Title: Protocols for Experimental Sjögren's Syndrome

AUTHOR(S) AND CONTACT INFORMATION

Harini Bagavant, Umesh S. Deshmukh*

Arthritis and Clinical Immunology, Oklahoma Medical Research Foundation, Oklahoma City, OK, USA. Tel: 405-271-2439. E. mail: harini-bagavant@omrf.org

*Umesh S. Deshmukh (Corresponding author)

Arthritis and Clinical Immunology, Oklahoma Medical Research Foundation, Oklahoma City, OK, USA. Tel: 405-271-2463. E. mail: umesh-deshmukh@omrf.org

SIGNIFICANCE STATEMENT

Experimental Sjögren's syndrome induced by the activation of specific innate immune pathways in mice mimics the salivary gland manifestations seen in Sjögren's syndrome patients, thereby providing insights into the disease pathogenesis.

ABSTRACT

Sjögren's syndrome (SS) is a systemic autoimmune disease affecting multiple organ systems. Salivary and lacrimal gland involvement cause dry mouth and dry eye and are the most common clinical presentations of the disease. SS patients also have autoantibodies targeting multiple nuclear and cytoplasmic antigens. Innate immune activation plays a critical role in SS pathogenesis. This manuscript describes the activation of specific innate immune pathways in mice to study SS's salivary gland manifestations. Methodologies for evaluating salivary gland inflammation and salivary function are described. This manuscript also describes protocols for in-house assays to measure autoantibody titers in serum.

Basic Protocol 1: Acceleration of SS by activating the Toll-like receptor 3 (TLR3) pathway.
Basic Protocol 2: Induction of SS by activating the stimulator of interferon genes (STING) pathway.
Alternate Protocol 1: Acceleration of SS by the administration of Freund's incomplete adjuvant (IFA).
Support Protocol 1: Evaluating the salivary gland function.
Support Protocol 2: Evaluating salivary gland inflammation.

Support Protocol 3: Measuring autoantibody titers by indirect immunofluorescence.

KEYWORDS

Sjögren's Syndrome, Innate immunity, Salivary glands, STING, TLR3

INTRODUCTION

Sjögren's syndrome (SS) is a systemic autoimmune disease affecting multiple organ systems (Vivino et al., 2019). SS most commonly presents with symptoms of dry mouth and dry eyes. On investigation, the patients show reduced saliva and tear production, high titers of circulating autoantibodies, and lymphocytic infiltration in the salivary glands. Involvement of other organs like the joints, lungs, central nervous system, and the co-existence of multiple autoimmune diseases like Systemic Lupus Erythematosus and Rheumatoid Arthritis offers significant challenges in modeling the entire spectrum of SS.

Mouse models focus predominantly on the salivary gland and lacrimal gland manifestations of SS characterized by salivary and/or lacrimal gland inflammation, reduced saliva and/or tear production, and circulating autoantibodies. It is now well accepted that chronic activation of innate immunity and type I interferons (IFN) plays a critical role in the initiation and progression of SS. This manuscript will discuss the activation of innate immune pathways linked to type I IFN responses for investigating the salivary gland pathology in mice. The materials and methods describe how to establish the model, measure salivary gland function, evaluate the salivary gland pathology, and measure serum autoantibodies. **Basic Protocol 1** describes SS acceleration in spontaneously autoimmune (New Zealand Black x New Zealand White) F1 mice (NZB/W F1) through the TLR3 pathway activation. **Basic protocol 2** describes the induction of SS-like disease in C57BL/6 mice by activation of the STING pathway. SS acceleration by non-specific activation of innate immunity using Freund's Incomplete Adjuvant **(Alternate Protocol 1)** is also described. Support protocols include the methods for evaluation of salivary gland function **(Support protocol 1)**, salivary gland inflammation **(Support protocol 2)**, and autoantibody detection **(Support protocol 3).**

STRATEGIC PLANNING

Mice: SS predominantly affects females. Hence, this protocol uses female mice from 8-10 weeks of age. The mice purchased from vendors should be allowed to acclimate for one week before starting the experiments.

BASIC PROTOCOL 1

ACCELERATION OF SS BY ACTIVATING THE TOLL-LIKE RECEPTOR 3 (TLR3) PATHWAY

Female NZB/W F1 mice spontaneously develop SS-like disease with salivary gland inflammation and glandular dysfunction at 7-8 months of age (Kessler, 1968). The onset of SS is significantly accelerated by injecting NZB/W F1 mice with a TLR3 agonist, Polyinosinic-polycytidylic acid (Poly(I:C)) (Nandula et al., 2011). By 9-12 weeks post Poly(I:C) treatment (or at 4 -5 months of age), mice develop severe salivary gland inflammation characterized by

the presence of large lymphocytic foci containing CD4+ T cells, B cells, dendritic cells, and macrophages. The salivary gland inflammation in Poly(I:C) treated mice is followed by a drop in saliva production. It should be noted that the NZB/W F1 mice also spontaneously develop fatal glomerulonephritis. Thus, monitoring mice for the development of severe proteinuria is highly recommended. The protocol is summarized in a schematic form in Figure 1A.

[*Insert Figure 1 near here]

Materials

NZB/W F1 female mice at 8-10 weeks of age (The Jackson Laboratory, stock no. 100008)

High molecular weight Poly(I: C) (Invivogen, cat. no. tlrl-pic, 10 mg vial)

Endotoxin-free saline for injection (Med-Vet International, cat.no. RX0.9NACL-10)

Multistix Siemens Urinanalysis test strips 10SG (Bayer Healthcare Model:2300)

Ethanol (70%)

Insulin syringes 29G X ½", 0.3 mL (BD, cat. no. 324702)

Endotoxin-free pipet tips, tubes

Water bath

Protocol steps

1) Prepare a stock solution of Poly(I:C) (1 mg/mL) by dissolving the entire contents of the 10 mg vial in endotoxin-free physiological water (0.9% NaCl, provided by the supplier with Poly(I:C)).

   Add the appropriate amount of endotoxin-free physiological water *to the vial containing lyophilized Poly(I:C) and mix by pipetting up and down. Place the vial in a water bath at 65-70^{o}C for 10 min. To allow proper annealing, remove from the water bath and let the solution come down to room temperature for 1 hour. Dispense the solution into 0.5 mL single-use aliquots in RNAse free tubes and store frozen at -80^{o}C till use.*

2) Before injection, remove a frozen aliquot of the Poly(I:C) stock solution and allow it to thaw gradually at room temperature. Then, dilute with an equal volume of endotoxin-free saline to obtain a 0.5 mg/mL working solution.

3) Inject 0.1 mL of working solution by the intraperitoneal route to deliver 50 μg Poly(I:C)/mouse. Inject 0.1 mL endotoxin-free saline in the control group. Return the mice to the caging.

   The use of insulin syringes (29G, 0.3 mL) with low dead volume is highly recommended for accurate dosing. Intra-peritoneal injection of Poly I:C does not cause any obvious changes in mouse behavior and does not appear to induce any major discomfort. Following a brief picking at the site of injection, the mice return to their normal activity.

4) Inject the mice every other day for 2 weeks (days 0, 2, 4, 6, 8, 10, 12, and 14) to give 8 injections.

   After 8 injections, no adverse effects have been observed. However, if the mice show signs of dehydration and ruffled skin, it is advisable to provide liquid gel as an additional water source.

5) Monitor salivary gland function starting one month after the first injection. Saliva production may be evaluated at monthly intervals (Support protocol 1).

6) Collect blood (0.075 to 0.15 mL) from the tail vein at monthly intervals, starting one month after the first injection. Separate the serum and store frozen at -20^{o}C for future autoantibody analysis (Support protocol 3).

   If the mice are kept for a longer period (>5 months of age), monitoring the mice for proteinuria every two weeks by putting a drop of freshly collected urine on a Urinalysis test strip is recommended. A reading of ≥ 2000 mg/dL (++++) on two consecutive days indicates that the mouse has developed severe lupus-like nephritis and is at risk of dying.

7) Euthanize the mice 5 months after the first injection and harvest the salivary glands for histopathologic evaluation. (Support protocol 2).

BASIC PROTOCOL 2

INDUCTION OF SS BY ACTIVATION OF THE STIMULATOR OF INTERFERON GENES (STING) PATHWAY

An SS-like disease is induced in C57BL/6 female mice injected with 5,6-Dimethylxanthenone-4-acetic acid (DMXAA), a cell-permeant ligand of the murine STING protein (Papinska et al., 2018). Activation of STING causes a rapid increase of IFNα and IFNβ levels in circulation and an elevation of interferon responsive genes in the salivary gland. By 4-6 weeks after DMXAA treatment, the mice show salivary gland dysfunction (Support protocol 1), salivary gland inflammation (Support protocol 2), and the presence of circulating autoantibodies (Support protocol 3). A schematic summary of this protocol is presented in Figure 1B.

Materials

C57BL/6 female mice at 8-10 weeks of age (The Jackson Laboratory, stock no. 00064)

DMXAA, 10 mg vial (TOCRIS Bioscience, cat. no. 5601/10)

Endotoxin-free 7.5% Sodium bicarbonate solution (Millipore Sigma, cat. no. S8761-100 mL))

Endotoxin-free sterile water (Invitrogen, cat no. 10977015)

Isoflurane inhalant anesthetic (Med-Vet International, cat. no. RXISO-100)

Ethanol (70%)

DMXAA solution (see reagents and solutions)

Ear tag/ clipper

Balance

Insulin syringes 29G X ½", 0.3 mL (BD, cat. no. 324702)

Endotoxin-free pipet tips, tubes,

Aluminum foil

Small animal anesthesia system (Kent Scientific or similar).

Protocol steps

1) Tag (using method approved by the Institutional Animal Care and Use Committee) and weigh the C57BL/6 female mice.

   Based on the disease penetrance (salivary gland inflammation and loss of function), 7-10 mice per group can be a starting point for the experiment.

2) Dissolve the entire contents of the DMXAA vial in 5% sodium bicarbonate solution to prepare a stock of DMXAA (10 mg/mL).

3) Calculate the DMXAA dosage for each mouse (20 mg/kg body weight) and prepare individual tubes with the appropriate volume of DMXAA stock solution. Add a 5% bicarbonate solution to get a final volume of 50 μL. Prepare a tube with 5% bicarbonate solution for injection into vehicle control mice.

   For example, a 20 gm mouse will get a dose of 400 μg DMXAA (40 μL of the DMXAA stock solution and 10 μL of 5% bicarbonate solution). A maximum of 500 μg/mouse is recommended since higher amounts were lethal in some mice.

4) Draw 50 *μL* of the prepared DMXAA solution into a syringe. Keep the solution and the syringe with DMXAA protected from bright light.

   Insulin syringes (0.3 mL) with attached needles (29G) have a low dead space volume and allow for accurate dosing.

5) For injection, anesthetize the mice in an isoflurane chamber following the institutional animal care and use committee (IACUC) guidelines. Remove one mouse at a time and place it on a flat surface. Spray the back of the mouse with 70% ethanol to disinfect. Inject subcutaneously at one site on the back between the shoulders. Return the mouse to its respective cage. The mouse should recover almost immediately. Repeat this process for each mouse in the experimental (DMXAA treated) and control (vehicle-treated) groups.

   After injecting the entire volume from the syringe, hold the needle in place for 5-10 seconds before withdrawing it to prevent the solution from leaking out.

   Anesthetizing the mice for subcutaneous injection on the back is optional. We have found the anesthesia allows for consistent and accurate dosing. Using isoflurane as the anesthetic agent allows for rapid recovery.

6) Monitor mice for at least one day post-injection to ensure that they are active and hydrated.

   Optional: place wet food on the floor of the cage. DMXAA injection induces a rapid increase of pro-inflammatory cytokines in the systemic circulation and may manifest as lethargy.

However, this increase is transient, and correcting dehydration in the mice allows rapid recovery.

7) On day 21, weigh the mice and recalculate the appropriate DMXAA dose. Typically, we observe that the mice gain 5-10% of their body weight over this time period. Repeat DMXAA and vehicle injections (follow step no. 3 to step no.6).
8) Evaluate mice for salivary gland dysfunction one month after the first injection. (Support protocol 1)
9) To terminate the experiment, euthanize the mice and harvest salivary glands to evaluate inflammation (Support Protocol 2). Collect blood and separate serum. The sera can be stored at -20^{o}C for autoantibody analysis later (Support protocol 3).

ALTERNATE PROTOCOL 1

ACCELERATION OF SS BY ADMINISTRATION OF FREUND'S INCOMPLETE ADJUVANT (IFA)

Innate immunity activation through specific pathways like STING and TLR3 described above influence SS development in mice. In susceptible mouse strains like NZB/W F1, Freund's Incomplete Adjuvant (IFA) administration also accelerates the onset of SS (Deshmukh et al., 2008). Although the precise mechanisms involved in this process are unclear, the activation of innate immune responses in macrophages and systemic pro-inflammatory cytokine production is implicated.

Materials

NZB/W F1 female mice at 7-9 weeks of age (The Jackson Laboratory, stock no. 100008)

Incomplete Freund's adjuvant (IFA) (Imject FIA Thermo Fisher Scientific, cat. no. 77145)

Endotoxin-free Dulbecco's PBS w/o Ca++ & Mg++ (Millipore Sigma, cat. no. TMS-012-A)

Isoflurane inhalant anesthetic (Med-Vet International cat. no. RXISO-100)

Ethanol (70%)

Sterile Syringes (3 mL with Luer lock and 1 mL Tuberculin syringes without needles)

Sterile Needles (18G, 25G)

Polypropylene female Luer x female Luer adapter (Cole-Parmer, cat. no. 45508-22)

Ice

Small zip-lock plastic bags

Small animal anesthesia system (Kent Scientific or similar).

Protocol steps

1) To prepare an emulsion, draw 1.0 mL of IFA through an 18G needle into one 3 mL syringe. Similarly, into another 3 mL syringe, draw 1.0 mL of PBS. Remove air from the syringes and connect the two syringes using a female Luer x female Luer adapter. Mix the two solutions vigorously to generate a water-in-oil emulsion. A mixing time of 30-40 min typically yields a good emulsion. When the emulsion is ready, transfer it to a 1 mL tuberculin syringe. Connect this syringe to a 25G needle, and inject mice by subcutaneous route.

   It is important to exclude air and air bubbles from the syringes before mixing. Air prevents the formation of a good emulsion.
   Keep the syringes cool and on ice while mixing back and forth. An ice-jacket (made out of a small zip-lock plastic bag filled with ice), wrapped around the syringes' barrel, maintains cold temperatures without interfering with the mixing motion. *Periodically replenish the melted ice in the plastic bag with fresh ice.*

2) Anesthetize 8-10 week old NZB/W F1 female mice with isoflurane and inject the IFA emulsion at two different subcutaneous sites (typically in one footpad and at the base of the tail). A total of 0.15 mL of the IFA emulsion/mouse. Inject the control group of mice with 0.15 mL of PBS at the same two sites.

   If IACUC regulations do not permit the footpad injections, IFA injection into the hock can be an alternative.

3) Prepare fresh IFA emulsions on day 30 and day 60. On these days, inject the mice with 0.15 mL IFA emulsions by the intraperitoneal route. Inject the control mice similarly with PBS (0.15 mL/mouse).

4) Monitor salivary gland function at monthly intervals (Support protocol 1).

5) Evaluate salivary gland inflammation by euthanizing the mice and harvesting salivary glands (Support Protocol 2). Collect blood (0.075 to 0.15 mL) from the tail vein at monthly intervals. Separate the serum and store frozen for future autoantibody analysis (Support protocol 3).

SUPPORT PROTOCOL 1

EVALUATING SALIVARY GLAND FUNCTION

Salivary gland dysfunction leading to reduced saliva production is a major cause of dry mouth in SS. In mice, saliva production in response to a sialagogue is used to measure salivary gland function (Bagavant et al., 2018). Pilocarpine hydrochloride, a muscarinic acetylcholine M3 receptor agonist, is used to induce salivation. The saliva appearing in the oral cavity is collected onto an absorbent material, and the amount of saliva produced is measured. This procedure can be carried out multiple times in the same animal. It allows

the measurement of sequential changes in salivary gland function over time. This method is most reliable in mice >20g bodyweight.

Materials:

Mice from basic protocols 1 or 2, or alternate protocol 1.

SalivaBio Children's Swabs (Salimetrics, cat. no. 5001.05)

Pilocarpine hydrochloride (5.63 mg/mL) in endotoxin-free saline (Millipore Sigma, cat. no. P6503)

Mouse Anesthetic Mixture (Reagents and Solutions)

Endotoxin-free saline for injection (MEDVET International, cat.no. RX0.9NACL-10)

Microcentrifuge Tubes 0.5 mL, 2 mL

Needles 18G

Sterile razor blade

Analytical Balance

Insulin syringes 29G X ½", 0.3 mL (BD, cat. no. 324702)

Board

Tape

Two timers set for 2 min and 15 min.

Microdissecting forceps (Roboz surgical store, cat. No. RS-5139) or similar

Mouse heating pad

Refrigerated microcentrifuge (Beckman Coulter Microfuge 20R or similar)

Protocol steps

1) Transfer the mice into a clean cage without any food for at least 2 hours before the procedure. This fasting prevents food particles from contaminating the saliva collection. Ensure that the mice have access to water ad libitum.

2) Prepare swabs for saliva collection. Take 0.6 mL microcentrifuge tubes and make a hole at the bottom of the tube using a heated 18G needle. Take an equal number of 2 mL microfuge tubes, detach their caps, and place the 0.6 mL tube into the 2 mL tube. Cut one Salimetrics Childen's swab into approximately 2 cm long pieces with a sterile razor blade. Cut each piece diagonally to obtain two cone-shaped swabs. Place one cone in each of the 0.6 mL tubes. Number each tube and weigh the 0.6 mL tube with the dry swab on an analytical

balance. Record the weights and place each of these dry cone swab tubes inside a 2 mL tube.

3) Dilute the stock solution of pilocarpine hydrochloride 100x in endotoxin-free saline to get a working solution of 0.0563 mg/mL.

4) At the end of the 2 hour fasting period, weigh each mouse and calculate the exact anesthetic (7 µL/gm body weight) and pilocarpine (0.375 mg/kg body weight) doses.

   A 30 g mouse will get 0.21 mL of anesthetic and 0.2 mL of pilocarpine hydrochloride. It should be noted that different anesthetics can stimulate or inhibit salivation and interfere with the results. This protocol uses a low dose combination of ketamine-xylazine that minimally affects saliva production. This method has been tested to give reproducible results for anesthetic use between 7-9 µL/gm body weight.

5) Set a timer for 2 min. Inject the mouse with the anesthetic solution by the intraperitoneal route. Start the timer.

   After 2 min, the mouse should be under the anesthetic's influence and not respond to gentle handling. *If the mouse is still awake, wait for an additional minute before proceeding to the next step.*

6) At the end of 2 min, inject the pre-calculated dose of pilocarpine hydrochloride solution by the intraperitoneal route. Reset the timer to 2 min.

7) Place the mouse, supine with the head downward, on a board with a 20-30 degree incline. Run an adhesive tape loosely across the middle to hold the mouse in position on the board during the procedure. At the end of two min, open the mouth using a pair of forceps and gently push the tongue aside. Use the forceps to place the conical tip of the swab in the mouth. Start the 15-minute timer.

   Make sure that the swab does not push the tongue back into the throat and obstruct breathing.
   If the procedure is carried out in a biosafety hood with the blower turned on, covering the mice with a small paper towel blanket can prevent the mice from getting cold. Placing the mice on a heating pad at this step can cause over-heating and is not recommended.

8) Keep the swab in position for 15 min. Any saliva produced is wicked into the swab. At the end of 15 min, use the forceps to hold the swab and sweep it around the oral cavity to mop up any residual saliva. Transfer the wet swab back into the same 0.6 mL tube.

9) Transfer the mouse to a heating pad for recovery. Once the mouse is awake, it can be returned to its cage.

10) Proceed to the next mouse. Save the tubes containing the wet swabs on ice until the procedure is complete for all the mice.

The mice should wake up within 10 min of completing the procedure. A subcutaneous injection of saline (0.1 to 0.2 mL), pre-warmed to 37^{o}C, can help the mice to wake up faster. Wet food can be placed in the bottom of the cage overnight to speed recovery from the procedure.

11) Weigh the 0.6 mL tubes with the wet swabs. Subtract dry weight from the wet weight of each tube to obtain the total weight of saliva produced.

If desired, the saliva can be recovered from the wet swab by centrifuging the 2 mL tubes containing the 0.6 mL tube with wet swabs for 1 minute at 10,000 rpm. The saliva will drain into the bottom of the 2 mL tube and be stored for biochemical analysis.

12) Express the results as the total amount of saliva produced (mg) or as the ratio of saliva weight (mg)/mouse body weight (gm).

This is a seemingly simple method. A well-organized work area with timers pre-set to the required times allows a seamless and efficient experiment execution. *Using this method in normal mice, depending on the operator, the variation in measuring saliva production can range from 14 to 20%. It is important to be consistent in dosing, timing, and all mouse handling for reproducible results. The procedure can be speeded up by working on two mice in tandem.*

A stock solution of pilocarpine saved in single-use aliquots at -80^{o}C significantly facilitates reliable results, especially in longitudinal studies.

For longitudinal studies, perform saliva collections at the same time of the day. The amount of basal saliva production in response to pilocarpine is strain-dependent. Hence, establishing baseline saliva for each strain is critical.

SUPPORT PROTOCOL 2

EVALUATING SALIVARY GLAND INFLAMMATION

Salivary gland inflammation in SS is characterized by dense aggregates of mononuclear cells, predominantly lymphocytes, and is defined as focal lymphocytic sialadenitis (Fisher et al., 2017). The lymphocytic foci are located in the peri-vascular and peri-ductal regions and may extend into the salivary acini. The methods for harvesting mouse salivary glands and evaluating disease severity are described.

Materials:

Mice from basic protocols 1 or 2, or alternate protocol 1.

Anesthetic mixture (see reagents and solutions)

10% neutral buffered formalin (Millipore Sigma cat no HT501128)

70% ethanol

Dissection boards and pins

Sterile forceps, scissors

Tissue cassettes (VWR cat no. 18000-134 or similar)

Equipment for tissue processing, embedding, sectioning, and staining (Leica Biosystems) or similar

Aperio CS2 digital slide scanner and Aperio ImageScope software (Leica BioSystems) or similar

Protocol steps

1) Anesthetize the mouse, confirm that the mouse is unresponsive to pain, and expose the salivary glands, as shown in Figure 2. Resect the right and left submandibular (SMG), sublingual (SLG), and parotid glands (PG). Place one piece of each gland in 10% neutral buffered formalin and store it at room temperature for 24 hours. Use these glands for histopathologic evaluation.

   Some mouse strains have excessive fatty tissue surrounding the submandibular and sublingual salivary glands. Cutaway the fatty tissue from these glands for easier processing and sectioning. Parotid glands in mice are poorly defined and difficult to dissect out from the surrounding fatty tissues. Therefore, collect the entire tissue in the area shown in figure 1. Glandular structures can be easily identified on histopathologic analysis. Note the presence of cervical lymph nodes (figure 1) embedded in the glandular tissue.

2) After 24 hours of fixation in formalin, transfer the salivary glands into 70% ethanol. Process the salivary glands for standard paraffin embedding, sectioning, and staining with Hematoxylin and Eosin. Two to three serial sections (5 μm thickness) of the salivary glands collected at two different levels allow the assessment of glandular inflammation.

   [*Insert figure 2 near here]

3) Examine the salivary gland sections. Representative images show the normal ductal and acinar arrangement in submandibular (Figure 3A) and sublingual (Figure 3B) salivary glands. Identify the areas with mononuclear cell infiltrates. A collection of >50 infiltrating cells is considered a lymphocytic focus. Mildly inflamed glands show lymphocytic infiltrates restricted to peri-ductal and peri-vascular areas (Figure 3C). The areas of lymphocytic infiltration increase in size and invade into the adjacent acini (Figure 3D). Lymphocytic infiltrates can occupy large areas of the salivary glands (Figure 3E), destroying acinar and ductal architecture (Figure 3F). A semi-quantitative assessment that evaluates the number of foci and the extent of parenchymal destruction is shown in Table 1, and the data are expressed as a number from 0-5.

Salivary gland inflammation is most common and severe in submandibular glands in mice. Sublingual and parotid gland infiltration is less common.

4) A quantitative method measures the disease severity either as the area fraction covered by lymphocytic infiltrates (Allushi et al., 2019) or as a focus score per 4 sq mm of salivary gland tissue (Fisher et al., 2017). For a quantitative assessment, scan the H&E sections using a digital pathology slide scanner. Using imaging software, draw margins around each lymphocytic focus and the entire section. Calculate the area (in sq mm) covered by lymphocytic infiltrates and the section's total area. Express the severity as the % area of inflammation = (area occupied by lymphocytic foci / total area of the section) x 100. Express the focus score as (number of foci/ total area of the section in sq mm) x 4. It is recommended that a minimum of 8 sq mm of the total glandular area should be evaluated. A representative image is shown in Figure 4.

Multiple commercial digital slide scanners and imaging software are available that can be used for quantitative scoring of salivary gland inflammation.

[*Insert figure 3 and 3 near here]

SUPPORT PROTOCOL 3

MEASURING AUTOANTIBODY TITERS BY INDIRECT IMMUNOFLUORESCENCE:

SS patients show antibodies to intracellular proteins Ro52, Ro60, and La, anti-nuclear antibodies, and rheumatoid factor. Different commercial assays employing recombinant proteins as target antigens coated onto ELISA plates or beads can be purchased for analyzing autoantibody specificities. In addition, immortalized cells grown on coverslips or slides are also commercially available for screening serum samples for circulating autoantibodies. The protocol described below can be established in-house and used to measure autoantibody titers and evaluate reactivity to nuclear and cytoplasmic antigens.

Materials

NIH/3T3 mouse fibroblast cells (ATCC, cat. no. CRL-1658)

Culture media: Dulbecco's Minimum Essential Medium (Lonza, cat. no. 12-614F),

Media supplements: 10% FCS, L glutamine, Penicillin Streptomycin (Life technologies).

Phosphate buffered saline

Ice-cold methanol

Diluent: PBS with 3% bovine serum albumin fraction V (Millipore Sigma, cat. no. 05470)

Goat anti-mouse IgG FITC (Southern Biotech, cat. no. 1030-02)

Normal goat serum (Southern Biotech, cat. no. 0060-01)

DAPI (nuclear stain) (Invitrogen, cat. no. D1306)

Prolong Diamond mountant (Thermo Fisher, cat. no. P36961)

24-well cell culture plates

Coverglass 12mm diameter (VWR, cat. no. 89015-725)

Pipets, tips,

Microscopy glass slides

Rocker/ shaker (Boekel scientific variable speed mini orbitron, cat. no. 201100) or similar

Fluorescence microscope

Equipment for sterile cell culture (biosafety cabinet, CO_2 incubator, centrifuge)

Protocol steps

1) Maintain a culture of NIH/3T3 fibroblast cells using standard cell culture protocol recommended by the ATCC (https://www.atcc.org/products/all/crl-1658.aspx#culturemethod)

   If the NIH/3T3 cell line is not available, the HeLa cell line (ATCC, CCL-2) can be used for autoantibody analysis. In this case, the autoantibodies recognize cross-reactive epitopes on the mouse and human proteins. In our experience, no significant differences in autoantibody titers or reactivity patterns were observed when using either NIH/3T3 or HeLa cells. To culture HeLa cells, follow the protocol recommended by ATCC (https://www.atcc.org/products/all/CCL-2.aspx#culturemethod).

2) Place glass coverslips in a beaker and sterilize by autoclaving. Using sterile forceps, transfer one coverslip/well of a 24 well culture plate.

3) Add NIH/3T3 cells (10k cells/well) in 0.1 mL of media to the center of each coverslip. Leave the plate undisturbed in the tissue culture hood for 20 min at room temperature. Then add 0.4 mL of media and transfer the plate to a 37^{o}C incubator with 5% CO2 and culture overnight.

   The incubation step at room temperature ensures that an optimum number of cells remain at the center of the coverslip.

4) The next day, wash the coverslips with PBS to remove loosely adherent dead cells.

   Each wash is carried out by adding PBS (0.5 mL/well), and shaking the plate gently on a platform rocker or shaker for 5 min, and then aspirating the PBS solution. All additions into the wells must be made promptly to prevent the wells from drying out at any stage of the assay.

5) Add ice-cold methanol 0.5 mL to each well to fix the cells for 10 min. Wash twice with PBS.

   Add blocking reagent, normal goat serum (1:50 dilution; 0.2 mL/well) in the diluent to each well, and incubate for 30 min at room temperature on a rocker.

   Make all solutions to be added on the coverslips to have at least 20 μL excess. Centrifuge at max speed for 3 min in a microcentrifuge to pellet down any particulate matter/ protein precipitates in the solutions. The precipitates can stick to the coverslip and create speckled background auto-fluorescence.

6) Aspirate and add sera at different dilutions 1:50, 1:100, 1:200 in the diluent. Coverslips with diluent alone serve as a negative control. Incubate for 1 hour at room temperature followed by 3 washes with PBS.

   From this step onward, protect the plate from exposure to bright light.

7) Add goat anti-mouse IgG-FITC (1:250 dilution) to each well and incubate for 30 min at room temperature. Wash 3 times with PBS.

8) Dilute DAPI solution in water (1:1000) and incubate with the cells for 10 min for nuclear staining. Wash twice with PBS.

9) Transfer the coverslips onto microscopy slides for imaging on a fluorescence microscope. For this, place a drop of Prolong gold mountant medium on the microscopy slides. Lift the coverslip from the culture dish using a pair of fine forceps, hold over lint-free paper, and allow excess fluid to drain. Then place the coverslip over the drop of mountant, taking care that the surface with grown cells now faces the mountant medium. Allow the mountant to cure overnight at room temperature. The slides can be evaluated the following day.

10) Observe at 20x on a fluorescence microscope for the intensity of staining and pattern. The highest dilution of the serum where the staining intensity is the same as the negative control is expressed as the end-point titer. Figure 5 shows representative results of autoantibody reactivity patterns in mice injected with DMXAA (Basic Protocol 2).

    This assay can be adapted to use with other adherent cell types. Very high cell densities can cause high auto-fluorescence backgrounds and confound the results. Therefore, it is recommended that a pilot experiment seeding different cell numbers be carried out to determine the optimal number of cells/coverslip. The indirect immunofluorescence assay allows the characterization of the autoantibodies as targeting the nucleus or cytoplasm. Readers are referred to the International Consensus on Antinuclear Antibody Patterns (ICAP) website (https://www.anapatterns.org/) for guidance on the interpretation of the staining patterns (Chan et al., 2015).

[*Insert figure 5 near here]

REAGENTS AND SOLUTIONS:

Mouse Anesthetic Mixture: Ketamine 10 mg/mL and Xylazine 1 mg/mL in sterile saline

Ketamine hydrochloride 100 mg/mL (Vet One cat no. C3N VT1)

Anased/Xylazine 20 mg/mL (Med-Vet International cat no. RXANASED-20)

Isotonic sterile saline (Vet One cat. No. 501032)

Mix 8.5 mL saline + 1 mL Ketamine + 0.5 mL Anased in a sterile glass vial. Store at 4^{o}C. The solution expires after 3 months from the date of formulation.

DXAA solution:

Mix endotoxin-free water (1 mL) with 7.5% sodium bicarbonate solution (2 mL) in a sterile tube to get a sterile, endotoxin-free 5% bicarbonate solution. Add the appropriate volume of the 5% sodium bicarbonate solution to the lyophilized DMXAA vial covered with aluminum foil to protect from light at all times. Ensure that the DMXAA has dissolved by pipetting and vortexing. It is best to reconstitute the whole vial and freeze the excess DMXAA solution at -80^{o}C in 0.25 ml single-use aliquots to avoid repeated freeze-thawing.

COMMENTARY

BACKGROUND INFORMATION

SS is a chronic, debilitating, autoimmune disorder affecting multiple organs, including exocrine glands (salivary and lacrimal), blood vessels, the respiratory system, gastrointestinal system, and central and peripheral nervous system (Vivino et al., 2019). A significant proportion of SS patients (~33%) have signs of other autoimmune diseases such as systemic lupus erythematosus, rheumatoid arthritis, inflammatory bowel disease, and autoimmune thyroiditis (Amador-Patarroyo et al., 2012). SS has a strong gender bias with a female to male ratio of 10:1. Genetic and environmental factors play an important role in susceptibility to SS. However, the exact cause for SS remains unknown, and an SS-specific cure has been elusive.

Over the past several years, multiple mouse models have been developed to investigate SS. However, no single experimental mouse model represents the entire clinical spectrum of the disease. The most common characteristics of SS recapitulated in mouse models are salivary gland and lacrimal gland inflammation, reduced fluid (saliva and tear) secretion, and the presence of autoantibodies in circulation. The mouse models provide insights into disease pathogenesis mechanisms and are valuable tools for testing novel therapeutic approaches. The mouse models for SS have been exhaustively reviewed (Xiao et al., 2019).

Some of the most commonly used mouse models and their salient characteristics are discussed here.

Genetic models of SS: Several mouse strains, including NZB/W F1 (Kessler, 1968) Non-obese diabetic (NOD) (Hu et al., 1992), B6.*Aec1Aec2* (Cha et al., 2002), and IL14α transgenic (Shen et al., 2009) mice that spontaneously develop SS-like salivary gland disease have been extensively used in the field to study SS.

The NZB/W F1 mice are the earliest described model for SS (Kessler, 1968). Female NZB/W F1 mice spontaneously develop a drop in saliva and tear production. The exocrine gland dysfunction is accompanied by lymphocytic infiltration in the salivary and lacrimal glands and circulating autoantibodies. The mice also develop lupus-like nephritis and represent the subset of SS patients with polyautoimmunity.

The NOD female mice are a popular model for type I diabetes mellitus. They also develop salivary and lacrimal gland inflammation, show a drop in saliva production with increasing age, and are used as a SS model (Hu et al., 1992). However, the glandular dysfunction in NOD mice correlates strongly with the level of hyperglycemia, a characteristic not common in SS patients (Allushi et al., 2019). Thus, while the glandular inflammation in NOD mouse may mimic that seen in patients, NOD mouse's utility in investigating salivary gland dysfunction is open to debate.

The B6.*Aec1.Aec2* strain was generated by crossing the NOD mice with the non-autoimmune C57BL/6 mice (Cha et al., 2002). The B6.*Aec1.Aec2* mice harbor the SS susceptibility genetic loci on chromosomes 1 and 3 from the NOD mice on a C57BL/6 background. These mice are protected from hyperglycemia and spontaneously develop the salivary and lacrimal gland manifestations of SS. However, the SS phenotype in this strain is notoriously susceptible to changes in the environment/microbiome. It has been a challenge to reproduce the phenotype in different animal facilities across the USA (personal communication).

The IL14α transgenic mice develop glandular inflammation, circulating autoantibodies, interstitial lung disease, and mild renal disease (Shen et al., 2009). In some mice, the salivary gland inflammation progresses to the formation of B cell lymphomas. This mouse strain demonstrates the effect of over-expression of a single gene mirroring SS patient characteristics and provides a tool for investigating SS's immune-mediated pathologies.

Each mouse model mimics some SS patients' features, and targeted deletion or overexpression of specific transgenes on these genetic backgrounds have been used to understand immune-mediated salivary gland injury.

Induced models of SS: The induced mouse models replicate some of the unique characteristics of SS patients to understand how these factors drive disease pathogenesis. The two main features of SS investigated in induced models are the role of SS-associated autoantibodies and innate immunity.

Over 75% of SS patients harbor high levels of autoantibodies to Ro52/TRIM21 protein, a cytosolic E3 ubiquitin ligase. Immunization of mice with the whole Ro52 protein or its fragments resulted in the generation of anti-Ro52 antibodies in circulation, associated with a drop in saliva production, without any salivary gland inflammation establishing the pathogenic role for anti-Ro52 antibodies in SS (Szczerba et al., 2016; Sroka et al., 2018). Interestingly, these studies demonstrated a dissociation between the severity of salivary gland inflammation and salivary gland dysfunction, a feature noted in several SS patients. Similar studies have been conducted using peptides from other autoantigens, including Ro60, carbonic anhydrase, and muscarinic 3 receptor (reviewed by Xiao et al., 2019).

A key feature in a significant number of SS patients is an elevated type I interferon signature, which is associated with increased disease severity (Vivino et al., 2019). The role of type I IFN in SS initiation has been demonstrated in a mouse model system (Szczerba et al., 2013). Genetic abrogation of signaling through the common type I Interferon α/β receptor in the B6.*Aec1.Aec2* mice protected them from salivary gland inflammation and dysfunction. As described in this unit, activation of type I interferons in spontaneous NZB/W F1 is associated with an accelerated onset of disease (Nandula et al., 2011).

The elevated type I interferons in SS patients forms the basis for a viral etiology for SS. However, despite extensive research, no single viral infection is associated with SS. Further, SS patients are not known to suffer chronically from concomitant viral infections. This has spurred interest in the role of sterile inflammation caused by the activation of innate immune pathways in SS's etiopathogenesis. Recent work shows that signaling through the cytosolic DNA sensing pathways, like those involving the STING protein, induced salivary gland manifestations of SS in mice (Papinska et al., 2018).

The discovery of newer molecular pathways and a rapidly evolving knowledge of innate immunity aided by cutting-edge methodologies like Next-Generation Sequencing and Spatial Transcriptomics will provide a better understanding of the disease.

CRITICAL PARAMETERS

Two of the most critical parameters for this unit's protocols are the mouse facility and saliva measurements.

Environmental factors strongly influence the mouse models of SS. The choice of mouse strain and a specific pathogen-free facility with good animal husbandry practices are critical for establishing SS mouse models.

Saliva measurements are influenced by environmental factors and require consistent procedures for reproducibility. As previously discussed, duration of fasting, accurate dosing, gentle mouse handling, and meticulous preparation of reagents facilitate reproducible results.

Proper dissection, fixation, and salivary gland tissue processing for good quality histopathology are important for accurate disease scoring.

There may be considerable variation in the severity of inflammation and saliva production. For each protocol described here, 7-10 mice are required per experimental and control groups to achieve statistical significance.

TROUBLESHOOTING

The methods described in this unit are relatively simple and could be easily adapted to most laboratory settings. However, it is known that environmental factors and gut microbiome changes in the colony can significantly impact the incidence and kinetics of SS and other autoimmune diseases. To maintain reproducibility, all mice described in this protocol are typically purchased at 5-8 weeks of age from The Jackson Laboratory. If experimental mice are to be bred in the investigator's colony, collecting and storing stool samples at -80^{o}C for future gut microbiome analysis should be considered to address any possible variations in the disease penetrance.

Lipopolysaccharide/endotoxin is a heat-stable, ubiquitous contaminant of laboratory glassware and chemicals unless specific precautions are taken. Endotoxin contamination and ensuing activation through TLR4 can confound the results of activation through specific innate immune pathways. Therefore, the use of endotoxin-free reagents and supplies are indicated throughout this unit.

UNDERSTANDING RESULTS

The SS models described here have been tested in female mice. Poly(I:C) induces a transient increase in circulating pro-inflammatory cytokines, including type I interferons and IL6, and a rapid and significant drop in saliva production (Nandula et al., 2011). In Poly(I:C) treated NZB/W F1 mice, inflammation appears at 12 weeks after treatment, and functional loss is seen at 20 weeks.

In non-autoimmune C57BL/6 mice, the functional drop is transient, and the mice show a complete recovery after the injections are stopped (Nandula et al., 2013). C57BL/6 mice fail to develop salivary gland inflammation. In contrast, the NZB/W F1 mice develop chronic salivary gland dysfunction and inflammation with Poly(I:C) treatment.

Disease induction is simple. Salivary gland inflammation and reduced saliva production are seen 30 days after the first injection of DMXAA in female C57BL/6 mice (Papinska et al., 2018). Using this protocol, the salivary gland inflammation persists up to 2 months after the first injection. The mice also develop inflammation of varying severity in the lacrimal glands and the lungs, reflecting the SS's multi-organ involvement (Papinska et al., 2020).

Following IFA injection, functional loss was seen in NZB/W F1 mice at 7 weeks after treatments, and inflammation was apparent at 12 weeks (Deshmukh et al., 2008).

TIME CONSIDERATIONS

All-time considerations are assuming experimental and control groups with 10 mice/ group.

Basic Protocol 1: Preparing Poly(I:C) 1 h and 30 min. Intraperitoneal injection of 20 mice: 1 h.

Basic Protocol 2: Dissolving DMXAA, preparing doses for each mouse: 1 h. Injection of 20 mice: 2 h.

Alternate protocol 1: Preparing Incomplete Freund's Adjuvant emulsion: 1 h. Injection of 20 mice: 3 h.

Support protocol 1: Saliva measurements for 20 mice: preparation time 30 min; saliva collection: 4 h.

Support protocol 2: Euthanizing mice, harvesting of salivary glands from 20 mice: 3 h. Tissue fixation, processing, sectioning, and staining: 3-4 days. Histopathologic scoring for 20 mice: 2 days.

Support Protocol 3: Assay carried out over 3 days. Day 1: 30 min, Day 2: 4 hour, Day 3: 1 hour.

ACKNOWLEDGEMENTS: Supported by grants from the National Institute of Dental and Craniofacial Research (DE025030, DE029707), National Institutes of Health, and Presbyterian Health Foundation, Oklahoma. Expert assistance from the OMRF imaging core facility is gratefully acknowledged.

CONFLICT OF INTEREST: The authors declare no conflicts of interest.

LITERATURE CITED:

Allushi, B., Bagavant, H., Papinska, J., & Deshmukh, U. S. (2019). Hyperglycemia and Salivary Gland Dysfunction in the Non-obese Diabetic Mouse: Caveats for Preclinical Studies in Sjögren's Syndrome. *Scientific reports*, *9*(1), 17969.

Amador-Patarroyo, M. J., Arbelaez, J. G., Mantilla, R. D., Rodriguez-Rodriguez, A., Cárdenas-Roldán, J., Pineda-Tamayo, R., Guarin, M. R., Kleine, L. L., Rojas-Villarraga, A., & Anaya, J. M. (2012). Sjögren's syndrome at the crossroad of polyautoimmunity. *Journal of autoimmunity*, *39*(3), 199–205.

Bagavant, H., Trzeciak, M., Papinska, J., Biswas, I., Dunkleberger, M. L., Sosnowska, A., & Deshmukh, U. S. (2018). A Method for the Measurement of Salivary Gland Function in Mice. *Journal of visualized experiments : JoVE*, (131), 57203.

Cha, S., Nagashima, H., Brown, V. B., Peck, A. B., & Humphreys-Beher, M. G. (2002). Two NOD Idd-associated intervals contribute synergistically to the development of autoimmune exocrinopathy (Sjögren's syndrome) on a healthy murine background. *Arthritis and rheumatism*, *46*(5), 1390–1398.

Chan, E. K., Damoiseaux, J., Carballo, O. G., Conrad, K., de Melo Cruvinel, W., Francescantonio, P. L., Fritzler, M. J., Garcia-De La Torre, I., Herold, M., Mimori, T., Satoh, M., von Mühlen, C. A., & Andrade, L. E. (2015). Report of the First International Consensus on Standardized Nomenclature of Antinuclear Antibody HEp-2 Cell Patterns 2014-2015. *Frontiers in immunology*, *6*, 412. https://doi.org/10.3389/fimmu.2015.00412

Deshmukh, U. S., Ohyama, Y., Bagavant, H., Guo, X., Gaskin, F., & Fu, S. M. (2008). Inflammatory stimuli accelerate Sjögren's syndrome-like disease in (NZB x NZW)F1 mice. *Arthritis and rheumatism*, *58*(5), 1318–1323.

Fisher, B. A., Jonsson, R., Daniels, T., Bombardieri, M., Brown, R. M., Morgan, P., Bombardieri, S., Ng, W. F., Tzioufas, A. G., Vitali, C., Shirlaw, P., Haacke, E., Costa, S., Bootsma, H., Devauchelle-Pensec, V., Radstake, T. R., Mariette, X., Richards, A., Stack, R., Bowman, S. J., … Sjögren's histopathology workshop group (appendix) from ESSENTIAL (EULAR Sjögren's syndrome study group) (2017). Standardisation of labial salivary gland histopathology in clinical trials in primary Sjögren's syndrome. *Annals of the rheumatic diseases*, *76*(7), 1161–1168.

Hu, Y., Nakagawa, Y., Purushotham, K. R., & Humphreys-Beher, M. G. (1992). Functional changes in salivary glands of autoimmune disease-prone NOD mice. *The American journal of physiology*, *263*(4 Pt 1), E607–E614.

Kessler H. S. (1968). A laboratory model for Sjögren's syndrome. *The American journal of pathology*, *52*(3), 671–685.

Nandula, S. R., Scindia, Y. M., Dey, P., Bagavant, H., & Deshmukh, U. S. (2011). Activation of innate immunity accelerates sialoadenitis in a mouse model for Sjögren's syndrome-like disease. *Oral diseases*, *17*(8), 801–807.

Nandula, S. R., Dey, P., Corbin, K. L., Nunemaker, C. S., Bagavant, H., & Deshmukh, U. S. (2013). Salivary gland hypofunction induced by activation of innate immunity is dependent on type I interferon signaling. *Journal of oral pathology & medicine 42*(1), 66–72.

Papinska, J., Bagavant, H., Gmyrek, G. B., Sroka, M., Tummala, S., Fitzgerald, K. A., & Deshmukh, U. S. (2018). Activation of Stimulator of Interferon Genes (STING) and Sjögren Syndrome. *Journal of dental research*, *97*(8), 893–900.

Papinska, J., Bagavant, H., Gmyrek, G. B., & Deshmukh, U. S. (2020). Pulmonary Involvement in a Mouse Model of Sjögren's Syndrome Induced by STING Activation. *International journal of molecular sciences*, *21*(12), 4512.

Shen, L., Suresh, L., Li, H., Zhang, C., Kumar, V., Pankewycz, O., & Ambrus, J. L., Jr (2009). IL-14 alpha, the nexus for primary Sjögren's disease in mice and humans. *Clinical immunology (Orlando, Fla.)*, *130*(3), 304–312.

Sroka, M., Bagavant, H., Biswas, I., Ballard, A., & Deshmukh, U. S. (2018). Immune response against the coiled coil domain of Sjögren's syndrome associated autoantigen Ro52 induces salivary gland dysfunction. *Clinical and experimental rheumatology*, *36 Suppl 112*(3), 41–46.

Szczerba, B. M., Rybakowska, P. D., Dey, P., Payerhin, K. M., Peck, A. B., Bagavant, H., & Deshmukh, U. S. (2013). Type I interferon receptor deficiency prevents murine Sjogren's syndrome. *Journal of dental research*, *92*(5), 444–449.

Szczerba, B. M., Kaplonek, P., Wolska, N., Podsiadlowska, A., Rybakowska, P. D., Dey, P., Rasmussen, A., Grundahl, K., Hefner, K. S., Stone, D. U., Young, S., Lewis, D. M., Radfar, L., Scofield, R. H., Sivils, K. L., Bagavant, H., & Deshmukh, U. S. (2016). Interaction between innate immunity and Ro52-induced antibody causes Sjögren's syndrome-like disorder in mice. *Annals of the rheumatic diseases*, *75*(3), 617–622.

Vivino, F. B., Bunya, V. Y., Massaro-Giordano, G., Johr, C. R., Giattino, S. L., Schorpion, A., Shafer, B., Peck, A., Sivils, K., Rasmussen, A., Chiorini, J. A., He, J., & Ambrus, J. L., Jr (2019). Sjogren's syndrome: An update on disease pathogenesis, clinical manifestations and treatment. *Clinical immunology (Orlando, Fla.)*, *203*, 81–121.

Xiao, F., Han, M., Wang, X., Gong, X., Huang, E., Zhu, Z., Zhao, F., Zhao, Y., Jiang, Q., & Lu, L. (2019). Animal models of Sjögren's syndrome: an update. *Clinical and experimental rheumatology*, *37 Suppl 118*(3), 209–216.

FIGURE LEGENDS:

Figure 1: Schematic representation of the timeline for Basic protocol 1 **(A)** and Basic Protocol 2 **(B)**.

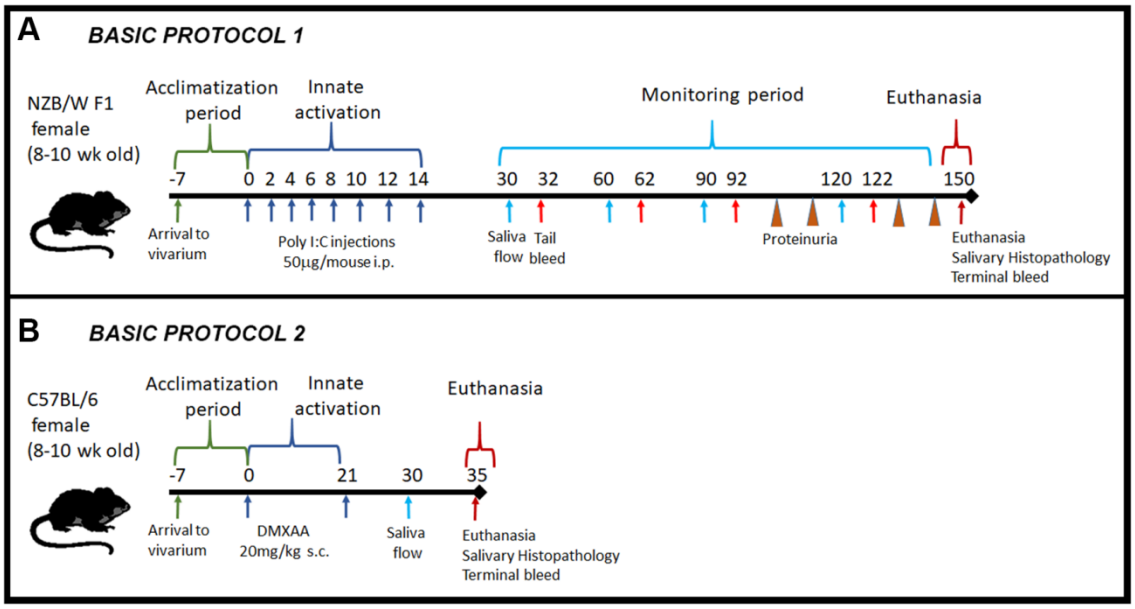


Figure 2: Dissection of salivary glands from mice. **(A)** Give a midline incision from the sternum to the lower lip's base and raise the skin flaps on either side to expose the salivary glands. **(B)** Identify the submandibular (left SMG- black outline), sublingual (left SLG - blue outline), and parotid (left PG – green outline) glands. Note that the cervical lymph nodes (CxLN – yellow dots) are embedded in this area and may be visible on dissection.

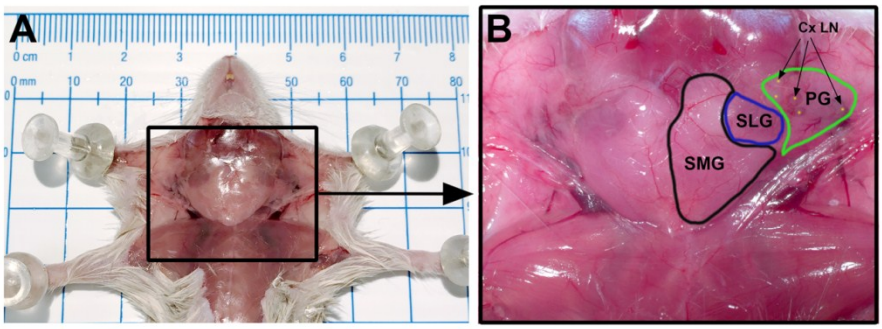


Figure 3: Evaluating salivary gland inflammation. Photomicrographs of formalin-fixed, paraffin-embedded, hematoxylin and eosin-stained mouse salivary glands showing normal ductal and acinar organization in SMG **(A)** and SLG **(B)**. SMG showing a small, single lymphocytic focus (arrow) in the peri-vascular, peri-ductal region **(C),** and a larger lymphocytic focus invading the surrounding acinar tissues (arrows) **(D)**. Extensive lymphocytic infiltration of the SMG **(E)**, with lymphocytes invading the glandular parenchyma **(F)** showing remnants of acinar and ductal tissue (arrows). Objectives used for capturing the Images A-D (20x), E (10x) and F (40x). Scale bar = 50 microns.

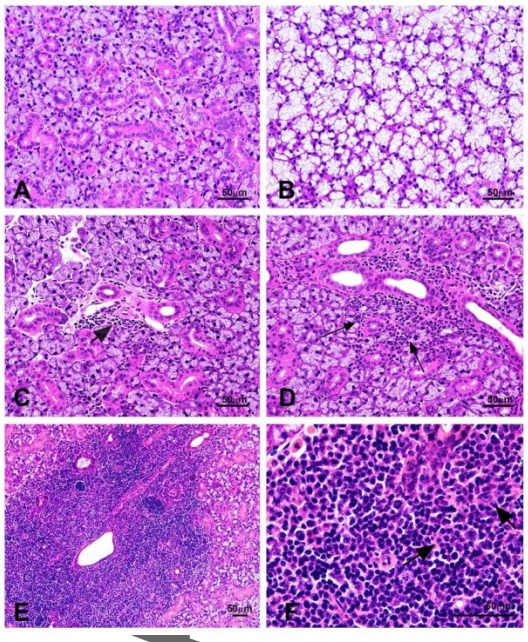


Figure 4: Quantitative evaluation of salivary gland inflammation. **(A)** The digitally scanned image of a cross-section through all the salivary glands harvested together showing SMG, SLG, and PG. **(B)** Areas covered by lymphocytic foci (n=17, green outline) and the total area of the section (blue outline). Severity of inflammation for this sample may be reported as (i) Area of inflammation (%) = area with lymphocytic infiltration (0.956 mm^2)/ total area of the section (23.379 mm^2) *100 = 4.09%; or (ii) Focus scores = [no. of foci (17)/ total area of the section (23.379)] *4 = 2.91.

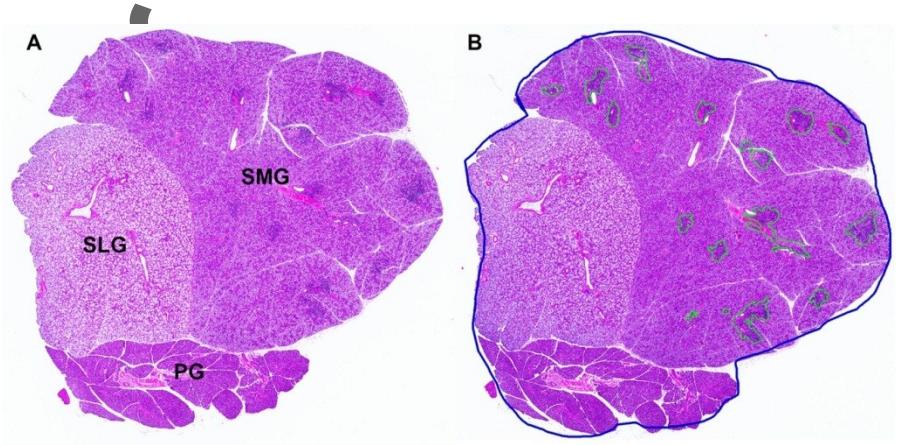
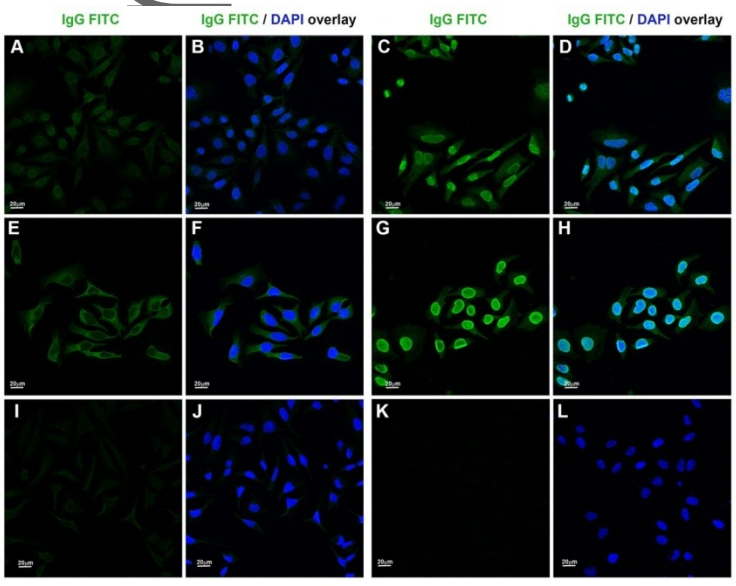


Figure 5: Autoantibody detection by indirect immunofluorescence. HeLa cells grown on coverslips were probed with sera from DMXAA injected mice (**A-H**) or control mouse sera (**I-J**). Bound antibodies were detected with goat anti-mouse IgG-FITC (Green), and nuclei were stained with DAPI (blue). Panel **K-L** show staining with only secondary antibody. Sera from DMXAA treated mice show weak **(A, B)** or strong **(C, D)** homogenous nuclear staining. Panels **(E, F)** show cytoplasmic staining, and **(G, H)** show prominent nuclear membrane staining. All the sera were tested at 1:50 dilution, and images were captured at 20x on a Zeiss LSM-710 confocal microscope. Scale bar = 20 microns.



TABLES:

TABLE 1: Semi-quantitative grading of salivary gland inflammation.

Grade	Description
0	No foci
0.5-1	1-5 foci Small, single, or multiple lymphocytic aggregates of >50 cells
2	>5 foci without parenchymal destruction Large peri-ductal and perivascular infiltrates without acinar involvement
3	Moderate parenchymal destruction Disruption of acinar basement membrane with lymphocytes infiltrating from into acini
4	Extensive parenchymal destruction Multiple large foci replacing glandular tissue
5	Complete destruction of the glandular tissue Rarely seen in this model.