THE FUTURE OF MYASTHENIA GRAVIS: EXPLORING THE ONSET, PROGRESSION AND IMPLICATIONS OF DISEASE

by

Chana Renee Paluszcyk

A Thesis Submitted to the Faculty of the

DEPARTMENT OF CELLULAR AND MOLECULAR MEDICINE

In Partial Fulfillment of the Requirements

For the Degree of

MASTER OF SCIENCE

In the Graduate College

THE UNIVERSITY OF ARIZONA

2016

STATEMENT BY AUTHOR

The thesis titled *The Future of Myasthenia Gravis: Exploring the Onset, Progression, and Implications of the Disease* prepared by *Chana Paluszcyk* has been submitted in partial fulfillment of requirements for a master's degree at the University of Arizona and is deposited in the University Library to be made available to borrowers under rules of the Library.

Brief quotations from this thesis are allowable without special permission, provided that an accurate acknowledgement of the source is made. Requests for permission for extended quotation from or reproduction of this manuscript in whole or in part may be granted by the head of the major department or the Dean of the Graduate College when in his or her judgment the proposed use of the material is in the interests of scholarship. In all other instances, however, permission must be obtained from the author.

SIGNED: *Chana Paluszcyk*

APPROVAL BY THESIS DIRECTOR

This thesis has been approved on the date shown below:

_____ *4/21/16*
Jean Wilson, Ph.D Date
Professor of Cellular and Molecular Medicine

_____ *4/21/16*
Lonnie Lybarger, Ph.D Date
Associate Professor of Cellular and Molecular Medicine

_____ *4/21/16*
David Elliott, Ph.D Date
Assistant Professor of Cellular and Molecular Medicine

_____ *4/21/16*
Michael Riggs, Ph.D Date
Professor of Animal and Comparative Biomedical Sciences

Table of Contents

The Future of Myasthenia Gravis: Exploring the Onset, Progression and Implications of Disease

by Chana Renee Paluszcyk

Abstract

Myasthenia gravis (MG) is an autoimmune disease whose name means "grave muscular weakness". MG is a rare disease affecting only 200-400 persons per million and the characteristic symptoms include muscle weakness, particularly in highly active voluntary muscles. MG affects the neuromuscular junction in an antibody-mediated manner, resulting in impaired nerve-muscle cell communication in affected individuals. Specifically, two main proteins are targeted: nicotinic acetylcholine receptors (ACh receptors) and a muscle-specific tyrosine kinase (MuSK). Previous studies have discovered the mechanism of MG pathogenesis but the exact mechanisms which cause the failure to maintain self-tolerance have not been discovered. Based on current knowledge of MG, this paper will explore potential causes of the disease and provide numerous hypotheses directed at future research opportunities.

Introduction and Overview

Myasthenia Gravis

Myasthenia gravis (MG) is an autoimmune disease whose name means "grave muscular weakness". MG is a rare disease affecting only 200-400 persons per million and the characteristic symptoms include muscle weakness, particularly in highly active voluntary muscles [1]. Initially, the extrinsic ocular muscles are often affected, which causes drooping eyelids [1]. There are multiple subtypes of MG, differing in factors such as the age of onset, genetic predisposition, clinical symptoms, presence or absence of thymic pathology, and autoantibody profile (See Table 1) [8]. There are congenital forms of MG; however these will not be the focus here. Instead, autoimmune forms of MG will be the primary focus. The factors involved in MG progression and maintenance will be discussed first, followed by an

exploration of the potential causes and future research in MG in the Synthesis and Future Work section.

As MG progresses, symptoms usually spread throughout the body and affect a wide number of muscles, causing generalized MG [1]. Generalized MG has the following characteristics [4]:

1. It usually affects cranial muscles, especially involving the eyelids, eye muscles and oropharyngeal muscles.
2. Often involves periods of remission or exacerbation which can vary in length from a single day, over the course of a few days or over an even longer period. This characteristic sets MG apart from most other diseases that affect the nervous or muscular system.
3. The disease symptoms are lessened when treated with drugs that inhibit acetylcholinerase, an enzyme that degrades acetylcholine at the neuromuscular junction.

MG is a disease that affects the neuromuscular junction in an antibody-mediated manner, resulting in impaired nerve-muscle cell communication in affected individuals. The disease symptoms manifest as a result of antibodies binding to proteins within the neuromuscular junction that are critically involved in signaling [1]. Specifically, two main proteins are targeted: nicotinic acetylcholine receptors (ACh receptors) and a muscle-specific tyrosine kinase (MuSK) [1]. The loss of acetylcholine receptors in the neuromuscular junction is the primary mechanism of disease, with loss of MuSK being much more rare [2] [9]. Approximately 20% of patients do not have antibodies against ACh receptors but instead target MuSK [9]. In patients with anti-MuSK antibodies, clinical symptoms and prognosis are usually more severe. Respiratory failure leading to death is more common in patients with

anti-MuSK antibodies. Patients with neither anti-ACh receptor antibodies nor anti-MuSK antibodies are known as seronegative. The exact prevalence of seronegative MG is unknown but it is hypothesized that these patients are actually anti-ACh receptor antibody-positive and the antibody titer is too low to be detected with current assays [8]. The last main subtype of MG is known as ocular MG. Patients who have ocular MG show muscle weakness that is limited to the eyes only and this subtype represents approximately 17% of MG cases [8]. The main subtypes of MG are summarized in Table 1 below.

Table 1: Subtypes of Myasthenia gravis

	Age of Onset	Histology of Thymus	Autoantibodies	Comments
Early onset	<40 years	Hyperplasia	ACh receptor	1:3 male:female ratio
Late onset	>40 years	Normal	ACh receptor	N/A
MuSK	<40 years (usually)	Normal	MuSK	Marked female predominance
Thymoma	40-60 years	Neoplasia	None identified	May be associated with other paraneoplastic disorders
Seronegative	Variable	Hyperplasia in some cases	None identified	N/A
Ocular	Adult in USA	Unknown	ACh receptor in 50% cases	Possible low affinity ACh receptor antibodies

Autoimmune diseases occur when the mechanisms fail that establish and/or maintain self-tolerance [51]. Self-tolerance is the term used to describe the immune system's learned ability to specifically "ignore" or "tolerate" self-antigens so that the body does not attack its own antigens [51]. In the case of MG, failure of the immune system to recognize the ACh

receptors or MuSK as self-antigens leads to the classical symptoms of muscle weakness and fatigue [1]. In the majority of MG cases, the ACh receptor on the muscle fiber is the self-antigen that is targeted by the body's own immune system. Antibodies bound to ACh receptors inhibit the binding of acetylcholine that is released in the neuromuscular junction upon receiving an action potential [1]. Free acetylcholine within the neuromuscular junction is quickly degraded by acetylcholinesterase (see Figure 1). The decrease in acetylcholine available to bind to ACh receptors inhibits action potential formation in the muscle fiber, resulting in decreased communication between nerve and muscle cells. As a result, patients with MG lose the ability to control muscle movements and the severity of the disease can differ between patients.

Current studies have discovered the mechanism of MG pathogenesis but the exact mechanisms which cause the failure to maintain self-tolerance have not been discovered. However, often the disease is associated with thymus hyperplasia or thymoma, providing insight into possible mechanisms of disease onset [2]. Approximately 15% of patients with MG have a thymic epithelial tumor; however, tumor resection typically does not cure MG in these patients [8]. The frequency of thymic abnormalities (not specific to thymic tumors such as thymic hyperplasia) in MG patients is even higher, at an estimated 75% occurrence rate [18]. Although no controlled trials are available to show the benefit of a thymectomy in MG, the potential of thymectomy in reducing the severity of symptoms and/or leading to disease remission is an attractive idea [18].

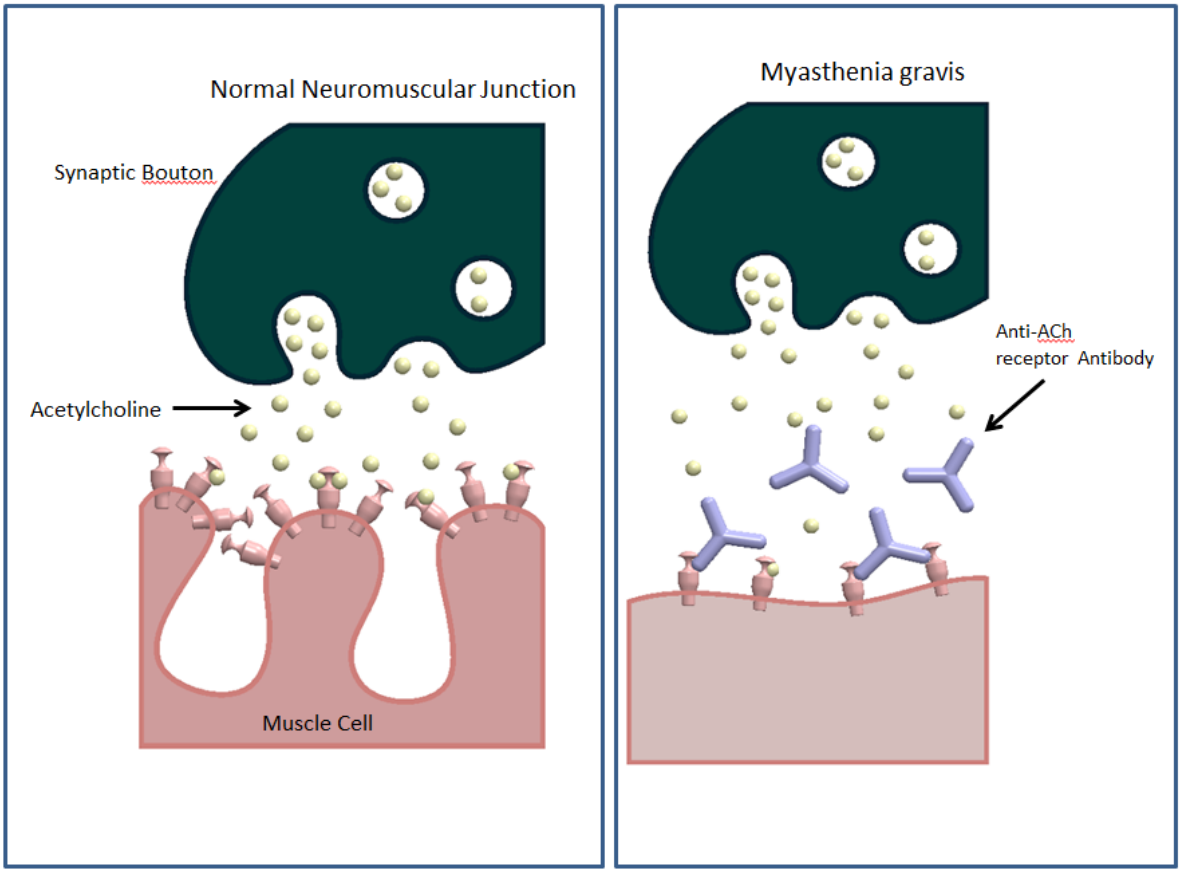


Figure 1: Nerve Transmission at the Neuromuscular Junction (normal vs. MG)

In normal nerve transmission, an action potential travels down the axon of a neuron. When the action potential reaches the neuron that is in contact with a muscle fiber, numerous voltage-gated ion channels open and allow ion concentration changes within the neuron. The result is acetylcholine release from the neuron into the neuromuscular junction. Acetylcholine binds its receptor (AChR) on the surface of the muscle fiber and triggers numerous downstream signaling pathways that ultimately lead to muscle contraction. In patients with MG, antibodies specific against AChR will bind to and inhibit acetylcholine from binding AChR, resulting in acetylcholinesterase rapidly degrading the acetylcholine.

Summary

Since MG is a rare and highly variable autoimmune disease, much research remains unexplored and unanswered. This paper will explore what is currently known about MG with respect to the clinical presentation and key molecular players and pathways involved. In addition, this paper will explore potential molecular mechanisms that may contribute to the onset and progression of the disease. Specifically, the following topics will be explored:

- The normal physiology of the nervous system (specifically the neuromuscular junction) at the cellular and molecular level to provide a foundation for understanding muscle impairment in the diseased state.
- The adaptive immune system and its role in the onset and progression of MG.
- Current theories on the causes of autoimmune diseases, with particular emphasis on MG.
- Current treatment for patients with MG, as this provides insight into disease mechanisms.

Finally, on the basis of our current knowledge of MG, as well as other autoimmune diseases that follow a parallel mechanism and progression, hypotheses and future research will be proposed to address some of the key unanswered questions about MG.

Nervous System

Nerve Impulse Generation

Neurons are cells of the nervous system that are able to carry information to target cells in the form of electrical and chemical signals [4]. Electrical signals are generated as the action potential travels along the neuron; they are formed and maintained by the flow of ions through voltage-gated ion channels in the neuron cell membrane [4]. This flow of ions produces a change in the electric current into and out of the cell, which drives an action potential across the cell membrane [34]. Chemical signals are generated once the action potential reaches the end of the axon and are used to communicate with the target cell (neuron or muscle cell). Chemical signals are transmitted when molecules such as neurotransmitters are released from the neuron, travel into the synaptic cleft and bind to receptors on the post-synaptic cell [34] (see Figure 1). The binding of neurotransmitter to the target cell membrane opens voltage-gated channels to propagate the action potential (in

neurons) or generates an endplate action potential in the muscle fiber that ultimately leads to muscle contraction [34].

Neuromuscular Junction: Signaling at the Nerve-Muscle Synapse

Signaling at the nerve-muscle synapse is relatively simple, consisting mainly of the release of neurotransmitter from the presynaptic membrane (neuron) which causes the opening of a single type of ion channel in the postsynaptic membrane (muscle fiber). When this channel is opened, it allows an influx and outflux of particular ions, such as Na^+, K^+ and Ca^{2+}. The overall flow of ions favors the influx of positively charged ions which, if threshold is met, causes a depolarizing synaptic potential in the muscle fiber that ultimately triggers muscle contraction [4] [31].

The neuromuscular junction is defined as a chemical synapse formed between a muscle fiber and a motor neuron [31]. The motor neuron innervates the muscle fiber at a region known as the endplate, which is a specialized region on the muscle membrane where the motor axon splits into smaller branches. The terminal end of these branches forms structures known as synaptic boutons, which are the sites where neurotransmitter is released from the motor neurons [1]. The synaptic boutons are positioned on regions of the muscle fiber known as junctional folds, which are the sites on the muscle fiber where the neurotransmitter receptors are located [31]. Upon stimulation, motor neurons release the neurotransmitter acetylcholine (ACh) which traverse the synaptic cleft and bind to nicotinic-type ACh receptors on the muscle fiber [31].

The synaptic bouton of the neuron represents the presynaptic membrane and the junctional fold of the muscle fiber represents the postsynaptic membrane [1]. The presynaptic and postsynaptic membranes are separated by a small gap known as the synaptic cleft, which is approximately 100nm wide [31]. The acetylcholine is released into the synaptic cleft where it travels to the postsynaptic membrane and binds to ACh receptors. The enzyme

acetylcholinesterase rapidly degrades ACh in the synaptic cleft and is anchored to collagen fibers of the basal laminae [32]. In patients with MG, the ACh cannot bind quickly to the ACh receptors because antibodies are bound to the ACh receptors, blocking ACh binding. As a result, the free ACh in the synaptic cleft is quickly degraded by acetylcholinesterase, resulting in impaired nerve-muscle communication. [34]

In summary, the neuromuscular junction is the most important location to study as it relates to MG. Understanding normal nerve impulse transmission and how the process specifically triggers muscle contraction is critical before researching new therapeutic options for MG. In addition, a solid understanding of the key players located at the neuromuscular junction is also critical, specifically for discovering potential drug targets. Therefore, as it relates to MG, a few of the important molecules concentrated at the muscle fiber endplate include acetylcholinesterase, membrane receptors for agrin (MuSK and low density lipoprotein receptor-related protein 4), utrophin (cytoskeletal-associated protein) and rapsyn (receptor-associated protein of the synapse) [29]. Acetylcholinesterase is the enzyme that degrades ACh in the synaptic cleft and is currently the target of drug inhibition in MG patients. Both MuSK and rapsyn are proteins that are required to form stable ACh receptor clusters on the cell membrane [29]. These proteins will be discussed in more detail in later sections. Utrophin is also a protein involved in ACh receptor clustering but also has been shown to be associated with nitric oxide synthetase [34]. It is hypothesized that nitric oxide at the postsynaptic surface serves a signaling function in addition to influencing synaptic formation [34]. MG patients have been observed to have mutations that alter expression of both rapsyn and utrophin, providing potential research options as it relates to genomic engineering (further discussed in the Synthesis and Future Work section).

Nicotinic Acetylcholine Receptors

Nicotinic acetylcholine receptors (ACh receptors) play a critical role in muscle contraction and are the main target of antibodies in patients with MG. Structurally, the ACh receptor is a pentameric membrane glycoprotein that is formed by five individual subunits: two α-subunits, one β -subunit, one γ -subunit and one δ –subunit [30]. The five subunits span the lipid bilayer membrane and form a pore through which ions can freely pass when the receptor is activated. The ACh receptor is in an inactivated (closed) state when there is no ACh bound to the receptor. The ACh receptor is activated (opened) when two ACh molecules are bound to the receptor. The ACh binding sites are located on the extracellular side of the cell, specifically on the two α -subunits where the α and γ /δ- subunits are connected [30] (Figure 2).

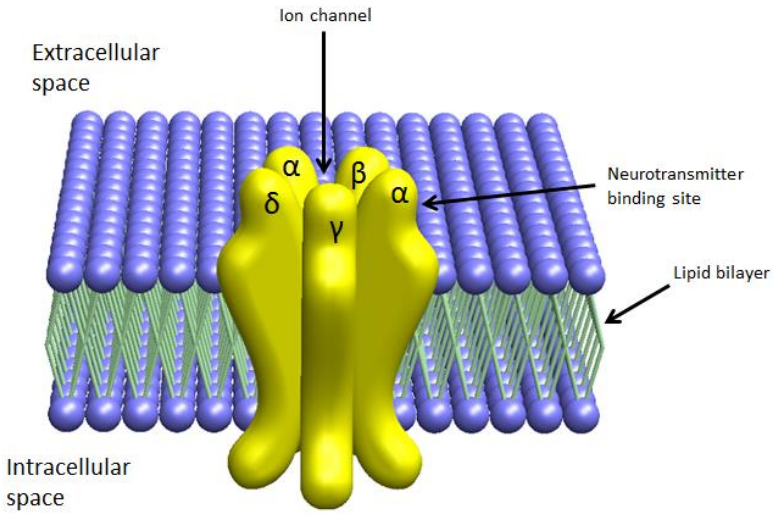


Figure 2: ACh Receptor

The ACh receptor is a five subunit protein that is arranged around a central pore. The ACh receptor has two acetylcholine binding sites in the α subunits. When two acetylcholine bind, there is a conformational change in shape, opening the pore. This allows the influx and outflux of particular ions, with a net flow inward of positively charged ions.

In patients with MG, antibodies directed against ACh receptors damage the postsynaptic membrane via complement-mediated lysis and antibodies crosslinking ACh receptor antigens [41]. Crosslinking between antibody-antigen is thought to induce a conformational change in the ACh receptor which promotes ACh receptor internalization (via endocytosis) and subsequent degradation [42]. If the rate of ACh receptor internalization and degradation is not sufficiently compensated by increased synthesis and insertion in the membrane, the result is decreased ACh receptor density and potentially impaired muscle fiber contraction during stimulation. Not all bound antibodies will be physically able to crosslink antigens; therefore, location of ACh receptors on the cell membrane influences the ability of crosslinking to occur [10].

During the development of the embryo, ACh receptors are initially scattered on the myotubes (developing skeletal muscle fibers) but a few weeks after birth the concentration of ACh receptors increases significantly (approximately 10,000/uM^2) [36]. Once the ACh receptors are inserted into the membrane, they form high-density clusters that tether with rapsyn, a 43kDa membrane-associated protein. Rapsyn is critical for ACh receptor clustering by anchoring the ACh receptor to the contractile protein F-actin via beta-dystroglycan [37]. Rat models for MG have been used to show the relationship between rapsyn and ACh receptor expression [38]. It has been experimentally observed that no ACh receptor clusters are formed in rapsyn-deficient mice [38]. Interestingly, the expression of rapsyn increases as the rat ages which stabilize the ACh receptors in the membrane. Further, increased expression of rapsyn at the neuromuscular junction induced resistance against antibodies for ACh receptors by decreasing the amount of antibody-induced internalization [39]. In contrast, significant overexpression of rapsyn at the neuromuscular junction in rats with chronic MG has a harmful effect since the endplates are already severely damaged, as will be further discussed in subsequent sections. Further, overexpression of rapsyn increases the

postsynaptic membrane turnover that is caused by the ACh receptor antibody binding [40]. These studies support the idea that the expression of rapsyn is critically related to both the ACh receptor levels and to the integrity of the endplate [10].

ACh receptors are the most common protein affected in MG patients; however, loss of ACh receptor-associated proteins is also linked to the development of muscle weakness and MG-like symptoms [10]. These proteins are rapsyn, MuSK, laminin and Dok-7. Further, in MG patients who have anti-ACh receptor antibodies, there is evidence that the additional loss of ACh receptor-associated proteins worsens the disease and causes delays in repair [40]. The roles of both rapsyn and MuSK will be discussed later but briefly, the protein Dok-7 will be discussed. Dok-7 is a member of a class of proteins involved in signaling complexes in the cell membrane [43]. Dok proteins regulate signal transduction as well as recruitment of other proteins by their phosphotyrosine binding domain and a C-terminal domain which contain binding sites for SH2 and SH3-containing proteins. Dok-7 is able to interact with the MuSK cytoplasmic domain, causing its autophosphorylation [43]. As will be discussed in the next section, MuSK is involved in forming the ACh receptor clusters on the muscle fiber membrane but when Dok-7 is silenced, these ACh receptor clusters fail to form. Dok-7 knock-out mice do not survive after birth and after analysis of the endplate, no ACh receptor clusters are observed, indicating they failed to develop prior to birth [43].

Muscle-Specific Tyrosine Kinase

Muscle-Specific Tyrosine Kinase (MuSK) is the target of antibodies in approximately 20% of diagnosed MG cases and is most commonly associated with respiratory failure, although the reason is unknown. MuSK is a protein that is required for the formation and maintenance of the neuromuscular junction, specifically by inducing the clustering of ACh receptors on the postsynaptic membrane. Functionally, MuSK plays a critical role in ACh receptor clustering at the membrane via the agrin/Lrp4/MuSK/rapsyn/ACh receptor pathway [29]. It is

speculated that antibodies directed against MuSK alter both the function in ACh receptor clustering and increase the MuSK turnover rate [10]. There has been no evidence to suggest that anti-MuSK antibodies recruit the complement system, result in loss of ACh receptor density on the membrane or cause a loss of integrity of the junctional fold, as is seen with anti-ACh receptor antibodies [10]. Thus, it may be the effects of MuSK on ACh receptor clustering that ultimately lead to disease caused by anti-MuSK antibodies.

It is currently speculated that patients who are anti-ACh antibody-negative and anti-MuSK antibody-positive follow a very different mechanism of pathology. There are both structural and functional differences between anti-ACh receptor antibodies and anti-MuSK antibodies. In contrast to what is observed with anti-ACh receptor antibodies, there is no impairment with junctional folding and no loss of ACh receptors on the postsynaptic membrane in MG patients who are positive for anti-MuSK antibodies [45]. Anti-MuSK antibodies are mainly of the IgG4 isotype which are functionally different in its' anti-inflammatory role, as compared to the other IgG antibody subclasses (refer to Figure 3 below) [10]. IgG4 antibodies are poor recruiters of the complement system because of low affinity for C1q and Fc receptors [10]. The ability of the Fc region to bind to an Fc receptor depends on the glycans at the conserved Fc region. IgG4 antibodies obtained from plasma have been observed to have two different antigen binding sites, a unique feature to this subtype and are a direct result of the Fab arm exchange (explained below) [10]. This feature is why one single antibody is unable to crosslink two identical antigens. The Fab arm exchange disrupts the once common belief notion of "one antibody, one antigen" and instead challenges our views about the role of IgG4 antibodies in MG [10].

As briefly mentioned above, IgG4 anti-MuSK antibodies undergo a unique process known as the "Fab arm exchange" which prevents cross-linking of antigens, therefore preventing the formation of immune complexes. The Fab arm exchange is a modification

occurring after translation (the conversion of mRNA into proteins). The entire exchange process involves IgG4 antibodies switching a heavy chain (and its' associated light chain) with a heavy and light chain from another antibody (refer to Figure 3 below) [10]. The result is an antibody that has two different variable antigen-binding sites within both the heavy and light chains; a unique characteristic that only antibodies able to undergo this process possess [46]. This exchange process has been observed both *in vivo* and *in vitro* but does require a reducing environment to facilitate the breaking of bonds between half-molecules. A reducing environment, such as the blood, contains reducing gases (e.g. hydrogen, carbon monoxide) in the absence of oxygen, therefore inhibiting oxidation reactions [46].

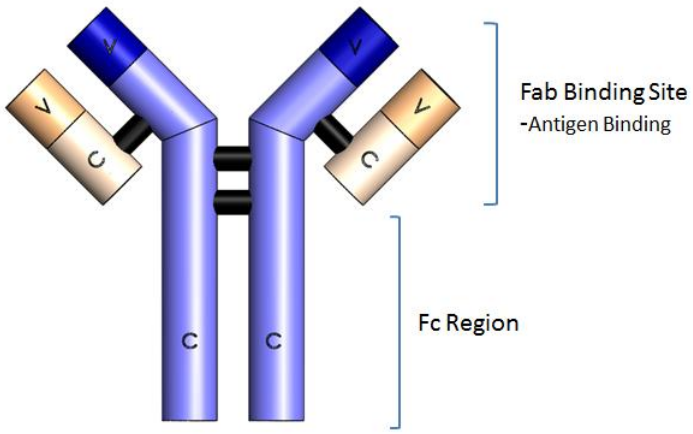


Figure 4: Structure of an IgG Antibody
An example of an IgG antibody with variable (v) and constant (c) antigen-binding sites. The light chains (gold) are connected to the heavy chains (blue) via disulfide bonds (black). Each antibody can bind a single antigen to the variable regions of the light and heavy chains (*exception*: *unless the antibody underwent the Fab arm exchange process).*

Summary of MG at the Neuromuscular Junction

There is great diversity in the number of motor neurons that innervate a single muscle fiber, depending on the level of motor control needed. For example, approximately 100 individual muscle fibers that control very fine movements (such as the ones controlling eye movement) are controlled by a single motor neuron. In contrast, nearly 1000 muscle fibers can be

controlled by a single motor neuron in the arm or leg since the movement is not finely controlled [4].

A “motor unit” is the term used to describe a motor neuron and the muscle fibers innervated by that particular motor neuron. Diseases that target the motor unit can be classified according to which component is primarily affected: 1) the cell body of the motor neuron 2) the axons 3) the neuromuscular junction or, 4) the muscle fibers innervated by a particular motor neuron [4]. In the case of MG, the neuromuscular junction is targeted, disrupting communication between the motor neuron and the muscle fibers which leads to impairment in muscle contraction. Synaptic transmission and neurotransmitter release are normal in patients with MG; however, there is not a sufficient amount of ACh binding to the ACh receptor to initiate an excitatory end-plate potential. Interestingly, the impaired ACh binding to its receptor is not simply due to antibodies being bound to the ACh receptor and physically inhibiting this binding, but rather the binding of the antibodies to the ACh receptors promote the destruction of the receptors, reducing the overall number of receptors on the postsynaptic membrane [26].

In many muscle fibers throughout the body, the total amount of ACh released during synaptic transmission can be decreased to nearly 25% of the normal amount released without impairing the initiation of the excitatory endplate potential [4]. This may help explain why some patients with MG still maintain fairly normal muscle contraction in many of their muscles. However, the number of available receptors is what is dramatically reduced in patients with MG, not the amount of ACh that is released [26]. When the density of available receptors is dramatically decreased, the probability that ACh will bind to an available receptor before being degraded by acetylcholinesterase is very low. Additionally, it has been observed that the physical morphology of the endplate is altered in patients with MG as well [26]. The normal depth of the junctional fold invagination is reduced and the synaptic cleft is

enlarged. Both of these changes promote the diffusion of ACh *away* from the postsynaptic membrane where the few available and functioning ACh receptors are located. This further reduces the probability that ACh will come into contact with its receptors. The overall result is a decreased ability for the muscle fiber to be stimulated. If the muscle fiber is not stimulated, no excitatory end-plate potential is generated resulting in impaired muscle contraction.

Adaptive Immunity

Summary of Key Players

The immune system is divided into two main categories: the innate immune system and the adaptive immune system. The innate immune system is composed of cells and mechanisms that we are born with that do not require an initial exposure to the pathogen to mount a response. Examples include physical barriers, macrophages, neutrophils and the complement system. Upon exposure to a pathogen, phagocytic cells such as macrophages immediately begin engulfing the pathogens to limit spread of the infection. In contrast, the adaptive immune system is composed of cells and molecules that are directed against very specific antigens. After an initial exposure to a pathogen, the adaptive immune system is responsible for creating "immunological memory" that leads to a stronger and faster immune response upon seeing that pathogen again. The key players are the T and B lymphocytes and autoimmune diseases occur as a result of adaptive immune cells reacting with self-antigens, through mechanisms that will be explored below.

MG is an antibody-mediated autoimmune disease, meaning that antibody-producing cells of the adaptive immune system are the primary players leading to disease onset [1]. Antibodies produced in patients with MG are usually of the IgG isotype and are capable of activating the complement system leading to further destruction of the neuromuscular junction, specifically at the endplate of the muscle fiber [8]. The IgG antibodies that are

produced have a very high affinity to the target epitope, indicating that the antibody response is T cell dependent [8]. Upon initial exposure to a pathogen, T cells are needed to fully activate B cells, which then differentiate into plasma cells and begin secreting IgM antibodies specific to the target antigen [44]. Over time, T cells secrete certain signaling molecules that allow the B cells to begin secreting different subtypes of antibodies, such as IgG, that are functionally more appropriate for the specific antigen [44]. In addition, the signaling molecules secreted by the T cells drive the production of antibodies to have a higher affinity to the target antigen, effectively promoting a stronger response. Lastly, the specific and tightly regulated communication between cells in the adaptive immune response is controlled by a class of signaling molecules known as cytokines, which also have been seen to contribute to the progression of MG disease and will be discussed later in this paper [44].

T cells

T cells are a part of the adaptive immune system and play a critical role in recognizing and eliminating (both directly and indirectly) specific antigens. In addition, T cells are critical in initiating a B cell response, through mechanisms that will be explored. Each person has millions of different T cells that specifically recognize a single antigen that must be processed and presented on MHC-I and/or MHC-II molecules expressed by antigen-presenting cells (APCs) such as dendritic cells and macrophages [11]. When a pathogen invades, only the T cells with the specific receptor to that antigen can recognize and bind to antigens presented on MHC molecules. Upon activation, the T cells proliferate and differentiate into effector lymphocytes which can lead to activation of B cells or direct elimination of the pathogen [11]. T cell activation is required to initiate a full B cell response that leads to B cell differentiation into antibody-producing cells known as plasma cells (see Figure 4 below). Both the T cell and B cell response are aimed to completely eliminate the invading pathogen.

The specific T cells of interest (as they relate to the development and progression of MG) are T helper cells (both Type 1 and Type 2), CD8+ T cells and CD25+ T regulatory cells [1].

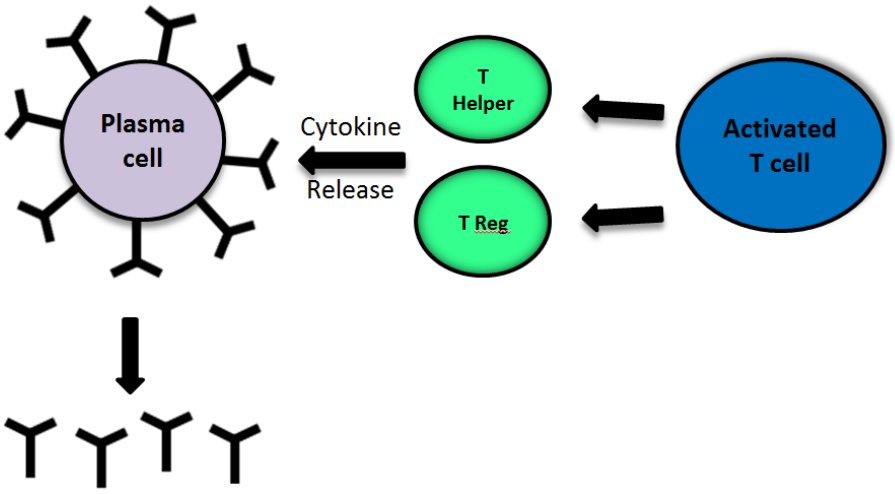


Figure 4: T and B cell Activation
Once activated, T cells can differentiate into different subtypes, including T helper cells and T regulatory cells. Cytokines released from these T cells leads to maturation of B cells into antibody-producing cells, known as plasma cells.

The great diversity of T-cell receptors (TCRs) that recognize specific antigens arises through a process of somatic gene rearrangement within the TCR genes [47]. If each TCR was encoded by an individual gene, the genome would need to contain thousands of additional genes. Instead, the variable region of the TCR is encoded by gene segments [47]. For a fully functional gene to be made and produce a specific protein, each of these gene segments must be brought together in an enzyme-catalyzed process known as gene rearrangement [47]. This rearrangement process creates a variable region that can be transcribed, translated and then checked to make sure it is functional. This process of rearrangement, splicing and bringing genes together creates the vast diversity seen in TCRs; further, the imprecise mechanisms that 'cut and paste' DNA nucleotides within the rearranged gene segments adds additional diversity to the TCR population.

An important category of signaling molecules that are involved in many pathways, including the T cell response, are cytokines [48]. Cytokines are small proteins that are released by cells, particularly cells of the immune system, and influence the behavior and fate of other cells [48]. Cytokines are critically involved in cell signaling; they bind to receptors on the cell surface of target cells and, through complex signaling pathways, influence a wide number of activities such as cell differentiation, proliferation, adhesion and many others [48]. Different cytokines are associated with the development of different cell lineages during an immune response. For example, when innate immune system cells such as macrophages encounter a foreign antigen, they begin secreting specific cytokines that drive the activation and differentiation of T cells. IL-12 released from cells such as macrophages and neutrophils promotes differentiation of CD4+ T cells into Th1 cells and IL-4 released from cells such as basophils promotes differentiation of CD4+ T cells into Th2 cells. Once differentiated, Th1 cells begin secreting IL-2 and IFN-gamma while Th2 cells begin secreting IL-4, IL-5 and IL-10 [6]. The function of the cytokines produced by Th1 and Th2 cells are discussed below [6].

Myasthenia gravis is an antibody-mediated disease specifically involving IL-12 and Th1 cells. IL-12 is a heterodimer composed of two glycosylated chains (p35 and p40) that are covalently linked together. IL-12 is a cytokine primarily released by activated monocytes, dendritic cells and neutrophils and drives the differentiation of activated CD4+ T cells into Th1 cells [6]. Th1 cells predominately activate macrophages, control the T cell-mediated hypersensitivity pathway, and promote IgG2a production [6]. The main role of IL-12 is to induce the differentiation of Th1 cells but it also enhances proliferation and cytolytic activity of NK and T cells. Additionally, IL-12 is critical in the production of complement-activating IgG antibody subtypes that are produced in MG. In contrast, Th2 cells are predominately involved in the immediate hypersensitivity pathway [6]. IL-4, which controls the differentiation of activated CD4+ T cells into Th2 cells, is also critically involved in the

production of IgE antibodies, which are the characteristic sign of immediate hypersensitivity. A number of additional studies have examined the role of both Th1 and Th2 cells (and the associated cytokines needed to drive differentiation and activation) in the development and progression of MG but will not be further discussed in this paper.

T cells and Self-Tolerance

There is a highly specific and precise method which ensures the TCR is functional prior to releasing the naïve T cell into the periphery. There are also mechanisms which eliminate naïve T cells in the thymus that contain a TCR that binds strongly to self-antigens [11]. In addition, there are many self-antigens in the periphery that are not seen during T cell development in the thymus so the T cells that bind strongly to these self-antigens need to be eliminated or inactivated after release from the thymus [11]. Although not fully known, it is hypothesized that failure of the thymus to eliminate T cells that bind strongly to self-antigens could lead to MG. Further, it is speculated that MG occurs later in life due to thymic hyperplasia or a thymus tumor, allowing T cells that strongly bind self-antigens to escape into the periphery through a mechanism not known [17]. In addition, the mechanisms in place to eliminate these naïve and/or activated T cells that bind strongly to self-antigens from the periphery may also fail, leading to MG in the absence of thymus involvement. Both the involvement of the thymus and failure to establish tolerance in the periphery will be discussed to fully understand potential causes of how MG arises and progresses.

The progenitor cells that will eventually give rise to T cells migrate from the bone marrow to the thymus for development into CD8+ and CD4+ naïve T cells that can enter the periphery and recognize antigen processed on MHC class I and MHC class II molecules, respectively. Initially, these progenitor cells do not contain the co-receptors CD4 or CD8 and are referred to as double-negative thymocytes [55]. Through a very complex process, gene rearrangement occurs and if successful, a functional T-cell receptor (TCR) is formed that has

both CD4 and CD8 co-receptors, known as double positive thymocytes. Cells that fail to form a functional TCR will die by apoptosis and are phagocytosed by thymic macrophages [55]. Double positive thymocytes have the advantage of having a TCR that can recognize antigen processed and presented on either MHC class I or MHC class II molecules. Which MHC class molecule the TCR binds to, and the affinity to which it binds, determines the T cell fate. If the TCR binds very weak or very strong to MHC+antigen complex, deletion of the T cell occurs [55]. If the TCR binds moderately to MHC class I+antigen complex, then differentiation into CD8+ T cells is favored. If the TCR binds moderately to MHC class II + antigen complex, then differentiation into CD4+ T cells is favored.

Once differentiated into a CD4+ or CD8+ T cell (but prior to release into the periphery), thymic selection occurs to ensure that the T cells do not attack self-antigens [55]. At this stage, a T cell with a functional TCR is formed but antigenic specificity has not been selected for yet. This next phase of T cell development involves eliminating TCRs that recognize self-antigens while favoring the T cells that recognize foreign antigens. Positive selection favors T cells that recognize antigens processed on self MHC molecules while negative selection eliminates T cells that are potentially auto-reactive [55].

Thymic cortical epithelium express both MHC class I and MHC class II molecules and therefore serve as the cells that "present" antigen+MHC complexes to double positive thymocytes undergoing positive selection [55]. If the antigen-MHC complex binds to a TCR, the cell is selected for further maturation; all double positive thymocytes that do not bind within a few days are eliminated by apoptosis. The cells that bound to antigen+MHC complexes are now "MHC restricted", meaning that the TCR can only recognize and bind to the particular antigen+MHC complex that it initially bound to. Negative selection occurs next and involves deletion of all cells that bind too strongly to self-antigens. T cells that have a TCR that bind too strongly to self-MHC class I molecules presented on APCs (and other

cells) in the thymus are deleted, while binding moderately is a signal for survival [55]. T cells that received the signal for survival are able to leave the thymus, enter the periphery and begin searching for foreign invaders.

Despite the complex and intricate mechanisms in place to eliminate T cells that recognize self-MHC+antigen complexes, the mechanism is not fail-proof and some auto-reactive T cells still escape into the periphery [22]. However, additional mechanisms are in place in the periphery to delete and/or inactivate these auto-reactive T cells. For example, when T cells react strongly with self-antigens in the periphery, tolerance can be established through mechanisms such as anergy (a state of "unresponsiveness"), deletion via apoptosis or suppression via T regulatory cells [22]. Under normal circumstances, T cells in the periphery are able to distinguish self-antigens from pathogenic antigens. In order to become fully activated, the MHC+antigen and co-stimulatory molecules must be present on the APC; in the absence of co-stimulation, the T cell will become anergic.

If tolerance is not established, self-reactive T cells will clonally expand and continue to attack the self-antigen which leads to autoimmunity [11]. A few molecules have been defined as important for the development of autoimmunity, specifically as it relates to T cell activation and/or deletion. For example, death receptor Fas (CD95) is important for inducing apoptosis (self-programmed cell death) [49]. It is speculated that the dysregulation of this protein, specifically as it is involved with T cell deletion, may contribute to the development of autoimmune disease [49]. In addition, some studies show that lack of the tyrosine kinase receptors (Try, Axl and Mer) can lead to autoimmunity [50]. These receptors are important in negatively regulating APCs. In mouse models lacking these receptors, APCs are not appropriately regulated and lead to over-activation resulting in autoimmunity [50]. Additional potential causes of MG, such as molecular mimicry, defects in the regulatory T cell pathways

and other potential breaches involved in maintaining self-tolerance will be further discussed in the Synthesis and Future Work section.

B cells and Plasma cells

At any given time, there are millions of different B cells circulating in each person, each recognizing a specific antigen. Once B cells are activated, they differentiate into plasma cells producing antibodies specific for the antigen that initially caused the B cell activation [7]. The B cell receptor that recognizes the antigen is called an immunoglobulin and is composed of two different polypeptides, called the heavy-chain and the light-chain. Under most circumstances, the Y-shaped immunoglobulin molecule contains two identical heavy-chains and two identical light-chains [7] (refer to Figure 3). Each immunoglobulin has a constant region which shares sequence similarity with other immunoglobulins and a variable region which differs in amino acid sequence between each B cell clone. The variable region of the immunoglobulin is the portion that specifically binds to the antigen [7]. When immunoglobulins are attached to the surface of the B cell, they are anchored in the membrane by two transmembrane regions. Secreted immunoglobulins, called antibodies, lack the transmembrane region but are otherwise identical to surface-bound immunoglobulins [7]. Similar to the T-cell receptor, the great diversity in immunoglobulins are due to gene rearrangement. The mechanism for generating diversity in immunoglobulins is very similar to that described above for T cells. With respect to self-tolerance of B cells, there is clonal deletion that can occur if developing B cells in the bone marrow react strongly to environmental (self) antigens [3]. Overall, however, tolerance in the B cell compartment is not as stringent since B cells generally require T cell help to become antibody-secreting cells [3].

Effector Mechanisms of Tissue Damage in MG

The binding of anti-ACh receptor antibodies (IgG1 and IgG3 subtypes mainly) results in strong activation of the complement system [10]. The complement system consists of over 30 serum proteins and cell surface receptors that have a variety of functions, including initiation of an inflammatory response, direct cell lysis, and activation of T and B cells [13]. The complement system is activated by three different pathways: classical, lectin and alternative. The classical pathway is the only pathway that will be discussed in this paper since it is the pathway activated in MG patients. The classical pathway is driven by the activation of the C1-complex after the production of antibodies in the B cell response [13].

As a result of antibody production against ACh receptors, a high density of IgG antibodies bind to the folds in the postsynaptic membrane, resulting in an area of tightly packed Fc regions (regions of the antibody that can bind to Fc receptors on the cell surface of some immune cells). Multiple IgG antibodies are needed to activate the classical pathway via binding of C1q [cite]. After the initial binding of Cq1 to the IgG antibodies, there is a series of conformational changes, proteolytic reactions and cleavage events in the other proteins involved in the complement system that leads to opsonization, chemotaxis and other processes that ultimately result in destruction of the postsynaptic membrane of the muscle fiber [23].

Activation of the complement system also leads to the formation of the membrane attack complex (MAC) which causes severe endplate destruction [10]. MAC is composed of C5b – 9 complement components that form a transmembrane channel within the plasma membrane [52]. The formation of this transmembrane protein can disrupt the cell membrane, resulting in lysis and death. In patients with MG, MAC promotes the degradation of the endplate by leading to destruction of the postsynaptic folding, loss of membrane potential, and loss of ACh receptor-associated proteins [10]. Additionally, the degradation at the endplate is

amplified because of the bivalent nature of the bound antibodies, causing ACh receptor degradation and endocytosis, as already discussed above. Interestingly, increasing the expression and synthesis of rapsyn results in stabilization of the ACh receptor and prevents ACh receptor internalization and degradation that occurs when bivalent antibodies bind, despite an active complement system [10]. Without activation of the complement system, it is speculated that the severe damage to the muscle endplate would not occur. Without the severe damage leading to ACh receptor destruction, it is also speculated that MG symptoms and progression may be reduced. Targeting the complement system in drug therapy to reduce MG symptoms and disease progression is discussed further in the Synthesis and Future Work section.

Development of Autoimmune Diseases

Hypersensitivity Disease Classifications

Autoimmune diseases result when the ability of "self-tolerance" is lost and self-antigens are attacked by the body's own immune system. Autoimmune diseases can be categorized within hypersensitivity pathways; these reactions involve an excessive immune response to foreign or self-antigens. There are four types of hypersensitivity reactions that can lead to pathology. When these reactions are directed against self-antigens, the result is an autoimmune disease. These four hypersensitivity types are:

1. Immediate hypersensitivity (*Type I*)

   This pathway involves mast cells binding to IgE antibodies using their IgE-specific Fc receptors. When the IgE encounters and binds to its antigen it becomes cross-linked which triggers mast cell degranulation and release of inflammatory molecules such as histamine. Diseases such as hay fever and allergic asthma fall under this hypersensitivity classification [5].

2. Antibody-mediated (*Type II)*

This pathway involves antibodies targeting and binding to antigens found on an individual's own cells and in some cases leads to: Natural Killer (NK) cell involvement, lysis of the cell *via* complement activation or receptor modulation. Additionally, there can be local production of anaphylotoxin (complement fragments such as C3a or C5a which cause reactions such as smooth muscle contraction and vascular permeability) and tissue injury following the release of hydrolytic neutrophil enzymes after autolysis [5]. MG follows this hypersensitivity pathway and will be discussed in further detail below.

3. Immune Complex (*Type III*)

   This pathway involves antigen/antibody complexes forming in the blood with subsequent deposition in the tissue, leading to complement and neutrophil activation which causes local damage and impaired function [5].

4. T-cell mediated (*Type IV*)

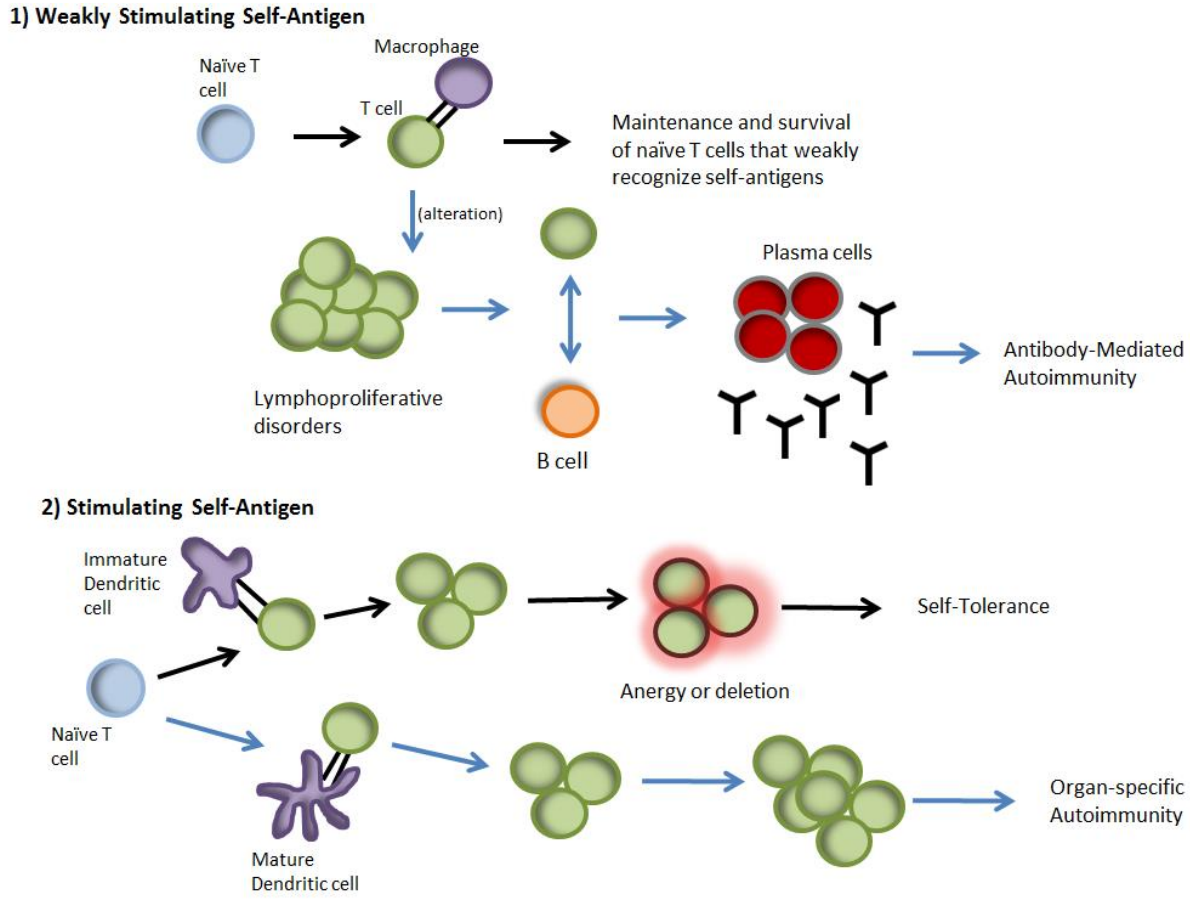
   This pathway induces an inflammatory response and activates macrophages as a result of T cell activation and cytokine release. Diseases such as insulitis fall within this classification of hypersensitivity reactions [5].

MG follows the antibody-mediated (Type II) pathway, as a result of antibody production specifically targeted at self-antigens. The mechanisms that generate and maintain self-tolerance in both T and B cells are manifold and complex, and failure of these mechanisms is necessary for autoimmunity to develop. To initiate a strong adaptive immune response, there are tightly controlled interactions between B cells, T cells and APCs. If these interactions are disrupted, autoimmunity may develop.

Potential Causes of Autoimmune Diseases

The development of an autoimmune disease is a very complex process and often involves alterations at multiple checkpoints that ultimately lead to disease [11]. Many factors are

thought to contribute to breaking self-tolerance which may result in the development of a number of different autoimmune diseases (see Figure 5). Some of these factors include genetic susceptibility, external environmental factors such as infections, and alterations in T cell signaling pathways [11]. Additionally, activities involved in cell cycle progression, glycosylation and the function of antigen-presenting cells (APCs) are also thought to be linked to the development of autoimmune diseases. The direct mechanism that causes MG is unknown but both molecular mimicry and pathology of the thymus are speculated to contribute to disease.



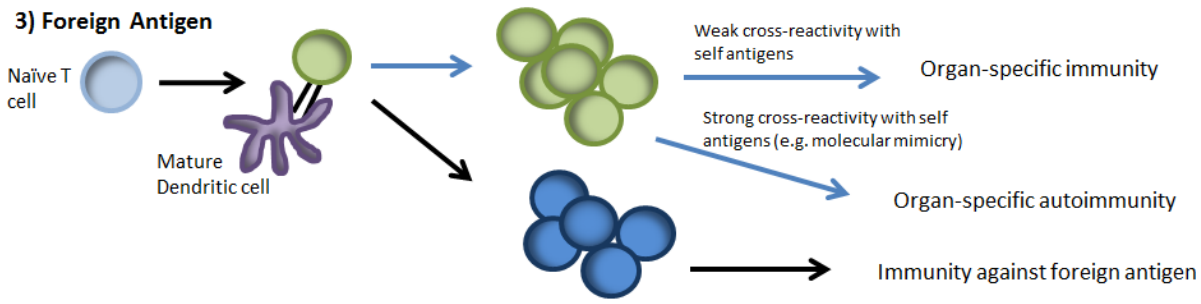


Figure 5: Possible Mechanisms of Autoimmunity

Black arrows represent the normal events that are involved during a T cell lifetime; blue arrows represent the abnormal events that occur when disruption leads to autoimmunity. **a.** Weak interaction of naïve T cells with self-peptide-MHC complexes results in the maintenance and survival of naïve T cells (black arrows). An alteration in T cell survival may cause lymphoproliferative disorders which can lead to autoimmune diseases when B cells are activated and begin secreting antibodies to self-antigens (red arrows). **b**. A naïve T cell may interact strongly with a self-peptide-MHC complex that leads to anergy or apoptosis of the activated T cell (black arrows). Disruption in the process of anergy or apoptosis may lead to T cell activation against self-antigens. Additionally, T cell interaction with inappropriate mature dendritic cells may result in activation of T cells specific for self-antigens (blue arrows). **c**. Pathogen invasion will result in activation of many different clones of T cells. Some of these clones may mistake self-antigens and foreign antigens (molecular mimicry) and this can lead to autoimmunity (blue arrows). Most activated T cells will only recognize the foreign antigen and lead to elimination and immunity against the foreign pathogen (black arrows). [7]

Many different environmental factors have been observed to break self-tolerance and lead to autoimmune diseases. For example, molecular mimicry occurs when self-antigens are mistakenly recognized by the adaptive immune system as foreign antigens due to their molecular similarity (Figure 6). The foreign antigen could be a virus, bacterium or other particle that the host mounts an immune response against. For example, a virus may infect a host and one or more of the virus antigens may share similar sequence homology with self-antigen(s) within the host. The host would mount an immune response against the pathogen and produce antibodies specific for that foreign antigen; however, these antibodies would also attack the host antigen and cause autoimmunity.

Molecular mimicry does not always lead to disease and in some cases it may induce tolerance [24]. Studies have shown that serum taken from MG patients can recognize virus and/or bacterial derived peptides, indicating that microbial antigens may in fact play in role in

the initiation of MG [25]. One report showed that MG can be induced in mice using a non-mouse derived molecule other than ACh receptor [25]. Here, bacterial antigens were used that share sequence and structural similarity to ACh receptor, providing compelling evidence that MG onset can be a direct result of molecular mimicry. In contrast, a different study attempted to show a correlation between infection from a particular bacterium and the potential protection of MG development and progression [24]. This study searched for microbial sequences that share similarities with T and B cell epitopes within the α-subunit of the ACh receptor [24]. A peptide from *Haemophilus influenza* showed 50% sequence homology to a T-cell epitope of the ACh receptor [24]. Test rats were injected with this peptide purified from *H. influenza* and weeks later were injected with ACh receptor purified from *Torpedo californica* in order to experimentally induce MG. A series of immunological assays were performed to analyze the results and they found that protection against MG symptoms was achieved in the rats that had been injected with the *H. influenza* peptide, in comparison to both the control group and the group injected with peptides that did not share sequence homology. The protection was associated with a down-regulation in ACh receptor antibody and decreased T-cell activation and proliferation. Determining if an immune response is pathogenic or induces tolerance depends on many factors, including the affinity of mimicry peptides for MHC molecules, the abundance and density within the host, the degree of cross-reactivity with the self-antigen, and the types of cytokines released [24].

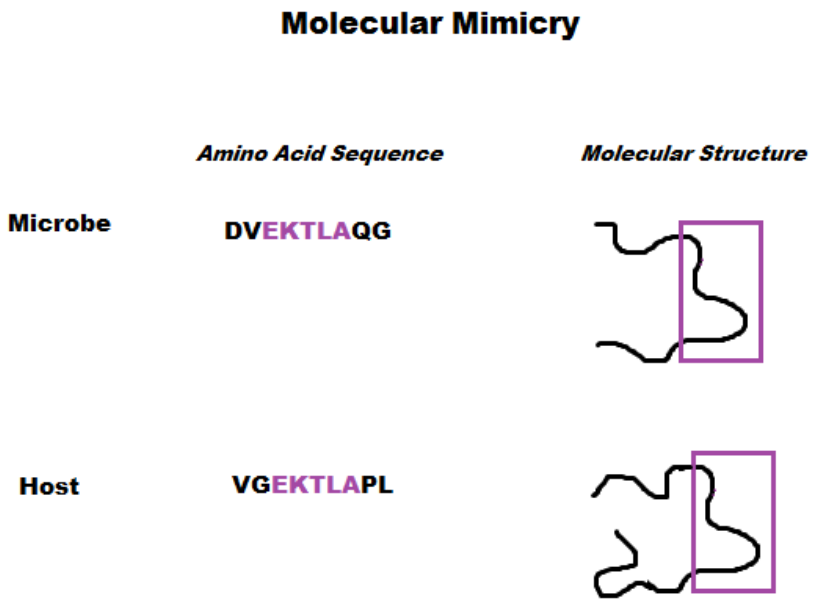


Figure 6: Molecular Mimicry
Cartoon schematic representing the similar amino acid sequence shared between a host and microbe protein. Autoimmunity may occur if the host immune system mounts a response to the microbe which then cross-reacts with self-proteins.

Thymus

The thymus is a primary lymphoid organ that is the site of T cell development [53]. The thymus is located in a region known as the anterior superior mediastinum which is behind the sternum and in front of the heart. The thymus is largest and most active into pre-adolescence and then begins to atrophy and decrease in activity. However, the thymus does remain functional throughout adult life, where T lymphopoiesis continues to occur [53]. Many studies over the years have been aimed at showing a direct correlation between the failure of the thymus to establish self-tolerance and the development of autoimmune diseases, specifically MG.

CD4+CD25+ regulatory T cells, derived primarily in the thymus, are critical in maintaining self-tolerance and if disrupted, can lead to autoimmunity [12]. These cells are a subset of CD4+ T cells that also constitutively express CD25, an interleukin 2 receptor

molecule [12]. MG is a CD4 + T cell-dependent autoimmune disease that is speculated to arise in the thymus in some patients [12]. One study examined the role of T regulatory cells in MG, specifically looking at CD4+CD25+ T cells. Overall, they found that there are a normal number of CD4+CD25+ T cells but that there is a defect in their function in regulatory activity. They also discovered that there is a decreased expression of Foxp3, a transcription factor critical for the function of T cell regulation [12]. Interestingly, a phenotypic analysis was performed on CD4+CD25+ T cells in MG patients and revealed an inability of these T cells to suppress the proliferation of responding T cells, indicating that self-tolerance in the thymus is lost due to the reduced function of CD4+CD25+ T cells [12].

Foxp3 (forkhead transcription factor) is critical for the development and functionality of CD25+ T cells in humans [12]. Specifically, Foxp3 is responsible for converting naïve $CD4^{+}CD25^{-}$ T cells into CD25+ T regulatory cells which suppress self-reactive T cells. Patients with MG were observed to have a decreased expression of Foxp3, resulting in impaired suppression of self-reactive T cells in some patients. Therefore, the dysregulation in Foxp3 provides a plausible explanation for how MG arises in the thymus as a result of non-functioning T regulatory cells [12].

The studies showing the role of both Foxp3 and CD4+CD25+ T cells in the initiation of MG are very insightful and potentially provide promising drug and/or treatment targets for MG patients. Attempting to re-establish normal levels of Foxp3 and regaining normal function of CD25+ T regulatory cells as a potential treatment option for MG patients will be discussed in the Synthesis and Future Work section.

Synthesis and Future Work

Current Treatment

There have been many advances in the treatment for MG over the last century, significantly decreasing the high mortality rate once associated with this disease [27]. During the early 1900's, respiratory failure was the primary cause of death in approximately 70% of patients diagnosed with MG. In 1965, multiple drug therapies were available (such as cholinesterase inhibitor) which drove the mortality rate down to approximately 15%. More recently, mortality rates have dropped even lower (approximately 5%) due to improved therapy that includes immunosuppressive drugs. Current MG treatments include [27]:

- Cholinesterase Inhibitors (inhibits the degradation of ACh at the neuromuscular junction)
- Corticosteroids (immunosuppressant)
- Immunosuppressive Therapy with agents such as Azathioprine and Cyclisporine
- Thymectomy
- Intravenous Immunoglobulin and Plasmapheresis

These current treatment options have shown success; however, there is room for much optimization to further improve the adverse effects still observed. In addition, there is currently no known cure for MG but multiple studies are currently examining novel treatment options in hopes to develop better treatments or even a cure for MG [27].

Future Research

Both the neuromuscular junction and the adaptive immune system pathways are critically involved in the onset, maintenance, and progression of MG. Having explored these pathways at the molecular level throughout this paper, we can now explore how these pathways may potentially be altered to lessen the disease symptoms of MG and provide grounds for future research. As described above, there are two main mechanisms that contribute to the loss of functional ACh receptors in patients with MG. First, the binding of antibodies to the ACh

receptor activates complement-mediated lysis of the muscle fiber endplate, resulting in a damaged postsynaptic membrane. This damage impairs the normal folding of the membrane and results in both functional impairment and a decrease in the number of voltage-gated Na^+ channels which decreases the ability of the muscle fiber to be depolarized [8]. Second, antibody binding and cross-linkage to ACh receptors leads to internalization and degradation of the receptors. This dramatically reduces the number of ACh receptors available for ACh binding, resulting in severe impairment in nerve-muscle communication [8]. Both of these mechanisms of pathogenesis provide multiple potential molecular targets for therapy.

Mouse Models in MG

Many of the studies that examine the progression of MG disease and potential treatment options use experimental mice models. MG can be induced in mice by immunizing with Torpedo californica acetylcholine receptors in complete Freund's adjuvant [54]. There is a dominant epitope within the ACh receptor α146-162 region which activates CD4+ T cells, ultimately leading to the production of anti-ACh receptor antibodies by B cells [54]. Experimental studies in mice provide a solid framework around designing pre-clinical studies that can be used to treat MG in human patients. The following sections propose continued use of the mouse model with subsequent clinical applications to human MG patients.

Complement System Inhibition

Multiple studies have shown evidence that the activation of the complement system is the main culprit that drives degradation and loss of ACh receptors at the neuromuscular junction [56]. Some of these studies included using antibodies to block critical components of the complement system, using inhibitors of complement components, and introducing genetic defects of complement components [58, 59]. Each of these studies were done in animals and showed that when the complement system is effected and/or inhibited, resistance and/or less susceptibility in developing MG was achieved [58, 59]. Interestingly, mice that are deficient

in IL-12 also develop minimal to no MG symptoms after being immunized with ACh receptor proteins [57]. In mice, IL-12 is critical for the production of IgG2a antibodies which are the antibodies that activate the complement system. The mice are left with only IgG1 antibodies, which are unable to activate the complement system (in contrast to humans). The lack of MG symptoms in these IL-12 deficient mice provides evidence to support that complement-activating antibodies are required for the symptoms of MG [57].

Prior to determining which players should be targeted for potential MG treatment options, it is important to understand any additional roles they have in other pathways. Inhibition of specific cytokines, such as IL-12 for example, may not be feasible drug or therapeutic targets due to the complicated and diverse function that many of them play in multiple processes. For example, IL-12 is critical for the production of IgG2a antibodies, but also plays a key role in promoting the differentiation of naïve T cells into T helper 1 cells that are involved in cell-mediated immunity [16]. Inhibition of cytokines involved in the adaptive immune response would likely result in failure to establish strong immunity to other infections and/or pathogens and cause detrimental side effects. As a result, other key players in the complement pathway will have to be targeted to provide feasible experiments with potential treatment outcomes.

Proposed Therapies for Complement-Inhibition

Since MG follows the classical pathway of the complement system, specific serum proteins involved in initiating that pathway could be inhibited to possibly lessen the symptoms of MG. For example, C1q is the serum protein involved in direct binding to the antibody-antigen complex, initiating the activation of C1r and C1s, which leads to activation of the rest of the complement cascade (and ultimately phagocytosis and activation of the membrane attack complex for cell lysis) [20]. Using a C1 inhibitor such as C1QBP (complement component 1 Q subcomponent-binding protein) will inhibit the activation of C1s and C1r. Therefore,

successfully inhibiting C1q formation will ultimately inhibit the damaging effects caused by the complement system. C1 inhibitor is normally found in the blood and has a role in inhibiting spontaneous activation of the complement system. In addition, C1 inhibitor has a role in controlling the kinin-kallikrein system which is involved in inflammation and coagulation. When there is a deficiency in C1 inhibitor, angioedema (swelling of the subcutaneous and mucous tissues) develops [14].

Under normal circumstances, C1 inhibitor is present in the body at an adequate concentration to inhibit the spontaneous activation of the classical complement system pathway. In patients who have a C1 inhibitor deficiency in addition to MG, it is hypothesized that MG symptoms would worsen. Since spontaneous activation of the complement system is no longer regulated properly, it is thought that deposition of the complement system proteins on tissue already potentially damaged (the muscle endplate) would increase inflammation and recruitment of macrophages, neutrophils and other inflammatory cells that would begin phagocytizing the complement-antigen complexes.

In MG patients who have normal levels of C1 inhibitor, it is hypothesized that significantly increasing the concentration of C1 inhibitor will decrease the antibody response against ACh receptors, leading to an overall decrease in ACh receptor endocytosis and subsequent degradation. This would ultimately lead to an overall reduction in localized inflammation and damage occurring at the muscle endplate. The expected outcome is that inhibiting C1q (and therefore inhibiting the classical complement pathway) will lessen the B cell response and overall reduce the amount of antibodies generated against ACh receptors. B cells have specific complement receptors, such as CR2, on their cell surface that upon binding to complement proteins will drive B cell activation and maturation. Since the complement system appears to play a strong role in B cell maturation, it is hypothesized that inhibiting the complement system would decrease the overall B cell response and result in

less antibody production. Other signals, including CD4+ T cell signaling and cytokines also drive B cell activation and maturation so antibodies would still be produced. However, since the complement system plays a large role in amplifying the inflammatory signal, it would be interesting to investigate whether the B cell response is decreased when complement is inhibited in patients with MG.

When inflammation is decreased, structural integrity at the muscle endplate may begin to be re-established, promoting the insertion of new ACh receptors in the membrane. Circulating antibodies are still available to bind to these ACh receptors; however, it is hypothesized that the plasma cells and memory cells will not produce as many antibodies, due to the reasons previously explained above, therefore decreasing the number of ACh receptors that are bound to anti-ACh receptor antibodies. In addition, IgG antibodies have an average half-life of 20 days, meaning that after just a few months some of these antibodies will have degraded. It is speculated that over time, less anti-ACh receptor antibodies will be circulating in the bloodstream due to the effects of inhibiting the classical complement pathway. If this occurs, there could be an overall decrease in ACh receptor endocytosis and degradation. As previously mentioned, ACh receptors must be in close enough proximity to allow crosslinking by antibodies. As a result of inflammation subsiding and an overall decrease in antibody production, it is hypothesized that the placement of some ACh receptors will not permit crosslinking of antibodies, therefore the ACh receptors will not be internalized. However, antibodies bound to the ACh receptors, regardless of whether they promote internalization and degradation of the receptor likely still impairs functionality of the ACh receptor. For this reason, inhibiting the complement system is just one part of re-gaining the communication between the motor neuron and skeletal muscle fiber in patients with MG.

Many studies have shown the serious effects of a deficiency in C1 inhibitor [14], but studies were not found that attempted to decrease MG onset, progression and/or symptoms by

increasing C1 inhibitor concentration. There have been studies that explored the effects of administering C1 inhibitor in patients with sepsis [15]. These patients were observed to have a reduction in complement system activation in addition to a reduction is neutrophil response which led to beneficial treatment effects [15]. This provides additional evidence to support that inhibition of the classical complement system pathway may provide a way to lessen MG symptoms that are caused by the association of complement fragments to bound IgG2a antibodies that ultimately leads to destruction of the muscle fiber endplate. Bound IgG2a antibodies may still impair muscle-nerve communication; however, the damage caused to the muscle fiber endplate would be reduced. This potential treatment option provides a means to maintain muscle endplate integrity, increasing the chances that acetylcholine will be able to bind to surface-bound ACh receptors.

Limitations of Complement Inhibition

There are risks involved with inhibiting the classical pathway of the complement system. The complement system is designed to enhance the innate and adaptive immune response by "complementing" the effects of antibodies and phagocytic cells to eliminate pathogens from the host. The classical pathway is driven by the binding of antibodies to antigen while the other two pathways (lectin and alternative pathways) are antibody-independent. When the classical pathway is inhibited, the response of phagocytic cells and antibodies may decrease; however, it is expected that different mechanisms would be used to compensate. For example, during invasion of a pathogen, phagocytic cells are releasing cytokines that will attract more phagocytic cells and members of the adaptive immune response. In addition, different subtypes of antibodies will bind to antigens on the pathogen and lead to degradation or phagocytosis. The lectin and classical pathways may also be activated and drive the elimination of the pathogen. Each of these pathways is independent of one another and will contribute to pathogen elimination in the absence of the classical pathway.

As is the case in most treatment regimes, the goal is that the positive effects outweigh the negative side effects. A number of studies have examined inhibition of the classical pathway of the complement system [20, 21], specifically to mitigate various immune diseases such as xenograft rejection [21]. One study examined over 42 different peptides with C1q binding sites to determine which peptide would inhibit the classical pathway via inhibition of C1q without affecting the lectin or alternative pathways [21]. They discovered one peptide (2J) that successfully inhibited the early steps of the classical pathway and they suggest this peptide as a promising option to mitigate the negative effects of the classical pathway in some immune diseases [21]. This provides further evidence to support designing an experimental study that specifically examines the effects of inhibiting the classical pathway in patients with MG. This paper proposes using the 2J peptide in MG mouse models to observe if there is an increase in ACh receptors over time and whether the muscle endplate damage is reduced, when compared to control mouse models. It may be that careful dosing of complement inhibitors can achieve a balance wherein MG symptoms are reduced, but some classical complement pathway activity is maintained.

Genetic Modifications

The use of current genetic modification strategies, such as the CRISPR/Cas9 system and microRNA, provide a solid framework to design studies for MG treatment. MicroRNAs are small noncoding regions of RNA that regulate gene expression at the post-transcriptional level. Mechanistically, microRNA works by binding to regions of messenger RNA prior to translation, which inhibits expression of those genes and therefore inhibits the synthesis of specific proteins encoded by those genes [19]. MicroRNA is expressed in normal cells; however, overexpression in cancerous or diseased cells has been shown to contribute to an ability to proliferate and resist apoptosis, potentially leading to immortal cells [19]. The

discovery of microRNA and its implication in disease has led to numerous innovative experimental proposals.

Similarly, the discovery of the CRISPR/Cas9 system has also led to the ability to modify specific genes in the genome, allowing the ability to remove harmful genes and/or add genes that may benefit the host. The CRISPR/Cas9 system is used in bacteria to confer resistance to foreign genetic elements, such as plasmids and/or genetic material from viruses. CRISPRS are DNA with short repetitions of DNA that were acquired from exogenous sources and inserted into the bacterial genome as spacers. These spacers recognize incoming foreign genetic material, similar to the RNAi (RNA interference) pathway in human cells. Incoming foreign DNA can be transcribed into spacers and long palindromic DNA into a long RNA molecule. Cas9 enzyme can then cut this long RNA molecule into crRNA (crispr RNA). crRNA can associate with interference complexes in order to degrade foreign genetic material. This system that has been identified in bacteria has now been used in genetic engineering to fix mutations in the genome, and potentially "cure" diseases that were once thought to be incurable. The next section of this paper proposes to incorporate genetic engineering models that could be applied to MG.

Proposed Therapies for Genetic Modifications

After discovering the correlation between microRNA and the development of cancer and/or diseases, many studies have devoted resources to discovering correlations between specific microRNAs and the regulation of tumor and/or disease-causing proteins. As already discussed, MG patients have been shown to have a decreased expression of Foxp3. Additional studies have shown a correlation between microRNA and Foxp3 expression in MG patients [17]. Specifically, MiR-125a-5p expression is increased in MG patients who have a thymoma, which is thought to contribute to the disease. A significant increase (6.72-fold) in MiR-125a-5p expression was seen after performing microarray and polymerase chain

reactions in thymic samples from MG patients and control samples [17]. Further, experimental evidence indicated that Foxp3 is a direct target of miRNA-125a-5p. Foxp3 was down-regulated in cells that were transfected with miRNA-125a-5p. When miRNA-125a-5p was inhibited, Foxp3 expression increased. Evidence supports that miRNA-125a-5p targets and degrades mRNA for Foxp3 [17].

As discussed above, Foxp3 is responsible for converting naïve $CD4^{+}CD25^{-}$ T cells into CD25+ T regulatory cells which suppress self-reactive T cells and B cells. Therefore, Foxp3 could be a link between thymus pathology and the development of MG and inhibition of MiR-125a-5p to re-establish normal expression of Foxp3 could be therapeutic. Specifically, the short-term and long-term effects of both downregulating and inhibiting MiR-125a-5p should be explored. MicroRNA complementary to MiR-125a-5p could be transfected in MG mice models to examine the effects of down-regulation while the CRISPR/Cas9 system could be used to examine the effects of complete MiR-125a-5p gene knockout. In addition, the correlation between disease state and altered MiR-125a-5p expression needs to be determined. For example, does altered MiR-125a-5p expression contribute to MG development? Or does altered MiR-125a-5p expression after disease onset contribute to disease progression? This will help answer the question "will re-establishing normal expression of Foxp3 help reverse the symptoms of MG?"

The initial hypothesis is that re-establishing normal Foxp3 expression will mainly be beneficial prior to disease development; therefore, this would not provide a promising treatment option for patients already diagnosed with generalized MG but may provide a way to inhibit the development of congenital MG. Once T-regulatory cells fail to suppress self-reactive T cells, the disease is initiated and symptoms are subsequently amplified by other players of the immune system, such as the complement system. However, re-establishing normal Foxp3 expression in patients who already have severe destruction to the muscle

endplate may still benefit if a cocktail treatment is given. In MG disease that is well-progressed, re-establishing normal Foxp3 expression may lead to suppression of additional self-reactive T cells, resulting in a decrease in activated self-reactive B cells and an overall decrease in ACh receptor antibody production. Re-establishing the Foxp3 expression may pose a more beneficial treatment option than removing the entire thymus since T cell maturity would no longer occur if the thymus was removed. Converting naïve $CD4^{+}CD25^{-}$ T cells into CD25+ T cells (which then suppress self-reactive T cells) is the critical step in this potential treatment plan and requires the thymus to remain intact. In addition, this treatment would be given in conjunction with suppressing the classical pathway of the complement system (as discussed above), effectively inhibiting further damage to the muscle endplate.

For patients who have a thymoma (or thymic hyperplasia), it is hypothesized that MG development occurs as a result of increased cell proliferation in the thymus which causes a loss of structural and functional integrity, specifically in the cortex and medulla where loss of structure is directly associated with loss of function. This allows developing and potentially auto-reactive T cells to escape into the periphery without passing the multiple checkpoints in place that would have deleted the auto-reactive T cells. This hypothesis leads into a very important question, which is "why is a thymoma associated with MG development and not in other autoimmune diseases such as multiple sclerosis"? Without knowing the answer to this question, it is speculated that T regulatory cells are a key player in MG development, whereas the T regulatory function in the development of other autoimmune diseases may be minimal. In patients who have a thymoma, T regulatory cells are impaired due to the decreased FoxP3 expression explained above. It is this altered expression, in combination with thymic abnormalities, which is thought to ultimately lead to the onset of disease in some patients. Auto-reactive T cells that escape into the periphery are normally suppressed by T regulatory cells, but because there is a genetic dysregulation in FoxP3 expression that impairs T

regulatory functionality, these auto-reactive T cells are able to begin attacking self-antigens. This is in contrast to other autoimmune diseases such as multiple sclerosis which is speculated to be caused by a combination of genetic, environmental and possibly infectious agents.

To determine the role that altered FoxP3 expression may have in other autoimmune diseases, designing an experiment using FoxP3 knockout mice and subsequent analysis of cancer and/or autoimmune disease development may reveal insight into the significance of T regulatory cells in disease development. It is hypothesized that normal FoxP3 expression is critical in keeping numerous autoimmune diseases from developing, not just MG. If this is the case, FoxP3 knockout mice would develop a wide range of diseases, both cancerous and autoimmune-related, due to the impaired function of T regulatory cells. This may provide additional insight into the role and potential therapeutic options that T regulatory cells provide in the onset and progression of numerous autoimmune diseases.

By leveraging the data gathered from the previous hypothesis, it would be interesting to explore whether re-gaining T regulatory function after MG disease development would suppress the severity and/or symptoms of the disease. Based on our current knowledge that T regulatory cells can suppress auto-reactive T cells, and that auto-reactive T cells are required to initiate and maintain a strong antibody response, it is hypothesized that re-establishing T regulatory cell functionality by increasing FoxP3 expression (from cells obtained from MG thymoma patients) of these T regulatory cells will drive the suppression of auto-reactive T cells. It is believed that even if T cells do not express FoxP3 during the developmental and/or naïve stage, they still have the ability to express FoxP3 later and exhibit the same suppressive properties as mature T regulatory cells. Ex vivo application of this theory could potentially be applied in vivo if these T regulatory cells could be injected back into the patient in order to suppress auto-reactive T cells.

T regulatory cells were once thought to have little or no role in the development of autoimmune diseases. More recently, research on T regulatory cells has increased substantially, and we have now learned that T regulatory cells are very involved in disease onset and progression. Future research around T regulatory cells will continue to grow and the new findings may be applied to numerous autoimmune diseases, not just MG.

Limitations of Genetic Modifications

Genetic modifications, such as microRNA and CRISPR, have their own unique limitations. First, the success rate of knocking out the target gene (with no impact to other genes) is often quite low. This can be due to many factors, including the state and conformation of the chromatin and the location of the gene to the start of transcription. In addition, non-specific binding of the spacer sequence can occur. The spacer sequence is only approximately 14 nucleotides long and may bind to regions of the DNA other than the target gene. For microRNA, complete gene knockout is not the goal but rather decreased expression of a protein is the goal. For some proteins, decreased expression may still result in enough functional protein produced that the desired effect is not achieved. MicroRNA is also highly unstable so the cost to ensure the microRNA does not degrade prior to introducing it into the patient is high, with risk that the efficiency is low. Additional studies would need to be performed to gather data regarding the efficiency and feasibility of genetic modifications as a cure to MG.

Neuromuscular Junction-Associated Proteins

Proteins located at the neuromuscular junction have been found to aid in the progression of MG and offer potential research and treatment opportunities. Multiple proteins involved in clustering ACh receptors on the muscle fiber have been identified such as MuSK, rapsyn and Dok-7. MuSK is required to form ACh receptor clusters and antibodies targeted at MuSK alter the ACh receptor clustering through mechanisms not clearly known. In patients who are

anti-MuSK antibody-negative, increased MuSK expression (and function) may be beneficial since MuSK stabilizes ACh receptors on the cell membrane. However, in patients who are anti-MuSK antibody-positive, increasing expression of MuSK may worsen the disease since it provides additional antigens for the anti-MuSK antibodies to bind. Increasing the number of antibodies binding to MuSK would likely lead to increased ACh receptor internalization and/or degradation.

Similarly, increasing the expression of ACh receptors in patients who are anti-ACh receptor antibody positive could increase the amount of available epitopes that the IgG antibodies could bind. This would likely lead to additional recruiting of the complement system and increase the damage at the endplate since an activated complement system would be activating additional responses that cause damage, such as the membrane attack complex. Overall, increasing expression of ACh receptors may result in a decreased amount of ACh receptors on the cell surface membrane since the damage caused would cause destruction and internalization of the receptors.

Proposed Therapies Involving Neuromuscular Junction-Associated Proteins

Due to the reasons just mentioned above, a different protein would have to be targeted to simultaneously achieve 1) decreased ACh receptor degradation and/or internalization 2) decreased antibody response 3) decreased complement system activation. Of the most feasible options to achieve these goals, rapsyn is seemingly the best target for numerous reasons. Without rapsyn, the junctional folds do not form properly which results in a non-functional synapse. In addition, rapsyn plays a critical role in maintaining an appropriate ACh receptor level and in maintaining the integrity and structure of the endplate. Since previous studies have revealed that an increased expression of rapsyn has shown to increase ACh receptor stabilization, altering the expression of rapsyn may be a feasible option. This could be achieved in numerous ways, including leveraging the genomic modifications explained

above. For example, mRNA that inhibits and/or degrades rapsyn (after production into proteins) should be identified so that microRNA complimentary to this inhibiting mRNA could be synthesized. Degrading the mRNA that ultimately results in inhibition of rapsyn should effectively increase the expression of rapsyn in the cell. Therefore, this will increase the ACh receptor stabilization at the cell surface in patients with MG.

Despite the potential beneficial effects of increasing ACh receptor stabilization in MG patients, increasing the expression of rapsyn may not always prove to be beneficial and it is important to consider the potential negative effects. Increasing cell surface receptors, in some cases, may result in decreased signal. This is due to the binding kinetics and availability of the ligand. For example, there is a limited amount of ACh that is released in the neuromuscular junction and it takes two ACh molcules to bind to a single ACh receptor. If there are now an increased number of ACh receptors on the cell surface (due to increasing rapsyn expression) there may be fewer ACh receptors that get two ACh molecules bound to them. If this occurs, less cations would flow in and potentially decrease the chances of depolarizing the muscle fiber. Further investigation into the normal ratio of ACh molecules: ACh receptors as well as the binding kinetics of ACh binding to ACh receptors should be explored to answer these questions.

It is also hypothesized that increasing rapsyn expression may worsen disease severity if the outcome results in stabilizing more ACh receptors that are in very close proximity to one another. As previously discussed, bound anti-ACh receptor antibodies that are very close to one another promote classical complement activation, further promoting inflammation and damage to the muscle endplate. Complement inhibitor may still have to be given under these circumstances to achieve both decreased complement activation and decreased ACh receptor internalization.

Another hypothesis is that increasing rapsyn expression will result in up to five bound rapsyn molecules to each single ACh receptor which may impair other cell surface signaling pathways. The physical space that would be occupied by these additional rapsyn molecules may sterically hinder adjacent proteins and molecules involved with cell surface signaling pathways. For example, rapsyn may now inhibit the ability of a kinase to autophosphorylate and activate a nearby protein. Additionally, rapsyn binds to f-actin inside the cell which, if too many rapsyn are bound, could impair cell transport and cell motility since f-actin is required in cytoskeletal stabilization, cell migration and transporting material within the cell. To explore this further, it would be interesting to increase rapsyn expression in both normal skeletal muscle and in motile cells such as macrophages. In normal skeletal muscle, it is hypothesized that muscle contraction would occur at undesired times due to the additional ACh receptors at the cell surface being able to bind to more ACh and cause depolarization of the muscle. It is speculated that this would be mostly observed in skeletal muscles that have just one single motor neuron, such as the muscles of the eyelid. Uncontrollable muscle twitching may be observed if the muscles are highly sensitive to depolarization. In cells that are highly motile, it is hypothesized that increasing rapsyn expression may impair the cell's ability to migrate, since the bound rapsyn may impair the cell's ability to polymerize and depolymerize actin during cell migration.

Limitations Involving Neuromuscular Junction-Associated Proteins

As previously mentioned, significant overexpression of rapsyn at the neuromuscular junction in rats with chronic MG has a harmful effect since the endplates are already severely damaged, promoting additional antigens for the anti-ACh receptor antibodies to bind. Further, overexpression of rapsyn increases the postsynaptic membrane turnover that is caused by the ACh receptor antibody binding [40]. Many experimental studies will have to be conducted to achieve the appropriate balance between increasing expression (which stabilizes ACh

receptors) and overexpression (which has harmful effects). If this balance can consistently and reproducibly be achieved, then this potential treatment option may be feasible for MG patients.

There is a lot to be discovered around rapsyn, specifically around the potential beneficial or negative effects of increasing rapsyn expression. Although it seems plausible that increasing rapsyn expression may have negative effects as mentioned above, there may be a correlation between moderately increasing rapsyn expression in MG patients and resistance to ACh receptor internalization.

Conclusion

In conclusion, this paper explored numerous options for potential research that may lead to a more beneficial treatment regime for patients with MG. Despite not knowing the exact underlying cause of MG, a substantial amount of research has already been performed that has shed light on the pathways that are altered in MG disease. The discoveries from these studies can be used to further explore novel treatment options that alleviate the symptoms of this progressive and life-changing disease.

References

1. Conti-Fine, B. M., Milani, M., & Kaminski, H. J. (2006). Myasthenia gravis: past, present, and future. *The Journal of Clinical Investigation. 116*, 2843-2854.

2. Vincent, A. (2002). Unravelling the pathogenesis of myasthenia gravis. *Immunology. 2*, 797-804.

3. Parham, P. (2009). *The Immune System.* New York: Garland Science, Taylor & Francis Group.

4. Kandel, E. R., Schwartz, J. H., Jessell, T. M., Siegelbaum, S. A., & Hudspeth, A. (2013). *Principles of Neural Science.* New York: The McGraw-Hill Companies.

5. Rajan, T. (2003). The Gell–Coombs classification of hypersensitivity reactions: a re-interpretation. *Trends in Immunology. 24*, 376-379.

6. Moiola, L., Galbiati, F., Martino, G., Amadio, S., Brambilla, E., Comi, G., . . . Adorini, L. (1998). IL-12 is involved in the induction of experimental autoimmune myasthenia gravis, an antibody-mediated disease. *European Journal of Immunology. 28*, 2487-2497.

7. Zhang, N., Zhang, X., Song, Y., Lu, X., Chen, D., Xia, X., Sunyer, J., Zhang, Y. (2016). Preferential combination between the light and heavy isotypes of fish immunoglobulins. *Developmental and Comparative Immunology.* 61, 169 – 179.

8. Meriggioli, M. N., Sanders, D. B. (2009). Autoimmune myasthenia gravis: emerging clinical and biological heterogeneity. *Lancet Neurology. 8,* 475 – 490.

9. Hoch, W., McConville, J., Helms, S., Newsom-Davis, J., Melms, A., Vincent, A. (2001). Auto-antibodies to the receptor tyrosine kinase MuSK in patients with myasthenia gravis without acetylcholine receptor antibodies. *Nature Medicine. 7,* 365 – 368.

10. Gomez, A. M., Van Den Broeck, J., Vrolix, K., Janssen, S.P., Lemmens, M. A. M., Van Der Esch, E.,…Losen, M. (2010). Antibody effector mechanisms in myasthenia gravis – pathogenesis at the neuromuscular junction. *Autoimmunity. 43,* 353 – 370.

11. Ohashi, P. (2002). T-Cell Signalling and Autoimmunity: Molecular Mechanisms of Disease.

12. Balandina, A., Lecart, S., Dartevelle, P., Saoudi, A., Berrih-Aknin, S. (2005). Functional defects of regulatory CD4+CD25+ T cells in the thymus of patients with autoimmune myasthenia gravis. *Blood. 105,* 735 – 741.

13. Carroll, M. C. (2004). The complement system in regulation of adaptive immunity. Nature Immunology. *Nature Immunology. 5*, 981 – 986.

14. Cicardi, M., Cugno, M., Spath, P., Agostoni, A. (1993). Autoimmune C1 Inhibitor Deficiency: Report of Eight Patients. *The American Journal of Medicine. 95,* 169 – 175.

15. Zeerleder, S., Caliezi, C., Van Mierlo, G., Eerenberg-Belmer, A., Sulzer, I., Hack, C. E., Wuillemin, W. A. (2003). Administration of C1 inhibitor reduces neutrophil activation in patients with sepsis. *Clinical and Diagnostic Laboratory Immunology. 10,* 529 – 535.

16. Scott, P. IL-12: Initiation of cytokine for cell-mediated immunity. (1993). *Science. 260,* 496 – 497.

17. Li J., Qiu D., Chen Z., Du W., Liu J., Mo X. (2015). Altered expression of miR-125a-5p in thymoma-associated myasthenia gravis and its down-regulation of foxp3 expression in Jurkat cells. *Immunology Letters.* http://dx.doi.org/10.1016/j.imlet.2016.02.005.

18. Jordan, B. , Kellner, J., Jordan, K., Bähre, M., Behrmann, C., Zierz, S. (2016). Thymic pathologies in myasthenia gravis: a preoperative assessment of CAT scan and nuclear based imaging. *Journal of Neurology.* 1 – 8.

19. Hiyoshi, Y., Kamohara, H., Karashima, R., Sato, N., Imamura, Y., Nagai, Y.,…Baba, H. (2009). MicroRNA-21 regulates the prolifieration and invasion in esophageal squamous cell carcinoma. *Clinical Cancer Research. 15,* 1915 – 1922.

20. Van den berg, R. H., Faber-Krol, M.C., Van Wetering, S., Hiemstra, P.S., Daha, M.R. (1998). Inhibition of activation of the classical pathway of complement by human neutrophil defensins. *Blood. 92,* 3898 – 3903.

21. Roos, A., Nauta, A. J., Broers, D., Faber-Krol, M. C., Trouw, L. A., Drijfhout, J. W., Daha, M. R. (2001). Specific Inhibition of the Classical Complement Pathway by C1q-Binding Peptide. *Journal of Immunology. 167,* 7052 – 7059.

22. Morlacchi, S., Soldani, C., Viola, A., Sarukhan, A. (2011). Self-antigen presentation by mouse B cells results in regulatory T-cell induction rather than anergy or clonal deletion. *Blood.* 118, 984 – 991.

23. Ricklin, D., Hajishengallis, G., Yang, K., Lambris, J. D. (2010). Complement: a key system for immune surveillance and homeostasis. *Nature Immunology. 11,* 785 – 797.

24. Im, S. H., Barchan, D., Feferman, T., Raveh, L., Souroujon, M. C., Fuchs, S. Protective molecular mimicry in experimental myasthenia gravis. (2002). *Journal of Neuroimmunology. 126,* 99-106.

25. Deitiker, P., Ashizawa, T., Atassi, M. Z. (2000). Antigen mimicry in autoimmune disease. Can immune responses to microbial antigens that mimic acetylcholine receptor act as initial triggers of myasthenia gravis? *Human Immunology. 61,* 255 – 265.

26. Vincent, A. (1980). Immunology of acetylcholine receptors in relation to myasthenia gravis. *Physiological Reviews.* 60, 756 – 822.

27. Kumar, V., Kaminski, H. J. (2011). Treatment for Myasthenia gravis. *Current Neurology and Neuroscience Reports. 11,* 89 – 96.

28. Catterall, W. A. (2000). Structure and Regulation of Voltage-gated Ca2+ Channels. *Annual Review of Cell and Developmental Biology.16,* 521 – 555.

29. Wu, H. Xiong, W. C., Mei, L. (2010). To build a synapse: signaling pathways in neuromuscular junction assembly. *Development. 137,* 1017 – 1033.

30. Miyazawa, A., Fujiyoshi, Y., Unwin, N. (2003). Structure and gating mechanism of the acetylcholine receptor pore. *Nature. 423,* 949 – 955.

31. Wood, S. J., Slater, C. R. (2001). Safety factor at the neuromuscular junction. *Progress in Neurobiology. 64,* 393 – 429.

32. Guerra, M., Cartaud, A., Cartaud, J., Legay, C. (2005). Acetylcholinesterase and molecular interactions at the neuromuscular junction. *Chemico-Biological Interactions. 157 – 158,* 57 – 61.

33. Kuffler, S. W., Yoshokami, D. (1975). The distribution of acetylcholine sensitivity at the post-synaptic membrane of vertebrate skeletal twitch muscles: iontophoretic mapping in the micron range. *Journal of Physiology. 244,* 703 – 730.

34. Hughes, B., Luisa Moro De Casillas, M., Kaminski, H. J. (2004). Pathophysiology of Myasthenia Gravis. Seminars in Neurology. 24, 21 – 30.

35. Bunge, R. P. (1968). Glial cells and the central myelin sheath. *Physiological Reviews. 48,* 197 – 251.

36. Bevan, S., Henry, S. J. (1977). The distribution of α-bungarotoxin binding sites of mammalian skeletal muscle developing in vivo. *Journal of Physiology. 267,* 195 – 213.

37. Moransard, M., Borges, L. S., Willmann, R., Marangi, P. A., Brenner, H. R., Ferns, M. J., Fuhrer, C. (2002). Agrin regulates rapsyn interaction with surface acetylcholine receptors, and this underlies cytoskeletal anchoring and clustering. *Journal of Biological Chemistry. 278,* 7350–7359.

38. Gautam, M., Noakes, P. G., Mudd, J., Nichol, M., Chu, G. C., Sanes, J. R., Merlie, J. P. (1995). Failure of postsynaptic specialization to develop at neuromuscular junctions of rapsyn-deficient mice. *Nature*. *377,* 232–236.

39. Losen, M., Stassen, M. H., Martinez-Martinez, P., Machiels, B. M., Duimel, H., Frederik, P., Veldman.,…De Baets, M. H. (2005). Increased expression of rapsyn in muscles prevents acetylcholine receptor loss in experimental autoimmune myasthenia gravis. *Brain*. *128,* 2327–2337.

40. Martinez-Martinez, P., Losen, M., Duimel, H., Frederik, P., Spaans, F., Molenaar, P., Vincent, A., De Baets, M. H. (2007). Overexpression of rapsyn in rat muscle increases acetylcholine receptor levels in chronic experimental autoimmune myasthenia gravis. *Americal Journal of Pathology. 170,* 644–657.

41. Sahashi, K., Engel, A.G., Lambert, E. H., Howard, F. M. (1980). Ultrastructural localization of the terminal and lytic ninth complement component (C9) at the motor end-plate in myasthenia gravis. *Journal of Neuropathology. 39,* 160–172.

42. Drachman, D. B., Angus, C. W., Adams, R. N., Michelson, J. D., Hoffman, G. J. (1978). Myasthenic antibodies cross-link acetylcholine receptors to accelerate degradation. *New England Journal of Medicine. 298,* 1116–1122.

43. Okada, K., Inoue, A., Okada, M., Murata, Y., Kakuta, S., Jigami, T.,…Iwakura, Y. (2006). The Muscle Protein Dok-7 is Essential for Neuromuscular Synaptogenesis. *Science. 312,* 1802 – 1805.

44. Guidotti, L., Chisari, F. (2001). Noncytolytic control of viral infections by the innate and adaptive response. *Annual Review of Immunology.* 19, 65 – 91.

45. Shiraishi, H., Motomura, M., Yoshimura, T., Fukudome, T., Fukuda, T., Nakao, Y.,…Eguchi, K. (2005). Acetylcholine receptors loss and postsynaptic damage in MuSK antibody-positive myasthenia gravis. *Annals of Neurology. 57,* 289–293.

46. van der Neut Kolfschoten, M., Schuurman, J., Losen, M., Bleeker, W. K., Martinez-Martinez, P., Vermeulen, E.,…Parren, P. W. H. I. (2007). Anti-inflammatory activity of human IgG4 antibodies by dynamic Fab arm exchange. *Science. 317,* 1554–1557.

47. Dik, W. A., Pike-Overzet, K., Weerkamp, F., de Ridder, D., de Haas, E. F. E., Baert, M. R. M.,…Staal, F. J. T. (2005). New insights on human T cell development by quantitative T cell receptor gene rearrangement studies and gene expression profiling. *The Journal of Experimental Medicine. 201,* 1715 – 1723.

48. Dinarello, C. A. (2000). Proinflammatory Cytokines. *Chest. 118,* 503 – 508.

49. Krammer, P. H. (2000). CD95's deadly mission in the immune system. *Nature.* 407, 789–795.

50. Lu, Q., Lemke, G. (2001). Homeostatic regulation of the immune system by receptor tyrosine kinases of the Tyro-3 family. *Science. 293,* 306–311.

51. Sakaguchi, S. (2000). Regulatory T Cells: Key Controllers of Immunologic Self-Tolerance. *Cell. 101,* 455–458.

52. Cybulsky, A., Takano, T., Papillon, J., Khadir, A., Liu, J., Peng, H. (2002). Complement C5b-9 Membrane Attack Complex Increases Expression of Endoplasmic Reticulum Stress Proteins in Glomerular Epithelial Cells. *The Journal of Biological Chemistry. 277,* 41342 – 41351.

53. Rose, N. R. (1994). Thymus function, ageing and autoimmunity. *Immunology Letters. 40,* 225- 230.

54. Christadoss, P., Poussin, M., Deng, C. (2000). Animal Models if Mysathenia gravis. *Clinical Immunology. 94*, 75 – 87.

55. Marrack, P., Lo, D., Brinster, R., Palmiter, R., Burkly, L., Flavell, R., Kappler, J. (1988). The effect of thymus environment on T cell development and tolerance. *Cell Press.* 53, 627 – 634.

56. Leite, M., Jacob, S., Viegas, S., Cossins, J., Clover, L., Morgan, B., Beeson, D., Willcox, N., Vincent, A. (2008). IgG1 antibodies to acetylcholine receptors in 'seronegative' myasthenia gravis. *Brain.* 131, 1940 – 1952.

57. Karachunski, P., Ostlie, N., Monfardini,C., Conti-Fine, B. (2000). Absence of IFN-gamma or IL-12 has different effects on experimental myasthenia gravis in C57BL/6 mice. *Journal of Immunology*. 164, 5236–5244.

58. Piddlesden, S., Jiang, S., Levin, J., Vincent, A., Morgan, B. (1996). Soluble complement receptor 1 (sCR1) protects against experimental autoimmune myasthenia gravis. *Journal of Neuroimmunology.*71, 173–177.

59. Christadoss, P. (1988). C5 gene influences the development of murine myasthenia gravis. *Journal of Immunology*. 140, 2589–2592.