

Analysis of sexual dimorphism in salivary gland toll-like receptors in Sjögren's Syndrome

Section 1: Specific Aims

Primary Sjögren's Syndrome (pSS) is an autoimmune disease targeting the salivary and lacrimal glands that results in dry mouth and dry eyes [1]. This chronic disease affects females to males in a 9:1 ratio, with middle-aged women primarily affected [2, 3]. Many autoimmune diseases have demonstrated elevated expression of Toll-like Receptors (TLRs), including SS [4-7]. While several lines of evidence suggest that TLR signaling is important in SS pathogenesis [2, 8-10], there are no studies to date examining sexual dimorphism of salivary TLRs in this disease. The **goal** of the proposed research is to determine whether TLR expression and activation are elevated in salivary tissue from female mice as compared to male, and whether these differences are more pronounced in pSS mice than in healthy controls.

We **expect** that TLR gene expression and activity will be higher in salivary tissue in both control and pSS female mice as compared to age and strain-matched males. Moreover, we predict that salivary TLR expression and activity will be elevated in pSS mice as compared to age and gender-matched controls. This work will positively **impact** the field because pSS is seen much more commonly in females than males and differential TLR activation may contribute to the strong female disease predilection observed. Identification of TLR signaling pathways that are hyperactive in women with pSS may lead to the discovery of novel pathways that can be targeted therapeutically.

We will examine TLR expression and function using the following **specific aims**:

1. To determine whether TLR gene expression is increased in female control and pSS mice as compared to age and strain-matched males, and whether these changes are greater in pSS mice as compared to age and gender-matched controls.
2. To determine whether specific TLR signaling pathways are hyperactive in control and pSS female mice as compared to age and strain-matched males, and whether these are elevated in pSS mice as compared to age and gender-matched controls.

Section 2: Research Strategy

2.1. Significance: Sexual dimorphism is seen in salivary tissue of healthy mice and many immune-related genes are elevated in females as compared to males [11]. SS is more common in women, although the

reasons for this are incompletely understood [12]. Activation of TLR signaling may be involved in SS pathogenesis as suggested by *in vivo* and *in vitro* studies [4, 8-10, 13]. However, it is not known at present whether there are differences in the expression and/or activity of certain TLR-induced signaling pathways between female and male salivary tissue. Therefore, it is important to determine whether TLR expression and responsiveness is increased in female salivary tissue. Results from this study may ultimately lead to identification of TLRs that may serve as therapeutic targets in SS. These may be particularly effective in women who are afflicted with this debilitating disease.

2.2. Innovation: Our work is innovative because it will establish whether salivary TLR expression differs between males and females. Our proposed study will enable us to directly examine TLR expression and function in salivary tissue from a pSS model. TLR signaling in both innate and adaptive immune cells plays a causative role in the autoimmune disease lupus [6, 7, 14], but has not been evaluated in depth in SS. TLR hyperactivity in female salivary tissue may be a seminal event in SS that is crucial for disease development and progression. Blockade of TLR signaling may effectively suppress salivary inflammation that is characteristic of SS. TLRs have been targeted successfully in several autoimmune diseases [15], and this may represent a novel therapeutic approach for SS patients. Currently, the causes of SS are unknown [12]. Blockade of TLR-related pathways may constitute a successful strategy to reduce SS disease.

2.3. Approach:

Overall strategy: We will examine salivary tissue that will be isolated from male and female NOD.B10Sn-*H2^b/J* (NOD.B10) primary SS mice and age-and gender matched controls. The NOD.B10 model develops spontaneous disease by the age of 26 weeks (wks) and all animals will be examined at this time point [16]. We will use C57BL/10SnJ (BL/10) mice as healthy controls, since NOD.B10 mice derive their MHC locus from the BL/10 strain [17]. Both NOD.B10 and BL/10 mice are commercially available. To determine whether TLR expression differs between male and female animals, we will examine transcript levels in submandibular gland (SMG) tissue from both healthy control and SS animals (Specific Aim 1). To determine whether specific TLR signaling pathways are hyperactive in control and pSS female mice as compared to age and strain-matched males, we will stimulate salivary tissue with TLR agonists and

compare production of an inflammatory cytokine (interleukin 6 (IL-6)) between males and females of each strain (Specific Aim 2). Expected results are provided in Table 1.

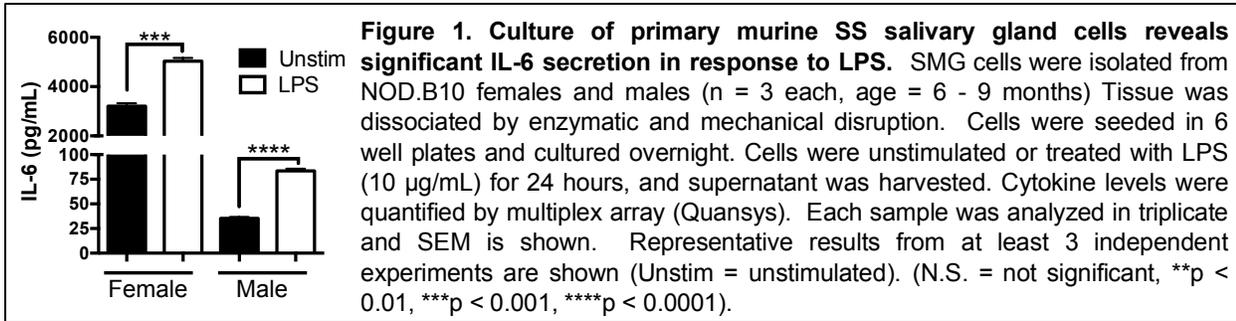
Table 1: Expected outcomes for specific aims 1 and 2

	BL/10 Male	BL/10 Female	NOD.B10 Male	NOD.B10 Female
Aim 1: TLR gene expression	++	+++	+++	++++++
Aim 2: Spontaneous IL-6 production	+	++	++	++++++
Aim 2: TLR-agonist induced IL-6 expression	++	++++	++++	++++++

Analyses used: We will use NOD.B10 female and male mice with clinical disease (26 wks of age) and age and gender-matched C57BL/10 controls for both aims. For specific aim 1, we will analyze 8 mice per group. Animals will be euthanized and SMG and spleen tissue harvested. We will use spleen tissue as a positive control. We will isolate mRNA and perform reverse transcription PCR. We will then synthesize cDNA. Quantitative PCR (qPCR) will be performed for TLRs implicated in SS (TLR1 – 4, 7, and 9) using primers specific to the mouse TLRs using SYBR green [4, 9, 18]. PCR settings will be as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. All samples will be analyzed in duplicate and expression normalized to β -actin. Relative expression will be calculated as $\Delta\Delta Ct$, where $\Delta\Delta Ct$ refers to ΔCt (TLR) - Ct (β -actin). Our lab has extensive experience in qPCR on salivary tissue [19, 20]. We will compare relative TLR levels in the following groups: control female to pSS female, control male to pSS male, pSS female to pSS male and control female to control male (Table 1).

For specific aim 2, we will determine whether SMG cells from female BL/10 and NOD.B10 mice secrete higher levels of IL-6 as compared to their male counterpart strains. We will also stimulate the SMG cells with the TLR agonists lipopolysaccharide (LPS) and polyinosinic:polycytidylic acid (poly(I:C)) to determine whether female SMG cells are more responsive to TLR ligands as compared to those derived from male. Previous studies by our group and others show salivary cells are responsive to these ligands (Figure 1 and [9]). We will also compare SMG tissue from age and gender-matched NOD.B10 and BL/10 mice to determine whether salivary tissue from SS mice is more responsive to TLR ligation, as measured by IL-6 secretion. We will pool SMG tissue from 3 animals of the same sex and strain, and each experiment will be repeated at least in triplicate. Our preliminary data demonstrate the feasibility of this approach (Figure 1). For aims 1 and 2, we will determine significance using the Mann-Whitney test.

Resource sharing: Findings will be shared by publication and at local, national and international scientific meetings. NOD.B10 and BL/10 mice are publicly available from The Jackson Laboratory.



Potential problems and alternative strategies: Since we have significant experience in the techniques proposed herein, we do not anticipate any significant problems. However, it is possible that we may fail to see amplification of specific TLR transcripts in both SMG and spleen tissue (our positive control). If this occurs, we will re-design our primers. We will use the publically available program from NCBI, Primer-BLAST, to aid in design.

Section 3: Resource Information

Kramer Laboratory: Dr. Kramer's laboratory is located in Foster hall and consists of approximately 1200 sq. ft. of space including a separate tissue culture area, a cold room and an autoclave facility.

Facilities

Animal: The animal facilities are located in the Biomedical Education Building adjacent to the School of Dental Medicine directly across the street from the Foster Hall building. The PI will use the Division of Comparative Medicine and Laboratory Animal Facilities (CM-LAF) at the State University of New York at Buffalo for housing mice. The CM-LAF veterinarians monitor the animals' health and currently maintain Dr. Kramer's mice colonies.

Core Facilities: Two BioRad CFX96 thermocyclers are available for qPCR.

Section 4: Other support for applicant and sponsor (mentor)

Support for this project will be provided by UB SDM start up funds to JMK.

Section 5: IACUC and IRB approval

This project does not involve human subjects.

Vertebrate Animals

This project involves the use of healthy control (C57BL/10) and SS mice (NOD.B10). The IACUC approval number for this project is ORB01102Y (Approval date 6/21/2016).

Literature Cited in Technical Proposal

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