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UNDERSTANDING THE FUNCTION OF ICOS/ICOSL COSTIMULATION IN EXPERIMENTAL AUTOIMMUNE MYASTHENIA GRAVIS

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UNDERSTANDING THE FUNCTION OF ICOS/ICOSL COSTIMULATION IN EXPERIMENTAL AUTOIMMUNE MYASTHENIA GRAVIS

by
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Dedicated to my loving wife, Erin, and to all of my family

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UNDERSTANDING THE FUNCTION OF ICOS/ICOSL COSTIMULATION IN EXPERIMENTAL AUTOIMMUNE MYASTHENIA GRAVIS

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The inducible costimulatory molecule (ICOS) is a relatively new member of the CD28 family of costimulatory molecules. For the first time, we have characterized the role of ICOS/ICOSL costimulation in experimental autoimmune myasthenia gravis (EAMG), a model of human MG. Following acetylcholine receptor (AChR) immunization, ICOS gene-deficient mice were resistant to the development of EAMG due to faulty germinal center formation, decreased levels of anti-AChR IgG of all isotypes tested, and a lack of IgG and complement binding to the neuromuscular junction (NMJ). Compared to control lymphocytes, lymphocytes from AChR-immunized ICOS-deficient mice proliferated poorly and produced significantly less IFN-γ and IL-10 following *in vitro* stimulation with AChR or the immunodominant AChR α-subunit peptide 146-162. *In vivo*, the lack of ICOS costimulation led to diminished B cell and plasma cell expansion, whereas the number of $CD4^+$ T helper cells was increased. Collectively, these results indicate that lymphocyte costimulation through the ICOS/ICOSL pathway is a vital component of the adaptive immune response to AChR in EAMG.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

ACh	Acetylcholine
AChE	Acetylcholinesterase
AChR	Acetylcholine receptor
APC	Antigen presenting cell
B6	C57BL/6
C-SMAC	Central supramolecular activation complex
CIA	Collagen-induced arthritis
CFA	Complete Freund's Adjuvant
DC	Dendritic cell
EAE	Experimental autoimmune encephalomyelitis
EAMG	Experimental autoimmune myasthenia gravis
ELISA	Enzyme-linked immunosorbent assay
EOM	Extraocular muscle
EPP	End plate potential
GC	Germinal center
HLA	Human leukocyte antigen
IBD	Inflammatory bowel disease
ICOS	Inducible costimulatory molecule
ICOSL	ICOS ligand
IFN	Interferon
IL	Interleukin
Ig	Immunoglobulin
KO	Knockout
LNC	Lymph node cells
MAC	Membrane attack complex
MG	Myasthenia gravis
MHC	Major histocompatibility complex
MIR	Main immunogenic region

MuSK	Muscle-specific kinase
NMJ	Neuromuscular junction
PBL	Peripheral blood lymphocyte
PBS	Phosphate-buffered saline
PNA	Peanut agglutinin
RIA	Radioimmunoassay
RNS	Repetitive nerve stimulation
SCID	Sever combined immunodeficiency
SFEM	Single-fiber electromyography
SLE	Systemic lupus erythematosus
SNMG	Seronegative MG
TEC	Thymic epithelial cell
TCR	T cell receptor
TGF	Tissue growth factor
TNF	Tumor necrosis factor
TNFR	TNF receptor

CHAPTER 1

INTRODUCTION

SIGNIFICANCE

In addition to antigen-specific signals delivered via the T cell receptor (TCR), signals delivered through costimulatory molecules are crucial for the optimal activation of T cells. Costimulatory signals are required not only for the initiation and maintenance of immunity to pathogens, but are also central to the maintenance of tolerance to self antigens and the prevention of autoimmune disorders. The CD28 family of costimulatory molecules includes the inducible costimulatory molecule (ICOS) and its ligand, ICOSL. In the current study, we have for the first time characterized the role of ICOS/ICOSL costimulation in experimental autoimmune myasthenia gravis (EAMG), which is a model of human myasthenia gravis (MG). Following AChR immunization, ICOS costimulation was required for T cell priming and germinal center formation in the secondary lymphoid organs. The lack of ICOS-delivered costimulatory signals resulted in defective immunoglobulin (Ig) class switching and production of both Th1 and Th2 cytokines by lymph node cells. Furthermore, we have examined the effects of blocking ICOS/ICOSL and CD28/B7 costimulation *in vivo* both to further compare the contributions of these pathways to the development of EAMG and to assess their plausibility as therapeutic targets.

MYASTHENIA GRAVIS: CLINICAL FEATURES AND RISK FACTORS

Clinical Features of MG

MG is a prototypic autoimmune disease of the nervous system characterized by weakness and excessive fatigability of primarily the ocular, bulbar, and proximal limb muscles. Clinical symptoms result from defective neuromuscular transmission, which is caused in the majority of patients by autoantibodies specific for the nicotinic

acetylcholine receptor (AChR) found on the post-synaptic membrane of the neuromuscular junction (NMJ). The prevalence of MG is 12 - 18 per 100,000 individuals, and the annual incidence is 1.1 – 1.5 per 100,000 (Neurology, 1998; Robertson et al., 1998). The symptoms of MG usually present after puberty and normally do not remit spontaneously, but half of prepubertal onset cases spontaneously remit (Evoli et al., 1998). The frequency of new (anti-AChR antibody positive) MG cases appears to be bi-modally distributed between women and men (Vincent et al., 2001). Most new early-onset MG cases tend to occur in women, whereas late-onset MG cases, particularly after the age of 70, are comprised of men.

MG can be manifested in a variety of forms, including early-onset, late-onset, neonatal, and congenital. Early-onset MG includes patients who present with symptoms before age 40, whereas late-onset MG includes patients who present with symptoms after age 40 (Vincent et al., 2001). Early-onset MG is associated with the presence of AChR antibodies and thymic hyperplasia, whereas late-onset MG is not associated with an enlarged thymus. Neonatal MG occurs in about 10-20% of infants born to myasthenic mothers and results from placental transfer of maternal antibodies, whereas congenital MG is not of autoimmune origin and is often due to mutations of the AChR or acetylcholine esterase (AChE) genes (Marx, 2002; Vincent et al., 2001).

Ocular muscle weakness (ptosis, diplopia) is the initial clinical symptom in most MG patients and ultimately occurs in more than 90% of myasthenic patients (Kaminski, 1998). Within six months of ocular symptom onset, about 50% of patients develop generalized MG. Typically, 75% of patients will develop bulbar or extremity weakness within one year, but after three years, very few patients (6%) will go on to develop generalized MG. Respiratory muscle weakness, although life-threatening in the event of respiratory failure, is not frequently observed (Boonyapisit et al., 1999; Grob et al., 1987).

There are several explanations for the observed heterogeneity of weakness among different muscle groups in MG. Differences in ion channel properties, sodium channel density, and safety factor of NMJs across various muscle groups are the most routinely

cited causes (Boonyapisit et al., 1999). The preferential weakness of the extraocular muscles (EOM) could be due to the expression of the fetal AChR isoform by the EOM (Kaminski, 1998; Kaminski and Ruff, 1997). Sera from ocular MG patients have the highest affinity for EOM AChR (Compston et al., 1980; Vincent and Newsom-Davis, 1982), and serum from MG patients contains antibodies that selectively react with fetal AChR (Tzartos et al., 1982; Vincent, 1987). However, the susceptibility of EOM to MG cannot always be linked with expression of fetal AChR (Marx, 2002), because 40% of patients with pure only ocular symptoms of MG are seronegative (Newsom-Davis, 1997). Other physiological differences between EOM and striated muscles may also account for the observed heterogeneity of weakness (Kaminski, 1998).

Diagnosis

When a motor nerve terminal is depolarized, voltage-gated calcium channels are opened, resulting in an influx of calcium ions into the nerve terminal. The calcium influx causes synaptic vesicles to fuse with the plasma membrane and exocytose acetylcholine (ACh) into the synapse. ACh then diffuses across the synapse and binds specifically to AChR on the plasma membrane of the motor end plate, which results in a transient depolarization (end plate potential, EPP) of the end plate. The hydrolysis of ACh by the enzyme acetylcholinesterase ensures that the EPP is transient. If the EPP is greater than the activation threshold, the subsequent electronic conduction results in muscle contraction, but if the EPP does not pass the threshold level, muscle contraction does not occur. This "all-or-none" process is the "safety factor" of neuromuscular transmission.

MG may be diagnosed through pharmacological, electrophysiological, and serological means. A pharmacological diagnosis of MG is based upon the administration of pharmacological drugs followed by clinical observation. The acetylcholinesterase inhibitors, Edrophonium chloride (Tensilon), neostigmine methylsulfate (Prostigmin), and pyridostigmine (Mestinon) temporarily causes relief from muscle weakness in MG due to a greater diffusion of ACh in the synaptic cleft, a lengthened interaction with AChRs, and a larger and lengthened EPP. Electrophysiological tests used in combination

with clinical observation include repetitive nerve stimulation (RNS) and single-fiber electromyography (SFEMG). (Meriggioli and Sanders, 2004).

Serological testing by radioimmunoassay is typically used to detect for the presence of serum autoantibodies to AChR, which are present in approximately 85% of patients with MG (Vincent and Newsom-Davis, 1985). Variations in amounts of antibodies can be seen from patient to patient and within the same patient through time, and antibody levels do not correlate with severity of symptoms. Almost always, patients with thymoma will have elevated levels of anti-AChR compared to patients without thymoma. Antibodies to non-AChR muscle proteins responsible for muscle contraction are present in 30% of all adult onset MG patients and in 80% of MG patients with thymoma (Cikes et al., 1988) and are often useful in diagnosing thymoma in patients with an onset before age 40. Other proteins to which some MG have antibodies directed toward include the ryanodine receptor and the intracellular protein, titin. Antibodies to titin are found in nearly all patients with thymoma and in half of patients without thymoma and with late onset. Anti-ryanodine receptor is found in 75% of patients with thymoma, especially in patients with malignancy (Skeie et al., 2003).

The remaining patients with generalized MG, but without detectable antibody to AChR, are termed "seronegative" (SNMG). As reviewed by Vincent *et al.*, a high proportion of SNMG patients have only ocular symptoms and make up approximately 20% of the MG population (Somner, 1993). This observation could be a result of undetectable amounts of antibodies or absorption of antibodies by the muscles. The involvement of serum immunoglobulin in SNMG has been firmly demonstrated (Evoli et al., 1996; Mier and Havard, 1985; Mossman et al., 1986), and the target of at least one of the culprit serum antibodies is MuSK. MuSK is a receptor tyrosine kinase expressed at the NMJ that drives the developmental clustering of AChRs on the postsynaptic membrane (Liyanage et al., 2002; Vincent et al., 2004). Antibodies to MuSK bind with high affinity to the extracellular Ig-like domains of MuSK and have been found in a large percentage of SNMG patients, but not in seropositive MG patients with generalized or ocular symptoms (Hoch et al., 2001; McConville et al., 2004). Anti-MuSK-positive

patients were often women with early onset (Evoli et al., 2003). SNMG patients with purely ocular symptoms did not have antibodies to MuSK, while 40% of generalized SNMG patients were positive for MuSK antibodies (Zhou et al., 2004).

Genetic Risk Factors in MG

HLA linkage

A growing body of evidence suggests that genetic factors are a crucial factor in MG susceptibility. MG is familial in 4-7% of all cases (Pirskanen, 1976), and the concordance rate is 40% between monozygotic twins (Murphy and Murphy, 1986). The genes of the human leukocyte antigen (HLA) complex are the most often associated with susceptibility to MG. The HLA complex, also known as the major histocompatibility complex (MHC), is located on the short arm of chromosome 6p in humans and is divided into the class I, II, and III regions. The class I region, which is located the most telomeric of the three, contains genes that encode the HLA-A, -B, and –C molecules. The class II region genes are situated the most centromeric of the three regions and encode HLA-DR, -DQ, and –DP. The class III region contains genes that encode complement system proteins. It is widely known that genes in the HLA complex are prone to linkage disequilibrium (non-random association), which has been documented in several disorders including, psoriasis (Calne et al., 1969b), coeliac disease (Calne et al., 1969a; Karoum et al., 1969; Sandler et al., 1971), systemic lupus erythematosus (SLE) (Mackay and Morris, 1972), and juvenile diabetes mellitus (Morris et al., 1976).

MHC class I gene linkage

The first examples of HLA disequilibrium in MG were seen in the early 1970s involving studies of the MHC class I genes. The frequencies of HLA-A1 and –A8 were increased (relative risk (RR) = 12.1) in Caucasians MG patients (Behan et al., 1973; Safwenberg et al., 1973), particularly in women with early onset (Pirskanen et al., 1972) and thymic follicular lymphoid hyperplasia (Fritze et al., 1974). These findings suggested that two forms of MG might exist—one that develops early in HLA-A8-

positive women without antibodies or thymoma, and one that develops later in HLA-A8-negative patients with antibodies and/or thymoma.

Numerous studies have also established a link between the MG in Caucasians and the MHC class I antigen HLA-B8. HLA-B8 confers a RR ranging from 5.12 (Safwenberg et al., 1978) to 9.56 (Matej et al., 1987), and patients with thymoma usually lack HLA-B8 (Keesey et al., 1978; Keesey et al., 1982; Naeim et al., 1978). HLA-B8-positive patients have higher anti-AChR titers than HLA-B8-negative patients (Keesey et al., 1982). Early onset female patients with high anti-AChR titers were the most common associated with HLA-B8 while female patients with low titers were usually HLA-B8 negative (Compston et al., 1980; Keesey et al., 1978; Naeim et al., 1978). Males with high titers were normally HLA-B8 negative (Keesey et al., 1978).

MHC class II gene linkage

HLA-B8 and the MHC class II allele, HLA–DR3, are in linkage disequilibrium in MG, as well as in normal populations (Naeim et al., 1978). HLA-DR3 was shown to confer a RR of 3.8 (Spurkland et al., 1991) to 8.84 in MG patients (Matej et al., 1987) and is significantly increased in females without thymoma, but with high anti-AChR titers (Kaakinen et al., 1975; Moller et al., 1976; Naeim et al., 1978). An HLA-DQ beta chain polymorphism is also strongly linked to MG and confers a very high RR of 32 (Bell et al., 1986). Numerous other HLA-DR3 and –DQ alleles are associated with MG patients with early onset MG and thymic hyperplasia, but reduced in those with thymoma (Compston et al., 1980; Giraud et al., 2001; Hjelmstrom et al., 1995; Spurkland et al., 1991). In a subgroup of patients without thymus abnormalities, but with antibodies to titin, there was an increase in HLA-DR7 and a decrease in –DR3 (Giraud et al., 2001).

The 8.1 ancestral haplotype (A1 B8 CW7 C4AQ0 C4B1 C2C BfS DR3 DQ2), which contains strong linkage disequilibrium between the listed alleles, has also been implicated in Caucasian MG patients with thymic hyperplasia, particularly in women with early onset (RR = 3.5). The association was primarily through the ancestral B8 DRB1*03 DRB3*0101 DQB1*0201 DQA1*0501 haplotype (Vieira et al., 1993). Degli-Esposti *et al*. mapped the location of candidate susceptibility genes in the 8.1 ancestral

haplotype to a region between *HLA B* and *TNF* and postulated the existence of a group of immunoregulatory genes located in that region (Degli-Esposti et al., 1992). The location of the 8.1 ancestral haplotype causative locus, designated *MYAS1*, was mapped to a 1.2-Mb segment that includes the class III and proximal class I regions and was associated with the increased autoantibody titers in patients with thymus hyperplasia (Vandiedonck et al., 2004).

The contributions of class II genes to the susceptibility of non-Caucasians to MG is distinctly different from that of Caucasians. In Chinese patients, disease onset was much earlier (50% before age 20), almost half of the juvenile cases were only ocular, and there was an association with HLA-DRw9 (RR = 17.1) and -Bw46 (RR = 3.9) (Hawkins et al., 1989). In Japan, there was no association with HLA-DR3 or –DQ2, but there was a strong association with HLA-DR9 (RR = 16.4) and –DR13 (RR = 7.1) in patients with an onset before age 3, particularly in DR9/DR13 heterozygotes (RR = 37.4) (Matsuki et al., 1990). The frequency of HLA-DR8 was increased in Japanese patients with a high anti-AChR titer (RR = 6.48), but HLA-DR9 was high in patients with a low titer (RR – 6.32) (Hayashi et al., 1988). No HLA-DR or -DQ associations were made in adult Japanese populations (Suzuki et al., 2001). African Americans with adult onset MG displayed a significant increase in the frequency of HLA–DR5, rather than the –DR3 allele (Christiansen et al., 1984).

Other MHC-linked risk factors

Abnormalities in the genes of complement protein C4 and TNF, which are located within the MHC class III region, have also been associated with MG (Dawkins et al., 1983; Hjelmstrom et al., 1998). The TNFa2 allele was positively associated with MG in patients, particularly in those with early onset. Additionally, the TNFB*1 allele was increased in female patients with early onset MG and thymic hyperplasia, but decreased in patients with later onset. Both the TNFa2 and TNFB*1 alleles are in linkage disequilibrium with alleles on the 8.1 ancestral haplotype and were associated with overproduction of TNF-α and/or LT-α in the peripheral blood of MG patients. It is still

unclear if overproduction of TNF-α or LT-α in the periphery and/or thymus contribute to thymus hyperplasia in MG (Hjelmstrom et al., 1998; Huang et al., 1999a; Manz et al., 1998).

Non-MHC risk factors

Among the most notable non-MHC candidates implicated are polymorphisms of the immunoglobulin genes of chromosome 6. Allotypes of Gm, which is a polymorphic marker on the Fc portion of immunoglobulins, have been associated with MG, but the contributions of Gm are heterogeneous throughout different ethnicities and in patients with varying clinical presentations (Nakao et al., 1980; Smith et al., 1983; Smith et al., 1984). The Km3 kappa light chain allotype was associated with increased anti-AChR serum titers in Caucasian MG patients (Dondi et al., 1994).

CHRNA1 is the gene that encodes the AChRα-subunit, which contains the main immunogenic region (MIR) to which many of MG anti-AChR IgG are directed (Tzartos et al., 1998). HB*14, a microsatellite marker allele of the CHRNA gene, has been associated with MG in Caucasians (Garchon et al., 1994) while the HB*15 CA repeat allele has been associated with South African Blacks (Heckmann et al., 1996). A combination of three loci, including HB*14, the class II allele DQA1*0101, and 8.1 ancestral haplotype locus, were found to be involved in susceptibility to MG (Djabiri et al., 1997; Garchon, 2003).

Polymorphisms of the interleukin 1 (IL-1β) and IL-1 receptor antagonist (IL-1Ra) genes, which are located on chromosome 1q12-22, have also been associated with MG (Huang et al., 1998b; Sciacca et al., 2002), thus indicating a possible pathogenic role for IL-1β in MG. Other non-MHC genes associated with MG include those encoding TAP (Hjelmstrom et al., 1997), T cell receptor α and β (Mantegazza et al., 1990; Oksenberg et al., 1988; Oksenberg et al., 1989), and CTLA-4 (Huang et al., 2000a; Huang et al., 1998a; Wang et al., 2002b; Wang et al., 2002c).

Treatment

Great progress has been made in the treatment of MG over the last century (Keesey, 2004). In the past, the anti-acetylcholinesterase drugs physostigmine, neostigmine, and Tensilon were used. Pyridostigmine chloride, a neostigmine analogue, is less toxic and has become one of the most widely used short-term drugs for MG (Saperstein and Barohn, 2004). For long-term treatment, the synthetic glucocorticoid prednisone was found to cause remission of MG symptoms in some patients (Jenkins, 1972; Warmolts and Engel, 1972). Although Prednisone is relatively inexpensive and causes clinical improvements within 2 to 3 weeks of treatment, it can be difficult to dose and continued usage can cause exacerbation of disease and other harmful side-effects such as hypertension and diabetes mellitus (Saperstein and Barohn, 2004). Azathioprine is an antimetabolite that inhibits T cell proliferation and ultimately leads to a decrease in anti-AChR production, but the benefits of azathioprine may not be observed for 6 to 18 months. Cyclosporine A, which operates by inhibiting helper T cells and enhancing suppressor T cells, is frequently used in refractory cases, but has severe side effects, such as hypertension, hepatotoxicity, and nephrotoxicity (Armstrong and Schumann, 2003).

Following the determination in the 1970s that MG was the result of antibody-mediated autoimmune attack on AChRs at the neuromuscular junction, plasmapheresis was accepted as a means for clinical improvement in MG patients (Dau et al., 1977; Pinching and Peters, 1976). Plasmapheresis is reserved for patients in critical condition and is used to help strengthen patients prior to thymectomy or the initiation of corticosteroids. Unfortunately, complications (thrombosis, pneumothorax, infection) arising from the use of an indwelling catheter may arise, and the benefits are short-lived. (Keesey, 2004; Saperstein and Barohn, 2004). High doses of intravenous immunoglobulin (IVIG), which has beneficial effects comparable to plasmapheresis and with lower risk of side effects, is effective in 60 to 70 percent of patients. The mechanism of action is unknown (Keesey, 2004; Saperstein and Barohn, 2004).

Thymectomy is a widely accepted surgical treatment for MG (Gronseth and Barohn, 2000), but the use of thymectomy in non-thymoma cases remains highly debated

due to a lack of controlled studies (Jaretzki et al., 2004; Saperstein and Barohn, 2004). Thymectomy is usually not performed in young children or the elderly, but is usually only performed in non-thymoma patients that are early onset with high titers of anti-AChR (Saperstein and Barohn, 2004). Thymectomy in ocular MG is controversial and is usually not performed without the presence of thymoma (Evoli et al., 2001). In SNMG patients with antibodies to MuSK, thymectomy was not beneficial (Evoli et al., 2003).

THE IMMUNOPATHOGENESIS OF MG

The factors that initiate autoimmunity to muscle AChR in MG remain unknown, but several theories have arisen (Lindstrom, 2000). One possible cause is the result of an exogenous or endogenous agent that alters AChR and renders it immunogenic. For example, penicillamine treatment of some rheumatoid arthritis patients induces reversible MG by a covalent reaction with thiol groups on AChRs, resulting in new antigenic sites. A second possibility is through a paraneoplastic autoimmune response. Generalized MG is frequently associated with thymic abnormalities, including thymoma. A third possible route MG initiation is through the loss of tolerance to native muscle AChR. Antibodies to fetal AChRs may often be found in MG patients (Vincent et al., 1998), and fetal AChRs expressed by the extraocular muscles may account for the enhanced susceptibility of extraocular muscles to MG (Kaminski, 1998; Kaminski et al., 2002). Molecular mimicry of AChR epitopes by bacterial or viral components may represent a fourth mechanism by which anti-AChR immunity is launched. It is hypothesized that immune responses to bacterial or viral components could react with AChR and lead to epitope spreading to include other AChR epitopes or epitopes of other muscle proteins. Bacterial DNA and proteins have been associated with autoimmunity (Bachmaier et al., 1999; Faller et al., 1997; Nachamkin et al., 1998), antibody epitope sharing has been documented between the AChR α-subunit and *K. pneumoniae* and *E. coli* membrane polypeptides (Stefansson et al., 1987), and human MG serum antibodies to AChR α-subunit cross-react with Herpes Simplex Virus glycoprotein D (Schwimmbeck et al., 1989).

AChR as an autoantigen

The nicotinic AChR at the NMJ and in the *Torpedo* fish is a ligand-gated ion channel and is a 290 kDa pentameric glycoprotein of five homologous subunits in the stoichiometry $\alpha_2\beta\gamma\delta$ (in adults, the γ-subunit is replaced by the ε-subunit upon innervation of the muscle) (Tzartos, 2000). The subunits are oriented around the central ion channel as $\alpha\gamma\alpha\beta\delta$. The N-terminus of each subunit is extracellular and is followed by four hydrophobic α–helices (M1-M4) that are thought to form the transmembrane regions and ion channel. The acetylcholine binding site is on the α-subunits near the disulfide-linked cysteine residues 192 and 193 (Kao et al., 1984; Kao and Karlin, 1986). The amino acid sequences of the muscle AChR α-subunit in humans and the *Torpedo* fish are approximately 80% identical, whereas the other subunits are about 55% identical. When intact AChR is used as an immunogen, most of the resulting antibodies are directed to conformational epitopes on the extracellular regions of the molecule, but when denatured AChR is used, the antibodies can also recognized cytoplasmic regions of the AChR. IgG binds to the MIR, which is located at the α-subunit sequence 67-76 in humans and *Torpedo* (Tzartos et al., 1998; Tzartos et al., 1988).

Pathogenic mechanisms of autoantibodies in MG

The MIR, consists of several anti-AChR antibody epitopes that are not shared with T cells, and the binding of anti-AChR antibodies to these epitopes is conformation-dependent (Tzartos et al., 1998). The mAb 35, which binds epitopes that include AChR residues α68 and α71, was used to narrow the location of the MIR (Beroukhim and Unwin, 1995) to the extreme synaptic ends of the two α-subunits. The ends of the α-subunit are the most accessible areas of the AChR, which may explain the high immunogenicity of the MIR. For this reason, anti-MIR mAbs passively transfer MG to animals and cause AChR modulation (Conti-Tronconi et al., 1981; Lennon and Lambert, 1980; Tzartos et al., 1987; Tzartos et al., 1985), but antibodies directed to non-MIR epitopes did not induce MG or cause AChR loss (Graus et al., 1995; Tzartos et al., 1998).

Anti-MIR antibodies inhibit neuromuscular transmission through (1) direct, complement-mediated lysis of the post-synaptic membrane (Nakano and Engel, 1993; Sahashi et al., 1980; Tuzun et al., 2003), (2) cross-linking adjacent AChRs, which increases their normal rate of internalization and lysosomal degradation, resulting in a net loss of AChR (Drachman, 1981), and (3) blocking or functional alteration of the ACh binding site, although this is not typically considered a significant source of MG pathology (Lindstrom et al., 1988) due to the heterogeneity of human sera (Drachman et al., 1982; Lennon and Griesmann, 1989; Vernet-der Garabedian et al., 1986). Univalent antibody fragments (Fab or Fv) of anti-MIR mAbs, on the other hand, are not pathogenic. Pre-treatment of a human muscle-like cells *in vitro* with anti-AChR Fabs protected the cells from human MG sera (Tzartos et al., 1985). Fab fragments of mAb 35 and mAb 195 protected rats from myasthenic antibodies (Papanastasiou et al., 2000; Trakas and Tzartos, 2001).

Characteristics of T lymphocytes in MG

The production of the high-affinity anti-AChR IgG in human MG requires the interaction of $CD4^+$ helper T cells with B cells. Thymectomy causes a rapid diminution in peripheral blood T cells, and treatment of MG patients with anti-CD4 mAbs causes electrophysiological improvement and inhibits the AChR-reactive proliferative response of peripheral lymphocytes (PBL) *in vitro* (Conti-Fine, 2000). When PBL from MG patients were depleted of $CD8^+$ T cells and transferred to SCID mice, the recipient mice developed antibodies to AChR and showed signs of disease. Mice that received CD4-depleted cells, however, did not develop myasthenic symptoms or have measurable anti-AChR levels (Wang et al., 1999).

AChR epitope recognition by $CD4^+$ T cells

The AChR α-subunit contains the majority of epitopes recognized by $CD4^+$ T helper cells in MG patients. There are a wide range of AChR α-subunit synthetic peptides (Berrih-Aknin et al., 1988; Harcourt et al., 1988; Hohlfeld et al., 1987) and non-α-subunit synthetic peptides (Conti-Fine, 2000) that induce proliferation of MG patient T

cells. The majority of MG patients in one study had $CD4^+$ T cells that responded to α48-67, α101-137, α304-322, and the carboxyl-terminal sequence α403-437 (Conti-Fine, 2000). The peptide sequences that induced proliferation by T cells were α1-14, α48-80, α101-154, α304-337, and α403-437. The sequence regions α48-80 and α101-154 are probably extracellular (Conti-Tronconi et al., 1994), whereas α304-337 is cytoplasmic (Conti-Fine et al., 1996). The α403-437 region is believed to contain hydrophilic peptide sequences that are exposed extracellularly and hydrophobic sequences that are transmembrane and comprise M4 (Conti-Fine et al., 1996; Conti-Tronconi et al., 1994).

Interestingly, the autoimmune $CD4^+$ T cells in generalized MG patient undergo significant change with time (Conti-Fine, 2000; Hughes et al., 2004). Non-thymoma MG patients with disease longer than four to five years had $CD4^+$ T cells that responded to all of the AChR subunits, whereas the $CD4^+$ T cells from patients with disease fewer than four to five years did not respond to all AChR subunits. Therefore, the number of AChR-reactive T cells and epitopes recognized by those T cells increases with time, which may be the result of epitope spreading.

$CD4^+$ T cell subsets and cytokines

T cells can be divided into subsets based on function and cytokine secretion. Th1 cells characteristically produce cytokines, such as IL-2, IL-18, and IFN-γ and drive B cells to produce IgG isotypes that fix complement, such as IgG_{2a}, IgG_{2b}, and IgG_3 in the mouse. Th2 cells secrete the anti-inflammatory cytokines IL-4 and IL-10 and promote the production of IgE and non-complement fixing IgG isotypes, such as IgG_1 in the mouse. Th3 cells are a regulatory subset of $CD4^+$ T cells that secrete TGF-β while Tr1 cells are another subset of regulatory cells that predominantly secrete IL-10. $CD4^+CD25^+$ regulatory T cells can either act through contact dependent mechanisms or by secreting cytokines like TGF-β or IL-10 (O'Garra, 1998; Roncarolo et al., 2001; Shevach, 2002).

IL-4 and IL-10 produced by unstimulated peripheral blood cells were lower in MG patients without thymectomy than in healthy controls (Huang et al., 2000b). However, the numbers of IL-4, IL-10, and IFN-γ mRNA-expressing cells was increased

in MG patients, as well those expressing the proinflammatory cytokine IL-6 (Link et al., 1995; Link et al., 1994a). IFN-γ–secreting and proliferating Th1 cells were increased, and IL-4- and IL-10-secreting Th2 cells were increased in MG patients (Huang et al., 1999b; Link et al., 1995; Yi and Lefvert, 1994). Increases in the serum IL-18 (IFN-γ inducer), IL-2, and IL-6 have also been reported (Hartung et al., 1991; Jander and Stoll, 2002; Shimada et al., 1993). Thus, both Th1 and Th2 cells are factors in MG due to their roles in promoting B cell activation and inducing the production of antibodies that fix complement, but their effects may variable due to the techniques used and natural variation in MG patient populations.

Polymorphisms of the TNF-α gene causing excess TNF-α and TNF-β secretion have been found in early onset MG patients with thymic hyperplasia (Hjelmstrom et al., 1998; Huang et al., 1999a) and in MG patients with anti-titin antibodies (Skeie et al., 1999). TNF-α and TNF-β are B cell growth factors, and TNF-α induces IL-6 production by thymic epithelial cells, so aberrant production of TNF-α and TNF-β could lead to thymic hyperplasia with thymic germinal centers, which are characteristic of early-onset MG. IL-6, which drives B cell maturation and IgG class switching, and IL-1β and IL-2 are overproduced by thymic epithelial cells (TEC) from MG patients with thymic hyperplasia (Cohen-Kaminsky et al., 1993a; Cohen-Kaminsky et al., 1993b; Emilie et al., 1991), further implicating these proinflammatory factors in a germinal center response to thymic myoid AChR and muscle AChR.

TGF-β, which is an anti-inflammatory cytokine that suppresses T and B cell immunity, is increased in patients who underwent thymectomy compared to non-thymectomized patients (Link et al., 1994b), suggesting that TGF-β plays a role in the suppression of clinical symptoms during thymectomy-induced remission of MG. Also, recombinant TGF-β suppressed the AChR-specific TNF-α, TNF-β, IL-6, and perforin responses of MG patient peripheral blood cells *in vitro*, but had no effect on IL-10 production (Link et al., 1995). However, there have been more recent reports that TGF-β is expressed by MG patient thymic tissue (Bernasconi et al., 2003) and increases the

production of IL-1α, IL-1β, and IL-6 by TEC cultures (Schluns et al., 1997), indicating that it could play a pathogenic role.

Although the roles of pathogenic anti-AChR T cells and their secreted factors in MG are becoming more clear, the contributions of regulatory $CD4^+$ T cells in MG are still in question. $CD4^+$ regulatory T cells (T_{regs}) develop in the thymus or in the periphery and are one of several safeguards in place to preserve immunological tolerance to self-antigens and maintain homeostatic equilibrium. $CD4^+$ T_{regs} are often identified by their expression of CD25, GITR, CTLA-4, and CD62L (Chatenoud et al., 2001; Sakaguchi, 2000; Salomon and Bluestone, 2001; Shevach, 2000), and their development is dependent upon the transcription factor, forkhead box p3 (Foxp3) (Ramsdell and Ziegler, 2003). Patients with Foxp3 deficiency develop multiorgan autoimmune disease (Kriegel et al., 2004), and deficiencies of T_{reg} effector function have been discovered in patients with multiple sclerosis (Viglietta et al., 2004), type 1 diabetes (Kukreja et al., 2002), and inherited thymic hypoplasia (Sullivan et al., 2002).

One study found no deficiency of $CD4^+CD25^+$ cells in MG and no change in the number of $CD4^+CD25^+$ cells in MG patients following thymectomy (Huang et al., 2004), but another study documented an increase in peripheral blood $CD4^+CD25^+$ cells following thymectomy, indicating their protective role (Sun et al., 2004). Also, there is evidence of defective $CD4^+CD25^+$ cell suppressive activity and decreased Foxp3 mRNA expression (Balandina et al., 2005) in MG. Collectively, these data suggest a possible protective role for T_{reg} cells in MG.

Thymus abnormalities in MG

Thymic hyperplasia

The thymus is a key organ in regulating the development of T cells and maintaining immunological tolerance. Thymocytes with low-affinity TCRs for self peptides are positively selected and migrate to peripheral lymphoid tissues. Thymocytes with high-affinity TCRs are negatively selected and undergo deletion or inactivation by anergy, although the escape of some autoimmune T cells is inevitable (Sprent et al.,

1988). The thymus has long implicated in MG due to the beneficial effects of thymectomy. Seropositive patients with early onset MG typically almost always exhibit thymic hyperplasia characterized often by the presence of numerous follicular germinal centers containing T and B lymphocytes (Schluep et al., 1988), whereas in late onset MG, the thymus is usually atrophic.

The basal membrane of the hyperplastic MG thymus is disrupted by the lymphoid follicles, resulting in the abnormal joining of the medulla and perivascular spaces, which are considered part of the peripheral immune system (Kirchner et al., 1986). This disruption of the normal architecture is marked by the presence of antigen presenting cells, such as dendritic cells (DC) (Schluep et al., 1988) and TEC, thymocytes, and muscle-like myoid cells that express nicotinic AChR α-subunits (Engel et al., 1977b; Fuchs et al., 1980; Kao and Drachman, 1977; Schluep et al., 1987; Wekerle et al., 1978). Myoid cells are rare muscle-like cells located in the medullary epithelium of both the normal and myasthenic thymus and express fetal and adult AChR, but rarely express MHC class II or costimulatory molecules, so it is unlikely that direct presentation of myoid AChR epitopes to T cells occurs (Kao and Drachman, 1977; Kirchner et al., 1986; Marx et al., 2003; Roxanis et al., 2002). Therefore, it is hypothesized that the TEC, in the presence of an abnormal thymic production of IL-1, IL-2 and IL-6, are the trigger of MG pathogenesis, because they possess the presentation and costimulatory molecules necessary for priming T cells with myoid AChR epitopes (Emilie et al., 1991; Le et al., 1987; Sadlack et al., 1993; Shiono et al., 2003). Early GC formation and antibody responses to myoid AChR α or ε subunits could then recruit professional APCs from the periphery, which following antigen uptake and migration out of the thymus, could result in determinant spreading to the skeletal muscle AChR epitopes. Since there is evidence indicating the bidirectional trafficking of resting and activated T cells to and from the thymus (Agus et al., 1991; Michie et al., 1988; Naparstek et al., 1983; Westermann et al., 1996), it has also been proposed that inflammation provoked in the thymic medulla induces the upregulation of MHC class II, costimulatory molecules, and AChR on TEC and/or APCs and prompts the entry of peripheral AChR α-subunit-reactive $CD4^+$ T cells

that previously escaped negative selection (Levinson et al., 2003). Once the anti-AChR T cells enter the thymic medulla, they encounter AChR peptide-laden APCs and become activated, resulting in the production of anti-AChR antibodies by thymic GCs.

Thymoma

Approximately 10% to 15% of MG patients have an epithelial tumor of the thymus known as a thymoma (Hohlfeld and Wekerle, 1994). A striking difference between thymic hyperplasia and thymoma in MG is the lack of myoid cells that express AChR in thymoma and a lack of anti-AChR production inside the thymoma (Fujii et al., 1984; Newsom-Davis et al., 1987). However, as reviewed by Marx *et al*., MG-associated thymomas contain autoreactive T cells specific for the AChR α-subunit and ε-subunit, which may form as the result of defective positive selection in the thymus (Nagvekar et al., 1998; Schultz et al., 1999). Second, potentially autoimmune, mature T $CD4^+$ and $CD8^+$ cells are exported from the thymus and can be identified in the periphery by the presence of T cell receptor DNA excision circles resulting from T cell receptor gene rearrangement (Buckley et al., 2001). Third, the expression of MHC class II by neoplastic TEC in thymomas is reduced, and thymopoiesis in thymomas is inefficient (Strobel et al., 2001), which may result in ineffective development of self-tolerance. Finally, thymomas in MG have been associated with multiple autoantigens, including AChR (Vincent, 1999), striational muscle antigens like titin (Aarli et al., 1998), neuronal antigens (Marx et al., 1992), cytokines like IL-12 and IFN-α (Meager et al., 1997), and ryanodine receptor (Mygland et al., 1995).

THE IMMUNOPATHOGENESIS OF EAMG

Much of our understanding of MG pathogenesis derives from the use of animal experimentation. MG was first recognized as an autoimmune disorder in 1973 when Patrick and Lindstrom observed muscle weakness in rabbits immunized two to three times subcutaneously with a Complete Freund's Adjuvant emulsion containing purified AChR from eel electric organ (Patrick and Lindstrom, 1973). Electromyography and

temporary clinical improvement following the injection of the AChE inhibitors proved the paralysis was due to abnormal neuromuscular transmission. All of the animals had antibodies to the eel acetylcholine receptor. Subsequent studies reproduced the induction of experimental autoimmune myasthenia gravis (EAMG) in Lewis rats, as well as guinea pigs, following one AChR immunization (Lennon et al., 1975). EAMG in rats consists of an acute phase, which occurs about one week following immunization and subsides within a few days, and a chronic phase that begins around four weeks and worsens with time, resulting in death. EAMG has also been induced by AChR immunization in other animals, including pigs, monkeys, and goats.

EAMG can be induced in rodents following the passive transfer of MG serum (Richman et al., 1980; Toyka et al., 1977) or peripheral blood cells (Wang et al., 1999), grafts of MG patient thymus tissue (Schonbeck et al., 1992), and bone marrow transplantation from MG patients (Damoiseaux, 2002). In some animals, such as cats, dogs, and horses, MG spontaneously develops and is the result of autoantibodies to AChR. Unfortunately, there is a lack of these animals for scientific experimentation.

Although the rat model of MG is a well accepted model of MG in humans (Link and Xiao, 2001), there is no analogous acute disease phase in humans. There are additional key reasons why mice remain the ideal species for the study of MG pathogenesis (Christadoss et al., 2000). First, there is a wide array of mice, including inbred, congenic, transgenic, and gene knockout (KO) strains available, as well as an ever-growing selection of murine recombinant proteins and antibodies. In addition, several characteristics of EAMG pathogenesis accurately reflect the pathogenesis of human MG. Like human MG, EAMG in the mouse primarily affects the skeletal muscles of the upper body, and the disease can be diagnosed by the tensilon test and a decremental response to repetitive nerve stimulation. Mice with EAMG also experience temporary improvement in symptoms following AChE inhibitor administration. The pathology of EAMG, as in humans, is the direct result of serum anti-AChR Ab binding to AChR at the neuromuscular junction and causing defective neuromuscular transmission with the aid of complement proteins. The AChR-specific IgG response in murine EAMG

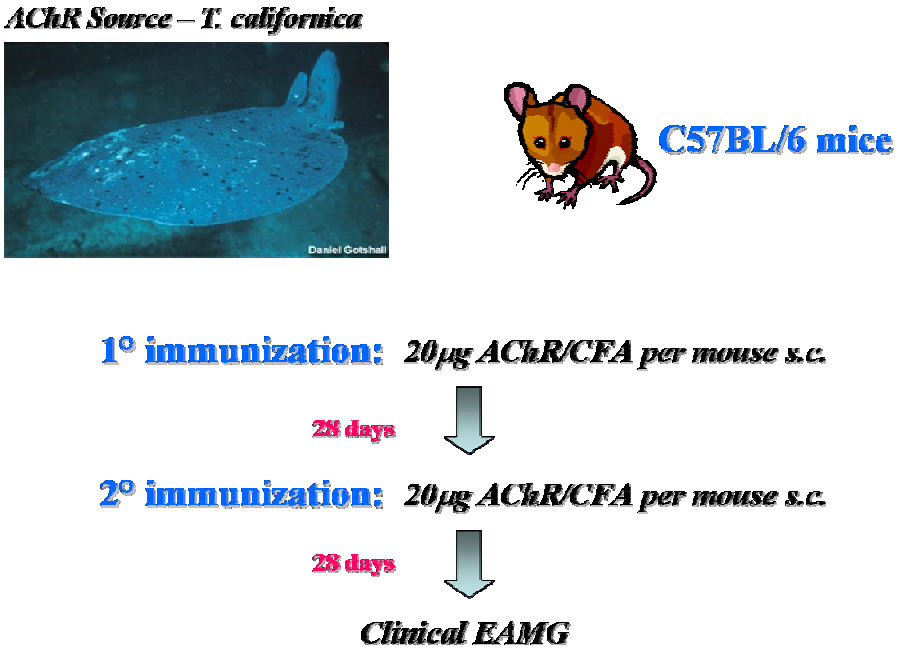


Figure 1. Method of EAMG induction in susceptible mice. Clinical EAMG is induced in susceptible strains of mice following two subcutaneous immunizations on days 0 and 28 with AChR emulsified in CFA.

is the result of the MHC class II-restricted presentation of AChR epitopes to $CD4^+$ T helper cells, which aid AChR-specific B cell differentiation in antibody-secreting plasma cells. As in humans, EAMG in mice is associated with certain MHC class II alleles. Together, these factors make the mouse an ideal species for experimental study of MG.

Introduction to EAMG

As shown in Figure 1, following two immunizations with purified *Torpedo californica* AChR emulsified in CFA, EAMG can be induced with a high incidence (50% – 100%) in mice of the $H\text{-}2^b$ haplotype, which includes the C57BL/6 (B6), C57BL/10, and C3H.SW strains. Although there may be variation in clinical incidence between experiments, strains of the $H\text{-}2^q$ haplotype (B10.Q) are typically less susceptible to

EAMG than H-2^b strains, followed by the H-2^k strains (B10.BR), and H-2^p strains (B10.P), which are nearly resistant (Berman and Patrick, 1980; Christadoss et al., 1981; Christadoss, 1979; Fuchs et al., 1976). It is rare for B6 mice to develop EAMG following only one immunization, because of the relatively unpronounced anti-AChR IgG response following the primary immunization compared to the secondary anti-AChR IgG response (Christadoss et al., 1985a). Furthermore, B6 mice appear to have a high "safety factor" of neuromuscular transmission and therefore can withstand a degree of muscle AChR loss (Christadoss et al., 1985a). Myasthenic symptoms usually appear in B6 mice beginning 7 to 10 days following the second immunization. Mice that develop intermediate muscle weakness (grade 1) often, but not always, progress to a more severe clinical status (grade 2). Mice with grade 2 EAMG (Figure 2) typically do not improve clinically and sometimes will die of respiratory distress or of starvation/dehydration if proper care is not given. In an individual mouse, there is no correlation between serum anti-AChR IgG level and muscle AChR loss or clinical incidence (Christadoss et al., 1985a).

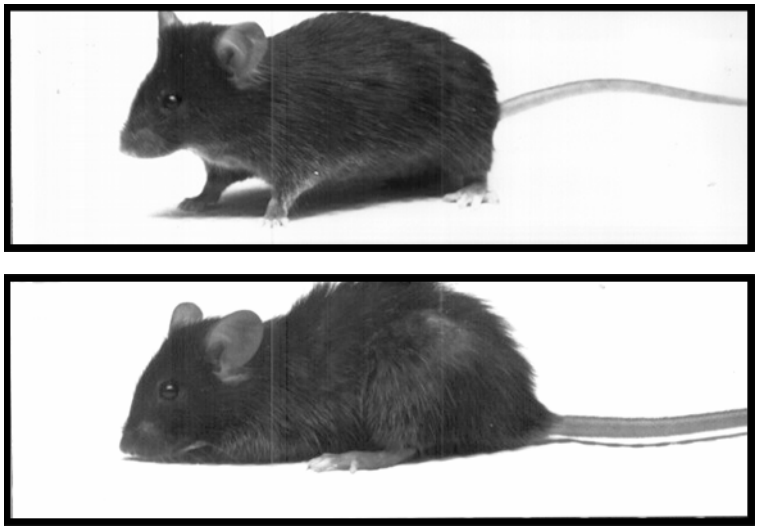


Figure 2. Visual comparison of a normal and myasthenic mouse. A clinically normal mouse (upper panel) is alert, active, and has a well-groomed coat. A mouse with grade 1 or grade 2 EAMG (lower panel) has weakness of the shoulder, neck, and forelimb muscles, displays with an inability to lift the head and/or difficulty walking and breathing, and has a poorly groomed coat.

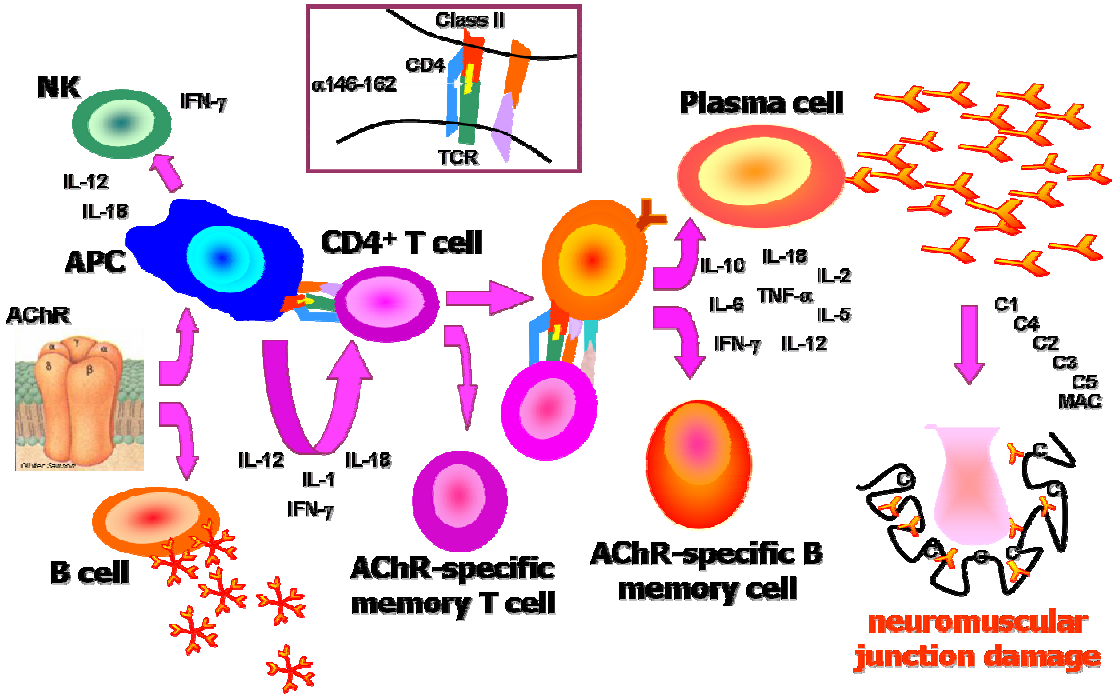


Figure 3. Proposed model of EAMG induction in B6 mice. Experimental evidence suggests that following s.c. immunization with *Torpedo* AChR in CFA, AChR protein is taken up by nearby antigen presenting cells and processed, and AChR-derived peptides are presented to T cells in the local lymph nodes (axillary, inguinal, popliteal). AChR-specific IgM and low levels of AChR-specific IgG can be detected soon after. After the first immunization, the clonally-expanded AChR-specific T and B cells form populations of memory cells that become reactivated later following the second immunization with AChR. In the germinal centers of the lymph nodes, AChR peptide-specific T cell-B cell interactions occur and result in the activation of B cells, IgG class switching, and plasma cell formation. Numerous Th1 and Th2 cytokines play an important role in the activation and expansion of AChR-specific T and B cells in the local lymph nodes. Ultimately, the secreted AChR-specific IgG binds to AChR on the motor end plate, activates the classical complement pathway, and causes defective neuromuscular transmission and clinical EAMG.

The Cellular Immunology of EAMG

Autoantibodies, complement, and the neuromuscular junction

The primary mechanism of neuromuscular transmission defects in MG is through antibody and complement-mediated destruction of AChR at the NMJ. IgG and complement components have been identified at the NMJ of MG patients (Engel et al., 1977a; Sahashi et al., 1980) and mice and rodents with EAMG (Sahashi et al., 1978). Anti-AChR IgG_{2b}, which fixes complement, has been implicated in the pathogenesis of

EAMG (Wu et al., 1997). The classical complement pathway is crucial to the development of EAMG, because when C4 is genetically deficient (Tuzun et al., 2003), EAMG is prevented, despite the presence of IgG deposition at the NMJ. Mice deficient in decay accelerating factor (DAF), which prevents C3 activation, develop more severe EAMG (Kaminski et al., 2003). Lytic components of the membrane attack complex are the final effectors in EAMG development, because mice deficient in C5 were resistant to EAMG induction by AChR immunization (Christadoss, 1989) and passive transfer of MG IgG (Toyka et al., 1977). Therefore, anti-AChR, as well as classic and alternative complement activation, are important for the development of clinical EAMG.

Role of MHC class II molecules

The genetic control of the immune responses to AChR in EAMG was initially localized to immune response genes located within the H-2A subregion of the MHC by a series of studies that utilized B10 congenic mice, recombinant mice, and F_1 hybrid mice backcrosses (Christadoss et al., 1979; Christadoss et al., 1982; Christadoss et al., 1981). Some of the first evidence demonstrating that the I-A molecule presents AChR peptides to $CD4^+$ T cells in EAMG came when antibodies to $I\text{-}A^b$ blocked AChR-induced proliferation of B6 lymphocytes *in vitro* (Christadoss et al., 1982; Christadoss et al., 1983) and when treatment of SJL ($H\text{-}2^s$) mice with mAb to the $I\text{-}A^s$ molecule prior to immunization suppressed anti-AChR production and the clinical incidence of EAMG (Waldor et al., 1983). B6.CH-2^{bm12} (bm12) mice, which possess a mutation in the $I\text{-}A^b$ β chain consisting of a three amino acid change in the $I\text{-}A^b$ β chain ($Ile^{67} \rightarrow Phe$, $Arg^{70} \rightarrow Gln$, $Thr^{71} \rightarrow Lys$) (McIntyre and Seidman, 1984; Widera and Flavell, 1984), results in decreased lymphocyte proliferation to AChR, anti-AChR Ab production, muscle AChR loss, and clinical EAMG incidence (Christadoss et al., 1982; Christadoss et al., 1985b). Likewise, MHC class II deficiency renders B6 mice completely resistant to EAMG (Kaul et al., 1994). On the other hand, MHC class I molecules and $CD8^+$ T cells do not play a significant role in EAMG pathogenesis, because B10 mice lacking the β2 microglobulin

gene, and thus lacking MHC class I and $CD8^+$ T cells, developed anti-AChR IgG and a normal EAMG incidence (Kaul et al., 1994).

Further evidence of the role of MHC class II in EAMG has been generated through the use of HLA transgenic (Tg) B6 mice, which do not express endogenous murine $I\text{-}A^b$ molecules. When immunized with a CFA emulsion containing human AChR purified from the TE671 cell line, HLA-DQ8 and –DR3 Tg mice developed clinical EAMG associated with a strong T cell proliferative response to the AChR α320-327 peptide (dominant T cell epitope) and the subdominant α304-322 and α419-437 peptides. HLA-DQ6 Tg mice were resistant to clinical EAMG and had diminished anti-AChR responses and reduced T cell proliferation and production of IL-2 and IFN-γ in response to AChR and α320-337 stimulation (Yang et al., 2002). The resistance of HLA-DQ6 Tg mice was linked with suppressed lymph node cell proliferation and the production of IFN-γ, IL-2, and IL-10 following stimulation with AChR (Poussin et al., 2001). Together, these studies illustrate the vital role of MHC class II molecule in the initiation of the immune response to AChR in EAMG.

Role of $CD4^+$ T cells and B cells

The necessity of $CD4^+$ T cells in EAMG was seen when mAbs blocked AChR-induced lymphocyte proliferation and both prevented and ameliorated EAMG (Christadoss and Dauphinee, 1986). $CD4^+$ T cell deficiency resulted in a nearly complete resistance to EAMG in B6 mice due to reduced AChR-specific T and B cell responses (Zhang et al., 1996). Following immunization with *Torpedo* AChR, $CD4^+$ T cells from B6 mice respond to immunodominant epitopes within the AChR α-subunit located within sequence regions 111-126, 146-162, and 182-198 (Yokoi et al., 1987). The α146-162 peptide evokes the strongest proliferative response by AChR-immune B6 T cells and contains the immunodominant T cell epitope, whereas α111-126 and α182-198 represent sub-dominant epitopes (Bellone et al., 1991; Infante et al., 1991; Shenoy et al., 1993). Furthermore, when B6 mice were primed with an immunization of AChR in CFA, a subsequent immunization with only the α146-162 peptide was sufficient to induce

myasthenic symptoms in 70% of the animals (Shenoy et al., 1994), thus solidifying the immunodominant role of the α146-162 peptide in EAMG and demonstrating the worth of AChR-specific $CD4^+$ T cells in the induction of EAMG.

B cells play an obvious role in MG and EAMG by differentiating into AChR-specific IgG-secreting plasma cells. B cell deficient mice (μ gene KO) do not generate an antibody response to AChR and do not develop EAMG (Dedhia et al., 1998). Although other reports have suggested that B cells are not important for AChR processing and peptide presentation in EAMG (Li et al., 1998), they may play a role in the presentation of AChR peptides since the primary lymph node cell response to AChR and α146-162 was impaired in μ gene KO mice (Dedhia et al., 1998).

T helper cell subsets and cytokines

Th1 cells and their secreted cytokines have been implicated in MG and have a strong impact in EAMG pathogenesis due to their ability to promote the production of the complement-binding IgG subclasses in mice (Abbas et al., 1996). IL-12 and IL-18 cooperate to drive the differentiation of IFN-γ-producing Th1 cells, and mice lacking genes for IL-12 and/or IL-18 have significant Th1 deficiencies (Ahn et al., 1997; Manetti et al., 1993; Okamura et al., 1995; Seder et al., 1993; Takeda et al., 1998; Yoshimoto et al., 1998). B6 mice deficient in IL-12 have decreased electrophysiological and clinical evidence of EAMG and display suppressed AChR-specific Th1 development and production of complement-fixing IgG subclasses (Karachunski et al., 2000; Moiola et al., 1998), and enhanced AChR-induced IL-4 and IL-10 production (Karachunski et al., 2000). Recombinant IL-12 administration increases AChR-specific IFN-γ-producing cells and the production of Th1-associated IgG subclasses, resulting in worsened clinical EAMG (Moiola et al., 1998; Sitaraman et al., 2000). IL-18 deficiency conferred a high degree of resistance to clinical EAMG in B6 mice due to a defect in Th1 differentiation and the synthesis of both Th1- and Th2-associted IgG isotypes specific for AChR (Scott et al., 2003; Shi et al., 2000). In the rat, anti-IL-18 treatment prevents EAMG and suppresses the progression of established disease (Im et al., 2001).

Understanding the direct contributions of IFN-γ to EAMG pathogenesis has proven somewhat difficult. Balasa *et al*. reported that IFN-γ-deficient (129/SvEv x C57BL/6) F_2 mice, despite normal lymphoproliferative responses to AChR and α146-162, were completely resistant to clinical EAMG development and had suppressed anti-AChR levels compared to wild-type mice (Balasa et al., 1997). IFN-γ receptor-deficient mice also had a reduced incidence of EAMG, and the resistance was due to low levels of Th1 AChR-specific IgG (Zhang et al., 1999). Unlike those studies, Karachunski *et al* showed that IFN-γ-deficient mice in the pure H-2^b background developed EAMG normally and had complement-fixing anti-AChR levels comparable to control mice.

The proinflammatory cytokine TNF-α is a pleiotropic mediator produced primarily by activated macrophages and is noted for its ability to promote inflammation, apoptotic cell death, and T and B cell differentiation (Eigler et al., 1997). TNF receptor-deficient (TNFR $p55^{-/-}p75^{-/-}$) B6 mice were highly resistant to the development of EAMG and displayed defects in lymphocyte proliferation to AChR and α146-162 and anti-AChR IgG production of all subclasses (Goluszko et al., 2002). B6 mice with moderately severe EAMG mice were treated for two weeks with soluble recombinant human TNFR:Fc, and the clinical status of the mice improved significantly compared to placebo-treated mice (Christadoss and Goluszko, 2002). TNF-α also promotes macrophages to release the proinflammatory mediator IL-6, which promotes the differentiation of B cells into plasma cells and regulates C3 production (Kopf et al., 1998; Taga and Kishimoto, 1997). TEC in MG patients overproduce IL-6, which in coordination with abnormalities in the TNF gene resulting in TNF-α hyperproduction, could be a factor in thymic hyperplasia (Cohen-Kaminsky et al., 1993b). IL-6 deficient B6 mice had marked defects in IgG class switching, AChR-induced lymphocyte proliferation and Th1 and Th2 cytokine production, lymphoid germinal center formation, and C3 synthesis (Deng et al., 2002). The contributions of these resultant defects rendered the IL-6-deficient mice largely resistant to EAMG. Together, IL-6 and TNF-α cooperate to drive both the adaptive and innate arms of the immune system to promote EAMG development.

The Th2 cytokine IL-10 has a broad range of functions, including anti-inflammatory activity, downregulation of Th1 responses, and B cell differentiation. IL-10 also plays an important role in driving EAMG development, because IL-10-deficient B6 mice were moderately resistant to EAMG. However, there was not a significant alteration of AChR-specific IgG levels. Rather, the resistance of these mice was associated with decreased complement-mediated cytotoxic potential of serum IgG and decreased lymph node B cells and MHC class II^+ cells (Poussin et al., 2000). Transgenic B6 mice with T cells that overproduce IL-10 are more susceptible to EAMG and have lower lymphocyte production of IFN-γ and normal serum anti-AChR IgG levels (Ostlie et al., 2001). Furthermore, recombinant IL-10 treatment caused an earlier disease onset and augmented clinical symptoms in Lewis rats (Zhang et al., 2001). Thus, Th2 cytokines with anti-inflammatory properties, such as IL-10, play a direct role in mediating EAMG development.

IL-4 is another Th2 cytokine that is anti-inflammatory mediator and is a growth factor for TGF-β-secreting suppressor T cells. Two separate studies demonstrated that the absence of IL-4 in B6 mice results in enhanced Th1-associated IgG_{2a} and reduced Th2-associated IgG_1 following immunization with AChR (Balasa et al., 1998; Milani et al., 2003). The lack of IL-4 has been shown not to alter the incidence of EAMG (Balasa et al., 1998), but resulted in a chronic form of the disease following only one immunization with AChR in CFA (Ostlie et al., 2003). The protective role for IL-4 in EAMG was further supported when nasal tolerance by administration of *Torpedo* AChR peptides did not protect IL-4 deficient B6 mice from anti-AChR production and clinical EAMG compared to peptide-tolerized B6 mice (Karachunski et al., 1999).

THE CD28 FAMILY OF COSTIMULATORY MOLECULES

The "two-signal model" of T cell activation holds that the TCR antigen-specific signal by itself is insufficient, so a second "costimulatory" signal is necessary for optimal T cell activation (Bretscher and Cohn, 1970). TCR stimulation without a second costimulatory signal renders the T cell anergic, or unable to respond upon re-exposure to

the original antigen (Schwartz, 1990). Many costimulatory molecules have been discovered since the characterization of the prototypic costimulatory molecule pair consisting of CD28, which is expressed by T cells, and B7, which is expressed by APCs. These costimulatory molecule pairs provide both positive and negative signals to the activated T cell, the balance of which determines the fate of the ensuing T cell response. Numerous therapeutic approaches utilizing costimulatory molecule blocking or inducing reagents have been aimed at restoring costimulatory "balance" to abnormal conditions such as that observed in autoimmunity.

The CD28/B7/CTLA-4 pathway

Members of the CD28 family are type I transmembrane glycoproteins in the immunoglobulin superfamily. CD28 is constitutively expressed by $CD4^+$ T cells, but CTLA-4 expression by T cells is rapidly expressed following activation and reaches a maximum at 48 to 72 hours following cell cycle entry. B7-2 is expressed constitutively at low levels on APCs and rapidly upregulated following activation, and B7-1 expression is induced after B7-2. The affinity of CTLA-4 for B7 is far greater than that of CD28. Ligation of CD28 by B71 or B72 results in T cell proliferation through the transcription and secretion IL-2, increased cell survival by the upregulation of the anti-apoptotic molecule Bcl-x_L, Th1 and Th2 differentiation, and enhanced B cell activation for antibody production. However, CTLA-4 delivers signals that inhibit IL-2 synthesis and cell cycle progression (Rothstein and Sayegh, 2003; Wang and Chen, 2004). Costimulation through CD28 thus appears to be involved in the initiation, but not reactivation, of immune responses, because naïve T cells are dependent on IL-2 as a growth factor, and the activation threshold (and requirement for costimulatory signals) are lower for the reactivation of memory T cells (Salomon and Bluestone, 2001).

TCR ligation by peptide-MHC class II activates protein tyrosine kinases that phosphorylate intracellular scaffolding proteins (LAT, Vav, SLP-76) necessary for the recruitment and activation of signaling molecules. Phospholipase C-γ1 (PLCγ1) and Grb-2:SOS are signaling molecules that ultimately lead to the activation of the transcription

factors, nuclear factor of activated T cells (NFAT), activator protein-1 (AP-1), and nuclear factor κB (NK-κB), which lead to IL-2 production and cell proliferation. CD28 ligation by B7 causes the recruitment and activation of Vav and phosphoinositide 3-kinase (PI3K). PI3K leads to the activation of PIP_2 and then PIP_3, which recruits other signaling proteins to the cell membrane, and Vav activates rac-1, a GTPase that enhances PI3K function. Through the actions of rac-1, cytoskeletal rearrangements occur that allow clustering of the TCR and lipid rafts in the central supramolecular activation complex (C-SMAC).

The CD28/B7 family plays an important role in maintaining Th1/Th2 balance. CD28 ligation induces IL-4 and IL-5 production, and the absence of CD28 costimulation results in a defect in IL-4, IL-5, and IL-10 production, but no defect in IFN-γ production (Rogers and Croft, 2000; Rulifson et al., 1997; Salomon and Bluestone, 1998; Schweitzer and Sharpe, 1998; Seder et al., 1994). NOD mice, which spontaneously develop diabetes due to infiltration of pathogenic Th1 cells into the pancreatic islets, are protected by treatment with stimulatory mAb to CD28, resulting in a predominantly Th2 infiltration into the islets (Arreaza et al., 1997), but a lack of CD28 costimulation augmented insulitis substantially as a result of defective differentiation of protective Th2 cells (Lenschow et al., 1996). In the NZB/NZW mouse strain that develops lupus-like disease, CTLA-4Ig or anti-B7 mAbs prevented disease development by inhibiting Th2 differentiation and the production of autoantibodies (Borriello et al., 1997; Finck et al., 1994; Linsley et al., 1992; Nakajima et al., 1995).

Costimulation through B7-1 or B7-2 can have differential outcomes on the immune response. EAE is an animal model of the demyelinating autoimmune disease multiple sclerosis (MS) and is mediated primarily by $CD4^+$ Th1 cells. Early treatment of anti-B7-1 with mAb prevented EAE due to a reduction of autoimmune $CD4^+$ T cells (Cross et al., 1995; Oliveira-dos-Santos et al., 1999), a shift toward the development of protective Th2 cells (Kuchroo et al., 1995), or the induction of anergy (Girvin et al., 2000). However, later during disease, the anti-B7-1 mAb treatment exacerbated disease (Vanderlugt et al., 1997) while anti-B7-1 Fab fragments blocked disease progression

(Miller et al., 1995). Anti-B7-2 treatments either had no effect (Racke et al., 1995) or augmented disease severity (Kuchroo et al., 1995). These results indicate that the CD28/B7 pathway can drive the cytokine balance to promote autoimmunity or protect from it.

The induction of peripheral tolerance requires CTLA-4 engagement by B7 molecules (Perez et al., 1997), and CTLA-4 deficiency in mice results in large polyclonal $CD4^+$ T cell expansion due to defective tolerance to peripheral autoantigens that results in early death of the animals (Tivol et al., 1995; Waterhouse et al., 1995). Anti-CTLA-4 mAb treatment exacerbates EAE in mice (Hurwitz et al., 1997; Karandikar et al., 1996; Perrin et al., 1996), increases the spreading of EAE relapse-associated epitopes (Karandikar et al., 2000), and exacerbates diabetes development when administered early (Luhder et al., 1998), indicating the suppressive role for CTLA-4 in autoimmunity. The mechanisms by which CTLA-4 inhibits T cell activation is somewhat unclear, but competition with CD28 for B7 is one proposed mechanism, as well as raising the TCR signaling threshold. CTLA-4 inhibits lipid raft formation the localization of LAT and TCRζ on the rafts and through its cytoplasmic tail, interacts with several signaling inhibitors, such as SHP-2 and PP2A (Rothstein and Sayegh, 2003; Rudd and Schneider, 2003). Furthermore, populations of regulatory T cells that prohibit autoimmunity constitutively express CTLA-4 (Salomon et al., 2000; Takahashi et al., 2000), CTLA-4 induces the secretion of suppressive cytokines, (Chen et al., 1998), and CTLA-4 raises the activation threshold of autoreactive T cells and may act independently of regulatory T cells (Eggena et al., 2004).

In humans, CTLA-4 gene polymorphisms have been associated with diabetes, thyroiditis, and rheumatoid arthritis (Awata et al., 1998; Lee et al., 2003; Marron et al., 2000), and soluble CTLA-4 and natural antibodies to CTLA-4 have been documented in autoimmune diseases (Matsui et al., 1999; Oaks and Hallett, 2000). The CTLA-4:Ig fusion protein was an effective therapy for lupus-prone B/W mice by effectively reducing immune complex deposition and lymphocyte infiltration in the kidneys (Cunnane et al., 2004). CTLA-4:Ig (abatacept) is currently in phase III clinical trials for the treatment of

rheumatoid arthritis, multiple sclerosis, and systemic lupus erythematosus (Dumont, 2004).

A recent study found that B7 was expressed by thymoma APCs of MG patients (Romi et al., 2002). Moreover, MG patients had increased B7-1^+ and B7-2^+ peripheral T helper cells and observed a direct correlation between the patients' age and the number of circulating B7-2^+ cells (Teleshova et al., 2000). Mice deficient in CD28 were less susceptible to EAMG and displayed a switch to a Th1 profile, characterized by an increase in IFN-γ-secreting cells and a decrease in AChR-specific IgG_1 production, suggesting that CD28 signaling is more important for Th2 differentiation in EAMG. In comparison, CD40L-deficient mice were completely resistant to EAMG and had even lower levels of AChR-specific IgG, as well as defects in both Th1 and Th2 cytokine production by lymphocytes (Shi et al., 1998). In contrast, mice genetically deficient in B7-1 were found to be highly resistant to EAMG development (0% incidence) and more resistant than B7-2-deficient mice (56% incidence). Compared to B7-2 deficient mice, B7-1-deficient mice also had a larger reduction in anti-AChR IgG production, as well as lymphocyte proliferation and IFN-γ and IL-10 production induced by AChR and α146-162 (Poussin et al., 2003).

CTLA-4 polymorphisms have been linked to MG (Huang et al., 2000a; Huang et al., 1998a; Wang et al., 2002c). Decreased T cell expression of CTLA-4 have been observed, and increased serum soluble CTLA-4 correlated with serum anti-AChR levels (Wang et al., 2002b). Anti-CTLA-4 treatment enhances T cell and antibody responses to AChR in B6 mice and causes a rapid onset of severe EAMG following AChR immunization due to diversification of the anti-AChR antibody repertoire and T cell epitope spreading (Wang et al., 2001). When rats were treated with CTLA-4Ig, anti-AChR production was stunted, and there was a shift in production from IgG_2 to IgG_1, suggesting that CTLA-4Ig blocked Th1 differentiation to AChR. Treatment during the acute phase of disease fully prevented EAMG while treatment during the chronic phase resulted only in partial disease prevention (McIntosh et al., 1998).

The ICOS/ICOSL pathway

ICOS is a member of the CD28 receptor family due to its high sequence homology and structural similarity to both CD28 and CTLA-4, and it is expressed by human and murine $CD4^+$ T cells only following TCR ligation (Hutloff et al., 1999; Wang et al., 2002a) and appears within 12 hours of TCR activation and reaches maximum expression at 48 hours (Hutloff et al., 1999; Mages et al., 2000; Wang et al., 2002a). It appears that ICOS expression is at least partially dependent upon prior T cell costimulation through CD28. ICOS expression by activated T cells from B7-1-deficient and B7-2-deficient mice was significantly reduced, but could be restored following anti-CD28 stimulation (McAdam et al., 2000). Furthermore, B7-2 blockade strongly inhibited ICOS expression by human $CD4^+$ T cells (Beier et al., 2000). The ICOS ligand, ICOSL (B7RP-1, B7h) is constitutively expressed by B cells, macrophages, and dendritic cells, although proinflammatory cytokines enhance its degree of expression (Aicher et al., 2000; Swallow et al., 1999; Yoshinaga et al., 1999). Though ICOSL is a type I transmembrane protein that shares amino acid sequence and structural identity to B7-1 and B7-2, it is not promiscuous, but forms a distinct receptor-ligand pair with ICOS (Hutloff et al., 1999; Swallow et al., 1999; Wang et al., 2002a; Witsch et al., 2002; Yoshinaga et al., 1999). In the secondary lymphoid tissues, ICOS expression is confined to the T cell-rich area of the GC (Hutloff et al., 1999), and ICOSL gene expression is localized to the B cell-rich follicle of the GC (Yoshinaga et al., 1999). The primary characteristics of ICOS-deficient mice are defective GC development and IgG class switching following immunization with protein antigen (Dong et al., 2001b; Tafuri et al., 2001). ICOSL-deficient mice also have defective IgG class switching following immunization with protein antigen, and ICOSL-deficient APC were poor inducers of T cell proliferation (Mak et al., 2003; Nurieva et al., 2003b).

Initial evidence suggested that costimulation through ICOS predominantly drives the generation of Th2 immunity. Stimulation through ICOS primarily enhanced the production of IL-4 and IL-10, but not IL-2, and ICOS antagonists caused biased Th1 differentiation (Hutloff et al., 1999; McAdam et al., 2000). ICOS blockade in mice

inhibited Th2-mediated, but not Th1-mediated, lung inflammation following adoptive transfer of antigen-specific effector cells (Coyle et al., 2000) and prevented Th2-mediated allergic airway disease (Tesciuba et al., 2001). ICOS deficient mice displayed defective Th2 humoral immunity marked by low levels of IgG_1 and IgE, as well as IL-4, and were more susceptible to Th1-mediated EAE (Dong et al., 2001a; Dong et al., 2001b; McAdam et al., 2000; Tafuri et al., 2001).

ICOS also plays an important role in driving Th1 differentiation. In the initial characterizing studies, ICOS costimulation not only promoted Th2 differentiation, but it also induced IFN-γ, TNF-α, and IL-2 production by T cells (Hutloff et al., 1999; Riley et al., 2001; Yoshinaga et al., 1999). In a more recent study, ICOS-ICOSL interaction was necessary for the priming of both Th1 and Th2 cells and humoral immunity development following immunization, but was not necessary for the migration of T cells into the B cell follicles of secondary lymphoid tissues (Smith et al., 2003). ICOS blockade with antibody or Ig fusion protein protected cardiac allografts from rejection in mice by reducing infiltrating $CD4^+$ and $CD8^+$ T cells and suppressing intragraft IFN-γ and chemokines (Ozkaynak et al., 2001). ICOS inhibition with ICOS-Ig prevented the development of EAE by increasing the apoptosis of autoreactive TCR-transgenic T cells and blocking T cell proliferation and production of IFN-γ and IL-10 (Sporici et al., 2001). ICOS-deficient mice were resistant to the development of collagen-induced arthritis (CIA) in mice, displayed reduced anti-collagen IgM and IgG_{2a}, and had reduced production of IL-17, an inflammatory cytokine commonly found in the synovium of RA patients that is produced by memory $CD4^+$ T cells and promotes osteoclastic bone reabsorption (Nurieva et al., 2003c). When ICOSL was blocked *in vivo*, CIA was prevented, and established disease was ameliorated due to decreased expression of synovium TNF-α, IL-1β, and IL-6 and reduced serum anti-collagen IgG_1, IgG_{2a}, and IgG_{2b}. Collagen-induced lymph node proliferation and secretion of IFN-γ and IL-10 were inhibited (Iwai et al., 2002).

Similar to B7 molecules, ICOS may have differential effects on the outcome of some autoimmune diseases. ICOS blockade early (days 1-10) after PLP immunization

caused Th1 polarization, enhanced CNS expression of proinflammatory cytokines and chemokines, and augmented leukocyte infiltration into the brain resulting in exacerbation of EAE. In contrast, ICOS blockade during the efferent phase (days 9-20) abrogated disease and T cells produced less IFN-γ production (Rottman et al., 2001). Anti-ICOS treatment accelerated the cell-mediated immune response of Th1-driven acute graft-vs.-host-disease (GVHD), but attenuated Th2-driven chronic GVHD (Oaks and Hallett, 2000).

Earlier reports of the Th2-skewed differentiation by ICOS during primary or secondary immune responses may be due to shared downstream signaling pathways with CD28, which drives Th2 differentiation by naïve T cells (Rudd and Schneider, 2003). The cytoplasmic tail of ICOS possesses a pYMNM motif similar to CD28 that recruits and binds the lipid kinase, PI3K (Coyle et al., 2000), but compared to CD28, ICOS was a much more potent activator of the PI3K-induced serine/threonine protein kinase Akt (Arimura et al., 2002). Interestingly, active Akt was expressed in higher levels by BALB/c mouse T cells than by B6 T cell cells. ICOS expression was much higher on Th2 cells than Th1 cells, and Th2 cells are more numerous in BALB/c mice (15%) than in B6 mice (7%) (Arimura et al., 2002; Coyle et al., 2000). The strain differences in Th2 differentiation and ICOS expression may be due to either high expression of the GATA-3 and c-maf transcription factors by Th2 cells, which increase ICOS expression or the Th2 transcription factor NF-ATc$_1$, which is increased in BALB/c mice and is important for ICOS-dependent IL-4 production (Arimura et al., 2004; Nurieva et al., 2003a; Rodriguez-Palmero et al., 1999). Despite these differences, however, phosphorylated Akt strongly promoted either Th1 or Th2 differentiation, depending on the local cytokine milieu at the time of activation (Arimura et al., 2004).

A homozygous deletion of the ICOS gene in humans has been associated with combined variable immunodeficiency (CVID), which is an inherited autosomal recessive disease characterized by panhypogammaglobulinemia (Grimbacher et al., 2003). The ICOS-deficient T cells from the four patients displayed normal subset distribution, activation, proliferation, and cytokine production, but there was a significant reduction in

naïve, switched, and $CD27^+$ memory B cells due to inadequate T cell help. In a more recent study, a homozygous ICOS deficiency was again found in CVID patients, but no abnormalities in the ICOSL gene were found (Salzer et al., 2004).

CD28 family members, including ICOS, have recently been implicated in the pathogenesis of human disorders. Genes for ICOS, CD28, and CTLA-4 are found on human chromosome 2q33 and are candidate susceptibility genes for type I diabetes due to their proximity with the type I diabetes susceptibility locus, *IDDM12* (Coyle et al., 2000; Marron et al., 2000; Nistico et al., 1996). Polymorphisms of the CTLA-4 and CD28 genes, but not the ICOS gene, were associated with type I diabetes in a Japanese population (Ihara et al., 2001). Similarly the murine CTLA-4 and ICOS genes are found on chromosome 1 within the type I diabetes susceptibility locus, *Idd5.1*. NOD.B10 *Idd5.*1 congenic mice were resistant to the development of diabetes, but developed a higher incidence of EAE than NOD mice. Activated T cells from NOD mice expressed higher levels of ICOS and produced more IL-10 than control B6 or B10 mice, which may explain the lower severity of EAE in NOD mice compared to *Idd5.1* congenic mice since IL-10 is protective in EAE (Greve et al., 2004).

ICOS has also been implicated in SLE, which is characterized by hyperactivation of the humoral immune system resulting in hypergammaglobulinemia and antibodies directed toward a number of autoantigens. An increased expression of ICOS was found on $CD4^+$ and $CD8^+$ T cells in patients with SLE, but there was a decrease in ICOSL expression by memory B cells. Clusters of B cells and plasma cells were also found in contact with $ICOS^+$ T cells in the patients of SLE patients, suggesting that ICOS is important for driving the development of autoreactive memory B cells and plasma cells in SLE, possibly in the periphery (Hutloff et al., 2004). Similarly, ICOS expression was increased on mononuclear cells from the inflamed mucosa of patients with inflammatory bowel disease (IBD), but not inflammatory or normal controls (Sato et al., 2004). ICOSL expression was upregulated on B cells, macrophages, and epithelial cells in the inflamed mucosa of IBD patients. In patients with rheumatoid arthritis, $ICOS^+$ T cells (both $CD4^+$ and $CD8^+$) were increased in the synovium and peripheral blood compared to controls,

and ICOS costimulation increased IFN-γ, IL-4, and IL-10 production by synovial fluid $CD4^+$ T cells (Okamoto et al., 2003).

Regulatory T cells are crucial for maintaining peripheral tolerance to autoantigens, and defects in T_{reg} function have been linked to autoimmunity in experimental models. In diabetes-prone mice, high ICOS and IL-10 expression were common on actively dividing T_{regs} within the pancreatic lesion prior to disease onset, and ICOS blockade resulted in the rapid progression from insulitis to diabetes due to a imbalance in T_{reg} activity (Herman et al., 2004). $ICOS^{hi}$ T_{regs} that secreted high levels of IL-10 were also observed in experimental models of Th1-mediated diabetes and Th2-mediated allergic airway hyperactivity in mice, and ICOS blockade abrogated their protective ability (Akbari et al., 2002; Kohyama et al., 2004). However, in a murine model of colitis, ICOS and CD28 collaborated in the pathogenic function of Th1 cells, whereas only CD28 was important for T_{reg} activity (de Jong et al., 2004).

SUMMARY AND SPECIFIC AIMS

In MG, AChR peptides are presented by APC to $CD4^+$ T helper cells in an MHC class II-restricted manner, and the cognate APC-T helper cell interaction is crucial to the development of EAMG. The exchange of costimulatory signals between APCs and lymphocytes are vital to the induction of EAMG. Although the importance of the CD28/B7 and CD40L/CD40 pathways have been established in EAMG, the regulation of the anti-AChR immune response by recently discovered members of the CD28 family members remain unknown. Therefore, we have characterized the contributions of the ICOS/ICOSL costimulatory pathway to the development of the humoral immune response to AChR that leads to clinical EAMG. Further, through the use of *in vivo* monoclonal antibody administration, we compared the impact of ICOS/ICOSL-delivered costimulation to that of CD28/B7-1 and CD28/B7-2.

Aim 1. Characterize how ICOS/ICOSL costimulation regulates the development of EAMG

ICOS has been shown to be a key mediator in the induction of T cell-dependent humoral immunity to protein antigens and have a strong impact on T helper cell differentiation. Following immunization with AChR, secondary lymphoid organs from normal and ICOS-deficient mice were analyzed for evidence of the germinal center reaction, and lymph node cells were examined by flow cytometry and restimulated *in vitro* for an assessment of AChR-induced proliferation and cytokine secretion. Mice were evaluated for clinical symptoms of EAMG, and the anti-AChR antibody response and neuromuscular junction pathology were measured.

Aim 2. Compare the effects of ICOS/ICOSL and CD28/B7 costimulation on T cell and humoral immunity to AChR

To our knowledge, there has not been an *in vivo* comparison of how ICOSL and B7 costimulatory signals regulate the development of humoral immunity. Furthermore, we do not know how blockade of these pathways would affect the outcome of an antibody-mediated autoimmune disease such as EAMG. To answer these questions, B6 mice were immunized twice with AChR, and groups were treated during both the primary and secondary immune responses with (1) control, (2) anti-ICOSL, (3) anti-B7-1, or (4) anti-B7-2 therapies. Mice were evaluated for clinical EAMG, and IgG production and class switching were analyzed. The effects of ICOSL and B7 costimulation on germinal center formation, lymphocyte proliferation, and Th1/Th2 cytokine production were also compared.

CHAPTER 2

MATERIALS AND METHODS

EAMG INDUCTION AND *IN VIVO* TREATMENTS

AChR purification and peptide synthesis

AChR was purified from the *Torpedo californica* electric organ with an α-neurotoxin affinity column (Lindstrom et al., 1981; Wu, 1997). The *Torpedo* AChR α-subunit peptide 146-162 was synthesized by the Synthetic Antigen Laboratory, The University of Texas M.D. Anderson Cancer Center, Houston, TX.

Ascites preparation and quality control

The anti-ICOSL mAb-producing hybridoma (clone HK 5.3) was provided by Dr. Hideo Yagita (Juntendo University, Tokyo, Japan). HK5.3 mAbs are rat IgG_{2a} isotypes and were previously shown to block the ICOS-ICOSL interaction leading to the prevention and amelioration of collagen-induced arthritis in mice (Iwai et al., 2002). The anti-B7-1 (clone 1G10) and anti-B7-2 (clone GL-1) hybridomas were provided by Dr. Arlene Sharpe (Harvard University, Boston, MA). The 1G10 and GL-1 mAbs are rat IgD isotypes and have been previously shown by our laboratory to block B7-1 and B7-2 costimulation during the primary immune response to AChR. Frozen HK5.3, IG10, and GL-1 hybridoma cell lines were provided to Abnova Corporation (Taipei City, Taiwan) for the production of ascites fluid in T and B cell-deficient SCID mice. Following production, ascites fluid were frozen and shipped to UTMB, thawed and centrifuged at 2,000rpm, and supernatants were frozen at -20°C until use.

Ascites fluid was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to verify the presence of the heavy and light chains of IgG under reducing conditions according to the procedure of Weber and Osborn (1969) and Laemmli (1970) (Laemmli, 1970; Weber and Osborn, 1969). 10 μl of the ammonium sulfate-precipitated and PBS-dialyzed ascites fluid (1mg/ml) was heated at 95 °C for 2

minutes along with an equal amount of 2X SDS sample buffer (16 mM Tris-HCl, pH 6.8, 2% SDS, 5% glycerol, and 0.01% bromphenol blue). The samples were loaded onto a 12% mini gel (Bio-Rad Laboratories Hercules CA) and were run for 1hr at 100 Volts using SDS-PAGE bufffer (25mM Tris, 192mM glycine, 0.1%SDS). Broad range molecular weight markers (Myosin, b-glycosidase, Bovine serum albumin, oval albumin carbonic anhydrase, soybeen trypsin inhibitors and aprotinin) from Bio-Rad Laboratories (Cat # 161-0318) were run along with the samples. Proteins were identified by staining the gel with Coumassie Blue staining solution (0.2% Coumassie Blue in 10% acetic acid and 40% methanol) for 2 hrs and excessive stain on the gel was removed from the gel by treating the gel with destaining solution (10% acetic acid and 40% methanol) for 2-4 hrs with 2-4 changes of the fresh solution.

To verify the binding of ascites fluid IgG to target antigens, a flow cytometry strategy was developed. To test the anti-ICOSL ascites, $1X10^6$ ICOSL-transfected L5178Y cells were suspended per tube in PBS-5% FBS and incubated for 20 minutes at 4°C with Fc-Block (BD Pharmingen), according to the manufacturer's recommendations, to eliminate non-specific binding with subsequent antibodies. Following a wash with PBS-5% FBS, the cells were incubated with 250 μl of PBS-5% FBS or anti-ICOSL ascites fluid for 20 minutes at 4°C. After washing, PBS-incubated cells were left unstained (unstained control), incubated with PE-conjugated anti-ICOSL (eBioscience) as a positive control, or treated with FITC-conjugated goat anti-rat IgG_{2a} as a negative control (BD Pharmingen), all according to the manufacturer's recommendations. Ascites-incubated cells were followed by treatment with FITC-conjugated goat anti-rat IgG_{2a} (BD Pharmingen). Cells were washed and suspended in PBS containing 1% paraformaldehyde and examined in the UTMB Flow Cytometry Core Facility on a Becton Dickinson FACSCanto. FlowJo software was used to analyze data and create histograms.

To verify the binding of anti-B7-1 and anti-B7-2 ascites, $1X10^6$ AChR-immune LNC from B6 mice were suspended per tube in PBS-5% FBS, incubated with Fc-Block, and washed. In a competitive binding assay, LNC were first incubated with ascites,

followed, washed, and then incubated with FITC-conjugated anti-B7-1 or B7-2 (BD Pharmingen). Control samples consisted of LNC left unstained, or LNC incubated directly with FITC-conjugated anti-B7-1 or anti-B72 (positive control). Experimental conditions are as described above, and samples were analyzed on a Becton Dickinson FACSCanto. FlowJo was used to analyze data and create histograms. Positive binding by ascites were interpreted as the ability of ascites to significantly prevent the binding of FITC-conjugated antibodies as compared to positive control values.

Mice

C57BL/6 mice were purchased from the Jackson Laboratory, Bar Harbor, ME. $ICOS^{-/-}$ breeding mice in the C57BL/6 background were previously described (Dong et al., 2001a). All animals were housed in the viral antibody-free barrier facility at the University of Texas Medical Branch according to Institutional Animal Care and Use Committee guidelines.

EAMG induction and clinical evaluation

For all *in vivo* experiments, mice were anesthetized and immunized on day 0 with 20 μg of AChR emulsified in CFA (Difco, Detroit, MI) s.c. at four injection sites (hind footpads and front shoulders). For clinical experiments, mice were immunized again on day 28 with 20 μg of AChR emulsified in CFA s.c. at four injection sites (front and hind shoulders). Mice were observed, and clinical EAMG was classified according to the following scale: grade 0, mouse with normal posture, muscle strength, and mobility; grade 1, normal at rest, but muscle weakness characterized by hunched posture, restricted mobility, and difficulty raising the head following exercise consisting of 30 consecutive paw grips on wire cage top; grade 2, symptoms equivalent to grade 1 without prior exercise; grade 3, dehydrated and moribund with grade 2 muscle weakness.

In vivo costimulatory molecule blockade strategy

In the pilot experiment to test the efficacy of the ascites fluid *in vivo*, 15 B6 mice were immunized s.c. with 20 μg of AChR emulsified in CFA on day 0 and divided into

groups consisting of the following numbers: (A) 6 control PBS-injected, (B) 3 anti-ICOSL injected, (C) 3 anti-B7-1 injected, and (D) 3 anti-B7-2 injected. On days -2, 1, 4, and 7, groups B-D were injected i.p. with 600 μl ascites fluid. Mice were euthanized on day 10 for experimental purposes.

To compare the effects of blockade of ICOSL, B7-1, and B7-2 on the development of EAMG, 40 B6 mice were immunized s.c. on days 0 and 28 with 20 μg of AChR emulsified in CFA, and 5 B6 mice were immunized with CFA alone as negative control animals. AChR-immunized mice were divided into groups consisting of the following numbers: (A) 10 rat IgG injected, (B) 10 anti-ICOSL injected, (C) 10 anti-B7-1 injected, and (D) 10 anti-B7-2 injected. On days -2, 1, 4, and 7 (primary immune response) and days 26, 29, 32, and 35 (secondary immune response), mice in group A were injected i.p. with 100 μg of rat IgG (100 μl PBS), and mice in groups B-D were injected i.p. with 500 μl ascites fluid. Mice were euthanized on day 28 for experimental purposes.

CHARACTERIZATION OF THE ANTI-ACHR HUMORAL IMMUNE RESPONSE

Germinal center staining

Germinal center staining on secondary lymphoid tissues was performed by the UTMB Histopathology Service Core. Four-micrometer-thick sections of paraffin-embedded spleens were prepared. Sections were de-paraffinized and rehydrated. Endogenous peroxidase activity was quenched with 3% H_2O_2 in PBS. Sections were blocked for nonspecific binding with normal goat serum diluted 1/20 in DAKO Ab diluent for 15 min. Ag retrieval was done with DAKO Target Retrieval Solution in steam for 20 min. Sections were then cooled down on the bench top for 20 min, rinsed two to three times with distilled water, and transferred to TBS. Slides were then incubated for 30 min with peanut agglutinin (PNA)-biotin (Vector Laboratories, Burlingame, CA) diluted 1/250 in DAKO Ab diluents and washed, followed by a second incubation with

streptavidin-HRP for the LSAB2 system (KO675). Bound conjugates were visualized with DAKO Liquid diaminobenzidine substrate-chromagen for 5 min when a brown color for PNA-positive cells was obtained. For all staining steps, a DAKO Autostainer was used. Slides were counterstained for 2 min with Mayer's modified hematoxylin diluted 1/5 in distilled water.

Radioimmunoassay for anti-AChR IgG Measurement

Sera were collected from mice following both the first and second immunizations with AChR in CFA. Mouse AChR-specific IgG was measured by RIA according to a previously described method (Lindstrom et al., 1981, Wu et al., 1997). Crude mouse muscle extract was incubated in Triton buffer with [^{125}I]α-BTX (5 x 10–9 M; Amersham Life Sciences Inc., Arlington Heights, Illinois, USA) for 1 hour. One μl of serum of each mouse was added. Normal mouse serum (bled from mice before first immunization) served as control. After overnight incubation at 4°C, goat anti-mouse IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, Pennsylvania, USA) was added to 1 ml of [^{125}I]α-BTX–labeled AChR. After 4 hours, the tubes were centrifuged, and the pellets were washed with 1 ml of Triton buffer, centrifuged again, and counted in a gamma counter. For each mouse, the AChR precipitated (minus control values) serum IgG amounts were expressed in nanomoles (nM) of α-BTX–binding sites bound per 1 L of serum.

ELISA for the measurement of anti-AChR IgG isotype switching

Affinity-purified mouse AChR (0.5 μg/ml) was coated onto 96-well microtiter plates (Dynatech Immulon 2, Dynatech Labs, Chantilly, VA) with 0.1 M carbonate bicarbonate buffer (pH 9.6) overnight at 4 °C. The plates were blocked with 2% BSA in PBS (pH 7.0) at room temperature for 30 min. Serum samples diluted 1:1,000 in PBS/0.05% Tween were added and incubated at 37°C for 90 min. After four washes, horseradish peroxidase-conjugated (HRPO) goat anti-mouse IgM, IgG, IgG_1, or IgG_{2b} (Caltag, San Francisco, CA) diluted 1:1,000 in PBS/0.05% Tween were added and incubated at 37 °C for 90 min. Subsequently, ABTS (indicator) solution in 0.1 M citric

buffer pH 4.3 in the presence of H_2O_2 was added, and color was allowed to develop at room temperature in the dark. For the detection of IgG_{2c}, biotinylated anti-mouse IgG_{2a}b (clone 5.7, BD Biosciences, San Diego, CA) diluted 1:1,000 in PBS/0.05% Tween was added after serum sample addition and washing for 45 min. at room temperature in the dark. After washing, 1:400 of 1mg/ml avidin-peroxidase was added and incubated in the dark, at room temperature for 30 min. After washing, ABTS solution in the presence of H_2O_2 was added, and color was allowed to develop. Absorbance values were measured at a wavelength of 405 nm using a Molecular Devices Emax, and the results were expressed as O.D. values.

Neuromuscular junction pathology

Mouse forelimb muscles were flash-frozen in liquid nitrogen and stored at -80°C. Five ten μm-thick sections from each mouse were air-dried, fixed in cold acetone for 10 min, and washed with PBS. To detect NMJ, sections were incubated with 1:100 tetramethylrhodamine-conjugated α-bungarotoxin (BTX) (Molecular Probes, Eugene, Oregon) for 1 hr at room temperature. To detect IgG or complement deposits at the NMJ, sections were then incubated for 1 hr at room temperature in the presence of FITC-conjugated goat anti-mouse IgG diluted 1:200 (Chemicon International, Temecula, CA), FITC-conjugated goat anti-mouse complement C3 diluted 1:200 (ICN-Cappel, Aurora, OH), or purified rabbit anti-human C5b-9 (MAC), which is cross-reactive with mouse MAC, diluted 1:200 (Calbiochem, San Diego, CA). To detect MAC deposits, sections were further incubated with Oregon Green-conjugated goat anti-rabbit IgG (Molecular Probes, Eugene, OR) (1:500). Sections were washed and viewed with an Olympus IX-70 fluorescence microscope and photographed with a DP-11 digital camera.

CHARACTERIZATION OF THE ANTI-ACHR CELLULAR IMMUNE RESPONSE

Flow cytometry analysis of lymph node cells

In all experiments, freshly isolated lymph node cells from individual mice were suspended in PBS-5% FBS and kept on ice at a concentration of $5x10^6$ cells/ml. 100 μl of cell suspensions were added to FACS tubes already containing 100 μl of PBS-5% FBS and the manufacturer's recommended concentration of FITC-, PE-, or PerCP-Cy5.5-conjugated mAb(s). For single- or double-staining experiments, FACS tubes contained any one or two, respectively, of the appropriate flourochrome-conjugated antibodies from BD Biosciences, including rat anti-mouse I-A^b, CD4, CD19, B7-1, and B7-2, and from eBioscience, rat anti-mouse ICOS and ICOSL. LNC were incubated with antibodies for 20 minutes at 4°C in the dark and then washed twice with 2ml PBS-5% FBS. LNC were in a final PBS containing 1% paraformaldehyde and examined in the UTMB Flow Cytometry Core Facility on a Becton Dickinson FACScan. Data analysis was performed with CellQuest software.

Lymphocyte proliferation assay

Seven days following AChR immunization or at the conclusion of clinical experiments, mice were euthanized and draining axillary, inguinal, and popliteal lymph nodes were collected. Lymph node cells (LNC) ($4x10^5$ cells/well) were seeded in triplicate onto 96-well, flat-bottomed microtiter plates in 0.2 ml of RPMI 1640 with and without AChR (2.5 μg/ml) or the AChR α–subunit peptide α146-162 (20 μg/ml). For *in vitro* costimulation blocking experiments, various dilutions (0.625 μg/ml – 10.0 μg/ml) of commercially available blocking Abs to ICOS (eBioscience) and various dilutions (1/16 – 1/2) of anti-B7-1 (clone 1G10), anti-B7-2 (clone GL-1), and mouse IgD (clone 11-26C) hybridoma culture supernatants (Arlene Sharpe, Harvard University, Boston, MA) were added to 96-well cultures at the time LNC were seeded. After 4 days, cells were pulsed

with [^{3}H] TdR (1 μCi/well) and harvested 18 hours later. ^{3}H incorporation was measured with a Beckman LS 6500 β scintillation counter.

Cytokine ELISA

AChR-primed LNC ($2x10^6$ cells/well) were seeded onto 48-well, flat-bottomed culture plates in 1.0 ml of RPMI 1640 with and without AChR (5.0 μg/ml) or the α146-162 peptide (50 μg/ml). Culture supernatants were collected after 72 hours for the detection of IFN-γ and IL-10 by ELISA. ELISA plates (Dynatech, Chantilly, VA) were coated with 2.0 μg/well of anti-IFN-γ and -IL-10 antibodies (BD Biosciences, San Diego, CA) overnight at 4°C. Plates were blocked with 10% FBS in PBS for 2 hr at room temperature. Culture supernatant samples or serially diluted recombinant cytokines (as standards) were added and incubated overnight at 4°C. The corresponding biotinylated anti-cytokine antibodies (1.0 μg/well) were added and incubated for 45 min. at room temperature in the dark. Avidin-peroxidase (2.5 μg/well) was added and incubated for 30 min. at room temperature in the dark. Then, 0.3 mg/ml 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) ABTS (Boehringer-Mannheim, Indianapolis, IN) was added, and color was developed at room temperature in the dark. Plates were read at 405 nm using a Molecular Devices Emax.

STATISTICS

Clinical EAMG incidence was analyzed using the Fisher's Exact Test, and clinical EAMG severity was analyzed using the Wilcoxon Two Sample Test. Data obtained using RIA, ELISA, proliferation assays, flow cytometry, and immunofluorescence were compared using the Student's *t*-test.

CHAPTER 3

COSTIMULATION THROUGH ICOS/ICOSL IS REQUIRED FOR HUMORAL IMMUNITY TO ACHR AND THE DEVELOPMENT OF EAMG

INTRODUCTION

During the APC-T helper cell interaction, costimulatory signals, as well as antigen-specific TCR-transduced signals, are required for the optimal T cell activation. The CD28 family of costimulatory molecules is the most well-characterized, and its contributions to both human and experimental autoimmunity have been studied extensively. CD28 ligation to B7 molecules predominantly drives Th2 differentiation (Rogers and Croft, 2000; Rulifson et al., 1997; Salomon and Bluestone, 1998; Schweitzer and Sharpe, 1998; Seder et al., 1994), while CTLA-4 ligation to B7 molecules results in the inhibition of activated T cells or the induction of tolerance (Perez et al., 1997; Tivol et al., 1995; Waterhouse et al., 1995). Aberrant CD28/B7-delivered signals can have differential outcomes on autoimmunity, resulting in either protection (Borriello et al., 1997; Cross et al., 1995; Finck et al., 1994; Girvin et al., 2000; Kuchroo et al., 1995; Linsley et al., 1992; Nakajima et al., 1995; Oliveira-dos-Santos et al., 1999) or augmentation (Kuchroo et al., 1995; Lenschow et al., 1996; Vanderlugt et al., 1997) of disease. CTLA-4 deficiencies result in lymphoproliferative disorders and augmentation of autoimmunity (Hurwitz et al., 1997; Karandikar et al., 2000; Karandikar et al., 1996; Luhder et al., 1998; Perrin et al., 1996; Tivol et al., 1995; Waterhouse et al., 1995), possibly due to the role of CTLA-4 in regulatory T cell activities (Chen et al., 1998; Eggena et al., 2004; Salomon et al., 2000; Takahashi et al., 2000). Evidence suggests that polymorphisms in the CTLA-4 gene or abnormal CTLA-4 (Huang et al., 2000a; Huang et al., 1998a; Wang et al., 2002b; Wang et al., 2002c) and B7 (Romi et al., 2002; Teleshova et al., 2000) expression are associated with susceptibility to MG. In mice, a lack of CD28 signaling caused resistance to EAMG associated with Th2 antibodies to AChR and a shift

to a Th1 cytokine profile (Shi et al., 1998) whereas deficiencies in B7 molecules caused resistance to EAMG and a defect in both Th1 and Th2 differentiation following AChR immunization (Poussin et al., 2003).

ICOS is a relatively new CD28 family member whose expression is induced on the surface of T cells following TCR ligation and possibly, CD28 costimulation (Hutloff et al., 1999). The ICOS ligand, ICOSL (B7RP-1), is expressed constitutively by APCs, but may be upregulated by proinflammatory cytokines (Beier et al., 2000; McAdam et al., 2000). Costimulation through ICOS has been shown to strongly impact germinal center formation in secondary lymphoid organs, humoral immunity and IgG class switching, both Th1 and Th2 differentiation, and regulatory T cell development (Dong et al., 2001b; Mak et al., 2003; Nurieva et al., 2003b; Sperling and Bluestone, 2001; Tafuri et al., 2001). Due to these reasons, the ICOS/ICOSL pathway has a strong influence on the development of autoimmunity, including EAE (Dong et al., 2001a; Rottman et al., 2001; Sporici et al., 2001), CIA (Iwai et al., 2002; Nurieva et al., 2003c), GVHD (Oaks and Hallett, 2000), diabetes (Greve et al., 2004; Herman et al., 2004; Kohyama et al., 2004), SLE (Hutloff et al., 2004; Okamoto et al., 2003), and colitis (de Jong et al., 2004). However, the effects of ICOS costimulation in EAMG are unknown, so the purpose of the current study was to characterize the impact of ICOS on the cellular and humoral immune response to AChR leading to the development of clinical EAMG.

RESULTS

ICOS deficiency protects C57BL/6 mice from EAMG development

To investigate the effect of the ICOS/ICOSL costimulatory pathway on the development of EAMG, B6 mice and ICOS KO mice were immunized on days 0 and 28 with 20 μg of AChR emulsified in CFA. As shown in Figure 2, in two separate experiments, ICOS KO mice were highly resistant to EAMG development compared to B6 mice. In experiment 1, 100% of B6 mice developed grade 1 or higher disease compared to only 11% of ICOS KO mice, and 50% of B6 mice developed grade 2 or

higher disease compared to 0% of ICOS KO mice. Results were similar in experiment 2 since 83% of B6 mice developed grade 1 or higher disease compared to 38% of ICOS KO mice, and 50% of B6 mice developed grade 2 or higher disease compared to 0% of ICOS KO mice.

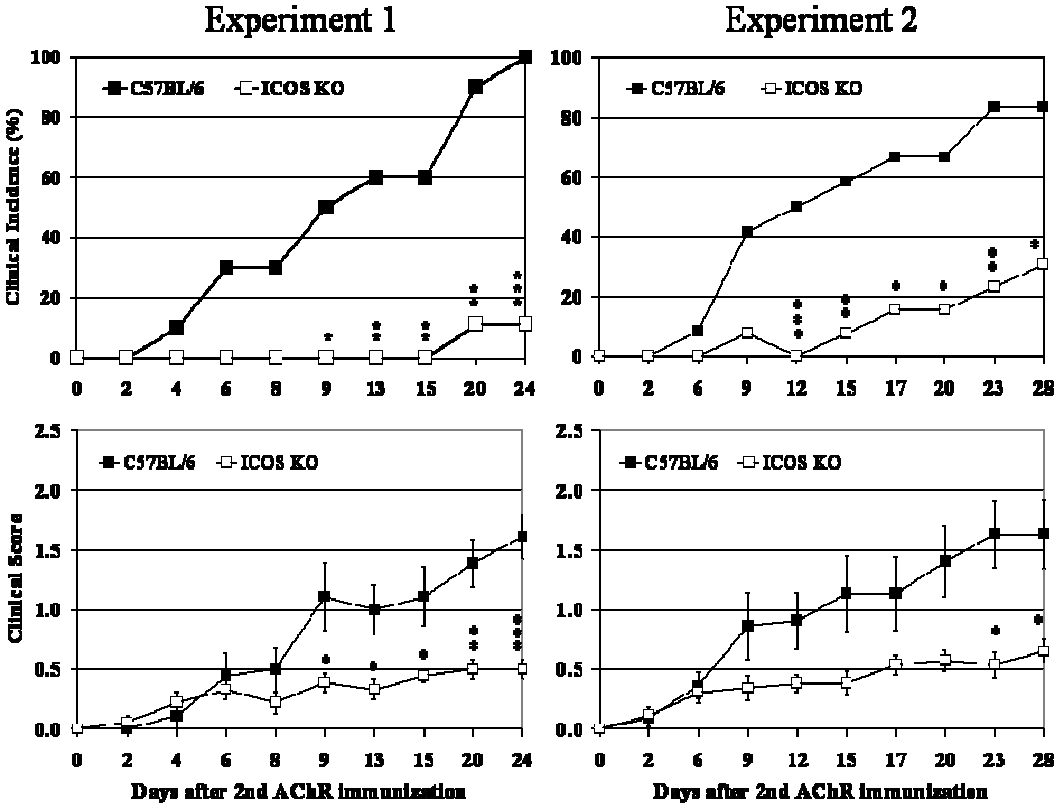


Figure 4. Effect of ICOS gene deficiency on clinical EAMG development. In two separate experiments, clinical EAMG was induced by immunizations with AChR in CFA on days 0 and 28. At the conclusion of both experiments, the clinical incidences (top) of EAMG and mean clinical scores (bottom) were significantly lower in ICOS KO mice than in control C57BL/6 mice. Statistical analysis of clinical incidences was performed using the Fisher's Exact Test, and the Wilcoxon Two Sample Test was used to analyze clinical scores. *$p < 0.05$, **$p < 0.01$, ***$p < 0.0005$.

ICOS deficiency impairs anti-AChR IgG class switching and production

Since anti-AChR IgG mediate the modulation and destruction of AChR at the NMJ of mice with EAMG, in both clinical experiments, sera were collected from B6 and

ICOS KO mice following the first and second AChR immunizations and examined by AChR-specific RIA. As seen in Figure 3, following both the first and second AChR immunizations, ICOS KO sera possessed significantly less circulating anti-AChR IgG than that of B6 mice.

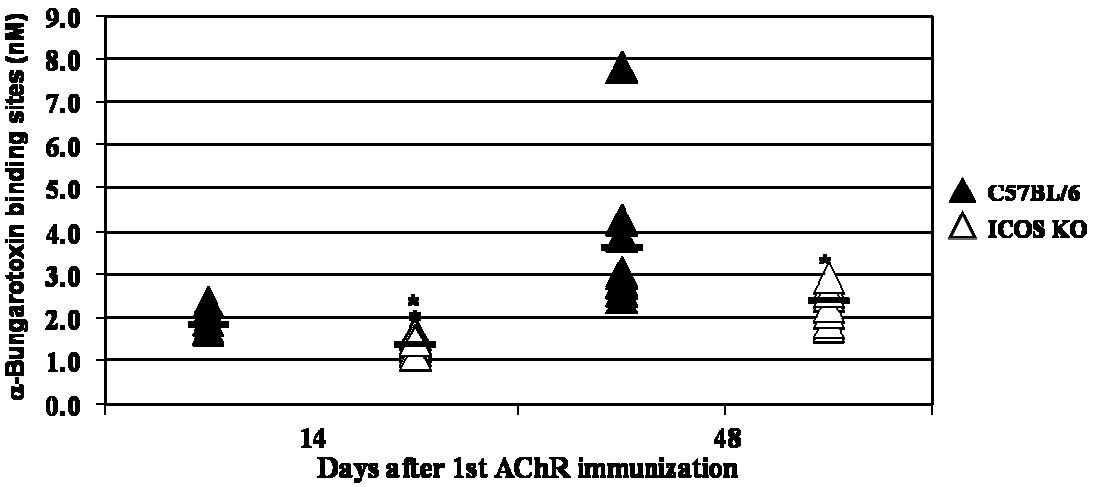


Figure 5. Effect of ICOS gene deficiency on anti-AChR IgG production. In both clinical experiments, sera were collected from C57BL/6 and ICOS KO mice following the first and second immunizations with AChR, and anti-mouse AChR IgG levels were measured by RIA. Data points represent samples from individual mice and are representative of both clinical experiments. *$p < 0.05$, **$p < 0.005$

An ELISA specific for IgG specific to mouse muscle AChR was used to examine the effect of ICOS deficiency on the production of Th1- and Th2-associated anti-AChR IgG isotypes. Figure 4 illustrates that after both the first and second AChR immunizations, sera from ICOS KO and B6 mice contained comparable concentrations of anti-AChR IgM, but sera from ICOS KO mice contained significantly lower levels of the Th1-associated anti-AChR isotypes IgG_{2b} and IgG_{2c}, and the Th2-associated anti-AChR isotype IgG_1.

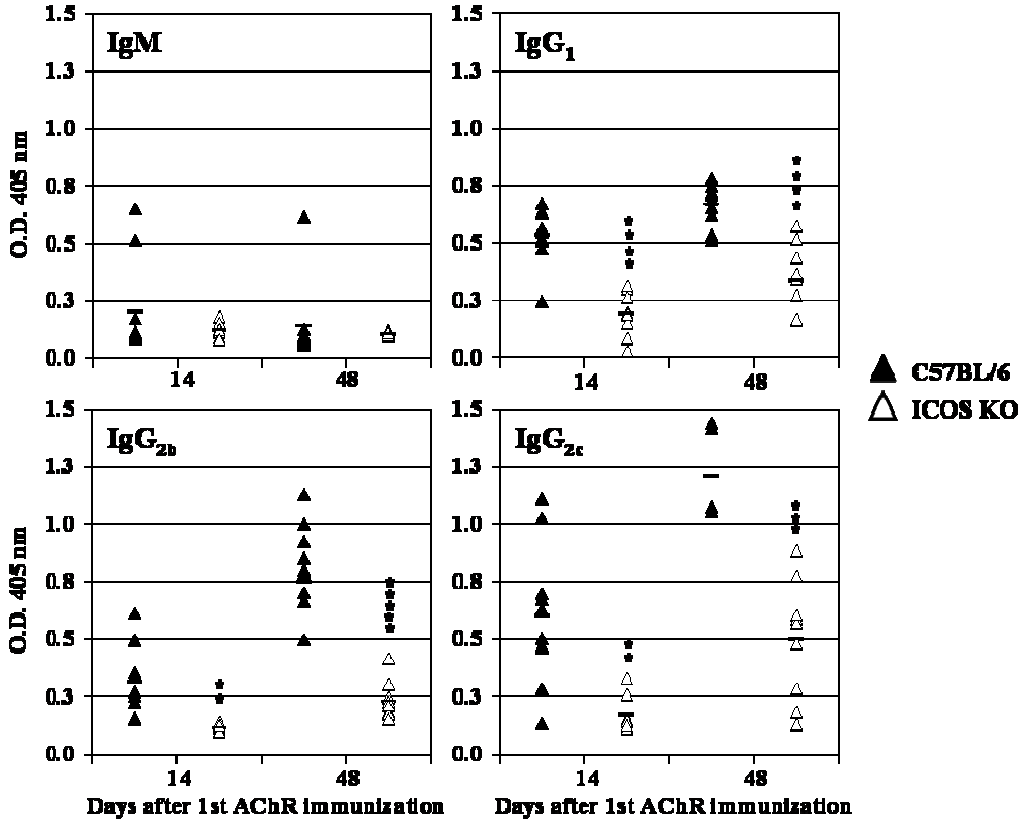
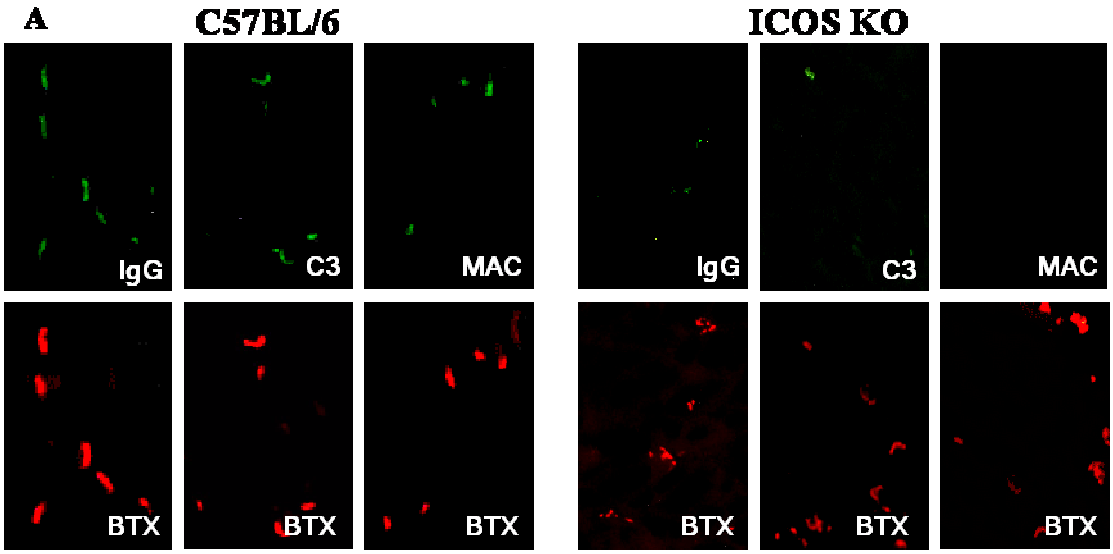


Figure 6. Effect of ICOS gene deficiency on anti-AChR IgG isotype production. In both clinical experiments, sera were collected from C57BL/6 and ICOS KO mice following the first and second immunizations with AChR, and anti-mouse AChR IgG isotype levels were measured by a mouse AChR-specific IgG isotype ELISA. Data points represent samples from individual mice and are representative of both clinical experiments. *$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$, ****$p < 5.0x10^{-5}$, *****$p < 5.0x10^{-6}$

ICOS deficient mice have reduced IgG binding and complement activation at the NMJ

To further illustrate how the defective humoral immune response to AChR rendered ICOS KO mice resistant to EAMG, five sections of forelimb muscle tissue from each B6 and ICOS KO mouse in clinical experiments were incubated with tetramethylrhodamine-conjugated α-bungarotoxin, which binds with high affinity to AChR at the NMJ. Through the use of immunofluorescence microscopy, FITC-conjugated mAbs specific for murine IgG, C3, or the human MAC were also used to

visualize pathogenic IgG and complement proteins bound to the NMJ. The findings in Figure 5 demonstrate that there were fewer NMJ bound by IgG, C3, and MAC proteins in ICOS KO mice than in B6 mice.



B

Number of NMJ bound by IgG, C3, and MAC

	C57BL/6	ICOS KO	P Value
C3	12.8 ± 4.2	6 ± 3.1	0.0109
IgG	4 ± 1.6	1.4 ± 0.9	0.0086
MAC	3 ± 1.0	0.8 ± 0.8	0.0029

Figure 7. Effect of ICOS gene deletion on IgG and complement deposition at the NMJ. At the conclusion of clinical experiments, 5 forelimb muscles from each C57BL/6 and ICOS KO mouse were frozen. Five frozen sections from each mouse were stained with tetramethylrhodamine-conjugated BTX (binds AChR at the NMJ) and FITC-conjugated antibodies specific for IgG, C3, or MAC. Photographs of representative frozen sections are shown (A), and NMJs and deposits of IgG, C3, and MAC were counted (B). Data shown are the mean counts per frozen muscle section from C57BL/6 and ICOS KO mice ± SE.

ICOS/ICOSL costimulation is required for the germinal center response to AChR

In the GCs of the secondary lymphoid organs, antigen-specific T cells drive B cell expansion, Ig class switching, and differentiation into memory cells. To examine the

effect of ICOS/ICOSL costimulation on GC reaction following immunization with AChR in CFA, spleens from B6 and ICOS KO mice were stained with PNA, which binds to GC B cells.

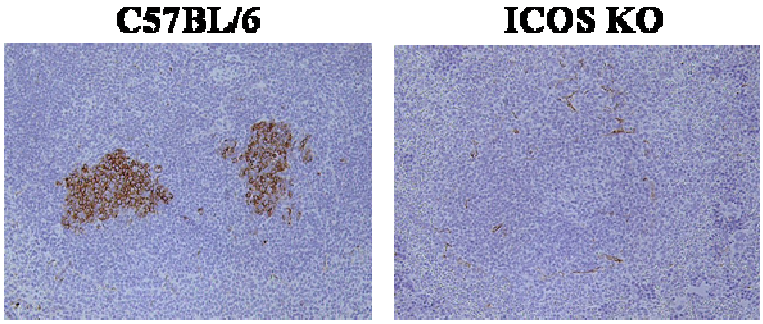


Figure 8. Effect of ICOS gene deficiency on germinal center formation following AChR immunization. Spleens were collected from C57BL/6 and ICOS KO mice 21 days following the first immunization with AChR. Slides were incubated with peanut agglutinin (PNA)-biotin, followed by streptavidin-HRP. PNA^+ B cells were visualized as a brown color following incubation with diaminobenzidine substrate-chromagen. Slides were counterstained for 2 min with Mayer's modified hematoxylin. Data are representative of 5 tissues sections from each of 5 mice per group.

As seen in Figure 6, the spleens of B6 mice contained numerous, well-developed PNA^+ B cell follicles, whereas the spleens of ICOS KO mice had only a few small GC. Since ICOS-delivered signals are important for the clonal expansion of helper T cells and B cells in secondary lymphoid tissues, LNC from B6 and ICOS KO mice at the conclusion of clinical experiments were analyzed by flow cytometry. Following the first AChR immunization, the number of ICOS KO $CD19^+$ cells was reduced (Figure 7) compared to B6 LNC, while the number of ICOS KO lymph node $CD4^+$ T cells was elevated. Control ICOS KO mice immunized with PBS in CFA also had fewer $CD19^+$ cells and increased $CD4^+$ cells in the lymph node compared to control B6 mice (Figure 7). In naïve animals and those immunized only with CFA, $CD19^+$ cells were also fewer in ICOS KO, and $CD4^+$ T cells were significantly more numerous (data not shown). In addition to possessing fewer follicular B cells, the lymph nodes of AChR-immunized ICOS KO mice were characterized by fewer IgG-secreting B cells (plasma cells) than B6 mice, which accounts for the observed decrease in serum anti-AChR IgG (Figure 7).

Following both immunizations with AChR, AChR-immunized ICOS KO mice possessed fewer IgM^+, IgG^+, IgG_1^+, IgG_{2b}^+, and IgG_3^+ cells than did B6 mice. Control ICOS KO mice immunized with PBS in CFA also showed a reduction of IgM^+ LNC, but not in any of the IgG subclass-expressing cells (Figure 7).

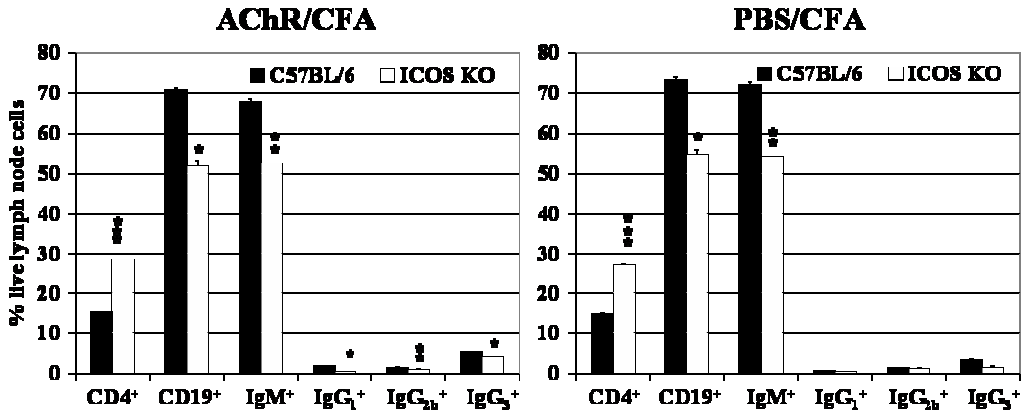


Figure 9. Effect of ICOS gene deficiency on lymphocyte expansion in the lymph node. LNC from mice immunized with AChR/CFA (left panel) or PBS/CFA (right panel) were incubated with fluorescently-labeled antibodies specific for mouse CD4, CD19, IgM, IgG_1, IgG_{2b}, and IgG_3 and analyzed by flow cytometry. Data bars represent the mean of two samples ± SE. *$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$

ICOS-deficient mice have a defect in AChR and α146-162-induced lymphocyte proliferation and Th1 and Th2 cytokine production

To evaluate the influence of the ICOS/ICOSL pathway on the T cell-mediated immune response to AChR, B6 and ICOS KO mice were immunized with AChR in CFA on day 0, and LNC were collected and restimulated *in vitro* on day 7. Figure 8 shows that without costimulation through ICOS, the proliferative response of LNC to AChR and the AChR α146-162 peptide was suppressed. Furthermore, IFN-γ and IL-10 production resulting from AChR and α146-162 peptide stimulation were significantly reduced in the absence of ICOS costimulation. Since ICOS KO mice could possess inherited abnormalities, LNC were collected from B6 mice 7 days after AChR immunization and restimulated *in vitro* in the presence of an ICOS-blocking mAb (clone 7E.17G9). Figure

9 demonstrates that all mAb concentrations, ranging from 0.625 μg/ml to 10.0 μg/ml, were sufficient to partially abrogate the lymphocyte proliferative response to AChR and

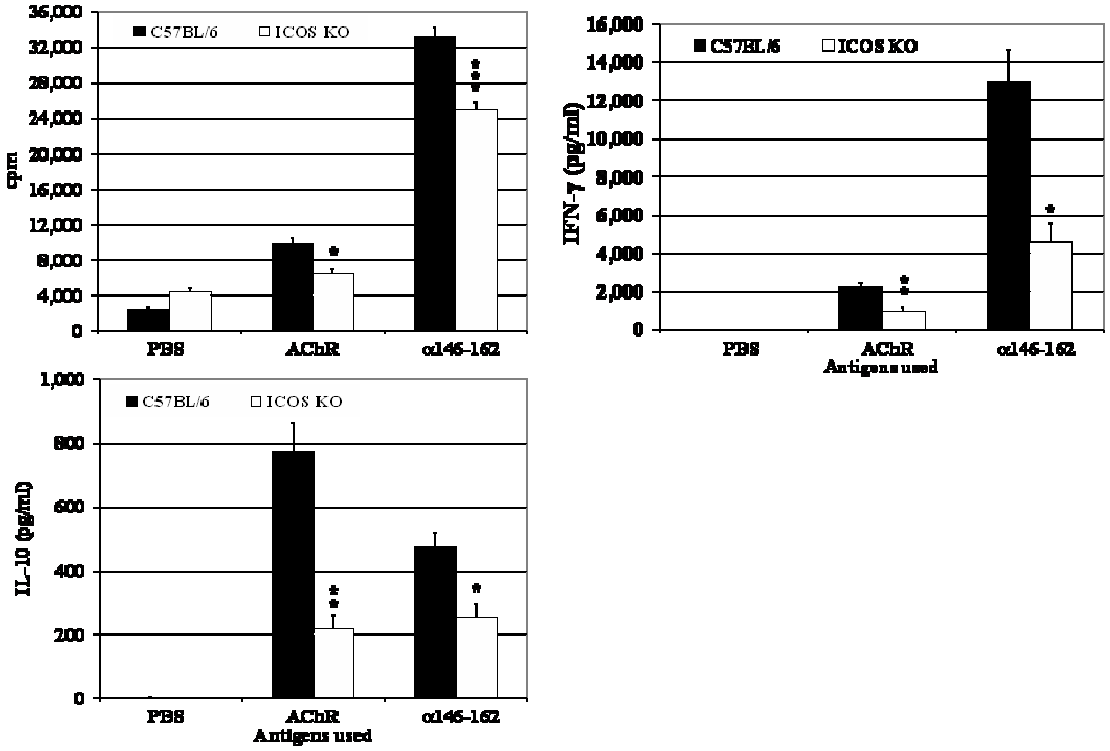


Figure 10. Effect of ICOS gene deficiency on LNC proliferation and cytokine production. On day 7, LNC from individual mice were restimulated in 96-well plates with PBS, AChR, or α146-162 for 5 days. Proliferation was measured by [^{3}H] thymidine uptake. LNC were restimulated in 48-well plates 3 days, and ELISA was used to measure supernatant IFN-γ and IL-10. Data are representative of 3 experiments. *$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$

the α146-162 peptide. To compare the effects of the ICOS/ICOSL and CD28/B6 pathways on the immune response to AChR, LNC were also restimulated in the presence of anti-B7-1 (1G10), anti-B7-2 (GL-1), or control (11-26C) hybridoma supernatants. B7-1 and B7-2 blockade inhibited AChR- and α146-162-specific LNC proliferation to a similar degree as ICOS blockade. ICOS costimulation is therefore required for proliferation of AChR-immune lymphocytes and for the production of both Th1 and Th2 cytokines.

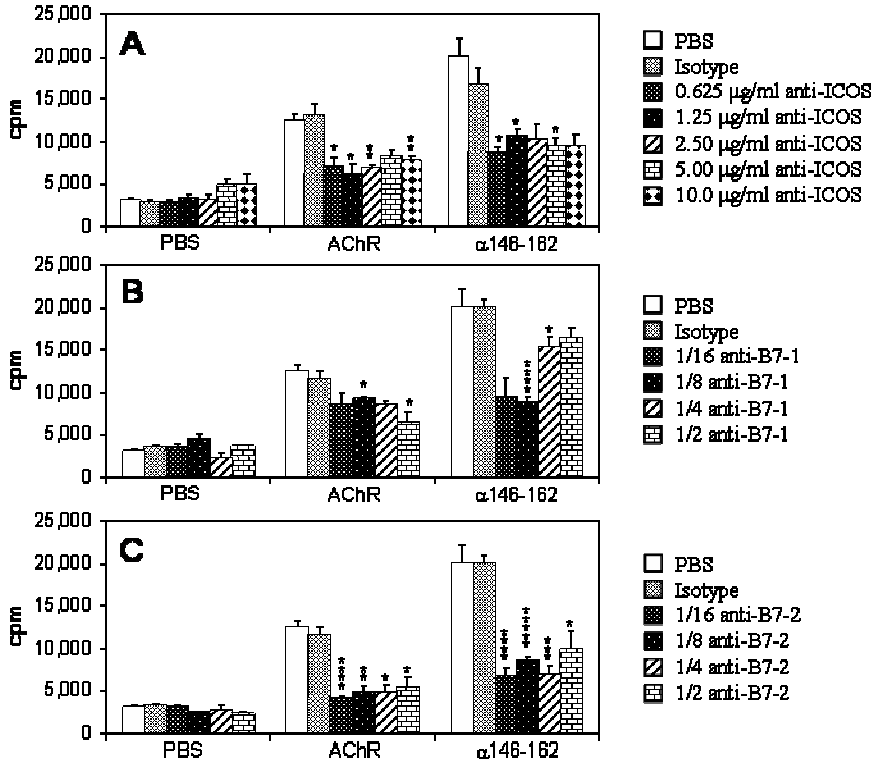


Figure 11. Effect of in vitro costimulatory blockade on LNC proliferation. Seven days after AChR immunization, C57BL/6 mice LNC were restimulated *in vitro* for 5 days with PBS, AChR, or α146-162. Isotype control mAbs or varying dilutions of blocking mAbs to ICOS (A), B7-1 (B), or B7-2 (C) were added to the cultures, and proliferation was assessed by measuring [^{3}H] thymidine incorporation. *p<0.05, **p < 0.005, ***p < 0.0005, ****p < $5.0x10^{-4}$, *****p < $5.0x10^{-5}$

DISCUSSION

The ICOS/ICOSL costimulatory pathway is crucial for the development of the GC reaction in secondary lymphoid tissues and the class switching and production of Ig (Dong et al., 2001b; Mak et al., 2003; Nurieva et al., 2003b; Nurieva et al., 2003c; Tafuri et al., 2001). Initial investigations revealed that ICOS might selectively promote Th2 immunity, because ICOS costimulation upregulated the production of IL-4 and IL-10, but not IL-2, and ICOS KO mice possessed pronounced defects in the production of Th2

cytokines and Th2 humoral immunity (Dong et al., 2001a; Tafuri et al., 2001). However, it is now accepted that ICOS costimulation is an equally important promoter of proinflammatory and Th1 cytokines (Sperling, 2001, Sperling and Bluestone, 2001) including IL-6, IFN-γ, and TNF-α. For example, ICOS blockade slows acute and chronic allogeneic heart allograft rejection in mice (Ozkaynak et al., 2001) and during efferent phase of EAE, ICOS blockade inhibits disease progression by reducing the production of IFN-γ and proinflammatory cytokines (Ozkaynak et al., 2001). During lymphocytic choriomeningitis virus (LCMV) infection, ICOS regulates both CD28-dependent and CD28-independent Th1 and Th2 responses, (Kopf et al., 2000) and in CIA, ICOS drives clinical disease progression and both Th1 and Th2 responses (Iwai et al., 2002, Nurieva et al., 2003). In the current study, we have provided the first evidence for the involvement of the ICOS/ICOSL costimulatory pathway in EAMG, an antibody-mediated model of human MG. Our results demonstrate that a deficiency in ICOS costimulation renders B6 mice resistant to the development of clinical EAMG due to an abrogated T cell-mediated humoral immune response to AChR. ICOS KO mice displayed a defect in anti-AChR IgG production and Ig isotype switching and a reduction of the *in vitro* proliferation of LNC and production of IFN-γ and IL-10 following restimulation with AChR and α146-162. Both Th1 and Th2 cytokines are required for the development of EAMG (Baggi, 2000) and it appears that the ICOS/ICOSL costimulatory pathway is at least one of several early T cell checkpoints that dictate the differentiation of both Th1 and Th2 populations in EAMG development. These results are similar to those found by our lab in which both B7-1 and B7-2 KO mice had stunted anti-AChR antibody production and IFN-γ and IL-10 production by LNC (Poussin et al., 2003). It is interesting, but not surprising, that CD28 KO mice had a decreased EAMG incidence associated with a Th1-skewed immune response marked by increased numbers of IFN-γ-secreting cells and a defect in AChR-specific production of the Th2-associated isotype, IgG_1 (Shi et al., 1998). CD28 costimulation has been shown to predominantly drive Th2 differentiation in other models of autoimmunity (Lenschow et al., 1996).

GC are the secondary lymphoid sites where T cells drive B cells to undergo class switching recombination and somatic hypermutation of Ig variable regions. Deficiencies in the ICOS/ICOSL pathway cause abnormal GC development in secondary lymphoid organs (Dong et al., 2001b) and defects in humoral immunity characterized by impaired Ig class switching (Dong et al., 2001a; Dong et al., 2001b; Iwai et al., 2002; Nurieva et al., 2003c; Rottman et al., 2001; Tafuri et al., 2001). In CIA, a genetic deficiency in ICOS resulted in complete resistance of mice to rheumatic joint inflammation due to inhibited anti-collagen $CD4^+$ T cell proliferation and IgG_{2a} production (Nurieva et al., 2003c). Further, anti-ICOSL mAb treatments in DBA/1J mice ameliorated clinical symptoms and inhibited collagen-specific T cell proliferation and anti-collagen IgG_1, IgG_{2a}, and IgG_{2b} secretion (Iwai et al., 2002). However, ICOS KO mice displayed an enhanced incidence of MOG-induced EAE characterized by an unusually high infiltrate of IFN-γ-secreting $CD4^+$ T cells in CNS tissues (Dong et al., 2001a). ICOS blockade in SJL mice early (days 1-10) after immunization with PLP augmented EAE, but when administered during the efferent immune response (days 9-20), EAE was abrogated, and CNS leukocyte infiltration and induction of proinflammatory cytokines and chemokines in the CNS was reduced (Ozkaynak et al., 2001). The present study of ICOS in EAMG clearly shows that following the primary immunization with AChR in CFA, PNA^+ GC were almost completely absent in ICOS KO mice. Flow cytometry analysis of LNC following the primary AChR immunization revealed fewer numbers $CD19^+$ B cells, but higher numbers of $CD4^+$ T cells. ICOS KO lymph nodes had fewer numbers of IgM^+, IgG^+, IgG_1^+, IgG_{2b}^+, and IgG_3^+ cells, and serum concentrations of these AChR-specific antibody classes were also reduced following two immunizations with AChR. The ICOS/ICOSL costimulatory pathway is therefore required for GC development following AChR immunization that results in B cell class switching and the production of both Th1- and Th2-associated anti-AChR IgG isotypes.

CD28/B7 costimulation is required for naïve T cells to overcome the activation threshold during their primary encounter with antigen (Chatenoud et al., 2001). It has been suggested that ICOS costimulation may be more crucial than CD28 costimulation in

governing late effector phases of the immune response rather than during the primary T cell clonal expansion following initial antigenic exposure. This theory is the result of studies that showed ICOS is expressed at marginal levels (or not at all) by naïve T cells (Hutloff et al., 1999) and that in the absence of B7-1 and B7-2, the upregulation of ICOS expression by TCR-activated T cells is reduced, but can be restored by CD28 stimulation (McAdam et al., 2000). However, the current study has demonstrated that ICOS-blocking mAbs can partially inhibit AChR- and α146-162-induced lymphocyte proliferation during the primary immune response to AChR to a degree similar to that resulting from blockade of B7-1 and B7-2. These findings suggest that the B7/CD28 and ICOS/ICOSL costimulatory pathways have an equally important role in the expansion of AChR-specific lymphocytes during the primary immune response to AChR and therefore could be an appropriate target for blockade as an MG therapy. However, since costimulation through the ICOS/ICOSL pathway can exert either a destructive or protective control in different murine models of autoimmunity, ICOS blockade may not be a suitable immunotherapeutic approach in all human autoimmune syndromes. Collectively, these studies have shown that the CD28 family of costimulatory molecules drives both Th1 and Th2 immunity to the AChR in EAMG and that deficiencies in any of the CD28 family costimulatory molecules can have a striking impact on the development of EAMG. Specifically, the ICOS, B7-1, and B7-2 molecules appear to drive both Th1 and Th2 differentiation in EAMG pathogenesis, whereas CD28 seems to primarily impact Th2 differentiation.

CHAPTER 4

FUNCTIONAL COMPARISON OF ICOSL, B7-1, AND B7-2 IN EAMG BY IN VIVO ANTIBODY BLOCKADE

INTRODUCTION

The *in vivo* blockade of CD28 family costimulatory molecules is a successful means of preventing or treating autoimmunity in mice and has led to a greater understanding of how these costimulatory pathways compare in their ability to promote the induction and/or maintenance of pathogenic effector lymphocytes. In mice, inhibition of B7 molecules *in vivo* prevents lupus-like disease (Borriello et al., 1997; Finck et al., 1994; Linsley et al., 1992; Nakajima et al., 1995). ICOSL blockade prevented and ameliorated CIA by reducing the anti-collagen inflammatory response (Iwai et al., 2002), and prevented EAE in transgenic mice by increasing apoptosis and blocking proliferation of autoimmune T cells (Sporici et al., 2001). However, B7 and ICOS blockade can have positive or negative effects on the development of EAE. In myelin-induced EAE, anti-B7-1 treatment early after immunization prevented EAE due by reducing autoimmune T cells (Cross et al., 1995; Oliveira-dos-Santos et al., 1999), but later during disease, anti-B7-1 treatment exacerbated disease (Vanderlugt et al., 1997). Anti-B7-2 treatments enhanced EAE severity (Kuchroo et al., 1995) or had no effect (Racke et al., 1995). In contrast, ICOS blockade early after PLP immunization enhanced the inflammatory infiltrate into the CNS resulting in exacerbation of EAE, but ICOS blockade during the late immune response alleviated CNS disease (Rottman et al., 2001). On the other hand, inhibition of the negative receptor, CTLA-4, worsens murine diseases like EAE (Hurwitz et al., 1997; Karandikar et al., 2000; Karandikar et al., 1996; Perrin et al., 1996) and diabetes (Luhder et al., 1998) due to uncontrolled expansion of lymphocytes.

Although genetic deficiencies in B7 or ICOS results in a relatively high resistance to EAMG, we do not know how *in vivo* treatment with anti-B71, anti-B7-2, or anti-ICOSL mAbs affect the development of clinical EAMG. Furthermore, the relative

contributions of the B7-1, B7-2, and ICOSL molecules to the anti-AChR immune response have not been characterized. The current study was performed in order to (1) analyze the ability of *in vivo* B7-1, B7-2, and ICOSL blockade to prevent the development of EAMG in C57BL/6 mice and (2) characterize the precise similarities and differences in the regulation of the T cell-dependent humoral immune response to AChR by B7-1, B7-2, and ICOSL.

RESULTS AND DISCUSSION

To investigate the relative effects of the B7/CD28 and ICOS/ICOSL costimulatory pathways on the development of EAMG, a treatment strategy was devised in which mAb injections were used to block B7-1, B7-2, and ICOSL *in vivo*. Hybridoma cell lines producing anti-ICOSL mAb (clone HK5.3), anti-B7-1 mAb (clone 1G10), and anti-B7-2 mAb (clone GL1) were provided to ABNOVA Corporation (Taiwan) for the commercial production of murine ascites fluid since culture of the hybridoma cell lines in our laboratory resulted in insufficient yields of the required mAbs. Since there was insufficient time remaining upon receipt of the ascites fluid from ABNOVA, we immediately began *in vivo* experiments, and at the same time, we also performed assays to confirm the presence of mAb in the ascites fluid. For qualitative testing, a small volume of ascites was precipitated with a saturated ammonium sulfate solution followed by SDS-PAGE gel analysis. SDS-PAGE analysis revealed the presence of light bands that corresponded with the molecular weight of mammalian IgG heavy (50 kDa) and light (29 kDa) chains. However, indirect flow cytometry analysis of $ICOSL^+$ L5178Y cells using HK5.3 ascites and a did not confer positive staining of a FITC-conjugated secondary antibody specific for the HK5.3 isotype (rat IgG_{2a}). Furthermore, anti-B7-1 and anti-B7-2 ascites fluid pre-incubation with AChR-immune LNC did not competitively inhibit the binding of FITC-conjugated mAbs to ICOSL, B7-1, and B7-2. Thus, we were unable to verify the presence of mAbs specific for ICOSL, B7-1, and B7-2 within the ascites fluids.

Despite the inability to confirm the quality of ascites fluid, a pilot experiment to test the *in vivo* costimulation blocking efficacy of the ascites was performed due to time constraints. B6 mice were immunized on day 0 with 20 μg of AChR emulsified in CFA. On days -2, 1, 4, and 7, groups of mice were injected i.p. with 600 μl of PBS, anti-ICOSL ascites, anti-B7-1 ascites, or anti-B7-2 ascites. On day 10, LNC were collected and restimulated *in vitro* with PBS, AChR, or α146-162. The AChR- and α146-162-specific LNC proliferation was significantly reduced in mice treated with ascites compared to control animals. To compare the effects of ICOSL, B7-1, and B7-2 blockade on the development of EAMG, B6 mice were immunized on days 0 and 28 with 20 μg of AChR emulsified in CFA. B6 mice immunized with CFA alone served as negative control animals. AChR-immunized mice were injected with rat IgG (control), anti-ICOSL ascites, anti-B7-1 ascites, or anti-B7-2 ascites on days -2, 1, 4, and 7 (primary immune response) and days 26, 29, 32, and 35 (secondary immune response).

The mean clinical incidence and clinical severity of ascites-treated mice did not significantly differ from rat IgG-treated control mice and did not alter the serum levels of anti-AChR IgG following the first and second immunizations with AChR. Furthermore, the ascites treatments had no effect on LNC proliferation *in vitro* or the expansion of MHC class II^+ICOSL^+ cells, $CD4^+$ cells, $CD4^+ICOS^+$ cells, $CD19^+$ cells, $B7\text{-}1^+$ cells, and $B7\text{-}2^+$ *in vivo*. The lack of success in the above experiments is almost certainly due to poor quality of the ascites since we were unable to obtain the expected high yield (1-3 mg/ml) of mAb or verify the specificity of the ascites. The poor quality ascites fluid could be due to ineffective production of ascites fluid or due to poor quality hybridoma cell lines.

We expect that successful anti-ICOSL blockade in mice would have significantly reduced both the incidence and severity of EAMG due to a reduction in anti-AChR IgG production by B cells. Further, we expect that successful blockade of B7-1 and B7-2 costimulation would have prevented EAMG to degrees comparable to that of ICOSL

blockade since B7-1 gene-deficient and B7-2 gene-deficient mice had disrupted anti-AChR T cell proliferation and antibody production resulting in resistance to EAMG.

CHAPTER 5

SUMMARY AND CONCLUSIONS

The CD28 family member of costimulatory molecules, which includes the CD28/B7/CTLA-4 pathway and the ICOS/ICOSL pathway, plays an important role in driving the activation and expansion of lymphocytes. In these studies, we have shown that the ICOS/ICOSL costimulatory pathway plays a crucial role in the generation of AChR-specific effector T and B cells in EAMG. Using B6 mice deficient in the ICOS gene, we demonstrated that costimulatory signals delivered through the ICOS/ICOSL pathway are critical for the formation of germinal centers in secondary lymphoid tissues and the production of anti-AChR IgG, including both Th1-assciated subclasses that fix complement and Th2-associated subclasses that do not fix complement. As a result, ICOS-deficient were highly resistant to the induction of EAMG following immunizations with AChR ultimately due to decreased binding of pathogenic IgG and complement proteins to the neuromuscular junction of skeletal muscles.

By treating B6 mice with ascites fluid containing monoclonal antibodies directed to ICOSL, B7-1, and B7-2, we hoped to make an accurate comparison of how these molecules promote the development of EAMG. Specifically, our aims in this experimental approach were (1) to prevent the development of EAMG through blockade of ICOSL, B7-1, or B7-2 and (2) to characterize how ICOSL, B7-1, B7-2 differ in their roles in driving the differentiation and expansion of AChR-specific T helper cells and B cells, the formation of lymphoid germinal centers, and the production of AChR-specific IgG. Ultimately, we anticipated that the successful completion of this *in vivo* study would lead to future experiments directed at designing an effective anti-costimulatory molecule therapy for EAMG consisting of a combination of anti-CD28 family antibodies. However, the treatment of B6 mice with anti-ICOSL, anti-B7-1, and anti-B7-2 ascites was ineffective at preventing the development of EAMG or the adaptive immune

response to AChR, most likely due to insufficient quantity or activity of mAb within the ascites.

Despite difficulties in performing the *in vivo* blockade of CD28 family member costimulatory molecules in an effort to prevent EAMG, significant progress was made in our knowledge of EAMG pathogenesis. An interesting result of this dissertation is the observation that ICOS costimulation in EAMG drives both Th1 and Th2 differentiation following AChR immunization. Lymph node cell cultures from ICOS-deficient mice displayed defective AChR-specific and α146-162 peptide-specific proliferation and production of the Th1 cytokine, IFN-γ, as well as the Th2 cytokine, IL-10. Furthermore, production of both the Th1-associated antibody classes IgG_{2b} and IgG_{2c} and the Th2-asscociated class IgG_1 were markedly reduced in the absence of ICOS/ICOSL-delivered signals to AChR-specific B cells. These findings are important, because they differ from early studies which demonstrated that ICOS deficiency or interference results predominantly in defective Th2 differentiation.

The observed inconstancies in how ICOS costimulation affects Th differentiation in EAMG compared to previous reports may be partially due to variations resulting from the differences in the antigen, adjuvant, and the source of lymphocyte used in experimentation. Both Th1/pro-inflammatory cytokines (IFN-γ, TNF-α, IL-6) and Th2 (IL10, IL-5) cytokines are for an effective anti-AChR humoral immune response and EAMG development. Whereas, the induction of EAMG in B6 mice requires the use of AChR emulsified in the pro-inflammatory adjuvant CFA, one early study of ICOS/ICOSL costimulation utilized ovalbumin (OVA) peptide with APC to stimulate OVA peptide-specific transgenic BALB/c mouse T cells in the presence of an ICOS-blocking reagent *in vitro* (McAdam et al., 2000). This report demonstrated that OVA-specific IFN-γ production was enhanced while OVA-specific IL-10 production was completely blocked. In another report, B6 mice were immunized with keyhole limpet hemocyanin (KLH) emulsified in CFA (Tafuri et al., 2001), and *in vitro* KLH-specific IL-4-producing cells were dramatically reduced by ICOS gene deficiency, while the numbers of IFN-γ-producing cells unaffected. Finally, an earlier investigation reported

that human peripheral $CD4^+$ T cells sub-optimally activated with anti-CD3 were activated by ICOS costimulation in a manner comparable to that of CD28 and produced exceedingly high levels of IL-10 compared to Th1 cytokines including IFN-γ and IL-2 (Hutloff et al., 1999). Thus, the role of ICOS costimulation in the differentiation of Th cells in B6 mice immunized with AChR in CFA can differ significantly from the role of ICOS costimulation in other mammals exposed to other T cell-dependent antigens.

The observed differences in the ICOS-mediated effects on Th differentiation between the current study and previous studies may also be explained by variations in downstream lymphocyte signaling between strains of mice. It is known that Th2 cells are twice as numerous in BALB/c mice than in B6 mice, and ICOS is expressed at higher levels on Th2 cells than on Th1 cells (Arimura et al., 2002; Coyle et al., 2000). The serine/threonine kinase, Akt, which drives Th2 differentiation, is expressed at a higher level on BALB/c T cells than on B6 T cells (Arimura et al., 2002). Additionally, the transcription factors GATA-3, c-maf, and NF-ATc_1 are expressed in high levels by Th2 cells, but not Th1 cells (Arimura et al., 2004; Nurieva et al., 2003a; Rodriguez-Palmero et al., 1999). Nonetheless, phosphorylated Akt can promote either Th1 or Th2 differentiation, depending on the local cytokine milieu at the time of T cell activation (Arimura et al., 2004), further implicating the role of the antigen and adjuvant used to generate effector lymphocytes via immunization.

Together with previously published reports, this investigation further demonstrates the complexity with which costimulatory molecules such as ICOS impact the immune response to autoantigens. Our results using ICOS KO mice have clearly demonstrated a "pathogenic" role for ICOS/ICOSL costimulation in EAMG. However, the pathogenic vs. protective role of ICOS in T cell-mediated autoimmune diseases such as EAE are less clearly defined. Furthermore, it has been suggested that ICOS may play an important role in maintaining peripheral tolerance to self-antigens, because IL-10-secreting $ICOS^{hi}$ T_{regs} are found in murine diabetes, and ICOS blockade ablated T_{reg} function (Akbari et al., 2002; Kohyama et al., 2004). This T_{reg} function of ICOS in EAMG is unlikely not only due our findings above, but also because IL-10 actually

promotes the development of EAMG (Poussin et al., 2000). For these reasons, it is crucial to further define the role of ICOS/ICOSL costimulation in the induction of maintenance of pathogenic effector cells and regulatory cells in autoimmunity. While anti-ICOS therapy might be a suitable venture for human antibody-mediated autoimmune diseases, such as MG and SLE, it may not be appropriate for the treatment of T cell-mediated diseases such as MS and diabetes.

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VITA

Benjamin G. Scott was born on October 23, 1975 to Greg and Vicki Scott. Ben attended Texas A&M University and received his Bachelor's degree in Biomedical Science in May, 1999. While at Texas A&M, Ben performed research with Dr. Jane Welsh examining the effects of stress on neuropathogenesis of Theiler's Virus Infection in the CNS and received two awards for the presentation of his findings. Ben also held jobs at the USDA and the Texas A&M University Department of Medical Physiology before matriculating to the University of Texas Medical Branch Department of Microbiology and Immunology in 1999.

During his graduate education at UTMB, Ben received numerous awards and scholarships including: The William and Madeline Welder Smith Foundation Award, The James W. McLaughlin Colloquium Travel Award, The Zhou Sisters Great Expectations Scholarship, The James W. McLaughlin Fellowship, The Christina Fleischmann Fellowship, The NIH Immunology and Mucosal Defense Training Grant, The Myasthenia Gravis Foundation of America Henry R. Viets Fellowship, The Graduate School of Biomedical Sciences Recruitment Award, and The James W. McLaughlin Recruitment Award.

Ben served on several committees while at UTMB and gained teaching experience by serving as a Bromberg Scholars Bench Tutorial mentor with a Ball High School student and by serving as a laboratory teaching assistant in the Pathobiology and Host Defense medical school course.

Ben has accepted a post-doctoral position in the laboratory of Dr. Phil Cohen at the University of Pennsylvania Department of Immunology.

During his time in Galveston, Ben met his bride, Erin Pauline Scott, and served as a Deacon in the First Baptist Church of Galveston.

PUBLICATIONS

Yang, H., Alagappan, D., Yu, X., **Scott, B.G.**, Tüzün, E., Ischenko, A., and Christadoss, P. IL-1 Receptor Antagonist-Mediated Therapeutic Effect in Murine Myasthenia Gravis is Associated with Reduced Serum IFN-γ, IL-2, TNF, IL-6, anti-AChR IgG_1, and C3. (Accepted for publication, *J. Immunol.*)

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