ROLE OF IGG1 M3R AUTOANTIBODIES IN SECRETORY DYSFUNCTION OF SJÖGREN'S SYNDROME

By

HUY HUYNH

A THESIS PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE

UNIVERSITY OF FLORIDA

2009

To everyone who supported my work.

ACKNOWLEDGMENTS

It is with sincere gratitude, humility, and thankfulness that I acknowledge the following individuals for their help and support during the last two years. Without their help, I would not have grown both professionally or personally to the potential that they saw within me to succeed. I would first like to thank my committee co-chairs, Dr. Gary Rodrick and Dr. Seunghee Cha, for accepting me as a student and guiding me through my efforts. I would like to thank Dr. Rodrick, for his patience and honest advice that helped guide me in the right direction. Also, I thank Dr. Cha, for her unwavering support and invaluable advice that not only helped me further my career and knowledge as a student, but also gave me the faith to push myself through the hardest of times.

Next, I would like to recognize my committee members for their support. I wish to thank Dr. Keith Schneider for his motivational talks about timeliness and his statistical expertise. Next, I wish to thank Dr. Ammon B. Peck for sharing his knowledge and passion for the field of autoimmunity. In addition, I would like to thank Dr. Minoru Satoh for his unforgettable humor and his never ending patience, while teaching me the intricacies of experimental technique, implementation, and understanding the reasoning behind our methods.

Lastly, I would like to thank my laboratory associates for always lending a helping hand and being such wonderful role models for me. I wish to thank Jason Weinstein, Dina Nacionales, and Tolga Barker in Dr. Satoh's lab for always taking time out of their schedules to teach me the nuances of research, as well as making my research experience enjoyable. Also, to my associates in Dr. Cha's laboratory: Kaleb Pauley, Marievic Bulosan, Kyumee Yo, and Christopher Chuong. I would like to thank them for being my support beam and a constant positive energy for me in the last two years. Without everyone, I would not have had the memorable experiences and knowledge that helped motivate me to succeed.

TABLE OF CONTENTS

page

LIST OF TABLES

LIST OF FIGURES

Abstract of Thesis Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Master of Science

ROLE OF IGG1 M3R AUTOANTIBODIES IN SECRETORY DYSFUNCTION OF SJÖGREN'S SYNDROME

By

Huy Huynh

August 2009

Chair: Gary Rodrick
Cochair: Seunghee Cha
Major: Food Science and Human Nutrition

Sjögren's syndrome (SS), first described in 1933 by the Swedish ophthalmologist Henrik Sjögren, is an autoimmune disorder that targets the exocrine glands, especially the lacrimal and salivary glands. Two main symptoms that characterize this disorder are xerostomia (dry mouth) and xerophthalmia (dry eyes). Studies have suggested that autoantibodies from SS patients reactive to type-3 muscarinic acetylcholine receptors (M3R) may account for defective secretion in these patients. Thus, the objective of this study was to investigate the effects of IgG1 anti-mouse M3R autoantibodies on saliva secretion and calcium release by utilizing *in vivo* and *in vitro* systems, respectively.

First, sera from C57BL/6J (control) mice and SS-prone NOD/ShiLtJ (experimental) mice were collected and purified into different isotypes of IgG (IgG1, IgG2b, IgG2c, and IgG3). ELISA analysis was performed for their quantification. Fractions of each isotype were injected intraperitoneally (IP) into immune-deficient B6.129S7-Rag1^{tm1Mom}/J recipient mice. After 3 hours, saliva was collected for 10 minutes following an injection of a secretagogue mixture (pilocarpine and isoproterenol) to measure saliva flow. This procedure was repeated each day over a 5-day period. In addition, to investigate *in vitro* if IgG1 anti-M3R autoantibodies inhibit

M3R-mediated intracellular calcium release, human salivary gland (HSG) cells were incubated with the purified fractions for 24 hours prior to agonist carbachol stimulation and subject to a calcium release assay.

In the *in vivo* experiment, both NOD and C57BL/6J IgG1 and IgG2c fractions had a stimulatory effect on saliva secretion on Day 2 and Day 3, respectively, but a decrease in saliva secretion was observed on Day 5 in the NOD fractions compared to B6 fractions. In the *in vitro* experiments, significant reductions ($p<0.05$) in calcium release were observed for both C57BL/6J IgG1 and IgG2b at 15 minutes of incubation, as well as for all C57BL/6J and NOD fractions (especially NOD IgG1) at 24 hours with an addition of 2 μL Ig for both time treatments, compared to carbachol-stimulated HSG cells alone.

The *in vivo* preliminary findings suggest that both NOD IgG1 and NOD IgG2c fractions can cause secretory dysfunction. This may be due to the binding of these fractions to M3R, thereby blocking the signaling pathway that normally controls saliva secretion. Conversely, C57BL/6J IgG1 and IgG2c fractions seemed to cause a stimulatory effect to increase saliva secretion. NOD IgG2b fractions did not appear to inhibit salivary flow. The *in vitro* experiments demonstrate that both C57BL/6J and NOD purified fractions cause a decrease in calcium release in HSG cells stimulated with carbachol, suggesting intracellular calcium release can be inhibited by chronic incubation (24 hours) with both C57BL/6J and NOD purified IgG isotypes. Also, it can be observed that HSG cell incubation with the same amount (0.2 μg) of Ig fractions can cause a similarly decreasing trend compared to using the same volume (2 μL). Overall, these results suggest that NOD IgG1 and IgG2c might have inhibitory effects on secretory function *in vivo*. Experimental optimization is needed to investigate if IgG1 is responsible for a decrease in carbachol-induced intracellular calcium release, *in vitro*, after 24-hour chronic incubation.

CHAPTER 1
INTRODUCTION

Sjögren's syndrome (SS) was first described in 1933 by the Swedish ophthalmologist Henrik Sjögren as an autoimmune disorder that targets the exocrine tissues of the body, especially the lacrimal and salivary glands. Other targets of the disease include symptoms localized in the cardiovascular, central nervous system, renal systems, lungs, and gastrointestinal tract (1). Two main symptoms that characterize the disorder are xerostomia (dry mouth) and xerophthalmia (dry eyes). Saliva secretion is an important process within the body because it is induced during food consumption and is vital in normal dietary function and oral health. Saliva is first produced by both ductal and acinar cells. Acinar cells are divided into two types: mucus and serous cells (2). Salivary secretion is mainly controlled by the parasympathetic and sympathetic nervous systems. The sympathetic nerve is mainly responsible for protein secretion including exocytosis found in acinar cells. Conversely, the parasympathetic nerve is held responsible for water and electrolyte secretion (2). It has been shown that alterations in parasympathetic neurotransmission can lead to problems within these secretory glands (3). With that understanding, it can be suggested that modifications of these cellular pathways can lead to dysfunction in saliva secretion.

Primary and Secondary Sjögren Syndrome

Sjögren's syndrome is an autoimmune disease that displays both characteristics of kertoconjunctivitis sicca and xerostomia classified as primary and secondary. Primary SS (pSS) exhibits aforementioned traits without underlying diseases, while secondary SS (sSS) displays them in conjunction with another autoimmune disease such as type 1 diabetes (T1D), systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), and scleroderma (3).

Nutritional Aspects

Being that SS is an oral disorder, nutrient intake before and after disease onset has been observed. Normally, with decreased salivation, many oral problems arise such as candidiasis, dental caries, as well as difficulty in swallowing and speaking. In women with primary SS, it has been reported that increased ingestion of lactose, thiamin, and riboflavin occurs due to higher milk intake (4). It has been found that milk is a good substitute to help balance out low saliva secretion in patients with xerostomia (4). Along with nutrient intake studies, other biological therapy methods have been applied to dampen autoimmune responses in SS. Such methods include investigating the effects of B-cell targeted therapies such as infleximab, rituximab, epratuzumab, or belimumab (5).

Autoantibodies and Muscarinic Receptors Detected in SS

Anti-Ro/SSA and Anti-La/SSB Autoantibodies

Not only are SS autoantibodies directed against IgG (rheumatoid factor), they are also directed against Ro/SSA and La/SSB antigens. As part of the classification criteria proposed by the American-European consensus group, anti-Ro and anti-La autoantibodies are important disease markers for SS (6). These autoantibodies were first discovered in 1975 where 3 different types were found within sera from SS patients. Since their discovery, they have been so named anti-Ro/SSA and anti-La/SSB (found only in patients with primary SS), as well as SS-C which was later found to be RA nuclear antigen, a nuclear antigen that was detected in cells associated with the Epstein-Barr virus. Anti-Ro and Anti-La autoantibodies are associated with numerous clinical symptoms such as hypergammaglobulinemia, parotid swelling, severe salivary gland dysfunction, rheumatoid factor, as well as lymphopenia (7).

These autoantigens are composed of a number of antigenic proteins that are coupled with small RNA molecules. The protein makeup of these particular antibodies is composed of four

small RNA's (hY1, hY2, hY3, and hY5). These RNA's are associated with 60-kD Ro/SSA or 48-kD La-SSB (7). Antibodies against Ro-60 kD occur in 50-90% of patients with SS, where their function is to bind to defective, small RNA's which eventually lead to their degradation (7), (8). Even though anti-Ro antibodies are composed of two subunits, Ro-52 kD is a structurally distinct protein whose physical interaction with Ro-60 kD has still not been shown (9). It is known that patients with SS and SLE have a surprisingly increased amount of 52-kD Ro/SSA in PBMC's (10). The La/SSB protein is found at a much higher concentration than both Ro/SSA proteins- about 50 fold higher in the cell (11). It is these RNA-protein molecules that can be found within all human cells as well as many other species of animals (12). Even though anti-Ro/SSA antibodies are not specific markers for SS, they are found in 60% patients. Anti-La/SSB antibodies, conversely, are found in about 40% of patients with SS, where the only other disease that they are shown to be present in SLE (12).

Role of Autoantibodies in the Pathogenesis of Sjögren's Syndrome

In a study by Bacman et al. (13), IgG in patient sera was shown to bind and affect muscarinic receptors (MR) of rat parotid glands. Non-competitively, the antibodies were shown to have an inhibitory effect on the MR through decreasing the binding capabilities of ^{3}H-quinuclidinyl benzilate (MR agonist), which also led to other alterations, such as the decrease in cAMP. This study suggested that patient IgG binds to MR and competes for the MR agonist. The role of Ig in SS was further investigated by using the NOD.Igμ^{null} mouse, a B cell deficient mouse model. Purified IgG obtained from SS patients or SS-prone NOD/ShiLtJ mice has been shown to alter normal salivary secretion in NOD.Igμ^{null} mice following intravenous infusions. Such changes do not occur if infused with serum IgG from healthy controls.

Anti-Muscarinic 3 Receptor Autoantibodies with Emphasis on IgG1 Isotype

Muscarinic acetylcholine type 3 receptors (M3R) are responsible for the neurological transmission of messages from extracellular to intracellular spaces, a process that plays an integral part in the normal saliva pathway found in salivary and lacrimal glands. These receptors are found in both exocrine and non-exocrine tissues, especially in acinar cells. It is understood that autoantibodies produced against M3R are responsible for disabling the normal saliva pathway and inhibits excitatory enteric neurotransmission (14),(15). In addition, many studies have suggested that M3R is the subtype that is responsible for smooth muscle dysfunction, strongly suggesting that it is the antibody's interaction with the receptor itself that causes these problems (16).

In a study by Cha et al. (17), responses from smooth bladder muscles stimulated with carbachol (a M3R agonist) were measured after incubation from anti-M3R positive and anti-M3R negative autoantibody containing sera from NOD/ShiLtJ mice or human patients that exhibited pSS. It was determined that a lower carbachol response was observed in bladder smooth muscle strips that were in an environment containing anti-M3R autoantibodies, compared to smooth muscle strips taken from anti-M3R autoantibody negative NOD/ShiLtJ and C57BL/6J control mice. It was also observed that chronic stimulation to M3R from anti-M3R autoantibodies resulted in receptor desensitization.

Sera of patients with SS have shown an increase of a variety of autoantibodies. To better justify that the M3R serves as a primary site for SS, a study was done by Nakamura et al. (18). In their study, a group of type 1 (M1R) and type 3 (M3R) receptor knockout mice were given dry food to ingest. After being fed, it was evident that the M3R receptor knockout mice showed signs of difficulty ingesting food, due to the decreased salivation in their mouths (18).

Therefore, autoantibodies functioning as an antagonist for M3R pathway would certainly play a key role in secretory function in autoimmunity SS (15).

Several attempts have been used to define an autoimmune disease, one of which is by Dawson et al. (16), in which 5 areas of interest are vital in defining a particular antibody as pathogenic in SS . In turn, these criteria can be used in better defining our study with SS, particularly focusing on anti-M3R autoantibodies (16),(19). The first, "Autoantibodies are present in patients with the disease (16)," suggests that there are serum IgG antibodies found in primary and secondary SS patients that are capable of influencing the functionality of muscarinic receptors on smooth muscle of bladder and salivary acinar cells. Due to ineffective and inefficient screening methods for these autoantibodies, further investigations must examine anti-M3R in SS.

The second criteria, "Antibody reacts with the target antigen (16)", explains the interaction where neurotransmission takes place. For SS, certain epitopes must be carefully examined to determine the exact location where reaction takes place. Previous studies suggest that the 2nd extracellular loop of M3R is the targeted epitope, but due to incomplete data, further investigation must be continued. The third criteria, "Passive transfer of antibody reproduces features of disease (16)," is notably the most important of the 5 criteria because the effects of the antibody to bring an onset of disease characteristics is vital to understanding disease pathology. Collected data from SS IgG transfer to mice have shown promising results with upregulation of M3R in bladder smooth muscles.

The fourth criteria, "Immunization with antigen produces a model disease (16)," is an important aspect that is still being considered. Because the target antigen of interest is still being investigated, this data is crucial in our study. Lastly, the fifth criteria, "Reduction in antibody

levels ameliorates the disease (16)," observes the decrease in pathogenicity of disease after antibody levels have been diminished. It was observed that anti-muscarinic receptor antibody activity was reduced *in vitro* by IgG anti-idiotypic antibodies pooled from healthy patients, leading to an improvement of bladder symptoms.

Recently, considerable attention has focused on the IgG1 subclass of IgG autoantibodies. In a study by Gao et al. (15), experiments using serum from SS patients confirmed the presence of anti-M3R autoantibodies, with results suggesting the presence of IgG1 autoantibodies. These antibodies are thought to identify a tertiary epitope detected on the extracellular domains of the receptor protein. High serum levels of IgG1 are usually found within SS patients, whereas IgG levels of other types such as IgG2, are found to be in lower concentrations (20). But this may only reflect the normal serum levels of these IgG isotypes in humans. Besides from looking at the effects of IgG1 antibodies, increased levels of IL-18 were found to be within acinar cells (20). This study proposes to address these criteria in hopes of gaining a better understanding of these autoantibodies and their effects.

Mouse Models

In order to monitor the effects of autoantibodies in SS, an *in vivo* study was performed in which three different mouse strains were utilized to observe and measure the effects of the proposed autoantibodies. C57BL/6J mice (B6), due to their non-SS disease phenotype, were used as the control mice. Their ability to resist autoimmune diabetes and exocrinopathy made them an ideal model for the control model (21). The NOD/ShiLtJ (NOD) mouse served as the experimental mouse exhibiting both T1D and SS. Besides from the aforementioned trait, the NOD mouse is prone to infiltration of mononuclear cells in the lacrimal and salivary gland, as well as symptoms such as inflammation of the pancreatic islets of Langerhans (21). Lastly, the

immunodeficient B6.129S7-Rag1^{tm1Mom}/J (Rag1KO) mouse served as the recipient mouse due to its lack of B and T cells, required for autoimmunity (22).

The ages for the mouse models used were specific in range so that SS-like disease characteristics were developed and present in sera. For example, previous studies have shown the NOD mouse, which served as the experimental condition, displays disease phenotypes in its sera and saliva reduction between the ages of 17-20 weeks of age (23). This age range was used to help determine what age range was needed to gather sera from both B6 (control) and NOD (experimental) mouse models for immunogloblulin purification. Also, in a study by Gao et al. (24), loss of salivary secretion was still evident in mice up to 36 weeks of age. Lastly, it was suggested that there are 3 phase developments that were found in the NOD-Aec1Aec2 mouse model (Figure 1-1 (25)) that we could correlate with our mice. This presented an age limit as to the length of antibody effects still present in the Rag1KO recipient mice after 36 weeks of age.

Defective Secretory Function in SS

There are many hypotheses as to how cellular functions are affected by SS. One of the main hypotheses is that autoantibodies that bind to M3R will cause an inhibition in the downstream control of fluid secretion. This can be invoked by an alteration in the normal calcium concentration release found within cells. As stated in Dawson et al. (26), IgG antibodies extracted from SS patients reduced the effects of carbachol (muscarinic agonist and calcium inducing agent) within both mouse and human acinar cells.

In normal patients, when parasympathetic neurotransmitters bind to the M3R, a signaling cascade is stimulated that results in the release of intracellular calcium. This release provokes cellular functions to occur such as the activation of chloride channels in salivary glands for fluid secretion or smooth muscle contraction in the bladder (17). It can be assumed that the interactions between anti-M3R autoantibodies and the receptor will lead to a disruption in the

normal intracellular signaling pathway that can alter calcium release, and thus disrupt normal salivary flow (17).

Specific Aims

In order to investigate the roles of IgG1 immunoglobulins in secretory function in SS, two specific aims were proposed and studied:

Specific Aim #1

Objective: To determine, *in vivo,* if decreased fluid secretion in Sjögren's syndrome is dependent on isotypic autoantibodies, especially IgG1 anti-mouse M3R autoantibodies.

Hypothesis: Infusion of the B and T-cell deficient Rag1KO mice with purified NOD IgG1 targeting M3R will be sufficient to induce loss of secretion in the recipient mice.

Rationale: To examine the effects of SS antibodies on Rag1KO mice, they were injected with varying isotypic mouse Ig fractions, where saliva secretion was collected and measured over a 5-day period to determine their effect.

Specific Aim #2

Objective: To investigate, *in vitro,* if the inhibitory effect of IgG1 anti-mouse M3R autoantibodies on M3R can be observed in a cell culture system.

Hypothesis: *Specific Aim #2 Hypothesis*- The binding of IgG1 anti-mouse M3R autoantibodies to the receptor is necessary to alter M3R mediated intracellular calcium release in vitro.

Rationale: To examine this, varying isotypic mouse Ig fractions were incubated with human salivary gland cells in order to induce inhibitory effects by monitoring intracellular calcium release in response to carbachol (muscarinic agonist).

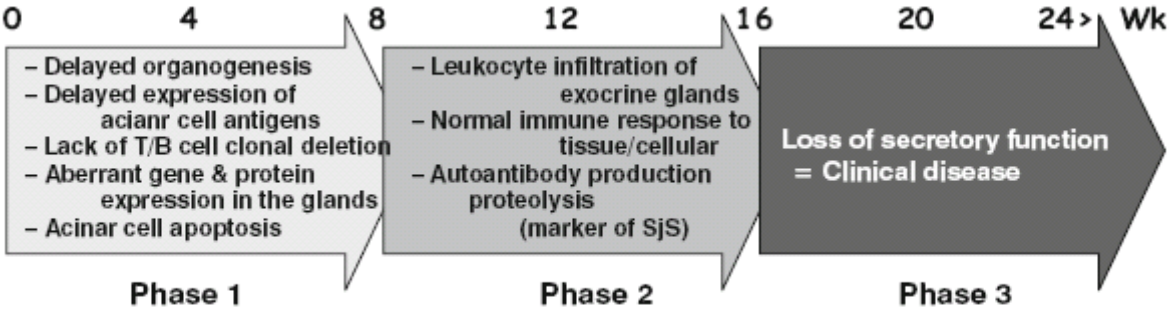

Figure 1-1. Description of the three phases of SS-like disease development in the NOD-Aec1Aec2 mouse model (Modified from Lee et al. (25)).

CHAPTER 2
MATERIALS AND METHODS

Specific Aim #1

Objective (In vivo)

To determine if decreased fluid secretion in Sjögren's syndrome is dependent on isotypic autoantibodies, especially IgG1 anti-mouse M3R autoantibodies.

Hypothesis

Infusion of the B and T-cell deficient Rag1KO mice with purified NOD IgG1 targeting M3R will be sufficient to induce loss of secretion in the recipient mice.

Rationale

To examine the effects of SS antibodies on Rag1KO mice, they were injected with varying isotypic mouse Ig fractions, where saliva secretion was examined over a 5-day period to determine their effect.

Mouse Model

In order to carry out the in vivo experiment, three mouse strains were needed that were purchased from Jackson Laboratory. The first two mouse strains served as donor mice for sera to purify IgG subclass fractions. The first strain used as the control was the C57BL/6J (B6-Jackson Laboratory, Stock Number: 000664). This mouse served as a control mouse due to the fact that it is a widely used general purpose inbred strain. B6 mice are used in many areas of research such as cardiovascular biology, diabetes, genetics, and immunology. Overall, B6 mice breed well, are long-lived, and have a low susceptibility to tumors. The second mouse strain used as the experimental mouse was the non-obese diabetic NOD/ShiLtJ (NOD-Jackson Laboratory, Stock Number: 001976) mouse. This is an appropriate mouse strain for our study due to its polygenic model for T1D and a characteristic for SS. Because the NOD parental strain

displays full disease phenotype characteristics around 17 weeks of age, sera were used from 16-20 weeks of age from both NOD (experimental) and B6 (control) strains. The third strain, B6.129S7-Rag1^{tm1Mom}/J (Rag1KO- Jackson Laboratory, Stock Number: 002216), served as the recipient mouse for the purified IgG fractions because of its immunodeficient background, where there is no production of mature B and T cells. The Rag1KO mice used in this study were obtained from the Cancer Genetics Research Complex (CGRC- Gainesville, Florida) and Dr. Minoru Satoh's laboratory. Saliva was collected and measured using mice between 16-28 weeks of age. All studies were approved by the Institute of Animal Care and Use Committee (IACUC) at the University of Florida.

Serum Collection

Before beginning purification, blood was collected from both donor mouse strains (B6 control mice and NOD experimental mice) between the ages of 16-20 weeks (approximately n=20-25/strain). Blood was collected from each mouse through orbital lobe excavation, where approximately 500 μL/mouse was collected. The blood was centrifuged for 10 minutes, at 4,500 rpm and 4ºC to obtain sera in the supernatant. The serum from both strains was then pooled together to a volume of approximately 4.5 mL/strain that were then used for purification.

Dialysis and Antibody Fraction Column Preparation for Purification

In order to obtain isotypic antibody fractions from the collected serum, a series of purification columns were assembled. To begin, dialysis was performed in order to create purification columns for the antibodies. Goat-anti mouse IgG1 (Southern Biotechnology, Birmingham, Alabama- #1070-01), IgG2b (#1090-01), IgG2c (#1079-01), and IgG3 (#1100-01) unlabeled 1 mg antibodies were purchased and filled into dialysis tubes (Fisher Brand, Nominal MWCO 12,000-14,000 #21-152-16). Tubes were dialyzed in 0.1M NaHCO3 with 0.5M NaCl coupling buffer for a period of 3-4 days, where the buffer was changed twice a day. Afterwards,

the contents of the dialysis tubes were transferred to centrifuge tubes and centrifuged at 4ºC for 15 minutes at 12,000 rpm. The supernatant's optical density (OD) was checked by spectrophotometer (Beckman DU-64 Spectrophotometer) to verify the amount of IgG used and confirm the efficiency of conjugation.

IgG concentration was estimated based on the conversion ratio of 1.42 OD280 = 1 mg/mL IgG. About 1g of CNBR-activated Sepharose 4B beads (GE Healthcare, Label No. 71-1181-00-ED, Lot. 311319) was mixed with 25mL of 10^{-3} M HCl and packed into 4, 10 mL Poly-Prep Chromatography Columns (Bio-Rad, Catalog 731-1550). Once the beads began packing, HCl was continually added to prevent dehydration from occurring. Once 0.5 mL of the column was packed with beads and approximately 30 mL of HCl had run through, the column was washed once more but with 30 mL of coupling buffer. After the coupling buffer ran through, the supernatant of the antibody centrifuge tubes were added into each respective column, mixed gently with a pipette, and rotated overnight at 4ºC to mix/homogenize with the beads.

The next day, the coupling buffer in the columns was drained and the OD of the eluate was measured by spectrophotometer to verify effective conjugation of IgG to the Sepharose beads. If the OD was very low, that meant that the protein bound to the beads, which was desired. The columns were then washed again with 30 mL of coupling buffer and drained. Next, 5 mL of 1.0 M ethanolamine hydrochloride was used as a blocking buffer and mixed and rotated for 1 hour at room temperature with the column beads. Once completed, the column was drained and a series of three wash cycles occurred. To start, 10 mL of phosphate-buffered saline, 1x, pH=7.4 (PBS-Cellgro, Cat.No. 21-040-CV) was washed through thoroughly to avoid any impurities found in the beads and columns. Next, the columns were washed with 10 mL of 0.5 M glycine elution (pH=2.5) buffer to detach any unwanted proteins from the beads. After the 3 wash cycles were

completed using PBS and glycine, the columns were restored back to neutral conditions by running 5 mL of PBS through the beads with 2mL left on top. This completed the column preparation and was ready for sera pass-through.

Purification of Whole IgG

Before isotypic fractions were collected, total IgG was purified from the serum of both B6 and NOD mice. In order to do so, a separate column was created for each mouse strain. Each column was packed with approximately 1 mL of Protein G Agarose (Sigma, Fast Flow from Streptococcus species P4691-5 mL) beads. Next, 10 mL of glycine buffer was washed through in order to eliminate any unwanted impurities. Lastly, neutral pH was restored by washing 10 mL of 1x PBS through the column. The serum was centrifuged for 15 minutes at 12,000 rpm at 4ºC. The supernatant was then run through the column for protein attachment. Once all sera was run through, approximately 10 mL of 1x PBS was passed through and collected as "earlier flow through" to clean the beads and return them back to their original white color. Columns were washed until the OD of the flow through returned back to the background level.

IgG was eluted from the beads using 5 mL of glycine buffer. To prevent the loss of antibody activity due to the acidic environment, 140 μL of 2 M Tris pH ~ 11 was added to every 1 mL of eluate immediately in order to neutralize the solution. The concentration of purified whole IgG was then measured using spectrophotometry as above.

Purification of Isotypic Immunoglobulins: IgG1, IgG2b, IgG2c, and IgG3

After draining PBS from the antibody fraction purification columns, the total IgG from the previous step was ready to be passed though each of the 4 columns in the order of: IgG2c → IgG1 → IgG2b → IgG3. The total IgG was run through one column at a time, collecting its pass through into a tube, which was then transferred to the next column. After the contents of the transfer tube had run through each column, 2 mL of PBS was run through each purification

column to wash out any residual from the whole IgG serum and again collected in the transfer tube. Doing so allowed the antibodies to bind to each respective purification column. Glycine buffer pH 2.5 was then run through each column to elute bound antibodies from the beads so that approximately 4 mL of isotypic fractions were collected. Because of the acidic environment of glycine buffer pH 2.5, 140 μL of 2 M Tris pH ~ 11 was also added per milliliter of elute to neutralize. This Glycine/Tris buffer became the environment that the Ig fractions were stored in, which would later on be used as one of the control treatments. Lastly, the purification columns were neutralized and restored back to original conditions by washing and draining each column with 10 mL of PBS.

Measurement of Concentration of Purified Ig Isotypes by ELISA

In order to quantify and determine the concentration for each purified antibody fraction, a sandwich ELISA was performed. Wells of 96 well microtiter plates (Nunc, Immobillizer Amino) were incubated for 2 hours at 22ºC with 50 μL/well of a mixture composed of goat anti-mouse kappa (Southern Biotechnology #1050-01, 1 mg/mL) and goat anti-mouse lambda (Southern Biotechnology #1060-1, 1 mg/mL) at a 9:1 ratio at 3 μg/mL. After incubation, both plates were washed once with TBS-Tween 20, after which they were blocked with 150 μL/well of 0.5% BSA buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 2 mM EDTA, 0.3% Nonidet P-40 (NET/NP40)) for 30 minutes at room temperature. Total IgG was diluted 1:10,000 and the purified fractions were diluted to 1:1,000, both in 0.5% BSA NET/NP40. Serial dilutions of the myeloma proteins (IgG1, IgG2b, IgG2c, IgG3, and IgM) were made from 10 μg/mL stock and served as a standard to create a standard curve to help calculate IgG concentration of samples. After the incubation period passed, the 0.5% BSA solution was discarded. Lastly, the serial dilutions were added 100 μL/well and stored overnight at 4ºC.

The next day, the 2º antibody mixtures were created using alkaline phosphatase-conjugated goat anti-mouse IgG1, IgG2b, IgG2c, and IgG3 (Southern Biotechnology, Birmingham, Alabama) at a 1:1,000 dilution with 0.5% BSA NET/NP40. After the plates were washed three times with TBS-Tween 20, the 2º antibody mixtures were added 100 µL/well and incubated for 2 hours in room temperature, after which they were washed three times again with TBS-Tween 20. The assay was developed using 100 µL/well diethanolamine/phosphatase substrate buffer (Sigma-Aldrich, St. Louis, Missouri) and absorbance was determined at 405 nm using a $VERSA_{max}$ ELISA plate reader (Molecular Devices, Sunnyvale, California).

Injection Strategy

The most obvious characteristic of the disease that can be detected, measured, and confirmed is through the measurement of saliva secretion from the injected mice. Rag1KO mice 16-28 weeks old were used to infuse with different isotypic antibody fractions. In order to accomplish this, 4 different categories of Rag1KO mice were injected with respective solutions or no solution: B6 fractions, NOD fractions, Glycine/Tris Buffer, and Non-Injected (Figure 2-1). The experimental groups used for this study were mice injected with fractions from B6 and NOD, while the control groups consisted of mice that were injected with a Glycine/Tris buffer and mice not injected with anything.

In order to determine how much was injected into each mouse, the ELISA results (Table 3-1 and Table 3-2) were analyzed. Because we wanted to recreate a real life in vivo situation, the serum data from the ELISAs was used to calculate a ratio, which was then used to determine the appropriate volumes of antibody fraction to inject into each recipient mouse (Table 2-1 and Table 2-2). After determining the injection amounts, a 5-day experimental plan was prepared (Figure 2-2). Only on the first day were the Rag1KO mice injected with purified Ig fractions. On this day, each Rag1KO mouse was injected through intraperitoneal injection (IP) with each

purified fraction (i.e., mouse 1=B6 IgG1, mouse 2= B6 IgG2b, mouse 3=B6 IgG2c, mouse 4= NOD IgG1, etc.). A three hour period was observed so that each fraction had time to incubate within each mouse. After the incubation period had passed, saliva collection was performed over a 10 minute span.

Saliva Collection

Saliva secretion was stimulated by injecting each mouse again through IP with a secretagogue mixture composed of 1 mg DL-Isoproteronol Hydrochloride (MP Biomedicals, Cat. No. 151368, Lot No. 9192C) and 2 mg Pilocarpine Hydrochloride (MP Biomedicals, Cat. No. 151892, Lot No. 4218F), mixed in 1 mL of 1x PBS solution. One minute after secretagogue injection, saliva was collected for a period of 10 minutes through the oral cavity using a 200 μL pipette. The Ig injections and saliva collection were measured and observed over a 5-day period. Afterwards, each saliva volume was measured and normalized by dividing each saliva volume collected by the weight of the respective mouse. Statistical analysis was performed once all measurements had been taken using both a t-test (non-parametric) and one-way ANOVA (non-parametric) with Tukey Test to compare all paired values.

Specific Aim #2

Objective (In vitro)

To investigate if the inhibitory effect of IgG1 anti-mouse M3R autoantibodies on M3R can be observed in an in vitro cell culture system.

Hypothesis

The binding of IgG1 anti-mouse M3R autoantibodies to the receptor is necessary to alter M3R mediated intracellular calcium release in vitro.

Rationale

To examine this, varying isotypic mouse Ig fractions were incubated with human salivary gland cells in order to induce inhibitory effects by monitoring intracellular calcium release in response to carbachol (muscarinic agonist).

Cell Culture

In order to monitor intracellular calcium release within a cell, human salivary gland (HSG) cells were incubated with different IgG isotypes of SS and monitored through a fluorescence reader. HSG cells were grown in 12 mL culture plates at 37ºC with 1x DMEM (Dulbecco's Modification of Eagle's Medium) as a medium. This medium consisted of 50 mL of heat inactivated 10% Fetal Bovine Serum (Cell Gro, Catalog Number: 35-011-CV) and 5 mL antibiotics Penicillin/Streptomycin Solution (Cell Gro, Catolog Number 30-002-CI).

Calcium Assay

After the culture was created and reached 80-90% confluency, it was re-seeded and transferred to 3, 96 well plates (Nunc, Catalog Number: 161093), where 36 wells were utilized each plate. They were then incubated at 37ºC with both control and experimental isotypic autoantibodies for a period of 15 minutes (short-term stimulation) and 24 hours (chronic stimulation) as the experimental treatments. Under each time point, 2 different treatments were applied: one plate using the same volume of 2 μL of each respective antibody for incubation, and one plate using the same amount of 0.2 μg of each respective antibody for incubation (Table 2-3 and Table 2-4). Also, in order to ensure that any alterations in calcium release were due to the fractions themselves, other treatments, used as controls, were monitored for calcium changes that consisted of: blank, dye only, cells and dye only (Negative Control), carbachol (CCh) and cells (Experimental Positive Control), and ionomycin (Iono) and cells only wells (Assay Control). Once incubated with Ig fractions, a Fluo-4 NW Calcium Assay Kit (Invitrogen, Lot Number:

537564) was utilized in order to measure calcium release. The protocol provided by the manufacturer was followed and calcium release was measured using a fluorescence reader (Molecular Devices Spectramax M5) set at 494 nm for excitation and 516 nm for emission.

In order to stimulate calcium release, 10 μL of 100 μM carbamoylcholine chloride (CCh) (Sigma-Aldrich, Catalog Number: C4382-1G), an experimental control and muscarinic receptor agonist, was injected into both control and experimental groups to begin calcium release. In order to ensure the assay was properly working, 1 μL of 5 μM ionomycin (Iono) Calcium Salt (Sigma-Aldrich, Catalog Number: I3909) was used, acting as another calcium inducing agent and serving as an assay control. Once injected with both inducing agents, calcium release was instantaneous; therefore fluorescence was read immediately after injection. Statistical analysis was utilized once all measurements had been taken using a one-way ANOVA (non-parametric) with Tukey Test to compare all paired values.

Table 2-1. B6 Ig fractions table that displays ratios used to calculate volume injected into each Rag1KO mouse *in vivo*

B6 Ig Fractions	IgG1	IgG2b	IgG2c
B6 Serum Ratio	1.000	0.5000	0.1250
Amount Ratio	40.00 µg	20.00 µg	5.000 µg
Volume Injected into each Rag1KO mouse	266.7 µl	303.9 µl	337.6 µl

Table 2-2. NOD Ig fractions table that displays ratios used to calculate volume injected into each Rag1KO mouse *in vivo*

NOD Ig Fractions	IgG1	IgG2b	IgG2c
NOD Serum Ratio	1.000	0.9200	0.1500
Amount Ratio	40.00 µg	36.80 µg	6.000 µg
Volume Injected into each Rag1KO mouse	250.0 µl	334.5 µl	193.5 µl

Table 2-3. B6 Ig fractions table that displays injection amounts and volumes that were used in each 15 minute and 24 hour incubation experiment *in vitro*

B6 Ig Fractions	IgG1	IgG2b	IgG2c
Amount Injected *in vitro* per well (constant volume-2 µl)	0.3010 µg	0.1320 µg	0.02960 µg
Volume Injected *in vitro* per well mouse (constant amount- 0.2 µg)	1.330 µl	3.030 µl	13.50 µl

Table 2-4. NOD Ig fractions table that displays injection amounts and volumes that were used in each 15 minute and 24 hour incubation experiment *in vitro*

NOD Ig Fractions	IgG1	IgG2b	IgG2c
Amount Injected *in vitro* per well (constant volume-2 µl)	0.3170 µg	0.2240 µg	0.06270 µg
Volume Injected *in vitro* per well mouse (constant amount- 0.2 µg)	1.260 µl	1.790 µl	6.380 µl

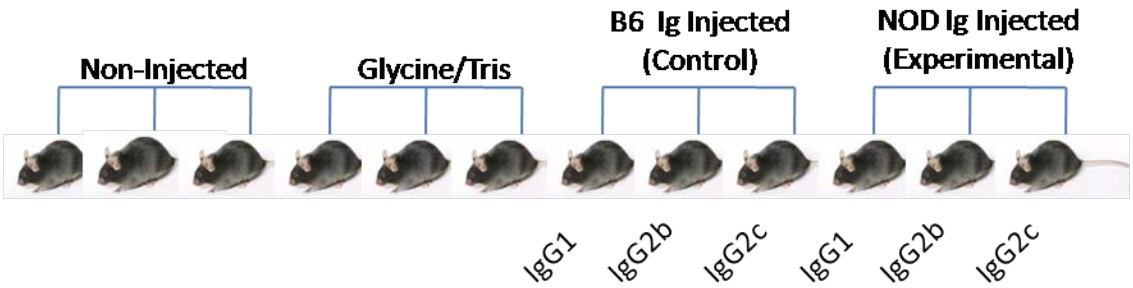

Figure 2-1. *In vivo* experiment with Rag1KO mice under four different treatment conditions: 1) Non-Injected Rag1KO mice (Control), 2) Rag1KO mice injected with Glycine/Tris buffer (Control), 3) Rag1KO mice injected with different B6 purified Ig fractions (Control), 4) Rag1KO mice injected with different NOD purified Ig fractions (Experimental).

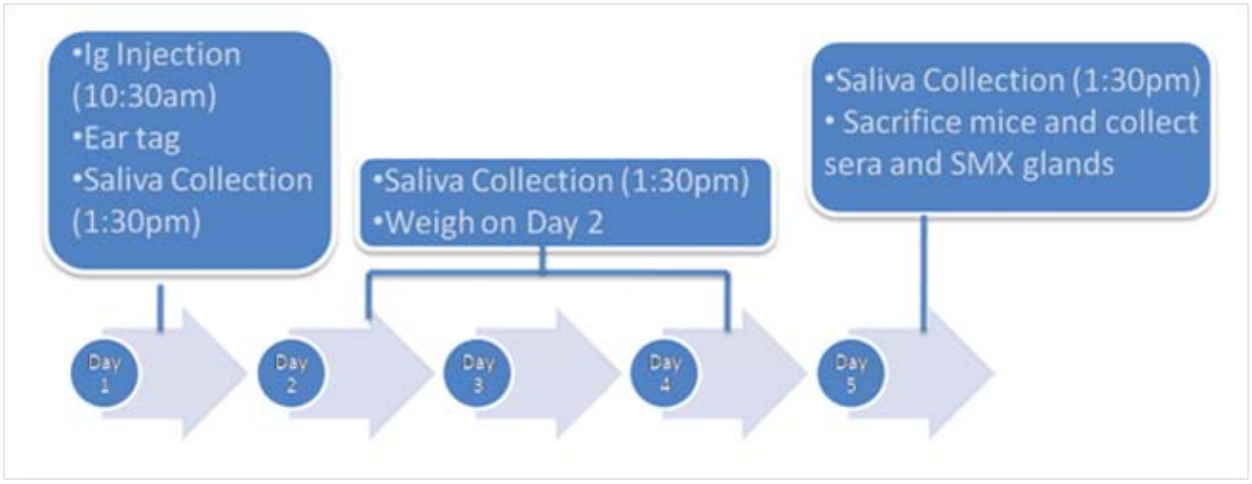

Figure 2-2. *In vivo* experimental timeline over a 5-day period.

CHAPTER 3
RESULTS

Specific Aim #1

Analysis of Purified Fractions Measured Concentrations of Ig Isotypes with IgG1 Being the Highest

In order to determine the amount of each IgG isotype needed to inject into each mouse in vivo, an ELISA was performed using the B6 and NOD purified Ig fractions. Analysis of each purified fraction revealed the concentration of each Ig fraction derived from B6 and NOD (Table 3-1 and Table 3-2). Using the concentration data, the amount of each Ig fraction to be injected into Rag1KO mouse was calculated. There was high reactivity (marked by asterisks) found in each purified fraction. From the B6 ELISA, the results were as follows: IgG1 = 150 μg/mL, IgG2b = 65. 81 μg/mL, IgG2c = 14.81 μg/mL, and IgG3 = <0.002. From the NOD ELISA, the results were as follows: IgG1 = 158.32 μg/mL, IgG2b = 111.75 μg/mL,, IgG2c = 31.34 μg/mL, and IgG3 = <0.002 μg/mL. The serum data from Tables 3-1 and 3-2 were used to create a ratio that was used in conjunction with the above values to determine how much Ig would be injected per mouse (Table 2-1 and Table 2-2). For example, the serum values in Table 3-1 were used to determine a ratio of 1.00: 0.500: 0.1250 for B6 Ig fractions. Next, this ratio was converted accordingly using the starting amount of 40 μg. This amount ratio was divided by the above concentrations (Table 3-1) to obtain the B6 Ig volumes that were injected into each Rag1KO mouse.

NOD IgG1 and IgG2c Inhibit Secretory Dysfunction in Immunodefecient Mice *In Vivo*

In order to gain a better understanding of the functional relationship of IgG1 and other autoantibody fractions to SS, an in vivo experiment was performed. To do so, 24 Rag1KO mice were injected, respectively, with purified fractions of mouse IgG1, IgG2b, and IgG2c (Table 2-1 and Table 2-2). This was performed in order to gather data to test our hypothesis that NOD IgG1

anti-mouse M3R autoantibodies causes saliva flow dysfunction. Note that IgG3 fractions from both B6 and NOD were not injected into mice due to their low concentrations defined by ELISA, probably due in part to the cryoglobulinaemic characteristics of IgG3, which could have been removed as precipitate during centrifugation at 4ºC. After injections of the appropriate isotypes, saliva was collected through the oral cavity and saliva volume was measured and data analyzed, as presented in Figure 3-1 – Figure 3-3. As shown in Figure 3-1, NOD IgG1 purified fractions caused increased stimulation on Day 2, but due to possible receptor desensitization, there was a decrease of saliva secretion over time due to IgG1. Mice injected with B6 IgG1 purified fractions showed an initial increase in saliva secretion on Day 2, followed by a decrease until Day 4. By Day 5, signs of increased saliva secretion occurred with mice treated with B6 IgG1 fractions. These data support the hypothesis that NOD IgG1, as well as NOD IgG2c, autoantibodies are effective suppressors of saliva flow.

In Figure 3-2, both B6 and NOD IgG2b fractions caused a stimulatory effect and showed max values on Day 2, but then B6 IgG2b fractions caused a rapid decrease in saliva volume though Day 5. NOD IgG2b fractions, on the other hand, also caused a decrease, but at a much slower salivary flow rate (flat line) compared to B6. As presented in Figure 3-3, B6 IgG2c fractions caused a stimulatory effect that peaked on Day 2, but slowly decreased through Day 5. NOD IgG2c fractions showed an increase peaking on Day 3 followed by a decrease in saliva secretion through Day 5. Lastly, both control groups, Glycine/Tris treated mice and non-injected mice, showed very similar patterns, and were used as a comparison in all three graphs over the course of the 5 days. Both groups showed decreases in saliva flow rates from Day 2-Day 4, but an increase on Day 5 for the non-injected mice, while Glycine/Tris-treated mice continued to show a decrease.

Specific Aim #2: Altered Intracellular Calcium Release in HSG Cells in the Presence of Ig Isotypes

A Fluo-4 NW Calcium Assay Kit (Invitrogen, Carlsbad, California) was utilized in order to monitor the alterations in calcium release (stimulated by carbachol (CCh)) through fluorescence readings with the incubation of B6 (control) and NOD (experimental) purified Ig fractions. This was performed to obtain data to test our hypothesis that IgG1 anti-mouse M3R autoantibodies would cause an alteration in the M3R mediated intracellular calcium release *in vitro*. In order to gain a better understanding of the effects of the antibodies, measurements were taken after two purified Ig incubation times of 15 minutes (short term stimulation) and 24 hours (chronic stimulation).

Under each incubation time point, 2 different treatments were applied (as defined in Table 2-3 and Table 2-4): one plate using the same volume of 2 μL of each respective antibody for incubation, and one plate using the same amount of 2 μg of each respective antibody for incubation. Carbachol, known to be a MR agonist and calcium inducing agent, was added to HSG cells that contained purified Ig fractions (experimental condition), as well as our control set-up of CCh-responsive cells to stimulate calcium release. To ensure that our calcium assay kit worked properly, ionomycin (a calcium ionophore) was utilized as an assay control to ensure that intracellular calcium was released. In all of our treatment studies in vitro, our ionomycin fluorescence readings ranged between 400-500 nm, which was a good indication that our assay properly worked and remained consistent throughout.

Two μL of each respective B6 and NOD Ig fraction was incubated with HSG cells for 15 minutes to monitor the effects under a short-term stimulatory environment. As shown in Figure 3-4, there was a significant difference ($p<0.05$) between both our negative and positive controls. Next, it was observed that all fractions, both B6 and NOD, caused an overall decrease in calcium

release compared to CCh-responsive cells. It was interesting that both B6 IgG1 and B6 IgG2b fractions caused a significant decrease ($p<0.05$) in calcium release compared to this positive control response. Lastly, it should be noted that NOD IgG1 caused a decrease in calcium release compared to the CCh-responsive cells even though it was not significantly different.

HSG cells were incubated with 2 μL of B6 and NOD Ig fractions for 24 hours in order to simulate a chronic environment. As shown in Figure 3-5, both positive and negative controls were significantly different ($p<0.05$) compared to each other. In addition, the overall effects of each B6 and NOD Ig fraction caused a decrease in calcium release that was significantly different ($p<0.05$) compared to the CCh-responsive cells.

Using similar amounts of Ig fractions (0.2 μg), HSG cells were incubated with the IgG isotypic antibodies for 15 minutes as a short term stimulation. It was observed that both negative and positive controls were significantly different from each other ($p<0.05$). Also, even though they were not significantly different ($p>0.05$) from the CCh-responsive cell responses, both B6 and NOD Ig fractions caused an observable decrease in calcium release. HSG cells were then incubated with 0.2 μg of each B6 and NOD Ig fraction for a 24-hour period as a chronic stimulation. As shown in Figure 3-7, the data collected indicated a decrease in calcium release in all B6 and NOD Ig fractions (except NOD IgG1), even though the data was not significantly different ($p>0.05$) compared to the responses CCh-responsive cells.

Table. 3-1. B6 Purified Ig Fraction ELISA results

B6	IgG1 (µg/mL)	IgG2b (µg/mL)	IgG2c (µg/mL)	IgG3 (µg/mL)	Total volume (mL)	Total Amount (µg)
IgG1	150.32*	1.07	0.51	<0.002	4.5	676.44
IgG2b	<0.03	65.81*	0.99	<0.002	4.5	296.145
IgG2c	1.52	3.68	14.81*	<0.002	4.5	66.645
IgG3	<0.58	<0.039	<0.013	<0.002	4.5	----
Serum	1637.6	773	204.6	<0.4	1.5	

Main reactivity denoted by asterisks (*). The data marked by asterisks (*) multiplied by the total volume resulted in the total amount of each respective B6 Ig fraction. The serum data was utilized to determine an *in vivo* ratio that was used to calculate Ig volumes injected into the Rag1KO mice (Table 2-1).

Table. 3-2. NOD Purified Fraction ELISA results

NOD	IgG1 (µg/mL)	IgG2b (µg/mL)	IgG2c (µg/mL)	IgG3 (µg/mL)	Total volume (mL)	Total Amount (µg)
IgG1	158.36*	1.61	0.53	<0.002	4.5	712.62
IgG2b	1.66	111.75*	1.46	<0.002	4.5	502.875
IgG2c	2.03	12.46	31.34*	<0.002	4.5	141.03
IgG3	0.03	0.01	0.01	<0.002	4.5	----
Serum	1628.8	1505.8	259.6	<0.002	1.5	

Main reactivity denoted by asterisks (*). The data marked by asterisks (*) multiplied by the total volume resulted in the total amount of each respective NOD Ig fraction. The serum data was utilized to determine an *in vivo* ratio that was used to calculate Ig volumes injected into the Rag1KO mice (Table 2-2).

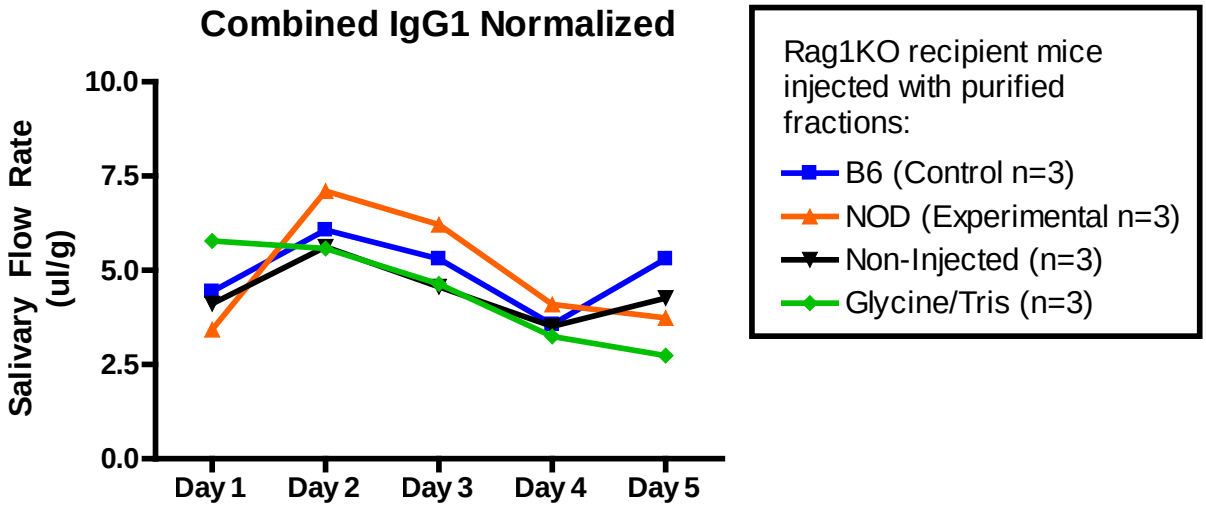

Figure 3-1. *In vivo* results of saliva secretion after 3 hour incubation (on Day 1) with IP injected B6 (control) and NOD (experimental) purified IgG1 into Rag1KO mice. Non-injected condition and Glycine/Tris (purified Ig storage buffer) were both used as controls conditions. Saliva was collected over a 5-day period. Statistical analysis was performed but data was not significant ($p>0.05$).

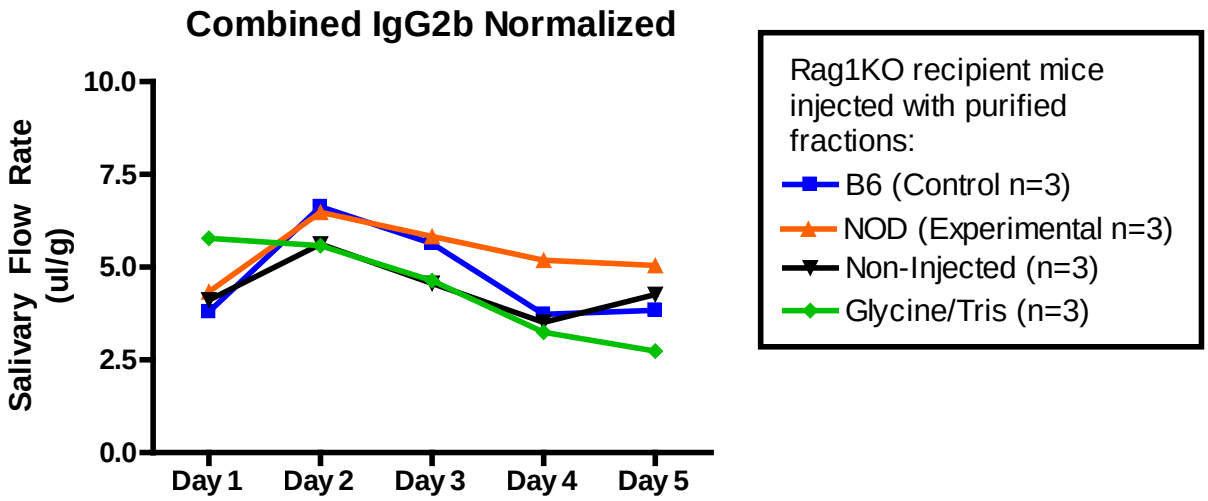

Figure 3-2. *In vivo* results of saliva secretion after 3 hour incubation (on Day 1) with IP injected B6 (control) and NOD (experimental) purified IgG2b into Rag1KO mice. Non-injected condition and Glycine/Tris (purified Ig storage buffer) were both used as controls conditions. Saliva was collected over a 5-day period. Statistical analysis was performed but data was not significant ($p>0.05$).

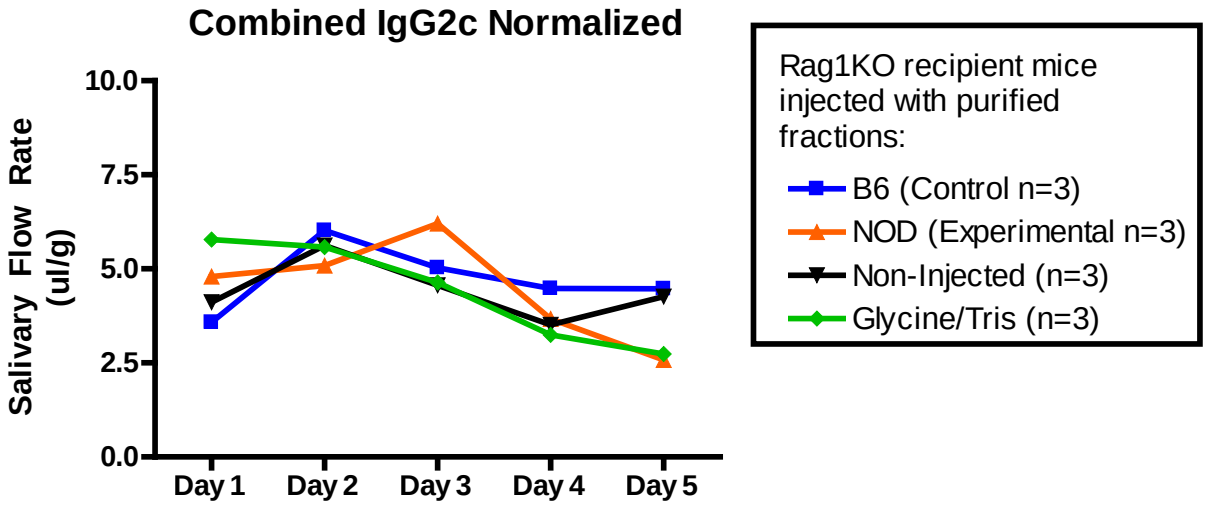

Figure 3-3. *In vivo* results of saliva secretion after 3 hour incubation (on Day 1) with IP injected B6 (control) and NOD (experimental) purified IgG2c into Rag1KO mice. Non-injected condition and Glycine/Tris (purified Ig storage buffer) were both used as controls conditions. Saliva was collected over a 5-day period. Statistical analysis was performed but data was not significant ($p>0.05$).

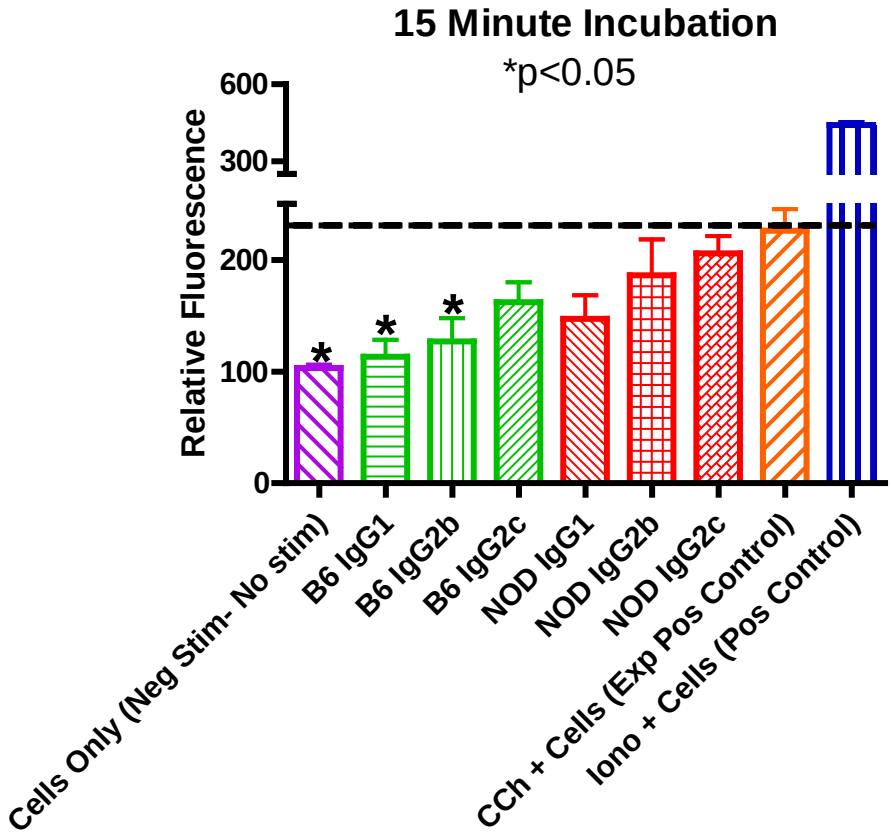

Figure 3-4. *In vitro* Calcium Assay with 15 minute incubation with 2 μL of each respective B6 (control) and NOD (experimental) Ig fraction. Asterisks (*) indicate $p<0.05$ compared to CCh + Cells (Positive Control).

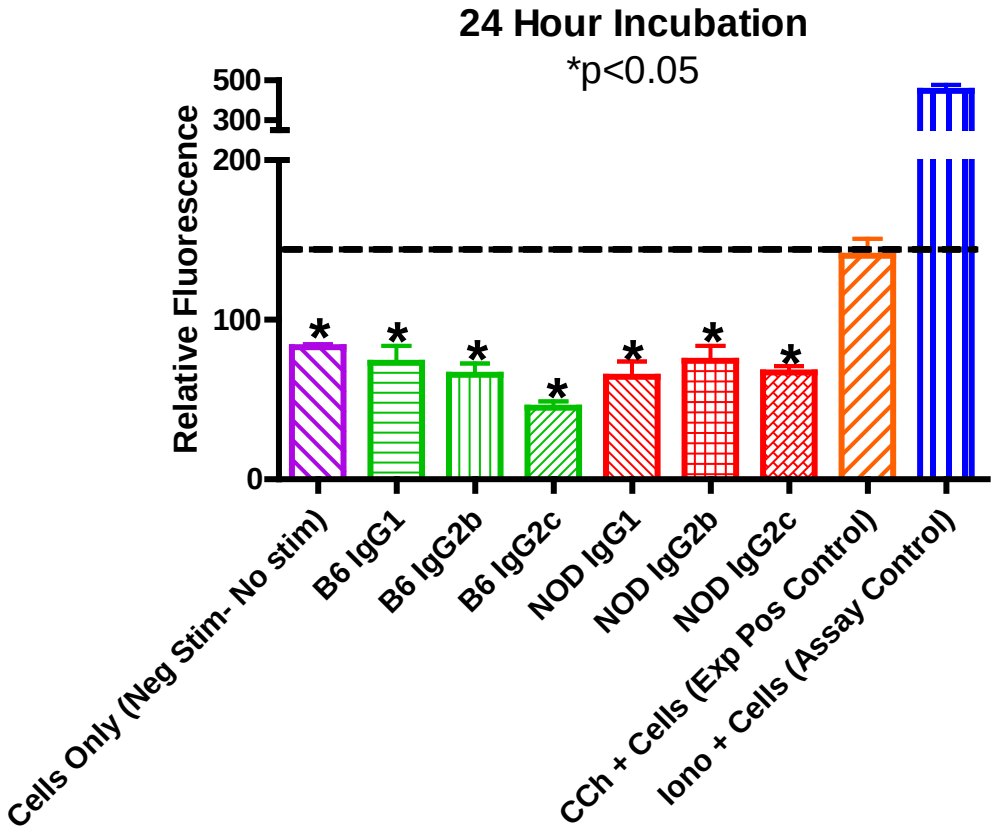

Figure 3-5. *In vitro* Calcium Assay with 24 hour incubation with 2 µL of each respective B6 (control) and NOD (experimental) Ig fraction. Asterisks (*) indicate $p<0.05$ compared to CCh + Cells (Positive Control).

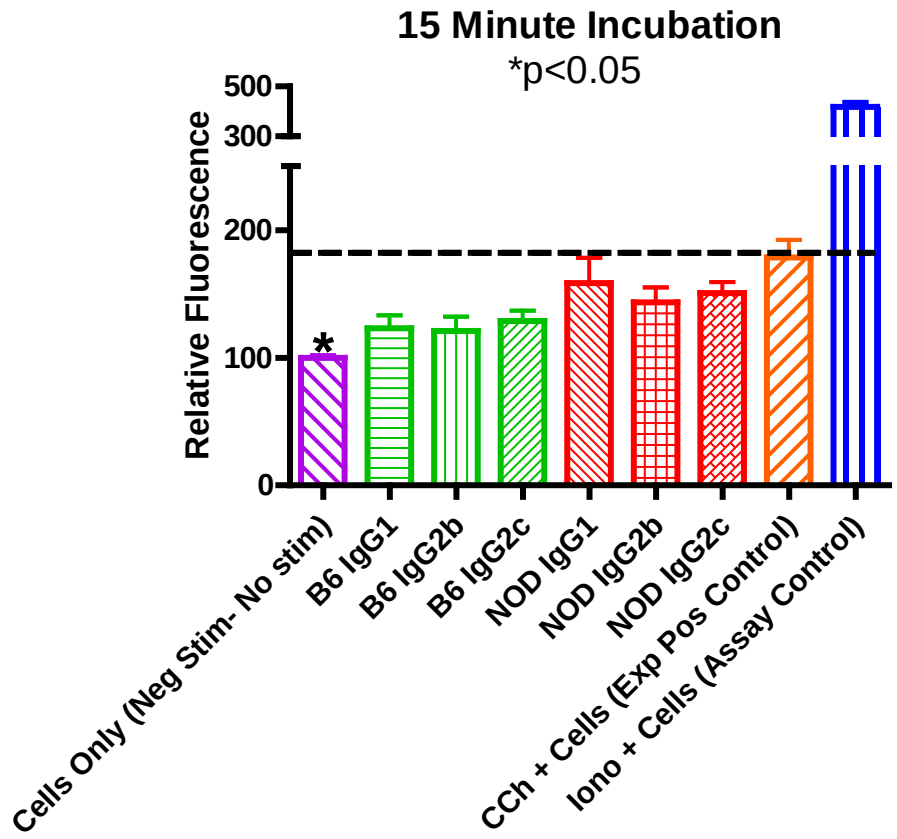

Figure 3-6. *In vitro* Calcium Assay with 15 minute incubation with 0.2 µg of each respective B6 (control) and NOD (experimental) Ig fraction. Asterisks (*) indicate $p<0.05$ compared to CCh + Cells (Positive Control).

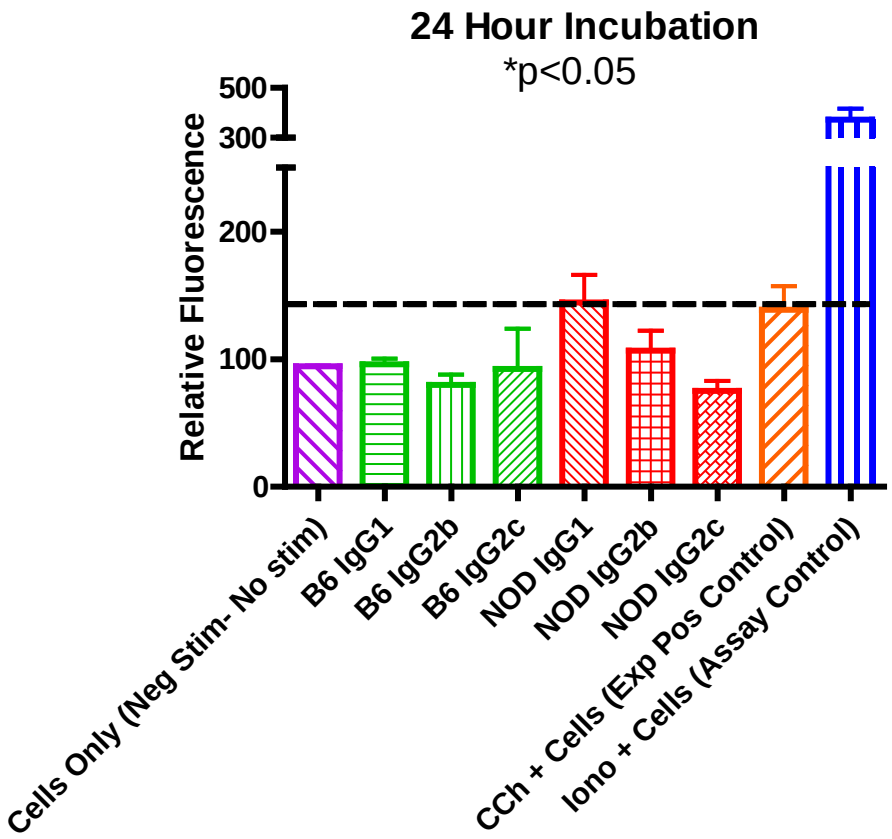

Figure 3-7. *In vitro* Calcium Assay with 24 hour incubation with 0.2 µg of each respective B6 (control) and NOD (experimental) Ig fraction. Asterisks (*) indicate $p<0.05$ compared to CCh + Cells (Positive Control).

CHAPTER 4
DISCUSSION

The purpose of this study was to measure and observe the effects of IgG1 subclass autoantibodies in secretory dysfunction in SS in both an *in vivo* and *in vitro* system. Different isotypic fractions of IgG1, IgG2b, IgG2c, and IgG3 were purified from sera from both B6 and NOD mice using affinity columns. Once completed, each fraction was analyzed through ELISA in order to determine the amount and concentration of each fraction for injection or *in vitro* study. For this project, two specific aims were addressed. The objective for Specific Aim #1 was to determine if decreased fluid secretion in SS was dependent on isotypic autoantibodies, especially IgG1 anti-mouse M3R autoantibodies. It was hypothesized that infusion of the B and T-cell deficient Rag1KO mice with purified NOD IgG1 fractions targeting M3R would be sufficient to induce loss of section in the recipient mice. The objective for Specific Aim #2 was to investigate and observe the inhibitory effects of IgG1 anti-mouse M3R autoantibodies on M3R in an in vitro cell culture system. It was hypothesized that the binding of IgG1 anti-mouse M3R autoantibodies to the receptor was necessary to alter M3R mediated intracellular calcium release within this system. Efforts at understanding the effects of purified Ig fractions, especially IgG1, identified two important findings pertaining to loss of salivary function in SS. First, it was observed that both purified NOD IgG1 and NOD IgG2c fractions caused secretory dysfunction *in vivo*. Second, preliminary results suggested that both B6 and NOD purified fractions, especially NOD IgG1, caused a decrease in calcium release in HSG cells after 24 hour incubation *in vitro*.

Specific Aim #1

For Specific Aim #1, it was proposed that injecting purified SS-prone NOD Ig fractions, not whole IgG, into Rag1KO mice *in vivo,* would be able to alter secretory flow, especially with

the use of IgG1 fractions. This hypothesis was based on the role of muscarinic receptors, more importantly M3R, in their functionality and relationship with IgG1 autoantibodies. Studies have shown that autoantibodies produced against M3R are responsible for inhibiting the normal saliva secretion pathway and interfering excitatory enteric neurotransmission (14), (15). This concept is based on considerable work previously carried out in our laboratory. In a paper published by Robinson et al. (27), it was shown that the use of NOD.Igμ^{null} mice, with the infusion of human serum whole IgG purified from primary SS patients, resulted in either the loss or a gain in secretory function in the exocrine tissues, most likely due to the state of immunity of the individual patient. Moreover, the fractions obtained from SS sera disabled the proper binding of muscarinic receptor agonist to the salivary gland membrane.

After exploring other studies and observing what they had found in regards to antibodies and SS, there was a general consensus that whole IgG from SS patients was able to produce an inhibitory effect on saliva secretion (13), (26),(27), (28). However, it had never been investigated before if the purification of whole IgG into its specific constituent parts would also have the same effect or a differing one. In addition, Gao et al. (24) showed that secretory function is maintained when IL-4 is deficient, thus failing to generate IgG1 isotypic Ig, in the NOD genetic background mouse, indicating that IgG1anti-M3R antibody is essential in secretory dysfunction. Therefore, this project provided a new direction with the use of purified Ig fractions. In this study, whole IgG from both B6 (control mice) and NOD (SS- like mice) were collected and purified into Ig fractions of IgG1, IgG2b, IgG2c, and IgG3 and injected into recipient mice to monitor their effects.

Due to previous findings using the NOD.Igμ^{null} mouse because of its many SS-like characteristics and lacking functional B cells, it was decided to find a similar mouse model in

order to proceed with my study (27). The Rag1KO mice, which served as our recipient mice, provided a model for injection because of its immunodeficient background. This was vital to this study because the introduction of a foreign agent into these recipient mice would avoid immunologic responses and generate antibodies. Therefore, this would ensure that the effects observed after injection of purified Ig fractions would originate not from antibodies or the function of T-cells, but from injected Ig fractions. After injection into the Rag1KO mice with the different purified IgG fractions, it was expected to observe a decrease in salivary flow from the effects of IgG1 fractions. But after analyzing the results (Figures 3-1 through 3-3), it was observed that each fraction appeared to cause varied results.

These results were gathered in order to gain a better understanding of the relationship of IgG1 and other autoantibody fractions to normal cellular functions. The experimental project prompted for the use of 24 Rag1KO mice to be injected with respective purified mouse sera fractions of: IgG1, IgG2b, IgG2c. This was performed in order to gather data to support our hypothesis that IgG1 anti-mouse M3R autoantibodies causes saliva flow dysfunction. However, IgG3 fractions from both B6 and NOD were not injected into mice due to their low concentrations based on the ELISA, which was resulted from their cryoglobulinaemic characteristics during centrifugation. After injection, saliva was collected through the oral cavity and saliva volume was measured and analyzed. Statistical analysis was performed once all measurements had been taken using both a t-test (non-parametric) and one-way ANOVA (non-parametric) with Tukey Test to compare all paired values but the data was not significant ($p>0.05$).

NOD IgG1 purified fractions caused increased stimulation on Day 2, but then due to possible receptor desensitization, there was a decrease of saliva secretion over time, thus

supporting our hypothesis. One speculation for this observation is that cells may undergo a compensatory process by up-regulating receptors, where initially there is a possible overstimulation at M3R on Day 2, but over time, the cells down-regulate receptors on the cell surface via endocytosis, thus there is a decrease in saliva secretion. At this time, it was assumed that reduced saliva secretion may be the consequence of IgG1 affecting M3R. The receptor, once blocked by IgG1, can lead to an alteration in the signaling pathway that leads to normal cellular functions. Both B6 and NOD IgG2b fractions caused a stimulatory effect and showed max values on Day 2, but then B6 IgG2b fractions caused a rapid decrease in saliva volume though Day 5. NOD IgG2b fractions, on the other hand, also caused a decrease, but at a much slower salivary flow rate (flat line) compared to B6 on Day 5. This suggests that the effects of NOD IgG2b are less inhibitory than those of NOD IgG1. This can be observed as NOD IgG2b playing a very minimal role in the blocking of the signaling pathway with M3R. In addition, it was observed that B6 IgG2c fractions caused a stimulatory effect that peaked on Day 2, but slowly decreased through Day 5. NOD IgG2c fractions showed an increase peaking on Day 3 followed by a decrease in saliva secretion through Day 5. This again, like NOD IgG1, displayed the same inhibitory effects that led to the decline in saliva secretion after Day 3.

In addition to observing the effects of NOD IgG1 and NOD IgG2c, there were unexpected results that need to be further investigated. For instance, when observing the data from B6 (control) fractions, as well as the control conditions of non-injected mice and Glycine/Tris treated mice, they also caused a decrease in salivary flow. This may be explained by the amount of stress that the mice were under for the 5-day period. The injections, handling, and saliva collection placed the mice in stressful and tiring conditions which can explain the decrease in saliva production in these mice. In addition, considering that the Rag1KO mice were derived

from both the CGRC and Dr. Satoh's laboratory, it is speculated that two different environmental conditions that the mice were housed in may have caused a discrepancy. This may have caused an unanticipated fluctuation in saliva flow that must be observed in the future. The difference between these effects and those caused by NOD IgG1 and NOD IgG2c is that both NOD IgG1 and NOD IgG2c caused a noticeable stimulation in saliva secretion, either on Day 2 or Day 3, that eventually led to the decrease in saliva secretion, whereas the B6 (control) fractions and both control conditions caused very little initial stimulations or no stimulation at all. Though the preliminary results support, in part, our original hypothesis that there is an inhibitory factor present in both NOD IgG1 and NOD IgG2c, further investigation must be completed.

Specific Aim #2

In addition to my *in vivo* study, this project also investigated the effects of the purified Ig fractions, more importantly IgG1, on the inhibition of M3R mediated intracellular calcium release in an *in vitro* system. In a study by Dawson et al. (26), it was found that whole IgG antibodies extracted from SS patients reduced the effects of carbachol (muscarinic agonist and calcium inducing agent) within both mouse and human acinar cells. When parasympathetic neurotransmitters bind to the M3R in normal individuals, a signaling cascade is stimulated and results in the release of intracellular calcium. This release provokes cellular functions to occur such as the activation of chloride channels in salivary glands for fluid secretion or smooth muscle contraction in bladder (17).

Many studies have shown the effects of whole IgG on intracellular calcium release but similar to Specific Aim #1, little has been studied as to the effects of isotypic Ig fractions on SS. Therefore, this project provided new insight as to how these fractions can alter the normal signaling pathway involving M3R that leads to the release of intracellular calcium. In order to monitor the effects of both B6 (control) and NOD (experimental) Ig fractions in an *in vitro*

setting, HSG cells were utilized as the target for incubation of each respective antibody. This was performed in order to demonstrate the inhibitory effects of NOD IgG1 on the binding of carbachol to M3R, thus leading to a dysfunction in calcium signaling. Both B6 and NOD fractions were incubated under two different time points of 15 minutes (short term stimulation) and 24 hours (chronic stimulation), in which two Ig injection treatments of 2 μL or 0.2 μg were performed at each time point.

Due to the fact that HSG expresses M3R and a mouse cell line expressing M3R was not available, HSG cells were utilized. A NCBI Blast search showed the sequence homology between human and mouse M3R is 86%. High sequence homology between both species suggests that the cross-reactivity of antibody may occur, which allowed me to utilize HSG cells for calcium release studies. Therefore, HSG cells were incubated with each purified fraction.

First, B6 IgG1 and B6 IgG2b caused a significant decrease in calcium release ($p<0.05$) compared to our CCh-responsive cells in Figure 3-4. Also, the comparison between positive (CCh + cells) and negative (No stimulation) controls showed a significant difference ($p<0.05$). This allowed me to assume that if the positive and negative controls were different, then any significant difference caused by the purified Ig fractions could be observed as acceptable and reliable. There was a general decreasing trend in calcium release in all B6 and NOD fractions in comparison to the CCh-stimulated cells.

Second, in Figure 3-5, the comparison between positive and negative controls displayed a significant decrease in calcium release ($p<0.05$, which meant any alterations caused by the Ig fractions could be accepted as having an inhibitory or stimulatory effect. After analyzing the results for each B6 and NOD fraction, it was observed that each treatment caused a significant ($p<0.05$) decrease in calcium release. This can be explained by assuming that each fraction

played an inhibitory role in blocking the M3R from the MR agonist (carbachol), thus affecting the signaling pathway downstream in the release of intracellular calcium. Further comparisons revealed that the longer the incubation time with purified Ig fractions, the greater the inhibitory effect on calcium release. This may be due to M3R desensitization because the longer the HSG cells are exposed to the purified fractions, the greater the inhibitory effect on calcium release. This was especially apparent for the 24-hour incubation time with 2 μL injections of fractions.

Third, the positive and negative controls were significantly different ($p<0.05$) in regards to a decrease in calcium release in Figure 3-6. The 15 minute incubation with the same amount of Ig (0.2 μg) again showed a decreasing trend in calcium release for all B6 and NOD fractions, similar to both Figure 3-4 and Figure 3-5 where the same volume (2 μL) of fractions were used. Even though the data for both B6 and NOD fractions (Figure 3-6) were not significant, a decreasing trend of inhibitory effects was noted. When comparing the 15 minute incubations, there was a greater inhibition of calcium release (Figure 3-4). This may have been due to the greater amount of protein that was present in the fraction used to incubate, compared to just using a constant amount but different volumes. This suggests that there was a sufficient amount of anti-M3R autoantibodies present to affect the binding of carbachol to the receptor, thus leading to a significant decrease in calcium release, which requires further verification

Fourth, shown in Figure 3-7, both B6 and NOD purified Ig fractions (with the exception of NOD IgG1) caused an inhibitory effect on calcium release, even though their data was not significantly different ($p>0.05$). In this example, the positive and negative controls were again not significantly different ($p>0.05$). This may have been due to not enough protein present in the fractions when incubated with the cells, thus causing a lesser effect compared to treatments. These data support the idea that because of a longer incubation time, each fraction was able to

desensitize and block the binding site of M3R from carbachol, thus affecting neurotransmitters from a normal signaling pathway. The decreasing trend was observed in all fractions, again supporting the fact that there was some sort of inhibition factor in the fractions that caused this to happen.

Lastly, NOD IgG1 was much different in Figure 3-5 when compared to Figures 3-4, 3-6, and 3-7 because of its inhibitory rather than its stimulatory effect. This can be explained again by not enough protein present in the fraction to block the binding of carbachol to M3R, thus leading to a surge of intracellular calcium being released similar to the responses by CCh-responsive cells (Positive control). When comparing the NOD IgG1 results of Figures 3-4 through 3-7, the greater amount of Ig used to incubate with the cells caused a greater inhibitory effect. High concentration of IgG1 caused an inhibitory effect, which supports our original hypothesis that IgG1 leads to inhibition of calcium release by affecting M3R signaling pathway.

Future Directions

To acquire better results for analysis for future experiments, the experimental conditions further needs to be optimized. In regards to the *in vivo* study, another step that should be performed is reconstituting the purified Ig fractions into whole IgG, and injecting that into the Rag1KO recipient mice. If the results mimic those of previous studies, then we could have a better interpretation of the effects of both whole IgG and purified Ig fractions on SS. In addition, a larger sample size of mice would greatly reduce statistical errors, as well as give us a better depiction of the population. As for the *in vitro* study, finding a suitable mouse cell line that expresses M3R may give a better representation of the effects of the mouse Ig fractions. Since the results suggested that both B6 and NOD fractions inhibited calcium release in HSG cells, further studies need to be performed to determine whether this effect was due to specific binding of anti-M3R autoantibodies or non-specific concentration dependent stimulation.

For example, knocking down M3R using small interfering RNA (siRNA), would allow us to determine if M3R expression is required for this inhibition. If inhibition is present in the absence of M3R, this would suggest that non-specific binding of antibody fractions can inhibit carbachol-induced calcium release in HSG cells, as observed with B6 fractions. This could be due to the higher protein concentration in B6 fractions compared to NOD. In addition, since using increased amounts of Ig fractions showed a decrease in calcium release, further investigation must performed to avoid discrepancies and errors. By doing so, dose effects of IgG1 can be analyzed while comparing it with the pathological inhibitory effects of anti-M3R autoantibodies. Lastly, an effective and reliable assay to detect the presence of anti-M3R autoantibodies and the effect of each fraction on the receptor should be performed to obtain a better understanding of its pathological effect on secretory dysfunction.

Conclusion

In conclusion, the current data suggest that NOD IgG1 and NOD IgG2c both cause secretory dysfunction *in vivo*, while NOD IgG1 causes an inhibition in calcium release *in vitro*. The *in vivo* findings propose that both NOD IgG1 and IgG2c can cause secretory dysfunction, probably due to their ability to block the M3R binding site, thus inhibiting the signaling pathway for normal saliva secretion. The *in vitro* findings suggest that both B6 and NOD fractions can cause a decrease in calcium release in HSG cells after 24 hours of chronic stimulation, and NOD IgG1 show inhibitory effects on the binding of carbachol to M3R, which is supportive of my hypothesis. By conducting further studies *in vivo* and *in vitro* using NOD purified fractions, it might be possible to gain a better understanding of functional implications of anti-M3R autoantibodies in autoimmune Sjögren's syndrome.

LIST OF REFERENCES

1. Cha, S., J. Brayer, J. Gao, V. Brown, S. Killedar, U. Yasunari, and A. B. Peck. 2004. A Dual Role for Interferon-γ in the Pathogenesis of Sjögren's Syndrome -Like Autoimmune Exocrinopathy in the Nonobese Diabetic Mouse. *Scandinavian Journal of Immunology* 60:552-565.

2. Mese, H., and R. Matsuo. 2007. Salivary secretion, taste and hyposalivation. *Journal of Oral Rehabilitation* 34:711-723.

3. Waterman, S. A., T. Gordon, and M. Rischmueller. 2000. Inhibitory effects of muscarinic receptor autoantibodies on parasympathetic neurotransmission in Sjögren's syndrome. *Arthritis & Rheumatism* 43:1647-1654.

4. Cermak, J. M., A. S. Papas, R. M. Sullivan, M. R. Dana, and D. A. Sullivan. 2003. Nutrient intake in women with primary and secondary Sjögren's syndrome. *European Journal of Clinical Nutrition* 57:328.

5. Ramos-Casals, M., and P. Brito-Zeron. 2007. Emerging biological therapies in primary Sjogren's syndrome. *Rheumatology* 46:1389-1396.

6. Vitali, C., S. Bombardieri, R. Jonsson, H. M. Moutsopoulos, E. L. Alexander, S. E. Carsons, T. E. Daniels, P. C. Fox, R. I. Fox, S. S. Kassan, S. R. Pillemer, N. Talal, and M. H. Weisman. 2002. Classification criteria for Sjogren's syndrome: a revised version of the European criteria proposed by the American-European Consensus Group. *Annals of the Rheumatic Diseases* 61:554-558.

7. Von Mühlen, C. A., and E. M. Tan. 1995. Autoantibodies in the diagnosis of systemicrheumatic diseases. *Seminars in Arthritis and Rheumatism* 24:323-358.

8. Wolin, S. L., and K. M. Reinisch. 2006. The Ro 60 kDa autoantigen comes into focus: Interpreting epitope mapping experiments on the basis of structure. *Autoimmunity Reviews* 5:367-372.

9. Chen, X., and S. L. Wolin. 2004. The Ro 60 kDa autoantigen: insights into cellular function and role in autoimmunity. *Journal of Molecular Medicine* 82:232(238).

10. Espinosa, A., W. Zhou, M. Ek, M. Hedlund, S. Brauner, K. Popovic, L. Horvath, T. Wallerskog, M. Oukka, F. Nyberg, V. K. Kuchroo, and M. Wahren-Herlenius. 2006. The Sjogren's Syndrome-Associated Autoantigen Ro52 Is an E3 Ligase That Regulates Proliferation and Cell Death. *Journal of Immunology* 176:6277-6285.

11. Fabini, G., S. A. Rutjes, C. Zimmermann, G. J. M. Pruijn, and G. Steiner. 2000. Analysis of the molecular composition of Ro ribonucleoprotein complexes. *European Journal of Biochemistry* 267:2778-2789.

12. JF, M., and S. RJ. 1992. Autoantibodies and their target antigens in Sjögren's syndrome. *Netherland Journal of Medicine* 40:140-147.

13. Bacman, S., L. Sterin-Borda, J. J. Camusso, R. Arana, and O. H. E. Borda. 1996. Circulating antibodies against rat parotid gland M3 muscarinic receptors in primary Sjögren's syndrome. *Clinical & Experimental Immunology* 104:454-459.

14. Nguyen, C., J. Cornelius, L. Cooper, J. Neff, J. Tao, B. Lee, and A. Peck. 2008. Identification of possible candidate genes regulating Sjogren's syndrome-associated autoimmunity: a potential role for TNFSF4 in autoimmune exocrinopathy. *Arthritis Research & Therapy* 10:R137.

15. Gao, J., S. Cha, R. Jonsson, J. Opalko, and A. B. Peck. 2004. Detection of anti-type 3 muscarinic acetylcholine receptor autoantibodies in the sera of Sjögren's syndrome patients by use of a transfected cell line assay. *Arthritis & Rheumatism* 50:2615-2621.

16. Dawson, L., A. Tobin, P. Smith, and T. Gordon. 2005. Antimuscarinic antibodies in Sjögren's syndrome: Where are we, and where are we going? *Arthritis & Rheumatism* 52:2984-2995.

17. Cha, S. H., E. Singson, J. Cornelius, J. P. Yagna, H. J. Knot, and A. B. Peck. 2006. Muscarinic acetylcholine type-3 receptor desensitization due to chronic exposure to Sjogren's syndrome-associated autoantibodies. *Journal of Rheumatology* 33:296-306.

18. Nakamura, T., M. Matsui, K. Uchida, A. Futatsugi, S. Kusakawa, N. Matsumoto, K. Nakamura, T. Manabe, M. M. Taketo, and K. Mikoshiba. 2004. M3 muscarinic acetylcholine receptor plays a critical role in parasympathetic control of salivation in mice. *Journal of Physiology* 558:561-575.

19. Drachman, D. 2003. Autonomic "myasthenia": The Case for an Autoimmune Pathogenesis *Journal of Clinical Investigation* 111:797-799.

20. Eriksson, P., C. Andersson, C. Ekerfelt, J. Ernerudh, and T. Skogh. 2004. Relationship between serum levels of IL-18 and IgG1 in patients with primary Sjögren's syndrome, rheumatoid arthritis and healthy controls. *Clinical & Experimental Immunology* 137:617-620.

21. Andersson, A. 2009. Genetic control of disease in an experimental model for Sjogren's syndrome. *Arthritis Research & Therapy* 11:102.

22. Currie, M., A. M. Zaki, S. Nejat, G. M. Hirsch, and T. D. Lee. 2008. Immunologic targets in the etiology of allograft vasculopathy: Endothelium versus media. *Transplant Immunology* 19:120-126.

23. Jonsson, M. V., N. Delaleu, K. A. Brokstad, E. Berggreen, and K. Skarstein. 2006. Impaired salivary gland function in NOD mice: Association with changes in cytokine profile but not with histopathologic changes in the salivary gland. *Arthritis & Rheumatism* 54:2300-2305.

24. Gao, J., S. Killedar, J. G. Cornelius, C. Nguyen, S. Cha, and A. B. Peck. 2006. Sjogren's syndrome in the NOD mouse model is an interleukin-4 time-dependent, antibody isotype-specific autoimmune disease. *Journal of Autoimmunity* 26:90-103.

25. Lee, B., M. Tudares, and C. Nguyen. 2009. Sjögren's syndrome: An old tale with a new twist. *Archivum Immunologiae et Therapiae Experimentalis* 57:57-66.

26. Dawson, L. J., J. Stanbury, N. Venn, B. Hasdimir, S. N. Rogers, and P. M. Smith. 2006. Antimuscarinic antibodies in primary Sjögren's syndrome reversibly inhibit the mechanism of fluid secretion by human submandibular salivary acinar cells. *Arthritis & Rheumatism* 54:1165-1173.

27. Robinson, C. P., J. Brayer, S. Yamachika, T. R. Esch, A. B. Peck, C. A. Stewart, E. Peen, R. Jonsson, and M. G. Humphreys-Beher. 1998. Transfer of human serum IgG to nonobese diabetic Igμ null mice reveals a role for autoantibodies in the loss of secretory function of exocrine tissues in Sjögren's syndrome. *Proceedings of the National Academy of Sciences of the United States of America* 95:7538-7543.

28. Waterman, S., T. Gordon, and M. Rischmueller. 2000. Inhibitory effects of muscarinic receptor autoantibodies on parasympathetic neurotransmission in Sjögren's syndrome. *Arthritis & Rheumatism* 43:1647-1654.

BIOGRAPHICAL SKETCH

Huy Huynh grew up and attended school in Winter Haven, FL, where he graduated high school in 2002. After high school, he began study at the University of Florida in Gainesville, where he obtained a Bachelor of Science in human nutrition in May of 2007. In the fall of 2007, Huy was admitted into the master's studies program at the University of Florida, where his degree was focused on food science. Under the co-mentorship of Dr. Gary Rodrick and Dr. Seunghee Cha, Huy was able to fulfill his requirements for his degree, as well as perform his autoimmune research that investigated the role of IgG1 anti-M3R autoantibodies on secretory dysfunction in Sjögren's syndrome. In the fall of 2009, Huy will enter dental school at the University of Florida. His ultimate professional goal is to become a practicing general dentist with an interest in teaching and academia.