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GENETIC AND IMMUNOLOGICAL CONTROL OF HUMAN MYASTHENIA GRAVIS

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Stockholm
2005

Published and printed by Reproprint AB
Box 2108S, SE-100 31, Stockholm, Sweden

ISBN 91-7140-494-5

TO MY FAMILY

ABSTRACT

Myasthenia gravis (MG) is a classical autoimmune disease characterized by muscle weakness due to an immune attack against the nicotinic acetylcholine receptor (AChR) on the post-synaptic membrane of the neuromuscular junction. Because of the involvement of multiple factors in the disease, the overall aims of this thesis were to uncover the regulation of candidate genes and the possible cellular mechanisms involved in the immuno-regulation of human MG.

MG patients showed some aberrations of the β2-adrenergic receptor (β2-AR), such as decreased density of β2-AR on peripheral blood mononuclear cells (PBMC), serum antibodies against β2-AR and aberrant distribution of SNPs of codon region within the β2-AR gene. I investigated the correlation of the promoter SNPs of the β2-AR gene with MG in the present study. I identified a low frequency of allele -468G, -367C, -47C and -20C in MG with thymoma, which confirms the notion that the pathogenesis of MG with thymoma appears to be different from that of other subgroups of the disease. The allele frequency of -468G, -367C, -47C and -20C was increased in MG patients with elevated levels of anti-AChR antibodies and severe generalized disease. It has been shown that this allele could significantly reduce transcription activity, resulting in a decreased density of the β2-AR on lymphocytes. Therefore, the finding provides an explanation at the molecular level for the decreased density of β2-AR on patients' lymphocytes.
The pro-inflammatory cytokines, chemokines and their receptors are considered to be important for the localization of immune cells. The chemokine receptor CCR2-64I (G/A) allele is associated with autoimmune diabetes in children whereas CCR5-**Δ**32 (32 bp deletion) is correlated to severity of rheumatoid arthritis and prolonged renal allograft survival. In the second study, the association of genetic polymorphisms of the chemokine receptors CCR2 and CCR5 with MG was investigated. The results revealed no association between allelic frequencies of CCR2-64I and CCR5-**Δ**32 and MG.
The cytolytic T lymphocyte-associated antigen-4 (CTLA-4) is a costimulatory receptor expressed mainly on activated T cells and mediates a negative regulation of T cell activation. The genotype G/G at +49 is in strong linkage with -318C/T and is more frequently present in MG patients with thymoma. MG patients carrying -318C showed significantly decreased expression of CTLA-4 mRNA in non-stimulated cells. In the present study, the relationship of the expression of *Ctla-4* with the promoter -318C/T SNP was studied in an *in vitro* system. Two constructs with a reporter gene driven by the *Ctla-4* promoter that contained either C or T alleles, were made and transfected in a Jurkat T cell line. The -318T allele showed higher promoter activity than the -318C allele, which confirmed the regulatory effect of this -318C/T SNP.
OX40 (CD134), a member of TNFR family, is a cell surface glycoprotein expressed on activated CD4+ T cells. Engagement of OX40 by its ligand enhances cytokine production by CD4+ T cells as well as the proliferation and the long-term survival of CD4+ T cells. In the present study, the expression of OX40 on CD4+ T cells from patients with MG was investigated. Results from thirty-six MG patients and twenty-eight healthy controls revealed that MG patients had more CD4+OX40+ T cells in freshly isolated PBMC than healthy individuals. These CD4+OX40+ T cells expressed CD25, but not CD69 and CTLA-4, implying that these T cells are not regulatory cells. High percentages of CD4+OX40+ T cells were found in patients with generalized disease, disease onset at an early age, thymic hyperplasia and high levels of anti-AChR antibodies. Upon activation by various concentrations of anti-CD3 antibodies, CD4+ T cells from MG patients showed a tendency towards higher levels of OX40 expression than cells from healthy individuals.

In summary, the association of β2-AR to MG patients with thymoma confirmed that these patients constitute a separate entity. The down-regulation of β2-AR in patients with MG, at least partially caused by genetic variants, might up-regulate the expression of CD25 on T cells and promote the expansion of autoreactive T and B cells. The up-regulation of OX40 and down-regulation of CTLA-4 lead to abnormal activation of T cells, especially the auto-reactive T cells. All of these abrrations can eventually contribute to the production of autoantibodies, the abnormal thymic histopathology and disease development in MG.

Keywords: Myasthenia Gravis (MG), β2-adrenergic receptor (β2-AR), CCR2, CCR5, cytolytic T lymphocyte-associated antigen-4 (CTLA-4), OX40

TABLE OF CONTENTS

LIST OF PUBLICATIONS

This thesis is based on the following publications and manuscripts:

I. Association of β2-adrenergic receptor (β2-AR) promoter SNPs to myasthenia gravis with thymoma, high levels of anti-AChR antibodies and severe generalized disease. **Zhao X**, Gharizadeh B, Wang XB, Ghaderi M, Pirskanen R, Nyren P, Garçon HJ, Lefvert AK (Manuscript, submitted).

II. Genotypes of CCR2 and CCR5 chemokine receptors in human myasthenia gravis. **Zhao X**, Gharizadeh B, Hjelmstrom P, Pirskanen R, Nyren P, Lefvert AK, Ghaderi M. Int J Mol Med. 2003, 12(5):749-53.

III. A CTLA-4 gene polymorphism at position -318 in the promoter region affects the expression of protein.Wang XB, **Zhao X**, Giscombe R, Lefvert AK. Genes Immun. 2002, 3(4):233-4.

IV. Expression of OX40 (CD134) on CD4+ T cells from patients with myasthenia gravis. **Zhao X**, Pirskanen R , Vivianne M, Lefvert AK (Manuscript, submitted).

ABBREVIATIONS

AChR	Acetycholine receptor
APC	Antigen presenting cell
BUP	Beta upstream peptide
β2-AR	Beta-2-adrenergic receptor
CD	Cluster of differentiation
ConA	Concanavalin A
CTLA-4	Cytotoxic T lymphocyte antigen 4
EAMG	Experimental autoimmune myasthenia gravis
EAE	Experimental autoimmune encephalomyelitis
ERK	Extracellular signal-regulated kinase
EtBr	Ethidium bromide
GVHD	Graft-versus-host disease
HTLV	Human T-cell leukemia virus
IBD	Inflammatory bowl disease
HC	Healthy control
HLA	Human leucocytic antigen
Ig	Immunoglobulin
IFN	Interferon
IL	Interleukin
ITIM	Immunoreceptor tyrosine-based inhibitory motif
ITAM	Immunoreceptor tyrosine-based activation motif
JNK	Jun N-terminal kinase
kDa	Kilo dalton
MCP	Monocyte chemotactic protein
MG	Myasthenia gravis
MIP	Macrophage inflammatory protein
MS	Multiple sclosis
MuSK	Muscle-specific receptor tyrosine kinase
MW	Moleuclar weight
RANTES	Regulated on activation, normal T-expressed and secreted
PAGE	Polyacrylamide gel
PBMC	Peripheral blood mononuclear cell
PMA	Phorbol 12-myristate 13-acetate
PTK	Protein tyrosine kinases
RA	Rheumatoid arthritis
RFLP	Restriction fragment length polymorphism
RIA	Radio-immunoprecipitation assay
RLK	Resting lymphocyte kinase
SDS	Sodium dodecyl sulphate
SHP-2	SH2-domin containing protein tyrosine phosphatase
SLE	Systemic lupus erethematosus
SLC	Secondary lymphoid-tissue chemokine
SF	Synovial fluid
SNP	Single nucleotide polymorphism
TNF/TNFR	Tumor necrosis factor/Tumor necrosis factor receptor
UTR	Un-translated region
ZAP-70	Zata-associated protein of 70 kDa

INTRODUCTION

1. General aspects of myasthenia gravis (MG)

In 1672, the earliest description of a MG patient was done by Thomas Willis, a physician in Oxford, who reported a woman with a paralysis that affected her limbs and tongue. The major clinical and immunological findings in MG research were reviewed by Professor Vincent A [1] (Figure 1a and 1b).

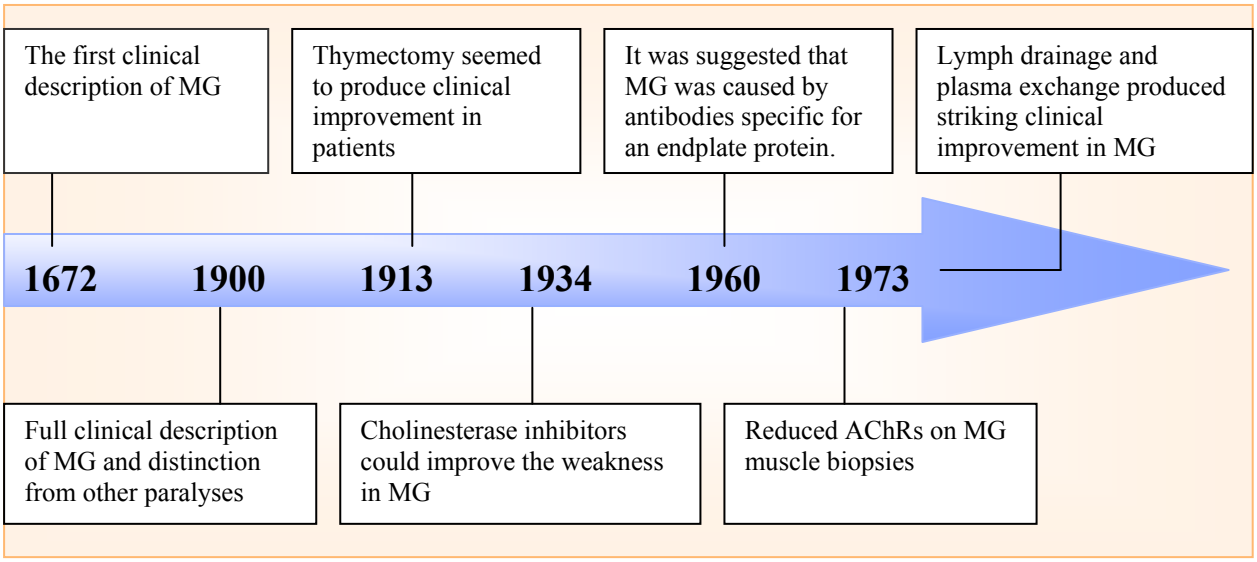


Figure 1a. Clinical developments in MG research (adapted from [1]).

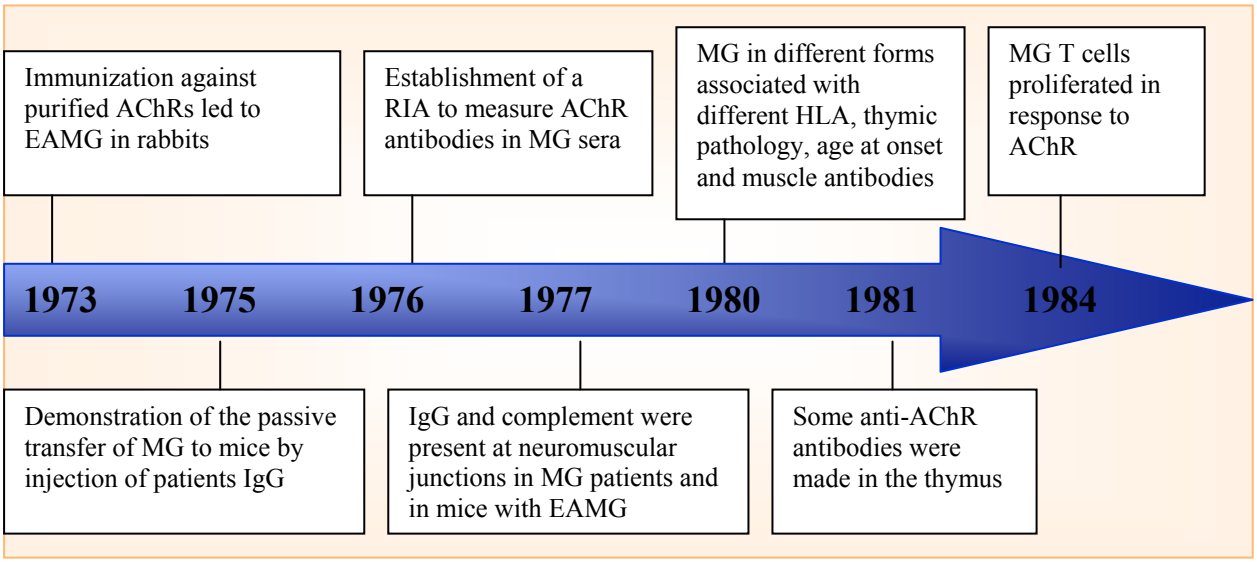


Figure 1b. Immunological developments in MG research (adapted from [1]).

The incidence of MG is between 0.25 and 2.00 people per million, and there is no difference between patients aged younger than 40 years and patients older than 40 years, but a substantially increased incidence in those over 60, with a bias towards men [2]. The prevalence is around 5 to 150 people per million in Caucasian populations. In Sweden, the prevalence is about 14.1 per million [3] and due to the modern therapies, the development of the MG can be well controlled and the life span of MG patients is equal to that of the healthy population.

The clinical presentation of MG patients has been reviewed and classified by Oosterhuis [4] (Table 1). The clinical classification was designed to identify subgroups of patients with MG that share distinct clinical features or severity of the disease that may indicate different prognoses or responses to therapy.

Table 1. Osserman-Oosterhuis' classification of MG

Class I	Ocular
Class II	
II a	Mild generalized
II b	Severe generalized
Class III	Acute fulminant
Class IV	Late severe weakness affecting other muscles than ocular

Thymic alterations occur so frequently in MG (90%) that a role for the thymus in the pathogenesis of MG is almost certain [5]. Hyperplastic changes are present in 60-70% and thymomas in 10-15% of the patients [5]. Thymic hyperplasia is characterized by numerous and prominent lymph follicles in germinal centers and T cell zones. The thymoma in MG is a neoplasm of the epithelial and lymphocytic origin and usually associated with a large number of nonmalignant lymphocytes. It should be removed by surgery in order to prevent local invasion. The presence of thymoma is often associated with high serum levels of anti-AChR antibodies and more severe disease [6]. About 5-10% of MG patients have normal thymic histology [7, 8].

2. Diagnosis

At present, a few findings in MG research have been applied to clinical diagnosis. Anti-AChR antibodies are present in about 85% of patients with generalized disease and are usually diagnostic for MG. The classical neurophysiological finding in MG is an increased decrement of the evoked compound muscle action potential in response to repetitive supramaximal nerve stimulation at 3 Hz [9]. More sensitive, but less widely available, is single fibre electromyography. This test measures the firing time of two muscle fibres within the same motor unit. In MG, there is increased variability (Jitter) and occasional blocking of impulses. In the edrophonium test, a short acting anticholinesterase is given intravenously. There is a rapid (within 2 min) but short-lived (less than 5 min) improvement in strength in most patients with MG.

3. Treatments

Oral anticholinesterase treatments, such as pyridostigmine, are the first line of treatment in patients with mild MG. Currently, thymectomy is usually done early in young-onset, anti-AChR antibody-positive patients with generalized MG. Evidence from uncontrolled studies suggests beneficial effects, but the results are not conclusive [2, 10]. Thymectomy is not usually beneficial in patients with late-onset disease and it is seldom done in patients with only ocular involvement [10]. If symptoms are not well controlled with such treatments, immunosuppressive treatments are indicated. Prednisolone is the most commonly used drug. Azathioprine can be added, either initially, or later, as an effective steroid-sparing agent. Cyclophosphamide and other immunosuppressive drugs are useful in severe MG. In severely affected patients, plasma exchange, or intravenous immunologlobulins (IVIg) may give a striking improvement.

4. Humoral immune responses in MG

4.1 Autoantibodies against AChR

MG is an autoimmune disorder mainly caused by antibodies against AChRs at the neuromuscular junction. Loss of functional receptors leads to a defect in neuromuscular transmission and consequently muscle weakness and fatigue. As shown in figures 1a and b, three experiments were crucial to demonstrate the pathogenic nature of human anti-AChR antibodies. First, injection of IgG from MG patients into mice led to a reduction in the number of AChRs at the neuromuscular junction and muscle weakness that mimics the symptoms found in MG [11]. Second, plasma exchange and lymph drainage, which removes circulating antibodies and other soluble factors, induced a marked clinical improvement [12-15]. Third, both IgG and complement were present at the neuromuscular junction of MG patients and they co-localized with the remaining AChRs [16, 17].

The AChR is a 290-300 kDa glyco-protein present at neuromuscular junction (Figure 2). In the presence of the detergent sodium dodecyl sulphate (SDS), AChR dissociates into four different subunits, which migrate on a polyacrylamide gel (PAGE) with apparent molecular weights (MW) of 38 (α), 49 (β), 57 (γ or ε) and 64 (δ) (Figure 3). There are two different forms of AChR. The fetal form is composed of 2α, β, δ and γ whereas the adult form is composed of 2α, β, δ and ε (Figure 3).

The majority of anti-AChR antibodies bind to the extracellular domain of AChR (Figure 3). The anti-AChR antibodies in MG patients are IgG but heterogenous in light chain, subclass, and their reactivity with different regions on the AChR [18]. A variable proportion in each patient binds to a region on the α-subunit, called the main immunogenic region (Figure 3) [19]. Some antibodies might also bind to the AChR γ-subunit. Antibodies against the cytoplasmic region of AChR in sera from MG are detected as well [20]. These autoantibodies can disturb neuromuscular transmission in at least three different ways. First, the autoantibodies cause complement-dependent lysis of the postsynaptic membrane [21, 22]. Second, the autoantibodies are able to cross-link AChRs on the surface of the membrane, which leads to an increase in the rate of internalization and degradation of AChRs [23]. Third, in some patients, autoantibodies directly block the function of AChRs. The increase of internalization or cross-linking by anti-AChR antibodies might be the most important determinant of loss of function of AChR [24].

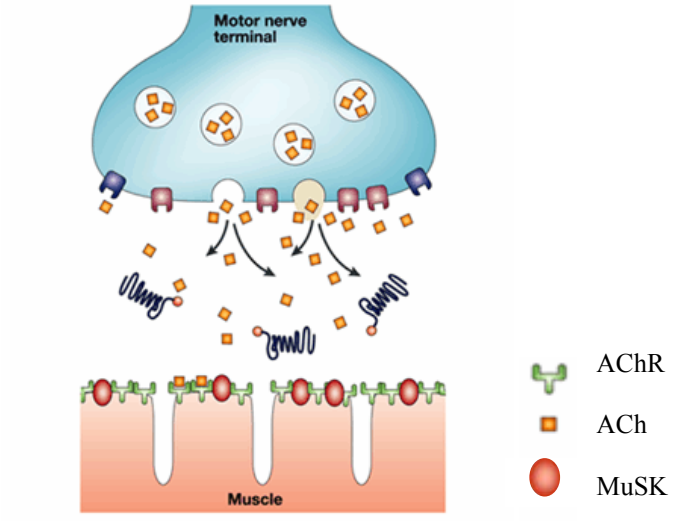


Figure 2. Neuromuscular junction.

The binding of ACh to AChRs opens the associated ion channels resulting in an action potential and contraction of the muscle (adapted from [1]).

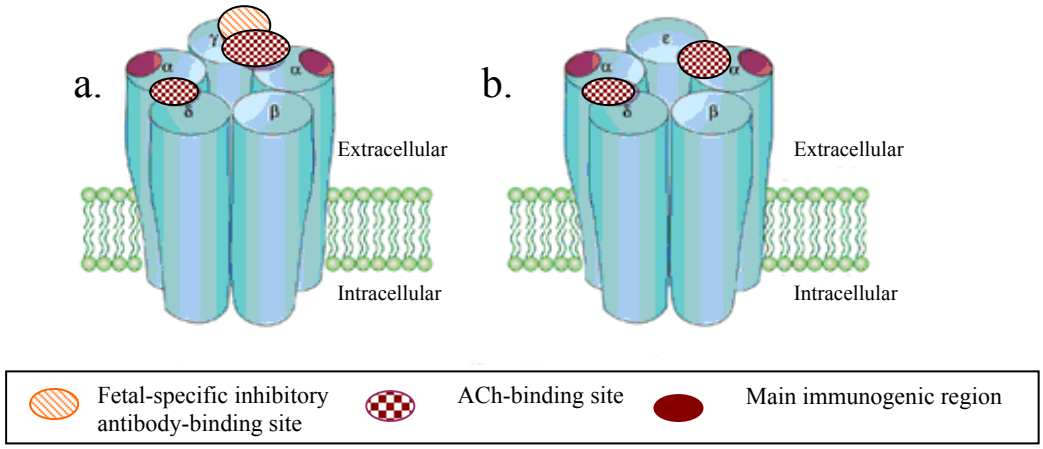


Figure 3. Fetal (a) and adult (b) forms of the AChR (adapted from [1])

4.2 Other autoantibodies in MG

Although 85% patients present anti-AChR antibodies, there are still 15% patients with generalized MG that are sero-negative for anti-AChR antibodies. Sero-negative MG is also an antibody-mediated disease [25]. Seventy percent of anti-AChR-antibody-sero-negative MG patients, but not anti-AChR-antibody-sero-positive MG patients, have serum antibodies against the muscle-specific receptor tyrosine kinase (MuSK) [26]. Other autoantibodies against striated muscle tissue sections, titin, actin, citric acid extractable antigen and ryanodine receptors have also been found in subgroups of MG patients [27, 28]. The anti-ryanodine receptor antibodies recognize a region near the N-terminus of ryanodine receptor important for Ca^{2+} release channel regulation. These antibodies inhibit Ca^{2+} release from sarcoplasmic reticulum in vitro. There is electrophysiological evidence for a disordered excitation-contraction coupling in MG patients [29]. The presence of anti-titin antibodies, which bind to key regions near the A/I junction and in the central I-band, correlates with myopathy in MG patients [29]. In addition, anti-nuclear antibodies, anti-thyroid antibodies and anti-beta-2 adrenergic receptor (β2-AR) antibodies are present in 20%, 30% and 18% of the patients, respectively [30, 31].

5. β2-AR protein and gene

The finding of anti-β2-AR antibodies in patients with MG inspired further studies of β2-AR in MG. Xu et al. demonstrated that patients with MG have a decreased density of β2-AR on peripheral blood mononuclear cells (PBMC) [32]. The decrease of β2-AR density seems to be caused by the autoantibodies against β2-AR that have an agonist action and down-regulate the expression of the receptor [33]. In clinical practice, β2-AR agonists are widely used agents in the treatment of MG and some patients do respond well with an improved clinical score, suggesting the beneficial effect of β2-AR agonist to MG patients [34].

The β2-AR is a glycoprotein that consists of 413 amino acid residues with MW 64 kDa [35] and contains an extracellular amino terminus, 7 clusters of hydrophobic amino acids and an intracellular carboxyl terminus. The β2-AR belongs to the G protein-coupled receptor family and its binding to norepinephrine increases the intracellular concentration of cAMP [36, 37] (Figure 4.).

The β2-AR is widely distributed on the membrane of skeletal muscle cells, cardiomyocytes and lymphocytes. The β2-AR expressed on the skeletal muscle regulates contractile force of the muscle whereas the β2-AR expressed on cardiomyocytes controls the heart rate. The β2-AR expressed on lymphocytes represents a linkage between the nervous system and the immune system. There is a growing amount of evidence showing that β2-AR is able to mediate suppression of the Th1 type cytokines such as IL-2 [38] and IL-12 [39], and stimulation of the Th2 type cytokines including IL-4 [40] and IL-6 [41] (Figure 4). The β2-AR agonist is able to inhibit the activation of both mouse and human T cells, as measured by proliferation and IL-2 secretion in response to anti-CD3 antibodies [38]. The β2-AR agonist also suppresses the proliferation PHA-stimulated PBMC and down-regulates CD25 expression on T cells [42]

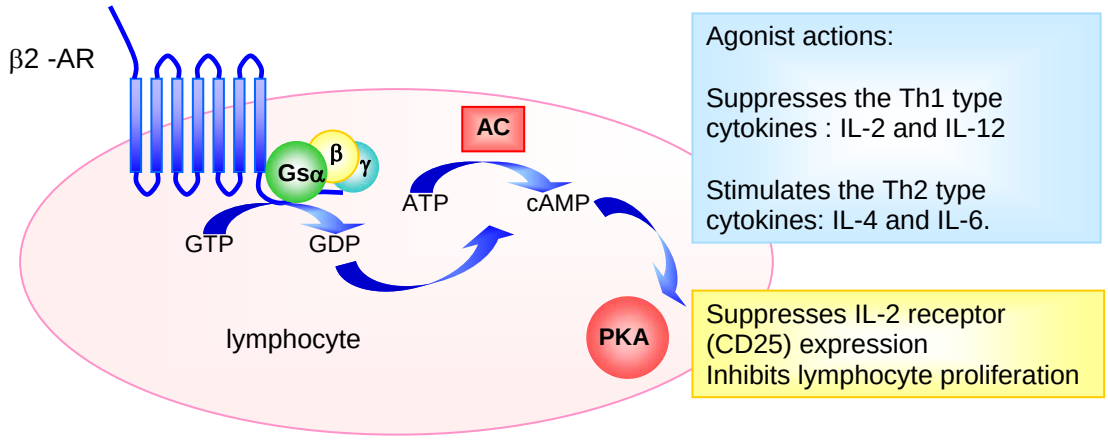


Figure 4. β2-AR and its function.

The β2-AR gene is localized to q31-q32 on chromosome 5. The cloning and complete nucleotide sequence of the cDNA for β2-AR was reported in 1987 [43]. The β2-AR gene has a conserved 5' leader region approximately 220 bp in length and contains no

introns in either the coding or untranslated sequences. Interestingly, the 5' region of the β2-AR gene contains several initiator ATG codons separated by short open reading frames and termination codons. This leading region encodes a beta upstream peptide (BUP) of 19 amino acids. The leader peptide is believed to inhibit translation of the β2-AR gene itself [44].

In the coding region, the β2-AR gene contains nine single nucleotide polymorphisms (SNPs), of which four are non-degenerative leading to amino acid changes (Arg-Gly16, Gln-Glu27, Val-Met34, Thr-Ile164). Three of them (codon 16, 27 and 164) in the β2-AR gene are functionally important. Gly16 is sensitive to agonist-mediated down-regulation whereas Glu27 is resistant to such down-regulation [45]. The frequency of homozygosity for Arg16 is increased in MG patients and Gln27 is associated with serum antibodies against β2-AR and AChR [33]. A high frequency of Gln27 was also reported in asthmatic children [46].The receptor homozygous for Ile 164 has markedly decreased capacity for ligand binding and properties for coupling compared to receptors homozygous for Thr164 [47]. Cardiac dysfunction was observed in transgenic mice having the Ile 164 form of β2-AR [47].

In the promoter region of β2-AR gene, a total of eight polymorphisms -20T/C, -47T/C, -367 T/C, -468 C/G, -654 G/A, -1023 G/A, -1343 A/G and -1429 T/A have been identified [48]. Four of these mutations, -20C, -47C, -367C, -468G significantly reduce β2-AR gene expression compared with -20T, -47T, -367T and -468C, respectively [48]. The SNPs at -47 and -367 introduce a non-conservative amino acid change in BUP, and 7/8 bp of a consensus AP-2 site [48]. The SNP at -47 is also associated with obesity and type 2 diabetes [49]. Polymorphisms at -20 and -47 are in linkage disequilibrium with codon 27 polymorphisms [49].

6. Cellular immune responses and costimulatory molecules in MG

The anti-AChR antibodies are produced by autoreactive B cells in a T-cell-dependent manner both in human MG and experimental autoimmune myasthenia gravis (EAMG) [50, 51], indicating a critical role of cellular immune responses in the pathogenesis of the disease. The possible roles of the cellular immune responses in MG are discussed in the following part.

6.1 T cells, B cells, DC and NK cells

The first demonstration of antigen-specific T cells in MG patients was made by Hohlfeld and colleagues, who observed proliferative responses of PBMC to purified Torpedo AChR in 1984 [52]. Recently, a pathogenetic role has been assigned to CD4+ T cells since they permit and facilitate the synthesis of high-affinity anti-AChR antibodies [53]. CD4+ AChR-specific T cell lines are isolated from the PBMC and thymus of MG patients [54-56]. Only if CD4+ T cells are included in the grafted cells, PBMC from MG patients grafted in severe combined immunodeficiency mice induce synthesis of human anti-AChR antibodies and myasthenic symptoms [57]. In contrast, depletion of the CD8+ T cells does not affect the ability of PBMC from MG patients to induce myasthenic weakness and the synthesis of anti-AChR antibodies. Additionally, treatment of MG patients with anti-CD4 antibodies causes clinical and electrophysiological improvements and abolishes the response of their T cells to the AChR *in vitro* [58].

Human B cells from PBMC are able to secrete antibodies with defined antigen specificities *in vitro*. In the presence of the anti-CD40 monoclonal antibody and cytokines, highly enriched B cells isolated from PBMC and thymic tissues from MG patients cultured on the adherent medulloblastoma cell line TE671 that expresses the functional AChR on its surface, produce anti-AChR antibodies [59, 60]. In contrast, B cells from the same donors cultured on other cell lines secrete large amounts of IgG without preference for anti-AChR antibody production [59, 60]. The antibodies in MG patients are mostly IgG, suggesting that the secretion of anti-AChR antibodies is T-cell-dependent. Anti-CD20 monoclonal antibodies, which are able to deplete all B cells, reduce the anti-AChR antibodies in the serum from treated MG patients [61].

In addition to T cells and B cells, DC and NK cells may participate in the disease development as well. DCs are professional APCs and play a major role in immune responses to foreign and autoantigens. DCs are able to stimulate antigen specific T cells, to induce proliferation of activated T cells and to present auto-antigens involved in autoimmune diseases. DCs isolated from patients activate autologous T cells and induce the production of IFN-γ, IL-4 and IL-10 [62]. On the other hand, DC modified by exposure to IL-10 [63] , IFN-γ [64], AChR [65] and TGF-β [66] modified DC are capable of ameliorating EAMG.

Even though Natural Killer cells (NK cells) are regarded as effector cells in innate immunity, IFN-γ-producing NK cells can prime APC for IL-12 production, indicating the involvement of NK cells in adaptive immunity [67, 68]. NK cells have recently been shown to interact directly with B cells via CD40-CD40L [69] and CD4+ T cells via OX40-OX40L [70] and contribute to the disease development of EAMG [71]. A low NK activity was firstly described in female MG patients [72]. This finding was later confirmed by other studies showing a markedly deficient NK activity and activated IFN system in not only female but also male MG patients [73]. On the other hand, the number of NK cells is significantly high in MG patients who are not thymectomized but returns to normal after thymectomy [74]. IFN-α induces NK cell proliferation, converts non-cytolytic NK precursor cells into functional NK cells and enhances the lytic efficiency of NK cells [75]. When MG patients are treated with IFN-α, they have increased NK cell cytoxicity but decreased NK cell count together with a mild improvement of the clinical score [76]. All above findings suggest that a deficient NK activity but increased NK cell count occurs in MG patients and it might be beneficial to the patients if the situation is reversed by treatment.

6.2 Costimulatory molecules on T cells and APCs

Since the secretion of anti-AChR antibodies is T cell dependent, T-B cell interactions play an essential role in the pathogenesis of MG. Thus, costimulatory molecules are studied intensively in MG.

The two-signal model of T cell activation proposes that the activation of T cells requires stimulation via both the T cell antigen receptor (TCR) (signal 1) and an additional costimulatory signal (signal 2) from APC such as B cells and DCs [77]. Among costimulatory pathways, there are two major families: the B7 family, including CD28 and cytotoxic T lymphocyte antigen (CTLA-4), and the tumour necrosis factor/tumour necrosis factor receptor superfamily (TNF/TNFR).

6.2.1 CTLA-4 (CD152)

The engagement of CD28 on T cells by CD80 and CD86 on the APC provides signal 2 [78]. CD28 shares extensive sequence homology with CTLA-4 [79]. CTLA-4 also binds to CD80 and CD86 with much higher affinity than that of CD28 (Figure 4). Therefore, CTLA-4 could inhibit T cell responses by competition with ligand binding with CD28. It is generally believed that CD28 provides positive signals to T cell activation whereas CTLA-4 delivers inhibitory signals.

A fundamental issue in the CD28-CTLA-4 system is how a process regulated by the integration of positive and negative signals, generated by receptors that bind the same ligands, can ever be activated when the affinity of the inhibitory receptor for the ligands greatly exceeds that of the stimulatory receptor. The answer is likely to involve the temporal separation of expression and protein stability. CD28 is constitutively expressed on the T cell surface, whereas CTLA-4 expression is up-regulated upon T cell activation and reaches maximum after 2-3 days [80]. CD28 is quite stable and has the potential to persist and mediate its function even after its protein synthesis is reduced or terminated [81]. In contrast, CTLA-4 undergoes fairly rapid degradation in activated T cells [81].

CTLA-4 is accumulated in lysosomes and secreted to the T cell-APC contact site upon TCR stimulation. Surface CTLA-4 is fairly rapidly degraded (it has a half-life of ~2 h) in activated T cells due to lysosomal targeting. The trafficking and the cell surface expression of CTLA-4 are dynamically regulated by clathrin-mediated endocytosis. This procedure is reciprocally modulated by tyrosine phosphorylation of the cytoplasmic region of CTLA-4. CTLA-4 can be phosphorylated at the Tyr residue by AP-2 adapter complex [82] and a number of typrosine kinases such as resting lymphocyte kinase (RLK), the Janus kinase Jak-2, the Src family tyrosine kinases Fyn, Lyn and Lck [83, 84]. Dephosphorylated cytoplasmic domain of CTLA-4 interacts with the medium chain of the AP-2 clathrin adapter protein and the interaction results in the rapid endocytosis of CTLA-4 from the cell surface, but when cytoplasmic domain of CTLA-4 is phosphorylated, CTLA-4 is stabilized on the cell surface [85]. Hence, the targeting of CTLA-4 to intracellular compartments may serve at least two functions. First, it provides constitutive delivery of CTLA-4 to the lysosomal compartment, which results in a rapid turnover rate. Second, it provides a point of regulation with which to spatially and temporally control the quantity of CTLA-4 on the T cell surface, thus placing restrictions on its access to CD80 and CD86 expressed by APCs.

CTLA-4 deficient mice suffer a severe lymphoproliferative disorder characterized by polyclonal T cell proliferation and early lethality, providing compelling supports for a critical role for CTLA-4 in down-regulation of T cell responses [86-88]. CTLA-4 regulates the T cell activation threshold and peripheral T cell tolerance, and attenuates T cell expansion (Figure 5). Importantly, CTLA-4 is highly expressed on CD4+CD25+ regulatory T cells and its blockage by antibodies appears to abrogate the regulatory function of these regulatory T cells [89].

CTLA-4 attenuates activation and proliferation of T cells by directly affecting the signalling transduction through TCR and CD28. Cytoplasmic domain of CTLA-4 recruits phosphatidylinositol 3-kinase, SH2-containing tyrosine phosphatase-2 (SHP-

2), and other molecules to the signalling apparatus associated with the activated TCR complex in order to directly antagonize TCR activity [83, 84] (Figure 5). CTLA-4 can affect TCR signals by bringing SHP-2 to the TCR complex and facilitating dephosphorylation of proximal signalling molecules [90]. The interaction of SHP-2 to CTLA-4 cytoplamic tail is Fyn dependent [83]. Even though TCR zeta-chain phosphorylation and subsequent zeta-associated protein of 70 kDa (ZAP-70) tyrosine kinase recruitment are not significantly affected by CTLA-4 engagement, CTLA-4 ligation selectively inhibits CD3-mediated phosphorylation of the ZAP-70 and the association of Lck with ZAP-70 [90, 91]. CTLA-4 crosslinking inhibits the activation of MAP kinase Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK) [92], and thus results in reduced activation of several transcription factors such as NF-KB, NF-AT and AP-1 [93, 94].

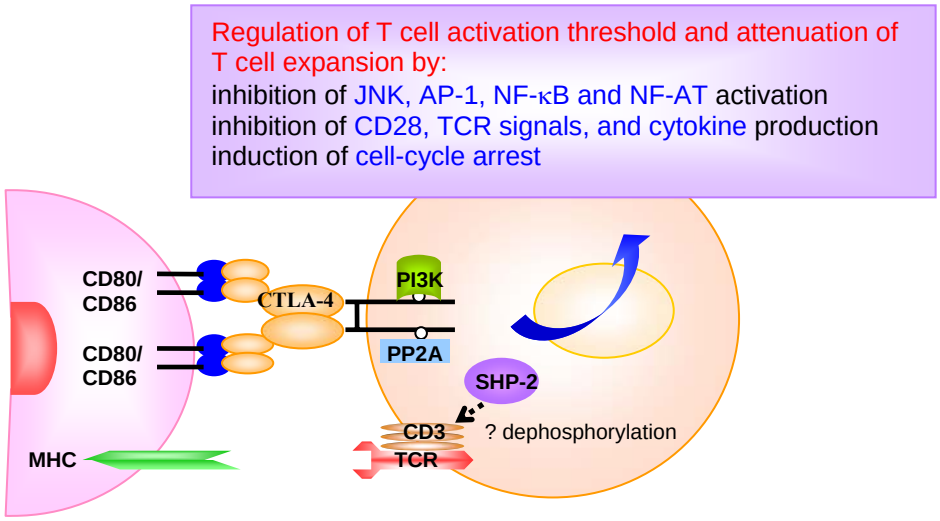


Figure 5. Model of the function of CTLA-4.

CTLA-4-mediated attenuation of the T cell expansion and the consequent effects on the antigen-driven selection are important for the regulation of T cell responses to foreign pathogens. Once autoimmunity is initiated, CTLA-4 may regulate its progression by restricting the expansion of auto-aggressive T cells. For instance, in experimental autoimmune encephalomyelitis (EAE)-susceptible and non-susceptible strains of mice, disruption of CTLA-4-CD80/CD86 interactions preferentially increases the number of autoantigen-specific T cells that produce pro-inflammatory cytokines [95]. This correlates with enhanced disease severity in EAE-susceptible strains. Thus, by regulating the magnitude and composition of an autoantigen-specific T cell response, CTLA-4 can affect the severity of autoimmunity.

MG patients have an abnormal expression of CTLA-4 on both freshly isolated and stimulated T cells from the peripheral blood. The aberrant CTLA-4 expression is characterized by low levels of CTLA-4 protein both on the surface of the cells and intracellularly, impaired up-regulation of CTLA-4 in T cells in response to ConA stimulation and high levels of soluble CTLA-4 in the serum from MG patients [96]. Moreover, CTLA-4Ig that blocks binding of CD28 to CD80 and CD86, is able to relieve EAMG [97].

The CTLA-4 gene is located on chromosome 2q33 [98]. Human CTLA-4 and CD28 gene are next to each other on the chromosome and separated by only 25 to150 kb [99]. The human CTLA-4 gene comprises 4 exons. The second exon encodes a V-like extracellular domain of 116 amino acids, the third a hydrophobic putative transmembrane region of 37 amino acids, and the fourth a putative cytoplasmic domain of 34 amino acids [98] (Figure 6).

RT-PCR amplification of the coding sequence of CTLA-4 in nonstimulated human T cells produces at least two transcripts with sizes of 650 and 550 bp. Sequencing analysis reveals that the large transcript codes for the membrane CTLA-4 and the small transcript is a spliced variant in which exon 3 coding for the transmembrane region is deleted. This spliced cDNA has been named as CTLA-4delTM and encodes a soluble form of CTLA-4, a frame shift introduced by the alternative splicing leads to addition of 22 extra amino acids before the translational termination [100]. In contrast to the accumulation of membrance CTLA-4 on stimulated T cells, CTLA-4delTM mRNA expression is restricted to nonactivated T cells. In fact, there is a third splice variant of CTLA-4, named ligand-independent CTLA-4 (liCTLA-4) in mice, lacking exon 2 including the MYPPPY motif essential for binding to the costimulatory ligands CD80 and CD86. liCTLA-4 is expressed as a protein in naive T cells and strongly inhibits T cell responses by binding and dephosphorylating the TCR zeta chain and ZAP-70. The expression level of liCTLA-4, but not full-length CTLA-4, is high in memory/regulatory T cells from diabetes-resistant NOD congenic mice compared to susceptible NOD mice [101].

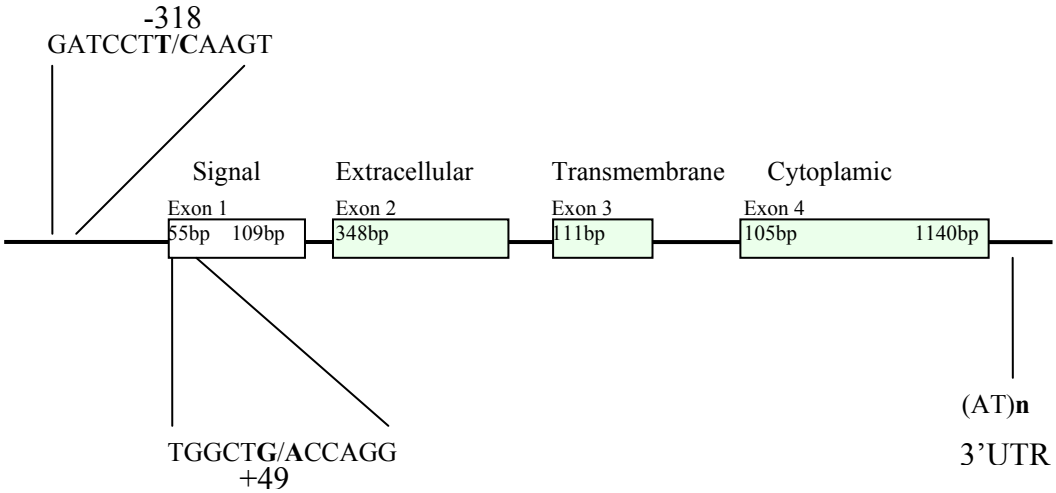


Figure 6. Schematic representation of known polymorphic sites within the CTLA-4 gene.

In addition to the different spliced variants, a few polymorphisms have been identified in the CTLA-4 gene (Figure 6). These include a C/T SNP at promoter -318 [102] and a G/A SNP at +49 of coding region. The -318C allele is associated with Wegener's granulomatosis [103] and autoimmune thyroid disease [104]. -318C is in strong linkage with +49G [105, 106]. The G/A transition at position +49 within first exon of CTLA-4 gene encodes an Ala/Thr substitution in codon 17 of the leader peptide [107]. The G/A polymorphism at +49 alters T cell activation and cellular localization of CTLA-4 [108]. Individuals carrying -318C and homozygous for +49G show

significant down-regulation of both cell-surface CTLA-4 after cellular stimulation and CTLA-4 mRNA in non-stimulated cells [109].

The G allele at position +49 is associated with several autoimmune diseases including type 1 diabetes, Grave's disease, Hashimoto thyroiditis, rheumatoid arthritis (RA), and multiple sclerosis (MS) [106, 108, 110-115]. It is more frequently present in MG patients with thymoma [105]. Patients with this genotype also have signs of immune activation manifested by elevated levels of the serum IL-1β and a high percentage of CD28+ T cells [105].

The CTLA-4 gene includes an (AT)n microsatellite within the 3'-untranslated region (3'-UTR) [116]. The allele with longer PCR product than 86 bp in the polymorphic (AT)n region in the CTLA-4 is in strong linkage with +49G [105] and confers susceptibility to Grave's disease [115], type 1 diabetes [117] and MG with thymoma [118]. It was speculated that mRNA from the alleles with longer PCR product than 86 bp might be instable [115, 117] and these longer alleles lead to T cell hyperreactivity via the CD28 pathway in MG [118]. Thus, these alleles with longer PCR product than 86 bp in MG patients with thymoma result in an instability of *Ctla-4* mRNA, a deficiency of *Ctla-4* expression, the net result being a triggering of T and B cell activation and autoantibody production *in vivo* [87, 96].

6.2.2 OX40 (CD134)

In addition to B7 family, TNF/TNFR superfamily members, such as CD27, OX40 (CD134), CD40L (CD154), 4-1BB (CD137), are important for the effective generation of many types of T cell responses. These molecules appear to provide signals to allow continued cell division initially regulated by CD28 and/or to prevent excessive cell death [119]. A blocking antibody against CD40L is able to ameliorate EAMG by downregulation of Th1 differentiation and upregulation of CTLA-4 [120].

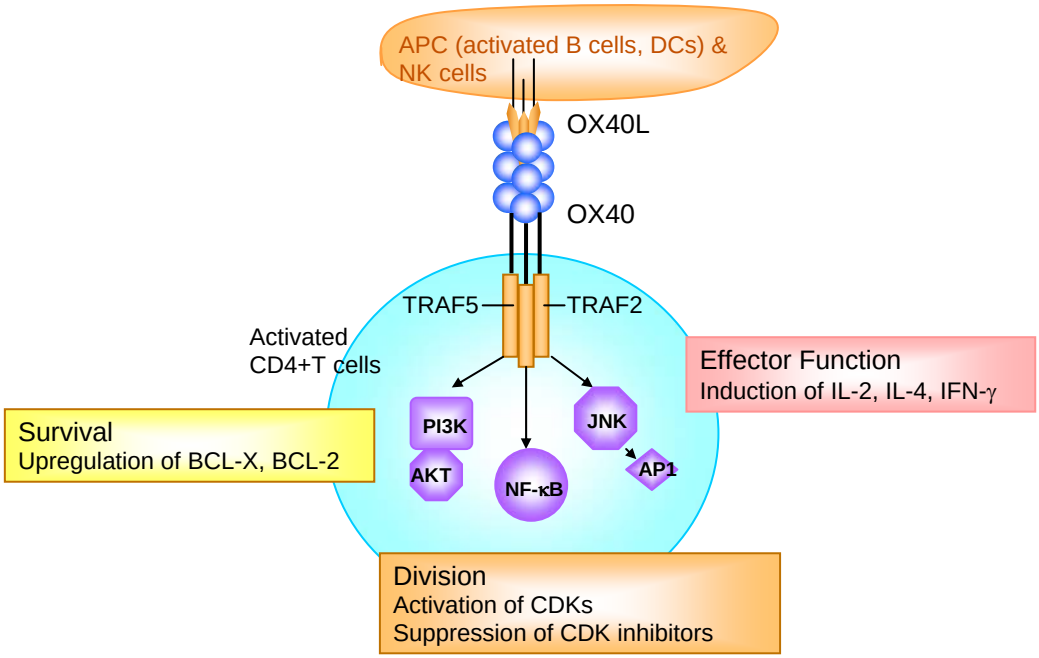


Figure 7. OX40 and its function.

OX40, a member of the TNFR family, is a 50 kDa cell surface glycoprotein expressed on activated T cells (Figure 7), but not on resting T cells. OX40 can be induced by TCR/CD3 signals and augmented by CD28 signals [121, 122], reinforcing the concept that OX40 and CD28 most likely co-operate together in a sequential manner. OX40 initially appears 12-24h after stimulation of naïve cells. Peak expression is seen after 2-3 days and then OX40 is downregulated [122]. Antigen-experienced effector/memory T cells can rapidly re-express OX40 within 4h of reactivation [119]. Though it is often quoted that OX40 is primarily expressed on activated CD4+ T cells [123], CD8+ T cells can also bear OX40 at least under certain condition [119]. Moreover, OX40 has now been visualized on other more diverse cell types including B cells, DCs and eosinophils, although as yet the physiological significance of this expression is unknown [119].

OX40 is not involved in the initiation of naïve T cell response [124]. However, OX40 signal enhances proliferation and both Th1 and Th2 cytokine production by CD4+ T cells [123, 125] as well as long-term survival of CD4+ T cells by promoting the expression of Bcl-xL and Bcl-2 [124, 126]. It also modulates late cell division and enhances cell cycle turnover of antigen-activated CD4+ T cells *in vivo* [124, 126] and leads to tumor necrosis factor receptor-associated factor 2 (TRAF2) and TRAF5 mediated NF-κB activation [127] (Figure 7).

OX40 ligand (OX40L) is expressed on the APCs (Figure 7), such as activated B cells [122, 128], DCs and macrophages many hours to days after activation [129]. In the case of DCs and B cells, Toll-like receptor signals induced by LPS can promote OX40L expression in addition to contributions from immunoglobin and CD40 signals [122, 125, 128, 129]. Additionally, OX40L has been visualized on activated endothelial cells in vitro, and in tissues from patients with lupus nephritis and inflammatory bowel disease (IBD), implying a role in promoting migration of OX40-expressing T cells into inflamed tissues, or providing signals to augment T cell activities in these peripheral sites [130-132]. The presence of OX40L on human NK cells and OX40-OX40L can function as an important ligand for cross-talking between NK cells and CD4+ T cells [70].

OX40L exhibits a potent costimulatory activity for T cell proliferation and IL-2 production [122]. Cross-linking of OX40L induces proliferation and differentiation of B cells [128] and maturation of immature DCs [129]. Blocking of OX40-OX40L interaction with polyclonal anti-OX40 antibody results in a profound decrease of the production of antigen specific, T-cell-dependent-antibodies and accumulation of CD4+ T cells [133]. Mice lacking OX40L show impairment of the APC function and reduction in T cell proliferation and production of both Th1 and Th2 cytokines [125, 134].

Collectively, these studies have suggested that a major role of OX40-OX40L interactions is to dictate the number of effector T cells that accumulate in primary immune responses, and consequently to govern the number of memory T cells that subsequently develop and survive.

Due to its involvement in T-B cell interaction, the OX40 signal is strongly implicated in autoimmune diseases. Signals from an agonistic antibody against OX40 can break an existing state of tolerance in CD4+ T cells [135, 136], implying the possibility that

a similar mechanism *in vivo* may exist and cause autoimmunity. Indeed, more and more recent findings confirm this notion. Excess interactions of OX40/OX40L result in autoimmune-like IBD [137]. OX40 signaling abrogates the disease-preventing activity of CD4+CD25+ regulatory T cells in an experimental IBD [138]. Abnormal expression of OX40 and/or OX40L has been demonstrated in the tissues of several autoimmune disorders such as EAE [139, 140], experimental IBD [141], atherosclerosis [142], human proliferative lupus nephritis [130], RA [143], human IBD [132], human inflammatory muscle disease [144] and human GVHD [145]. Blocking the OX40:OX40L pathway ameliorates ongoing EAE [146], diabetes in NOD mice [147], murine models of asthma [148], collagen-induced arthritis [143], murine GVHD [149] and experimental IBD [141]. Polymorphisms in OX40L affect the risk of myocardial infarction in humans [142].

In MG patients, OX40 is up-regulated in thymic tissues adjacent to the germinal center and thymoma and might interact with OX40L in the germinal center to enhance anti-AChR antibody production [150]. A case control study demonstrated that a patient who developed MG 25 months after allogeneic bone marrow transplantion had prominently elevated levels of CD4+OX40+ T cells in the peripheral blood one month before the onset of MG [151].

6.3 Cytokines and chemokines

In addition to the humoral and cellular immune responses, other complementary factors such as cytokines and chemokines may contribute to the pathogenesis of MG.

6.3.1 Cytokines

Cytokines are widely secreted by T cells, B cells, macrophages and NK cells. They are divided into two sub-populations: Th1 and Th2. Th1 cytokines include pro-inflammatory cytokines, such as IL-2, IL-18 and IFN-γ. They are involved in cell-mediated immune responses. Th2 cytokines, such as IL-4 and IL-10, may reduce the intensity of immune responses and provide help to B cells (Figure 8).

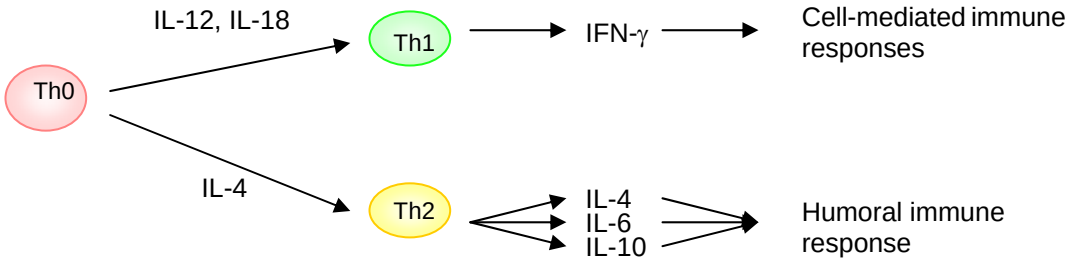


Figure 8. Differentiation of CD4+ T cells.

Both Th1 and Th2 cytokines participate in the development of human MG [152]. The anti-AChR CD4+ T cell lines propagated from MG patients and specific for AChR subunits or individual epitopes secreted both IL-2 [153, 154] and Th2 cytokines such as IL-4 [152], when challenged with the relevant AChR antigen. IFN-γ genes are highly expressed in thymic tissues of MG and the up-regulation of IFN-γ can increase the transcription of AChR in thymic epithelial cells which may initiate the autoimmune anti-AChR response in thymus [155]. The expression of IL-1β is

increased in hyperplastic thymus from MG patients [156]. IFN-α induces NK cell proliferation, converting non-cytolytic NK precursor cells into functional NK cells and enhancing the lytic efficiency of NK cells [75]. IFN-α production is correlated to the severity of MG. When MG patients are treated with IFN-α, they have mild improvements of the clinical score [76]. The serum levels of IL-18 are significantly elevated in MG, especially in patients with generalized disease [157]. IL-10, which is a potent growth and differentiation factor for B cells and able to enhance the production of IgM, IgG and IgA by PBMC, may play a pathogenic role in MG. The number of AChR specific IL-10-secreting cells is increased in PBMC from MG patients [158].

However, the susceptibility of mice to EAMG is most likely related to Th1 cytokines [159]. Th1 CD4+ cells are especially important because they drive the synthesis of anti-AChR antibodies [53]. Transgenic mice that constitutively express IFN-γ in neuromuscular junction exhibit features strikingly similar to human MG [160] whereas IFN-γ knockout (-/-) mice are resistant to EAMG [161]. In addition, mice that are deficient of IL-1β are resistant to the induction of EAMG [162]. IL-12, a crucial cytokine for differentiation of Th1 cells, is necessary for the development of EAMG [53, 161]. Th2 cells secrete different cytokines, with different effects on the pathogenesis of EAMG. Among them, IL-10 facilitates the development of EAMG [163]. IL-4 is not necessary for EAMG development [53]. However, IL-4 has a role in the establishment of mucosal tolerance to AChR [53].

6.3.2 Chemokines and chemokine receptors

6.3.2.1 Chemokines

In contrast to numerous studies of cytokines in MG, the study on chemokines in MG is limited. Chemokines are low MW proteins (6-14 kDa) that play essential roles in recruitment and activation of leukocytes at the site of inflammation. Some of the chemokines may also regulate the growth, activation and differentiation of lymphocytes [164]. Two subfamilies of chemokines, α and β, have been classified based upon the arrangement of the first two cysteines, which are either separated by one amino acid (C-X-C, α-chemokine) or next to each other (C-C, β-chemokine). The C-C chemokines include RANTES (regulated on activation, normal T-expressed and secreted), macrophage inflammatory protein (MIP)-1α, MIP-1β and monocyte chemotactic protein (MCP)-1 [165].

The cells that express C-C chemokines RANTES, MCP, MIP-1α and MIP-2 mRNA are not detectable in muscle tissues over the course of EAMG in rats [165]. However, after exposure to anti-AChR antibodies, MCP-1 can be detected in skeletal muscle cells [166]. In the hyperplastic MG thymus, the expression of secondary lymphoid-tissue chemokine (SLC) markedly goes up particularly around blood vessels, where the CD44-high mature DCs accumulate. SLC has been shown to be a relatively selective chemoattractant for T cells, including memory T cells, and to be involved in lymphocyte re-circulation. The lymph stream directed by SLC on vessels potentially induces mature DCs to interface with re-circulating T cells [167, 168]. These findings suggest that DCs may migrate into the hyperplastic thymus from the vascular system via mechanisms that involve CD44 and SLC. DCs present self-antigens, thereby

promoting the priming and/or boosting of potentially autoreactive T cells against the AChR [168].

6.3.2.2 Chemokine Receptors

Chemokines exert their biological functions through binding to seven transmembrane G-protein coupled receptors. CC receptors bind to CC chemokines and CXC receptors bind to CXC chemokines. Four human CXC chemokine receptors (CXCR1 through CXCR4), eight human CC chemokine receptors (CCR1 through CCR8), and one human CXXXC chemokine receptor (CX_3CR1) have been identified [165]. Among these receptors, CCR5 and CCR2 will be addressed in detail.

CCR5 is expressed on monocytes, activated T cells, DC and NK cells and binds to MIP-1α, MIP-1β and RANTES [165]. The human CCR5 gene is located on chromosome 3p21 and encodes a 352-amino acid protein with a MW of 40.6 kDa [169]. CCR5 is preferentially expressed by polarized Th1 cells. Interestingly, chronically inflamed tissues contain an increased number of CCR5 positive mononuclear cells, and the number of immunocompetent cells is correlated with histopathological signs of inflammatory severity [170]. CCR5-Δ32 is a 32 bp deletion form of the receptor and encodes a truncated inactive receptor that is not expressed on the cell membrane. The CCR5-Δ32 heterozygotes express also low amounts of the protein. The CCR5-Δ32 homozygotes are resistant to macrophage-tropic strains of HIV-1 infection [170] and are linked with slower disease progression towards AIDS [171, 172]. Homozygosity for the CCR5-Δ32 is positively associated with prolonged renal allograft survival [173], reduced risk of asthma [174], decreased severity of rheumatoid arthritis [175], and decrease risk of early-onset myocardial infarction [176].

CCR2 is expressed on monocytes, activated T cells, DC and NK cells and binds to MCP-1 [165]. Smith et al. identified a Val64-to-Ile substitution (190G/A mutation) in the first transmembrane region of CCR2 [177]. They analyzed the CCR5 and CCR2 genes and found that the 32-bp deletion in the CCR5 locus and the 64I allele at the CCR2 locus were in strong linkage disequilibrium with each other, suggesting that CCR5-Δ32 consistently occurs together with allele CCR2-64V, whereas CCR2-64I is present together with the wild-type allele at the CCR5 locus. Deficiency of CCR2 expression has been shown to be associated with human ovarian carcinoma [178]. The CCR2-64I allele is associated with T1D in children and diabetic nephropathy in Japanese population [179, 180]. These findings suggest that CCR5-Δ32 and CCR2-64I alleles might be important for protection or susceptibility to immmune-mediated disorders.

7. Genetic susceptibility to MG

MG is a heterogeneous disorder. There is strong evidence that the individual's genetic make-up is an important predisposing factor for development of the disorder.

The strongest evidence for an immuno-genetic predisposition to development of MG is that identical twins have a 30% concordance rate of MG [181]. Another evidence is that patients with early-onset and late-onset MG have different HLA association [182]. Early-onset MG is defined as presenting disease symptoms before age 40 and more

common in women. About 60% of early-onset patients are HLA-B8 and DR3 positive. Late-onset MG is usually defined as first presenting in people older than 40 years, with a small bias to men and related to HLA-B7 and DR2 [182]. In Chinese and Japanese populations, up to 30% of patients develop MG in early childhood, many of them with ocular MG only [183, 184]. Ocular MG is associated with HLA-BW46 [185]. MG patients with thymoma and hyperplasia display different HLA association. The frequencies of HLA-B8, DR3, DQA and DQB are high in patients with hyperplasia and low in patients with thymoma [186].

In addition to HLA association, other genetic markers have been demonstrated as well. As mentioned previously, cytokines play a critical role in the disease development of MG. A positive association of IL-1β TaqI restriction fragment length polymorphism (RFLP) allele 2 carriage is more pronounced in MG patients without HLA-B8 [187]. In the TNF-α gene, the -308 allele 2, which strongly associates with HLA-B8, is associated with female patients with both early-onset disease and thymic hyperplasia. There is no association between polymorphisms in the IL-4 gene and MG [188]. The prevalence of a 'high secretor' phenotype of IL-10 is higher in individuals with 'high secretor' phenotype of IL-1β in healthy controls, but no such balance was found in MG patients [189]. The microsatellite IL10.R allele 112 was associated to patients having normal thymic histopathology, while microsatellite IL10.G allele 134 with patients having high levels of serum antibodies against AChR. This indicates that the IL-10 gene has diverse mechanisms in MG [189].

8. Non-genetic factors

The hypothesis that MG may be triggered by molecular mimicry, which is an immune response to an infectious agent that resembles the AChR, has acquired some support. Antibodies obtained from 6 of 40 patients with MG bind to a peptide sequence of herpes simplex virus that is homologous to a sequence of AChR α subunit [190]. Other studies showed that hepatitis C may lead to MG via a mechanism of cross-reactivity between viral epitopes and AChR [191, 192].

It has been shown that 92.5% of MG patients are detected positive for human T cell leukemia virus type I/II (HTLV-I/II) *tax-rex* genes and 55% for *pol* gene, indicating that HTLV-I or a part of the virus genome is involved in the etiopathogenesis of MG [193]. In addition, the presence of human foamy virus (HFV) genome in thymus tissues of MG patients has been determined [194].

AIMS OF THE STUDY

MG is a complex disease with multiple factors contributing to its pathogenesis. The overall aims of this thesis are to disclose the influence of some candidate genes and cellular mechanisms involved in the immunoregulation of human MG. The following issues are clarified:

1. The correlation between the promoter SNPs of the β2-AR gene and MG

2. The association of chemokine receptors CCR2 and CCR5 gene polymorphisms with MG

3. The regulatory effect of –318C/T SNP in *Ctla-4* on transcription and protein expression

4. The expression pattern of OX40 on naïve and activated CD4+ T cells MG and healthy individuals

MATERIALS AND METHODS

1. Study groups

Patients with MG and healthy controls (HC) were age and sex matched in the studies. The diagnosis of the disease was based on a typical case history, clinical signs, positive responses to edrophonium, a decremental response following repetitive nerve stimulation and in most cases, a typical single fibre EMG and the presence of antibodies against AChR. All MG patients were recruited in the MG Centrum at the Department of Neurology, Karolinska Hospital, Stockholm, Sweden. For the OX40 study, none of the patients had ongoing treatments with immunomodulatory drugs including corticosteroids, azathioprine, cyclophosphamide, methotrexate. The majority of the patients received cholinesterase inhibitors. The subject patients were classified into different groups according to the thymic histopathology, age of on-set, gender or concentration of anti-AChR antibodies. Anti-AChR antibodies in sera from MG patients were determined by standardised RIA [6].

2. DNA extraction, PCR and pyrosequencing

Genomic DNA was extracted from EDTA preserved whole blood by a standard proteinase K digestion followed by phenol/ chloroform extraction.

For four SNPs of β2-AR, target sequences containing the polymorphisms at positions -468, -367, -47 and -20 were amplified by PCR. The primers used were as described in paper I. The temperature cycling was 94°c 30s, 55°c 45s, 72°c 30s for 50 cycles. Target sequences containing G/A SNP at position 190 of CCR2 gene and polymorphisms of CCR5-64I were amplified by PCR and primers for CCR2 and CCR5 gene were shown in paper II.

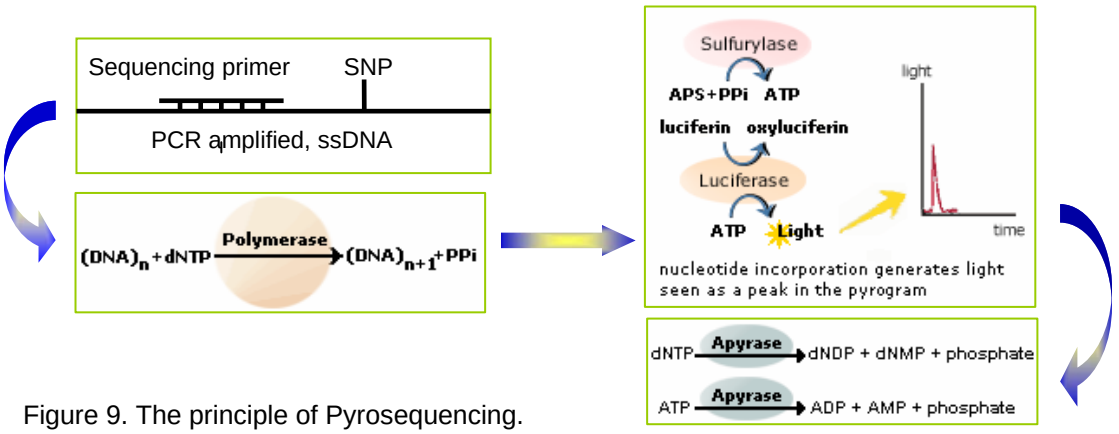


Figure 9. The principle of Pyrosequencing.

The SNPs of CCR2 and β2-AR were analyzed by an automated bench-top PSQ™ 96 system (Pyrosequencing AB, Uppsala, Sweden) according to the manufacturer's manual. A detailed description of the pyrosequencing technology can be found at http://www.pyrosequencing.com. The pyrosequencing method is a sequencing-by-synthesis DNA sequencing technique that specifically analyses SNPs and polymorphism of a DNA sequence less than 200 bp [195] (Figure 9). In short, the sequencing reaction starts by the addition of one of the four dNTPs. DNA polymerase catalyzes an incorporation admitted by the DNA base-pairing rule. The PPi generated

after each successful incorporation event is converted to ATP by ATP sulfurylase. The produced ATP is detected by the luciferase that produces light. ATP and dNTPs are degraded by apyrase prior to the next dNTP addition. dNTPs are added iteratively and the sequence result is presented in the form of a program (PSQ^{TM}96 system, Pyrosequencing AB, Uppsala, Sweden).

Genetic variation is the basis for human diversity and plays an important role in human diseases. Over decades, methods for screening and mapping the genetic variability were based on RFLP and microsatellite markers. More recent efforts have focused on the most common type of human genetic variation, the SNPs. A position is referred to as a SNP when it exists in at least two variants with frequency of more than 1% for the least common alternative [196]. SNPs are distributed across the human genome with an approximate frequency of 1 SNP per 1000 bp [196]. Similar to microsatellite markers, SNPs can be used in linkage studies to identify disease susceptible genes and in clinical genetic testing. The properties that make SNP analysis preferable compared to microsatellites are that SNPs are more prevalent than microsatellites and that many SNPS are located within the promoter and the coding region, directly affecting the expression and function of a gene.

As the number of identified SNPs increases, there will be an increasing demand for efficient methods to assess the biological impact of this kind of genetic variation. The golden standard method for SNP scoring is conventional Sanger DNA sequencing. However, sequencing based on gel electrophoresis generates more information than necessary and is time-consuming, laborious and labelling requiresed Significant efforts have been made to improve SNP analysis with alternative techniques. Many of these techniques use allele-specific oligonucleotide hybridization to discriminate between allelic variants such as high-density microarray chips [197], padlock probes [198] or allelic discrimination PCR [199]. The mini-sequencing approach is an attractive alternative because of the direct interrogation of the variable position by DNA polymerase extension using different means of detection of the extended product [200].

Compared to other sequencing techniques, pyrosequencing has a few advantages. It is a fast and easy method because PCR products from 96 samples can be directly sequenced simultaneously and the time for the sample preparation is only around 1.5 hours per 96 well plate. Additionally, a unique property with pyrosequencing is that each allele combination (homozygous or heterozygous) will give a specific pattern. This feature makes genotyping accurate and easy. Simple manual comparison of predicted SNP patterns and the obtained raw data from the pyrosequencing can score SNPs. Moreover, the predicted pyrosequencing pattern depends on the order of nucleotide additions. Thus, one has the possibility of changing the order of nucleotide additions if discrimination of the patterns is not satisfactory [201].

3. Construction of plasmids and transfection

Genomic DNA was used as template to amplify a 329-bp fragment covering the -318 polymorphic site of CTLA-4. The used primers were shown in paper III. The amplified products (-318C and -318T alleles) were cloned into a TA cloning vector (In Vitrogen) and then subcloned into PGL3-basic vector at the KpnI/XhoI sites to drive the firefly luciferase reporter gene (Promega, Madison, WI, USA).

CTLA-4 is mainly expressed by T cells. Jurkat T cell lines were chosen as the target of the transfection since CTLA-4 is not expressed by these lines and the expression of the alleles of CTLA-4 in cell lines would not be affected. Transfection experiments were conducted using the FuGENE 6 Transfection Reagent from Roche Molecular Biochemical (Stockholm, Sweden). The cells were stimulated 24 h with ConA (5 mg/ml) and Phorbol 12-myristate 13-acetate (PMA) (10 ng/ml) and harvested after 48 h. Dual-Luciferase Report assay system (Promega) was applied according to the manufacturer's instruction. The luciferase activity was measured using a Turner luminometer (TD-20/20).

Genetic reporter systems are widely used to study eukaryotic gene expression and cellular physiology. Applications include the study of promoter activity, regulation of transcription factors, intracellular signaling, mRNA processing and protein folding. Firefly luciferase is a 61 kDa monomeric protein that does not require post-translational processing for enzymatic activity. Thus, it functions as a genetic reporter immediately upon translation. Photon emission is achieved through oxidation of beetle luciferin in a reaction that requires energy, Mg^{2+} and O_2. As a result, the reaction generates a "flash" of light that rapidly decays after the substrate and enzyme are mixed. The signals are thus detected by the monitors. Dual reporters are commonly used to improve experimental accuracy. The term "dual reporter" refers to the simultaneous expression and measurement of two individual reporter enzymes within a single system. This system minimizes the experimental variability caused by differences in cell viability or transfection efficiency. Thus, dual-reporter assays often allow more reliable interpretation of experimental data by reducing extraneous influences.

4. Preparation and culture of PBMC

The heparinized blood samples were collected from controls and MG patients. PBMC were prepared by Ficoll density gradient centrifugation (Amersham Biosciences, Uppsala, Sweden). The cells were collected and washed three times in RPMI-1640 (Gibco, Paisley, Scotland) containing antibiotics.

Increasing levels of immobilized α-CD3 antibodies were used to stimulate PBMC. Briefly, α-CD3 antibody (OKT3) was coated on 24-well cell culture plate (Sarstedt, Sweden) and incubated overnight at 4^{o}C. The plate was then washed three times with PBS and ready for cell culture. RPMI-1640, supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine and 10% (V/V) FCS (Life Technologies, Rockville, MD) was used as the culture medium. PHA (5 μg/ml) (Life Technologies, Rockville, MD) was used as a positive control.

5. Antibodies and flow cytometry

The following monoclonal antibodies (mAbs) were purchased from Becton & Dickinson (Mountain View, CA, USA): FITC-labelled anti-OX40, anti-CD28; PE-labelled anti-CD69, anit-CD25, anti-CD28, anti-CD152; PerCP-labelled anti-CD4, anti-CD3; irrelevant isotype-matched mouse mAbs were FITC-IgG1, PE-IgG1.

Cell suspensions were triple stained with FITC, PE- and PerCP-conjugated mAbs. Cells were incubated with mAb for 30 min at room temperature in the dark and analysed in a Becton-Dickinson FACScalibur flow cytometer using CellQuest software (Becton-Dickison). For freshly isolated PBMC, only small lymphocytes were gated and analysed. For stimulated PBMC, a large gate was set according to the forward and side scatter.

6. Statistical analysis

The nonparametric Mann-Whitney tests were used to compare MG, subgroups of MG and HC. Reported p-values were two-tailed and considered statistically significant at $p<0.05$. The Chi-square and Fisher-test were used when needed.

RESULTS AND DISCUSSION

1. Certain β2-AR promoter SNPs were associated with thymoma and others with high levels of circulating anti-AChR antibodies and severe generalized disease (Paper I).

Distribution of β2-AR promoter polymorphisms in MG patients and healthy controls

There are no differences in the frequencies of genotype and alleles between patients and healthy controls and the observed frequencies are comparable to what has been reported for other Caucasian populations [202]. The distribution of genotypes and alleles is in accordance with the Hardy-Weinberg formula in both patients and healthy controls.

Association of β2-AR promoter polymorphism with high concentrations of anti- AChR antibodies, severe generalized disease and thymoma

The patients were stratified into different groups according to gender, levels of serum anti-AChR antibodies, severity of the disease and thymic histology (Tables 2A, B and 3A, B).

The frequencies of the allele -468C, -367T, -47T and -20T in patients with severe generalized disease and increased levels of anti-AChR antibodies in serum were thus significantly decreased, compared to healthy individuals (Figure 1 and Table 3B) and patients with low anti-AChR antibodies in serum (Fig. 2 and Table 3B), respectively. Since the SNP at Gln27 is also associated with MG with anti-AChR anibodies [33], linkage analysis was performed between Gln27 and promter SNPs. We found that Gln27 was strongly linked to -468C, -367T, -47T and -20T and this finding was consistent with a previous study [49].

The frequencies of the allele -468C, -367T, -47T and -20T in Swedish patients with thymoma were significantly increased (Table 3B). The two p-values are not significant after correction, suggesting that studies of larger population should be conducted to confirm the notion. Indeed, results from 62 French patients with thymoma confirmed the association between thymoma and -468C, -367T, -47T and -20T when compared with French patients with thymoma and Swedish patients with other thymic histology (Table 3B).

The positive association between β2-AR and the level of anti-AChR antibodies in the circulation and with severe generalized disease suggested that β2-AR could contribute to the production or induction of specific anti-AChR antibodies and disease severity. This notion was in agreement with the study carried out by Xu et al. [33], who showed that MG patients with anti-AChR antibodies were more often homozygous for Gln27 and the patients with generalized MG exhibited increased frequencies of homozygosity for Arg16. Indeed, Gln27 is found to be strongly linked to -468C, -367T, -47T and -20T in both our study and a previous study [49]. Gln27 is sensitive to agonist-mediated down-regulation [45]. Alleles of -468C, -367T, -47T and -20T can significantly enhance the transcription activity as described by Scott et al [48]. The increased allele frequencies of Gln27 and the decreased allele frequencies of -468C, -367T, -47T and -20T occurring in MG patients might therefore lead to

decreased density of β2-AR on lymphocytes in these patients during the agonist stimulation mentioned by Xu et al [32]. Together with previous findings, our study thus provides an explanation at the molecular level for the decreased density of β2-AR on patient's lymphocytes.

There is a growing amount of evidence showing that the activation of β2-AR suppresses CD25 expression [42], inhibits lymphocyte proliferation and down-regulates Th1 type cytokines, but stimulates the Th2 type cytokines including IL-4 [40] and IL-6 [41]. Even though CD25 is also expressed by regulatory T cells, CD25 expressed on most T cells is an activation sign for T cells. *In vivo* activation of the CD25 promotes the clonal expansion of the activated T cell population. In human MG and EAMG, the AChR specific T cells direct the synthesis of anti-AChR antibodies produced by autoreactive B cells [50, 51]. Therefore, the low expression of β2-AR may lead to up-regulation the expression of CD25 on T cells in MG patients and subsequently excess activation of B cells and production of anti-AChR antibodies. We can speculate that decreased density of β2-AR caused by genetic variants result in the imbalance of Th1/Th2 cytokines, with a bias to Th1, the expansion of B cells with autoreactive properties and eventually higher levels of anti-AChR antibodies and more severe generalized disease.

The promoter sequence of β2-AR is in a GC rich region [48] and it is therefore logical to predict that transcription factors of Sp family might bind to this region. If transcription factors of Sp family do bind to this region as proposed, they may bind to the -468C, -367T, -47T, -20T and -468G, -367C, -47C, -20C with different binding activity which thus result in different transcription activity that was observed Scott et al [48].

Additionally, β2-AR agonists, which stimulate β2-AR, are widely used agents in the treatment of MG and some patients do respond well with improved clinical score [34], indicating that the elevated levels of β2-AR is beneficial for MG patients whereas the down-regulation of this receptor is harmful. The β2-AR expressed on the skeletal muscle regulates contractile force of the muscle. The decreased expression of β2-AR on muscle cells caused by genetic variation may directly affect the contractile force of the muscle and aggregate the disease symptom whereas β2-AR agonists restore the function of β2-AR and improve the muscle function of the MG patients.

2. No association between genetic variants of CCR2 and CCR5 with MG (Paper II).

There are no differences in allelic frequencies of CCR2-64I and CCR5-Δ32 in MG patients in comparison to healthy controls (Table 1). Allelic frequencies of CCR5 and CCR2 in total patients and control groups are similar to those observed in other European population study reports. No differences in the frequencies of the CCR2-64I and CCR5-Δ32 alleles are observed among subgroups of patients, stratified by thymic histopathology, age of onset and levels of anti-AChR antibodies.

The combination frequency of CCR2-64I and CCR5-Δ32, indicating strong linkage disequilibrium of these alleles in the Swedish population was shown in Table 2. The CCR2-64I G/G genotype is associated with the wild-type CCR5.

Analysis of knockout mice and genetic studies has revealed significant new information about the normal biological function of CCR2 and CCR5. For example, CCR2 is a major regulator of induced macrophage movement in mice [203]. CCR2 is involved in inflammatory infiltration into the central nervous system in EAE mice [204]. The CCR2 gene can be a new candidate for the susceptibility locus of type I diabetes [205]. Analysis of CCR5 knockout mice in several infectious disease models has revealed that the expression of CCR2 and CCR5 is an important event in inflammatory responses in the central nervous system, a novel finding that also could have important clinical and therapeutic implications [206]. CCR5-Δ32 is related to asthma and a slower rate of disease progression in MS [207, 208].

In MG, thymic alterations are so frequent (90%) that a role for the thymus in the pathogenesis of MG is certain [5]. Thymic hyperplasia is characterized by numerous and prominent lymph follicles in germinal centers and aggregation of T and B cells and has been found in 60-70% of the patients [5]. Whether there is anything abnormal regarding the homing of these T and B cells remains unknown. It is therefore of interest to investigate the correlations between chemokine receptors such as CCR2 and CCR5 and MG, since chemokine receptors play a critical role to direct the development of inflammation and migration of lymphocytes.

Our study showed no increased risk for susceptibility to MG influenced by chemokine receptor gene CCR2 and CCR5 polymorphisms. Li et al. investigated the expression of C-C chemokines, type 1 and type 2 cytokines in the EAMG in Lewis rats. They did not find any C-C mRNA expression in the skeletal muscle tissue during course of EAMG [209]. However, other reports indicated that the cytokine environment provided by leukocyte trafficking through muscle may play a pivotal role in disease progression. Using a rat model of MG, it was observed that exposure to anti-AChR antibodies also induced increased levels of chemokine MCP-1 production by skeletal muscle cells [210]. Therefore, we cannot exclude the possible importance of CCR5 and CCR2 chemokine receptors in MG based on our present findings and larger materials are required to draw reliable conclusions.

3. The -318T allele exhibits a higher promoter activity than the -318C allele (Paper III)

Homozygosity for -318/T in the promoter of *Ctla-4* is rarely present in the Caucasian population. In the previous study, it was discovered that the frequencies of individuals with genotypes -318C/C, C/T, and T/T were 86%, 17% and 0%, respectively, in healthy individuals (n = 122) and 80%, 19%, 1%, respectively, in patients with MG (n = 101) [105].

Even though -318 SNP was not directly associated with MG, CTLA-4 mRNA levels were significantly higher in non-stimulated PBMC from subjects with MG carrying the -318T allele than in cells from subjects lacking the allele, indicating the SNP at -318 regulates the expression of *Ctla-4 in vivo* and may affect the disease development in patients [109]. To test this, two *Ctla-4* promoter reporter plasmids that contain either C or T alleles were constructed. As shown in Figure 1, the transcription activity of allele -318T was higher than allele -318 C. This result was also confirmed in the

THP-1 cell line transfected with these two reporter constructs, and stimulated with IFN-γ (5 mg/ml) and PMA (10 ng/ml).

We confirmed the regulatory effect of -318 C/T SNP as shown in previous study. -318C is more commonly observed among patients with MS [112], Grave's disease, Hashimoto's thyroiditis [104] and Wegener's granulomatosis [103], implying that CTLA-4 serves as a useful marker for genetic association studies of autoimmune diseases and -318C might contribute to the pathogenesis of autoimmune diseases.

4. More freshly isolated CD4+ T cells from MG patients expressed OX40 than cells from healthy individuals (Paper IV).

OX40 expression on freshly isolated PBMC from MG and HC

Results from thirty-six MG patients and twenty-seven healthy controls revealed that more CD4+ T cells in freshly isolated PBMC from MG patients express a higher percentage of OX40 than CD4+ T cells from healthy individuals.

All CD4+OX40+ T cells expressed CD28 (100%) but very few of them expressed CTLA-4 (CD152) (<1%) (Figure 2b, 2c). CD25, normally expressed on activated T cells, was co-expressed on 91% of CD4+OX40+ T cells (Figure 2d), which was in agreement with the previous studies [150]. In contrast, only 54.5% CD4+OX40- T cells were CD25 positive (Figure 2d).

Interestingly, patients in stage IIA-IIB (generalized disease), with early onset MG and having hyperplasia of the thymus and high levels of anti-AChR antibodies expressed OX40 on a higher percentage of CD4+ T cells. Patients without anti-AChR antibodies had the same frequencies of OX40+ T cells as healthy controls.

OX40 expression on activated CD4+ T cells from MG and HC

We next investigated the initiation of the expression pattern of OX40 and whether prolonged expression of OX40 occurs in MG. In the present study, we stimulated PBMC with increasing concentration of α-CD3 antibodies for 24 and 96 hours. CD4+ T cells from MG patients (N=5) showed a tendency to elevated OX40 expression compared to cells from healthy individuals (N=5) even though the difference is not significant (figure 3a). Consistent with previous findings, α-CD3 antibody alone could induce robust expression of OX40 on CD4+ T cells [123]. Stimulated CD4+OX40+ T cells co-expressed activation markers such as CD69 and CD152 (Figure 3c and 3d).

Altered expression of OX40 was demonstrated in MG. Biopsies from thymus with hyperplastic changes from MG patients showed more OX40 cells around the germinal centers (GC). A considerable number of OX40+ cells were detected in the thymus adjacent to the thymoma tissue [150]. Importantly, a patient who developed MG 25 months after allogeneic bone marrow transplant displayed a prominently increase of CD4+OX40+ T cells in the peripheral blood one month before the onset of MG [151]. However, correlation of peripheral blood OX40+ T cells with GVHD who underwent allogeneic hematopoietic stem cell transplantation was also observed [145]. The last two notions caused us to question whether up-regulation of OX40 on PBMC was due

to the effect of transplantation or MG, and whether enhancement of OX40 expression could be a representative feature of human MG.

Indeed, our results demonstrated that the circulating CD4+OX40+ T cell populations were increased in patients affected with MG, and especially in patients with thymic hyperplasia, compared to healthy controls. These CD4+OX40+ T cells exhibited high levels of CD25, suggesting at least some of them are activated autoreactive T cells [211]. CD69 expression on those cells was not detected, indicating that the cells were not very recently activated T cells. The lack of expression of CTLA-4 on the surface of those CD4+OX40+ T cells indicated that the CD4+OX40+ T cells were not regulatory cells. Indeed this is further substantiated by using CD25 and OX40 double staining showing that the OX40+CD25+ cells were never CD25 bright, which was to expect for naturally arising regulatory T cells in humans [212].

One of the most important events in the development of MG is the activation of self-reactive T cells and abundant production of IgG autoantibodies. Co-stimulation through OX40 is crucial for the induction of an allo-reactive T cell response both in humans and mice and involved in T-cell-help for B cells in the development of an IgG response [128, 133, 213]. We found that MG patients with high levels of anti-AChR antibodies have elevated levels of CD4+OX40+ T cells (Table 1 in paper IV), compared with healthy controls whereas patients who do not have anti-AChR antibodies had the same levels of CD4+OX40+ T cells as healthy individuals. The finding suggests that the up-regulation of OX40 in MG patients induces the activation of autoreactive T cells and the production of T-cell dependent autoantibodies. This notion was further confirmed by animal experiments, which showed that constitutive OX40-OX40L interactions triggered autoimmune-like disease [137], and that an agonist anti-OX40 antibody allowed anergic, autoreactive T cells to acquire effector cell functions [135, 136].

HYPOTHESIS OF PATHOGENESIS OF MG WITH THYMOMA

MG comprises a few disease subgroups, as shown in the introduction part of the thesis. Different thymic histophathological variations occur in MG. The thymoma in MG is a neoplasm of the epithelial and lymphocytic origin and usually associated with a large number of nonmalignant lymphocytes. Patients with thymoma constitute a distinct clinical subgroup and usually develop severe disease starting from middle age. These patients frequently have a broad spectrum of autoantibodies against muscle proteins and frequently have mononuclear cell infiltration in skeletal muscles [214]. MG patients with thymoma also have a marked decrease in the number of CD4+CD25+ regulatory T thymocytes in the thymus [215] and a low frequency of NK cells in PBMC [216]. All above findings indicate more aggressive activations of both humoral and cellular immunity in thymoma patients than patients from other subgroups of MG.

We found that specific alleles of β2-AR gene were associated with MG patients with thymoma, MG with high levels of anti-AChR antibodies and severe generalized disease. The allele -468C, -367T, -47T and -20T significantly up-regulated β2-AR gene expression [48] and was increased in MG patients with thymoma. β2-AR binds to norepinephrine to induce an increase in the intracellular concentration of cAMP [36]. Therefore, elevation of β2-AR protein induces increased cAMP inside cells during β2-AR stimulation and triggers the secretion of Th2 cytokines such as IL-4 and IL-6 [40, 41, 217]. IL-4 enhances the IL-1-induced IL-6 production in cultured human thymic epithelial cells [218]. IL-6 acts as an autocrine growth factor for thymic epithelial cells [219, 220] and induces the differentiation of cytotoxic T cells from immature thymocytes [221]. Elevated IL-6 may thus result in the aggregation of epithelial and lymphocytic cells observed in thymoma tissue and contribute to the formation of thymoma. Indeed, high IL-6 gene expression and protein production by cultured human thymic epithelial cells from patients with myasthenia gravis was demonstrated in previous studies [222, 223].

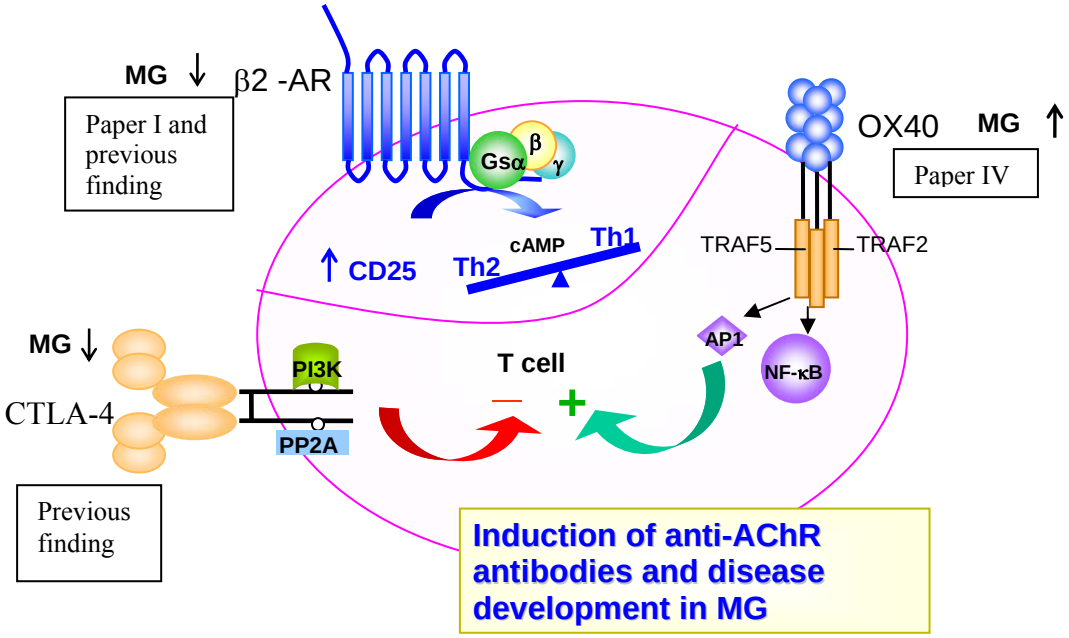
Totally three polymorphic sites within the CTLA-4 gene are well characterized, -318 T/C, +49G/A and the long/short (AT)n repeats in 3'-UTR. The allele +49G and longer (AT)n repeats in the 3'-UTR are associated with MG with thymoma [105, 116]. Even though the -318 SNP of CTLA-4 was not directly associated with MG, the CTLA-4 mRNA levels were significantly higher in non-stimulated PBMC from subjects carrying -318T allele than in cells from subjects lacking the allele, indicating the SNP at -318 regulates the expression of *Ctla-4 in vivo* [109].

We confirmed the regulatory effect of SNP at -318 by *in vitro* study, which demonstrated that the allele -318C down-regulated CTLA-4 gene expression. -318C and +49G are in strong linkage as the +49G allele and longer (AT)n repeats in 3'UTR are in marked linkage [105, 109]. Individuals carrying -318C and homozygous for +49G showed significantly decreased expression of both cell-surface CTLA-4 after cellular stimulation and CTLA-4 mRNA in non-stimulated cells [109]. Moreover, longer alleles at 3'UTR of CTLA-4, which were associated to MG patients with thymoma, strikingly reduced stablility of *Ctla-4* mRNA [116]. Taken together, the haplotype -318C, +49G and longer (AT)n repeats in 3'UTR down-regulate both CTLA-4 gene and protein expression. Considering the critical role of CTLA-4 in the immune system, the combination of -318C, +49G and longer (AT)n repeat in 3'UTR

thus may contribute to the disease development and the formation of thymoma in MG patients.

In summary, the data support the notion that MG with thymoma is a distinct form of the disease compared to other subgroups of MG.

HYPOTHESIS OF PATHOGENESIS OF MG WITHOUT THYMOMA



Down-regulation of β2-AR occurring in MG, which might be caused by either genetic variants in patients (Paper I) or autoantibodies against β2-AR [33] or both, up-regulates CD25, induces imbalance of Th1 and Th2 cytokine production and activates autoreactive T and B cell. OX40, a positive signal to T cells, is up-regulated in MG (Paper IV). In a previous study, cells from patients showed low levels of CTLA-4 protein both on the surface of the cells and intracellularly, and impaired up-regulation of CTLA-4 in response to ConA stimulation [96]. The up-regulation of OX40 and down-regulation of CTLA-4 lead to abnormal T cell activation, especially autoantigen-specific T cell activation, and uncontrolled cytokine productions. The combination of these effects eventually leads to production of autoantibodies and induction of the disease.

ACKNOWLEDGEMENTS

Many thanks to you all who have helped and encouraged to make this thesis possible. Especially, I would like to acknowledge:

Prof. Ann Kari Lefvert, my supervisor, for the supports, encouragements, freedom you gave me through these years, for allowing me try plenty odd ideas and training me as an independent scientist. I will never forget the enthusiasm and optimism you always show me.

Former and present colleagues from Immunological Research Unit, CMM: Maria**,** Ricardo, Doina, Ryan, Elisabeth, Paola, Priya, Natasha, Heba, Haiting, Elin, Thair, Rajender. For making the lab a fun place with their great sense of happy-go-luck attitude. Thank you for assistance, lunch, coffee break, cake, diner, and the most important, the friendship!!! Thank you Ricardo for teaching me the flow cytometry and sorry that I am such a curious girl that always wants to try myself which will drive everyone mad. Thank you Maria for all kind of helps, cake recipes and everything else, and so sorry that I always bother you whenever I could not find things, including tubes, cell culture flasks and even my glasses, which are always lying with my scissors but I can never see them by my own naked eyes. I am too blind. Doina and Priya for accompanying me during lunch, for the knowledge of Romania and India that I learn from you. Ryan for English correction of my thesis.

My co-authors! Without you all, this thesis may never be real! They are: Baback from KTH, Mehran, Xiongbiao, Ming, Pawan from KS. For all of the enthusiasm and optimism you gave to me and I do learn a lot from you all! My patients! Thank you all for your generous donation!!!

Ritva and Gorge, for always being ready for helping me with the clinical data, being patient to read my papers. Without you, I could never distinguish any clinical symptoms from those patients and my paper would never be real.

My supervisor of 20-point exam work, Manucheu Abedi, who led me to this fantastic immunology field and made me to consider it as my PhD career, even though I was so regretful of my choice at the beginning. Thanks for being so kind and nice.

Special thanks to Vivi, whom I wish to be my co-supervisor, but cannot be true since she is too busy. I deeply appreciate all of the collaboration with you and thank you so much for reading my thesis. I also appreciate your clear and sharp scientific thinking and your modesty, patience and warm heart. It is so nice to know you. And Dujia, for all of the helps and funs to talk to you, and Tina, for having party with you and nice course that you organized and Anderas, for helping me running the flow and analysis. Prof. Göran Hansson, for letting me borrow your instruments and for concern.

Patrick, for your funny cartoon books and language corrections, for being ‘Lei feng’ help us, even though you don’t like him so much. Thank you for correcting my papers! People in the corridor, floor 03: Dexiu, for your supervision of my tennis skill and all of the fun in tennis court and study together in the library, for all of your friendship, which made me feel warm deeply in the heart! Thank you Stina, Lotta, Anna Karin, Emmanuel, Yuri and all of the members in the journal club, and sorry that I could not

list them all, but you know who you are, for the cakes, young and happy face, fresh discussions and scientific atmospheres, which I really enjoyed. Thank you Meta for always helping me with flow cytometry. It was you that released my stress from this iron machine when I had the passion to beat it, break it and die with it. Annika, for your patience to assist me with all of the sorting staffs, clear and clean way to work, happy discussions, lending me the book about Budapest and baby care. I am very glad to work with a nice person like you. ZhongQun, for reading my papers. I would also like to thank 00 floor people in CMM. For all of the nice cakes, friendly talks, presentations, helping me to deal with the computer and projector during my presentation, everything.

My friend Yuemei and Xuyan and Yuemei's mum, who make me feel as I was in China whenever I visit your home. Thank you all for the nice food, the happy time we have been together, baby bed and a lot of gifts that I received from you. It is you, my friends, who convert the cold and long winters in Stockholm to brighter, shorter and enjoyable ones. My friend Linxi and dear Anni, our angel, a smart young girl with a kind heart, for always intending to invite me to play with your little fellows even though I was born 24 years earlier than you. Thank you my friend Cheng fei and Peng Zirong, for nice talking and giving me so much baby clothes.

My food club, even though I was absent very often, so often that you guys almost forgot to invite me anymore. They are Zongpei, Zhao Qing, I-hui, Li Yu, Jin Yuxuan, Chaorui, for all of the funs from food and listening to you guys about your adventures around the world. Special thanks to Zongpei and Chaorui for deep concerns.

My grandpa and grandma from both my Pa and Ma side, for feeding me until 16 years old, for loves and supports. My aunts and uncles from my mother side, for feeding me, playing with me, loving me and more and more. My 3 aunts from my father side, for love, and considering me as your daughter, for plenty bottles of Chinese medicines and food you mailed to me. My parent and sisters in-law, for endless, non-selfish supports and more. My Pa, Ma and Xiaopu, for constant love and non-selfish supports, for deep concerns over the years. Limin, for being a family, for being my best friend, for traveling around Stockholm, Sweden and Europe, for common interests in history and art, for open mind, for playing tennis together, for advising me to choose immunology as my master and PhD topic, for fresh discussions about interesting developments in biology, reading my thesis and all of my papers carefully, for always being ready for helping me and taking care of me during these years, for cooking food, washing the dishes. My coming son, though now I can only see you by ultra-sound, but I know you are a smart, cute little baby. You cannot imagine how much happiness I get from you.

This work was supported by grants from the European Comission, the Fifth Framework Programme (grant no QLG1-CT-2001-01918), the Swedish Research Council (grant no 05646), the foundations of the Karolinska Institutet, the King Gustaf V:s 80-years Foundation and the Palle Ferb foundation.

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