-B-box (79.18±26.47 mg) and MBP (77.54±28.24 mg) immunised

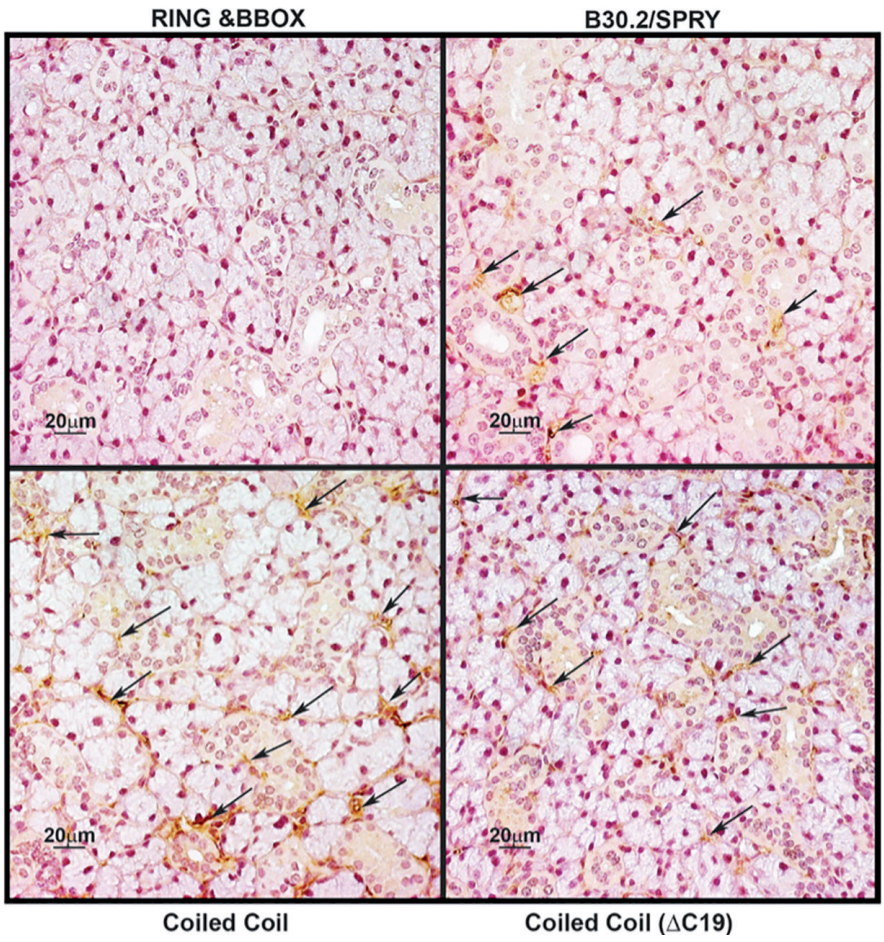

Fig. 3. IgG deposits are detected in between ducts and acini in submandibular salivary glands predominantly in mice immunised with CC and CC(ΔC19) domains. Representative photomicrographs of submandibular salivary glands stained for IgG deposition by immune-histochemistry. Intra-lobar IgG deposits (yellow/brown) are indicated by arrows.

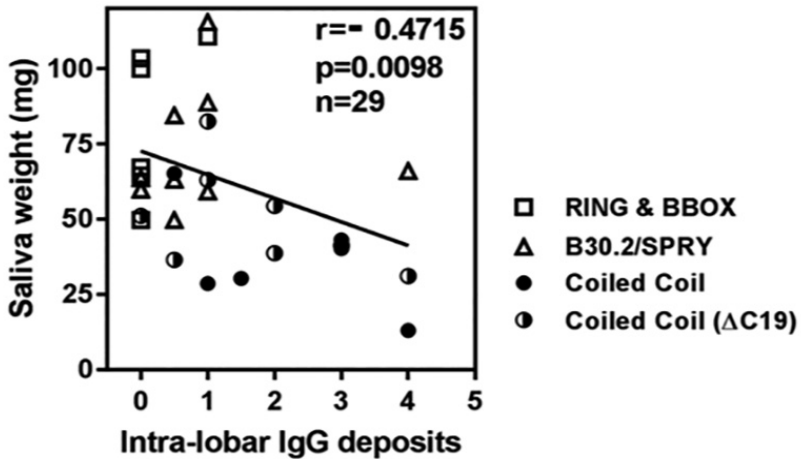

Fig. 4. Strong negative correlation between the amount of intra-lobar IgG deposition and pilocarpine-induced saliva in mice immunised with Ro52 domains. The correlation between pilocarpine-induced saliva (mg) measured at 7-8 weeks post immunisation and severity of intra-lobar IgG deposits for each mouse immunised with Ro52 domains was evaluated by Pearson method. The solid line represents the best fit curve determined by linear regression.

mice. In contrast, mice immunised with the B30.2/SPRY (72.77±20.36 mg), CC (40.12±18.43 mg), and CC(ΔC19) (49.85±16.89 mg) proteins showed significantly lower saliva production. In comparison to the baseline, the maximum mean drop in saliva was seen in the CC (57%) and CC(ΔC19) (46%) groups, followed by the B30.2/SPRY (22%), MBP (16%) and RING-B-box (15%) groups. However, only mice immunised with the CC and CC(ΔC19) immunised groups had significantly lower saliva than MBP immunised mice, indicating that an immune response to the CC region had the largest pathogenic effect on salivary gland function.

The submandibular glands from all mice were examined for the presence of characteristic lymphocytic foci. As we have reported previously in the whole Ro52 immunised mice (13), none of the mice immunised with Ro52 domains showed any significant inflammatory foci in their submandibular glands. Whether these mice would develop classical lymphocytic foci within their salivary glands, and whether the glandular dysfunction would become permanent at later time points are critical questions and they will be investigated in future.

Salivary glands of immunised mice show distinct distribution of IgG deposits in submandibular salivary glands

Our previous studies employing whole Ro52 immunisation has demonstrated antibody deposition within the salivary glands (13). Thus, submandibular salivary glands from Ro52 domain immunised mice were studied for IgG deposits using immuno-histochemistry. IgG was detected along the inter-lobar connective tissue septa in all mice. A surprising finding was the distinct distribution of IgG in the intra-lobar regions in between the ducts and acini (Fig. 3). A semi-quantitative analysis showed higher amounts of IgG deposited in the CC and CC(ΔC19) mice and some IgG deposits in SPRY immunised mice. Surprisingly, most of the RING-B-box immunised mice showed no such IgG deposits.

An analysis of the association between the salivary gland IgG deposits and pilocarpine-induced saliva showed a highly significant (p=0.0098) negative correlation (Fig. 4). These data suggest that increased amount of IgG deposited in the intra-lobar regions is associated with salivary gland hypofunction.

Discussion

In this report we demonstrate that immune response against the CC domain of mouse Ro52 is sufficient to induce salivary gland hypofunction in mice. Although immunisation with other Ro52 domains, the RING-B-box and the B30.2/SPRY, generated an equivalent amount of immunoprecipitating anti-Ro52, these mice did not develop significant glandular dysfunction.

Several reports in the literature have demonstrated that antibodies targeting the CC domain are found in patients with SjS (9, 10). Studies from the Wahren-Hernelius group demonstrate that immune response against this region plays an important role in the pathology of congenital heart block (CHB) (17). Monoclonal antibodies recognising epitopes within amino acids 200-239 could experimentally induce CHB (11, 18). Interestingly, monoclonal antibodies targeting the RING and the B30.2/SPRY regions were not efficient in inducing CHB (11). Similarly, in our study, the antibodies against these 2 domains reacted equally well with whole Ro52 but did not induce salivary gland hypofunction.

The CC domain of human Ro52 spans amino acids 128-238 (Fig. 1A). Whereas, the mouse Ro52 CC domain spans amino acids 188-250 (Fig. 1A), and is smaller than the corresponding human domain. However, it should be noted that the pathogenic B cell epitope region mapped on human CC region by the Wahren-Hernelius group is present in the mouse CC domain used in this study. In addition, considerable homology is observed between the CC region of mouse and human Ro52 proteins (Supplementary Fig. 4). Considered together, these data clearly demonstrate the importance of antibody responses targeting the CC region of Ro52 in pathogenesis of two distinct disorders in humans and in mice.

Analysis of sera from CHB mothers has demonstrated that antibodies predominantly recognise the C-terminal region of the human CC domain (18). Thus, to determine the relative role of immune response against this C-terminal region in salivary gland disease, we immunised mice with CC(ΔC19), which is a truncated form of the mouse CC domain, lacking 19 amino acids from the C-terminal. Both the full length and truncated forms induced robust antibody responses capable of immunoprecipitating the whole Ro52. The immune response against the full length peptide induced a higher drop in saliva production (57%) compared to the truncated form (46%). However, this difference was statistically not significant. These data suggest that immune response against the C-terminal part of the CC domain plays an additive role but is not absolutely essential for the induction of glandular dysfunction in mice.

Functionally Ro52 is an E3 ubiquitin ligase and it is mainly involved in the regulation of innate immune responses, particularly the down modulation of type I IFN production by ubiquitination of IRF3 (19). The RING domain of Ro52 interacts with the E2 ubiquitin conjugating enzyme UBE2E1. It has been reported that SjS and SLE patients harbour antibodies recognising the RING region, and *in vitro*, these antibodies blocked the interaction between Ro52 and UBE2E1, thereby inhibiting the function of Ro52 (5). In our study, immunisation of mice with a combined RING-B-box domain generated anti-Ro52 antibodies, but it did not induce glandular dysfunction. Thus, immune response against the RING-B-box domain might not be critical in the pathogenesis of SjS, particularly for salivary gland disease.

The mice immunised with Ro52 domains failed to develop sialoadenitis. This was similar to our previous report with whole Ro52 immunisation (13). Thus, in these model systems, the role of lymphocytic infiltrates in glandular dysfunction can be ruled out. Our previous studies employing whole Ro52 immunisation suggests that anti-Ro52 antibody deposition in salivary glands is responsible for the induction of glandular dysfunction (13). The present study demonstrates a strong correlation between intra-lobar IgG deposition and functional loss. Both of these phenomena were predominantly seen in CC immunised mice, which highlights epitope specificity of anti-Ro52 antibodies as an important criterion for their pathogenic potential. It is possible that antibodies from CC immunised mice recognise cross-reactive antigen(s) and thereby deposit in the inter-lobar regions in the submandibular glands. This deposition induces production of pro-inflammatory factors causing glandular dysfunction (Supplementary Fig. 5). This thesis is supported by the literature demonstrating the role of cross-reactivity between anti-Ro52 antibodies and cardiac proteins in the induction of CHB (20-22).

Unlike lupus-nephritis, SjS is not considered an immune-complex mediated disease. Immune cell infiltration and formation of lymphocytic foci within the salivary glands are considered to be critical for the induction of glandular hypofunction. However, a significant number of SjS patients are minor labial salivary gland biopsy negative, and they still have severe dry mouth (13). Furthermore our group has recently reported a group of SjS patients who were anti-La positive, anti-Ro negative, and biopsy negative but had objective dry mouth (whole unstimulated saliva <1.5ml/15 min) (23). Indeed, it would be of great interest to investigate whether antibody deposits are present in the salivary glands of these patients and whether these deposits correlate with the extent of dry mouth.

Antibodies specific to salivary gland antigens have been reported in SjS patients (24-26). Isolated clinical reports have also previously described antibody deposition in salivary glands of SjS patients (27, 28). However, the clinical relevance of these deposits remained unexplored. The tissue distribution of IgG deposits can be good predictors of disease severity in other autoimmune diseases. In SLE, immune complex deposition in the glomerular mesangium are seen with mild proteinuria, whereas, sub-endothelial immune deposits are associated with active destructive lesion (29). Thus, it would be of interest to determine whether presence and location of antibody deposits in salivary glands of SjS patients can be developed as prognostic markers for the disease. The present study provides a strong rationale to conduct such investigation.

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References

1. GHILLANI P, ANDRÉ C, TOLY C *et al*.: Clinical significance of anti-Ro52 (TRIM21) antibodies non-associated with anti-SSA 60kDa antibodies: results of a multicentric study. *Autoimmun Rev* 2011; 10: 509-13.
2. OKE V, WAHREN-HERLENIUS M: The immunobiology of Ro52 (TRIM21) in autoimmunity: a critical review. *J Autoimmun* 2012; 39: 77-82.
3. OZATO K, SHIN DM, CHANG TH, MORSE HC: TRIM family proteins and their emerging roles in innate immunity. *Nat Rev Immunol* 2008; 8: 849-60.
4. WADA K, KAMITANI T: Autoantigen Ro52 is an E3 ubiquitin ligase. *Biochem Biophys Res Commun* 2006; 339: 415-21.
5. ESPINOSA A, HENNIG J, AMBROSI A *et al*.: Anti-Ro52 autoantibodies from patients with Sjögren's syndrome inhibit the Ro52 E3 ligase activity by blocking the E3/E2 interface. *J Biol Chem* 2011; 286: 36478-91.
6. ESPINOSA A, OKE V, ELFYING 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.
7. MALLERY DL, MCEWAN WA, BIDGOOD SR, TOWERS GJ, JOHNSON CM, JAMES LC: Antibodies mediate intracellular immunity through tripartite motif-containing 21 (TRIM21). *Proc Natl Acad Sci* 2010; 107: 19985-90.
8. MCEWAN WA, JAMES LC: TRIM21-dependent intracellular antibody neutralization of virus infection. P*rog Mol Biol Transl Sci* 2015; 129: 167-87.
9. WAHREN-HERLENIUS M, MULLER S, ISENBERG D: Analysis of B-cell epitopes of the Ro/SS-A autoantigen. *Immunol Today* 1999; 20: 234-40.
10. ROUTSIAS JG, TZIOUFAS AG: B-cell epitopes of the intracellular autoantigens Ro/SSA and La/SSB: tools to study the regulation of the autoimmune response. *J Autoimmun* 2010; 35: 256-64.
11. AMBROSI A, DZIKAITE V, PARK J *et al*.: Anti-Ro52 monoclonal antibodies specific for amino acid 200–239, but not other Ro52 epitopes, induce congenital heart block in a rat model. *Ann Rheum Dis* 2012; 71: 448-54.
12. BURBELO PD, CHING KH, HAN BL, BUSH ER, REEVES WH, IADAROLA MJ: Extraordinary antigenicity of the human Ro52 autoantigen. *Am J Transl Res* 2010; 2: 145-55.
13. SZCZERBA BM, KAPLONEK P, WOLSKA N *et al*.: Interaction between innate immunity and Ro52-induced antibody causes Sjögren's syndrome-like disorder in mice. *Ann Rheum Dis* 2016; 75: 617-22.
14. 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.
15. KEECH CL, GORDON TP, MCCLUSKEY J: The immune response to 52-kDa Ro and 60-kDa Ro is linked in experimental autoimmunity. *J Immunol* 1996; 157: 3694-9.
16. GORDON TP, KINOSHITA G, CAVILL D *et al*.: Restricted specificity of intermolecular spreading to endogenous La (SS-B) and 60 kDa Ro (SS-A) in experimental autoimmunity. *Scand J Immunol* 2002; 56: 168-73.
17. STRANDBERG L, WINQVIST O, SONESSON SE *et al*.: Antibodies to amino acid 200–239 (p200) of Ro52 as serological markers for the risk of developing congenital heart block. *Clin Exp Immunol* 2008; 154: 30-7.
18. HOXHA A, RUFFATTI A, AMBROSI A *et al*.: Identification of discrete epitopes of Ro52p200 and association with fetal cardiac conduction system manifestations in a rodent model. *Clin Exp Immunol* 2016; 186: 284-91.
19. HIGGS R, GABHANN JN, LARBI NB, BREEN EP, FITZGERALD KA, JEFFERIES CA: The E3 ubiquitin ligase Ro52 negatively regulates IFN-β production post-pathogen recognition by polyubiquitin-mediated degradation of IRF3. *J Immunol* 2008; 181: 1780-6.
20. XIAO GQ, QU Y, HU K, BOUTJDIR M: Down-regulation of L-type calcium channel in pups born to 52 kDa SSA/Ro immunized rabbits. *FASEB J* 2001; 15: 1539-45.
21. EFTEKHARI P, SALLÉ L, LEZOUALC'H F *et al*.: Anti-SSA/Ro52 autoantibodies blocking the cardiac 5-HT4 serotoninergic receptor could explain neonatal lupus congenital heart block. *Eur J Immunol* 2000; 30: 2782-90.
22. KAMEL R, EFTEKHARI P, CLANCY R, BUYON JP, HOEBEKE J: Autoantibodies against the serotoninergic 5-HT 4 receptor and congenital heart block: a reassessment. *J Autoimmun* 2005; 25: 72-6.
23. DANDA D, SHARMA R, TRUONG D *et al*.: Anti-La positive, anti-Ro negative subset of primary Sjögren's syndrome: anti-La is a reality but is the disease? *Clin Exp Rheumatol* 2017; 35: 438-44.
24. BERTRAM U, HALBERG P: A specific antibody against the epithelium of the salivary ducts in sera from patients with Sjögren's syndrome. *Acta Allergol* 1964; 19: 458-66.
25. MACSWEEN RN, GOUDIE RB, ANDERSON JR *et al*.: Occurrence of antibody to salivary duct epithelium in Sjögren's disease, rheumatoid arthritis, and other arthritides. A clinical and laboratory study. *Ann Rheum Di*s 1967; 26: 402-11.
26. CHISHOLM DM, MASON DK: Labial salivary gland biopsy in Sjögren's disease. *J Clin Pathol* 1968; 21: 656-60.
27. FRIEDMAN H, EGAMI M, KILMAR V: Necrotizing lesions in Sjögren's syndrome. I. An immunofluorescent study. *Z Rheumatol* 1980; 39: 28-32.
28. FERNANDES JD, NICO MM, AOKI V *et al*.: Xerostomia in Sjögren's syndrome and lupus erythematosus: a comparative histological and immunofluorescence study of minor salivary glands alterations. *J Cutan Pathol* 2010; 37: 432-8.
29. WEENING JJ, D'AGATI VD, SCHWARTZ MM *et al*.: The classification of glomerulonephritis in systemic lupus erythematosus revisited. *Kidney Int* 2004; 65: 521-30.