UNIVERSITY STUDY OF MILAN

Graduate School in Pharmacological Sciences
Faculty of Medicine and Surgery
Department of Medical Pharmacology
PhD in Medical Pharmacology, Chemotherapy and Toxycology
XXIV Cycle
BIO/14 - Pharmacology

PhD Thesis

PRECLINICAL STUDIES OF PIXANTRONE AS IMMUNOSUPPRESSIVE DRUG IN THE ANIMAL MODEL OF MYASTHENIA GRAVIS

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Academic year 2010 – 2011

To my father
Thank you for teaching me the value of laughter

Index

Summary

Myasthenia Gravis (MG) is an antibody-mediated T-dependent autoimmune disease, involving the neuromuscular junction. Experimental autoimmune Myasthenia Gravis (EAMG) is induced in female Lewis rats by immunization with Acetylcholine Receptor from *Torpedo Californica* (TAChR) in Complete Freund's Adjuvant (CFA). The injection induces the activation of specific Th1 cells in response to the antigen of immunization, stimulating the production of anti-TAChR antibodies (Abs) that are able to cross-react with self-rat AChR, generating specific anti rat AChR Abs, which activate complement system inducing the receptor and endplate degradation and damaging the transmission from nerve to muscle. Therapeutic strategies for MG include immunosuppressive drugs, that exert an aspecific immune dysfunction. These treatments are effective in a large amount of patients, however, clinical responses to conventional therapies appear ineffective in some patients or the severity of side effects can limit their prolonged administration. Thus, new specific autoimmune therapies are needed for treatment of unresponsive or intolerant MG patients. Pixantrone (PIX), an aza-antracenedionic derivate, is a drug with antitumor activity due to its cytotoxic effect, via DNA intercalation and topoisomerase II inhibition, linked to its cumulative dose. Owing to this mechanism of action, PIX presents also an immunosuppressive activity and was recently tested in the EAMG, in which have demonstrated to improve both preventive and therapeutic treatments when administered at the dose 16.25 mg/Kg q7dx3 for a cumulative dosage of 48.75 mg/Kg [1].

In the present study we have focused our attention to the therapeutic treatment of EAMG; in particular, the aim of our study has been to evaluate different PIX schedules, in order to identify the lowest dosage able to ameliorate ongoing EAMG, thus further lowering the risk of toxic effects.

We first evaluated PIX effect on the *in vitro* proliferative response of lymphoid cells, to determine the pharmacologic properties of the drug in presence of different cells subpopulations, characterized by different proliferative responses: R97-116 specific T cell line, lymphocytes (LNCs) and splenocytes (SPNs) from

R97-116 primed rats, SPNs from HD rats and peripheral blood mononuclear cells (PBMCs) from human healthy donors. For this purpose, by dose-response curves we demonstrated that PIX (0.1 pM - 1 µM) inhibited lymphoid cell responses in a dose dependent manner. Moreover our data demonstrated that a lower concentration of PIX was sufficient to inhibit 50% of maximal response of R97-116 specific T cell line, represented by an homogeneous pool of cells (more than 90%). In mixed populations, such as LNCs and SPNs isolated from R97-116 primed rats, composed to 20-40% of antigen-specific cells, or even in SPNs from HD rats and PBMCs from healthy human donors, the IC_{50} for PIX is found to be increased. Complete suppression of proliferative response, was achieved with nM PIX concentrations for cells in active proliferation (i.e. R97-116 T cell line) and raised to µM PIX concentrations in presence of SPNs from HD rats and PBMCs from healthy human donors. Our *in vitro* experiments suggested that PIX efficacy is higher for T cells that are able to actively proliferate.

We also tested *in vitro* whether PIX (0.1 nM, 1 nM, 10 nM, 10 µM) was able to interfere with differentiation of myeloid precursors cells (MPCs), from bone marrow of HD rats, in immature dendritic cells. The drug resulted toxic (reduction in cell viability ≤ 50%) already at PIX 1 nM and the cell viability was dramatically reduced to 0% when the cells were exposed to PIX concentrations 10 nM and 10 µM.

Then we investigated *in vivo* PIX efficacy, firstly on TAChR-immunized rats treated 24 h post immunization (p.i.) with a single dose of PIX (16.25 mg/Kg x1, 8.12 mg/Kg x1, 4.06 mg/Kg x1) and sacrificed after 10 days. A single dose of PIX was able to reduce the number of total live cell count of LNCs from PIX treated rats compared to vehicle, accordingly with a decrease of *ex vivo* TAChR specific T cell proliferation (90%). In parallel we evaluated the effect of the drug in physiological conditions, administered a single dosage of PIX (16.25 mg/Kg) in HD rats or HD rats immunized only with CFA and compared to HD controls, but the drug had no effect on the number of total live cells on LNCs. These data demonstrated that lower PIX doses were able to modulate selectively the TAChR specific proliferation *in vivo* and allowed us to tested alternative schedules, on the base of previous studies [1], in the therapeutic treatments.

We studied *in vivo* the effect of long-term treatments with PIX in EAMG rats, immunized with TAChR/CFA and treated 4 weeks p.i with PIX, at two cumulative

dosage 48.75 mg/Kg (16.25 mg/Kg q14dx3 as positive control, 8.12 mg/Kg q7dx6) and 24.36 mg/Kg (4.06 mg/Kg q7dx6, 8.12 mg/Kg q14dx3). Vehicle treated rats was administered with a sterile saline solution. In parallel we evaluated the effect of a chronic administration of PIX in HD rats (16.25 mg/Kg q14dx3) compared to HD controls, but PIX treatments did not affected *in vivo* the body weight of the HD animals and had no effect on the number of total live cells of LNCs. At the end of the experiments (11 weeks p.i.) only PIX cumulative dosage 48.75 mg/Kg shown a pharmacological effect, modulating clinical signs of EAMG (gain of body weight and decrease in the clinical score), affecting the number of total live cells of LNCs and decreasing the TAChR specific proliferation in the same organ. Moreover, qPCR revealed a reduction in IFNγ mRNA transcription levels in SPNs, accordingly with the decrease in anti-Rat Abs titre in the sera of the PIX treated rats. On the contrary the cumulative dosage 24.36 mg/Kg resulted ineffective in the modulation of classical monitoring parameters of EAMG. Although we observed an effect on TAChR specific proliferation of LNCs, these treatments resulted ineffective to inhibit the production of IFNγ and, as consequence, of pathogenic Anti Rat AChR Abs. We also tested *in vivo* the chronic toxicity of the drug on MPCs and both of treatments at the cumulative dosage 48.75 mg/Kg resulted myelotoxic in HD and EAMG rats compared to HD controls, but the protocol 8.12 mg/Kg q7dx6 presented a lower toxicity compared to the 16.25 mg/Kg q14dx3. Thus following the protocol 8.12 mg/Kg q7dx6 we observed major efficacy in the disease modulation with fewer myelotoxic effects. Conversely the cumulative dosage 24.36 mg/Kg did not interfere on cell viability compared to HD.

In conclusion we speculated that though both of treatments for the cumulative dosage 48.75 mg/Kg were able to improve EAMG, PIX pharmacokinetic profile in the schedule 8.12mg/Kg q7dx6 overlaps well with its clearance (8 days), thus allowing the achievement of a systemic steady-state, with the consequent improvement of the efficacy of this treatment compared to 16.25mg/Kg q14dx3 schedule. The cumulative dosage of 24.36 mg/Kg instead resulted ineffective to modulate EAMG because it didn't reach the systemic effective dose in rats.

On the basis of our results we proposed PIX as promising immunomodulatory agent suitable for clinical investigation in MG patients, that present severe form of the disease or major contraindications to the classical therapies.

Chapter 1 – Introduction

1.1 Immunological tolerance and autoimmunity

A key issue in immunology is to understand how the immune system is able to discriminate between self and non-self, inhibiting the autoimmune responses and activating the immune responses against foreign antigens, respectively [2].

The immunological self-tolerance is an active process that controls the unresponsiveness to the auto-antigens at central level, by the primary lymphoid organs, and in the periphery, by the secondary lymphoid organs [3, 4]. The central tolerance occurs in the thymus and leads to the clonal deletion of the auto-reactive T cells, representing the main mechanism involved in maintaining the tolerance to self-antigens [5].

T lymphocyte progenitors are produced in bone marrow and migrate to the thymus, where they will be selected and matured. Firstly, they are double negative thymocytes, then productively rearrange α and β T cell receptor (TCR) chains, which are first expressed by cluster of differentiation 4 and 8 (CD4+CD8+) double positive thymocytes. These cells are selected by positive and negative selection processes.

Selection processes occur in the thymus, due to the capability and the affinity level of immature thymocytes to recognize and bind self-antigens, processed and presented by major histocompatibility complex (MHC) molecules on thymic antigen presenting cells (APCs). CD4+CD8+ thymocytes are positively selected, and rescued from programmed cell death, due to low-affinity interactions between their rearranged TCR and the self MHC molecules on thymic epithelial cells in the thymic cortex. If the MHC molecules belong to the class I or the class II, double positive thymocytes will lose the expression of CD4 or CD8 molecules, respectively, and they will give rise to single positive thymocytes (CD8+ or CD4+). Instead, the negative selection results in the immature thymocytes deletion via apoptosis, due to high-affinity interactions between their rearranged TCR and the self-antigens presented by MHC molecules on APCs in the thymic medulla or in the corticomedullary junction. The thymic negative selection is the main

mechanism of self-tolerance: failures in this process can lead to autoimmune diseases.

The selected thymocytes further undergo maturation, leave the thymus and migrate to the peripheral lymphoid organs [6-9]. However, auto-reactive T cells may escape the thymic clonal deletion and recognize peripheral tissue antigens, effecting autoimmunity. Despite these escaped cells are normally present in all individuals, autoimmune diseases affect only about 5% of the population, suggesting that peripheral self-tolerance mechanisms must exist to control potential pathogenic T cells [10].

The peripheral tolerance is a continuous process that occurs when mature lymphocytes, escaped from negative selection during ontogeny, encounter self-antigens in secondary lymphoid organs. This process is mainly controlled by specific regulatory T (Tr) cell subsets and includes different mechanisms as ignorance, deletion by apoptosis, anergy induction and active suppression [4, 5]. Several types of Tr cells play the role of immunosurveillance: from the CD4+ repertoire, naturally occurring CD4+CD25+ regulatory T (Treg) cells and induced CD4+ Tr cells, including Tr1 and T helper 3 (Th3) cells; from other cell subsets, naturally occurring regulatory NKT cells and CD8+ regulatory T cells [8].

1.2 Myasthenia Gravis

Myasthenia Gravis (MG) is a relatively rare neuromuscular disease, characterized by the loss of nicotinic acetylcholine receptors (AChRs), key molecules for the neuromuscular transmission and the consequent muscle contraction. The loss of AChRs leads to muscle weakness and fatigability, the main symptoms of the pathology [11]. In the 1980s, several immunologists studied the potential implication of antibodies (Abs) in MG and found in the plasma of myasthenic patients anti-AChR-specific Abs (in particular polyclonal immunoglobulin G, predominantly belonging to IgG1 and IgG3 subtypes), reactive to different epitopes on the surface of AChRs [12]. More than 85% of MG patients develop antibodies against AChRs [13].

Acquired MG is an uncommon disorder, although prevalence has increased over time and is recently estimated around 50-125 cases per million [14]; this correlates with an improvement in diagnosis techniques and with a major life span. The

annual incidence rate widely varies from 1.7 to 10.4 per million, depending on the location of the studies [15, 16].

The disease onset is influenced by both sex and age: the incidence is higher in women than in men, (3:1 ratio) during early adulthood (age <40 years), is roughly equal during puberty and after 40 years of age, instead is higher in men than in women after 50 years of age [17]. Childhood MG is uncommon in Europe and North America, comprising 10–15% of cases [15]; it is much more common in Asia, such as in China, where up to 50% of myasthenic patients develop the disease before 15 years of age, mainly with the typical symptoms of ocular MG [18].

1.2.1 MG subtypes

Several Myasthenia Gravis subtypes can be identified on the basis of differences in clinical presentation, age at onset, autoantibody profile, and presence or absence of thymic pathology.

Patients with generalized MG can be distinguished for early- and late-onset disease, usually defined as beginning before or after 40 years of age, respectively [19]. Early-onset patients are usually females, with anti-AChR antibodies and enlarged hyperplastic thymus glands. They may develop other organ-specific autoantibodies and be affected by other autoimmune pathologies, most commonly autoimmune thyroid diseases [20]. Late-onset patients are usually males, with normal thymic histology or thymic atrophy at last. They can be also affected by ocular symptoms, typically develop a more severe disease than early-onset MG patients and rarely go into spontaneous remissions [21]. Furthermore, these patients usually present antibodies reactive to striated muscle proteins, such as titin and ryanodine receptor (RyR) [22]. The presence of anti-RyR antibodies, is associated with severe, generalized and oropharyngeal weakness and frequent myasthenic attack [23].

About 10–15% of MG patients are affected by thymic epithelial tumor, known as thymoma,. present in men and women with equal incidence; it can occur at any age, with an onset peak at 50 years of age [24]. Clinical manifestations, commonly characterized by progressive generalized and oropharyngeal weakness, result more severe than in early-onset MG patients, without thymoma.

Myasthenic patients with thymoma present a high titer of anti-AChR Abs and usually develop antibodies against titin and ryanodine receptor [25]. In these patients, the thymectomy often completely and permanently removes the tumor, but myasthenic symptoms usually persist and require chronic immunotherapy [16].

Approximately 15% of patients with generalized MG do not show anti-AChR Abs, while in 40% of these, antibodies directed to Muscle Specific Kinase (MuSK), a postsynaptic neuromuscular junction (NMJ) protein, are detected [26]. MuSK-positive MG onset appears earlier and patients are predominantly females [27]. Subjects with anti-MuSK antibodies can show symptoms similar to anti-AChR-positive MG patients, but commonly develop atypical clinical features, such as selective facial, bulbar, neck, and respiratory muscle weakness and marked muscle atrophy, occasionally with relative sparing of ocular muscles [27]. In anti-MuSK-positive MG, respiratory attacks are more common than in generalized anti-AChR-positive disease, while thymic alterations are usually absent [28].

MG patients without both anti-AChR and anti-MuSK Abs (seronegative MG patients) show heterogeneous clinical signs and can develop purely ocular, mild generalized, or severe generalized disease. Seronegative patients are essentially indistinguishable from patients with anti-AChR-positive MG for clinical features, pharmacological treatments response and even thymic abnormalities in some cases [29].

When myasthenic symptoms involve exclusively ocular muscles, the disease is defined ocular Myasthenia Gravis, and represents 17% of all MG subtypes in white populations [17]. Ocular MG seems to be more common in Asia (up to 58% of all myasthenic patients), with prevalence in children [18]. In 90% of cases the disease do not generalize, if the muscular weakness remains confined to the ocular muscles for at least 2 years [17]. Up to 50% of ocular MG patients develop anti-AChR Abs, but a high antibody titer do not necessarily predict a potential generalization of the disease [30]. Anti-MuSK antibodies are rarely detected in ocular MG [31].

Transient neonatal Myasthenia Gravis is characterized by muscle weakness, due to trans-placental transfer of maternal pathogenic auto-antibodies, and occurs in approximately 10–15% of infants born to mothers with MG [32]. Myasthenic symptoms usually develop a few hours after birth, but may be delayed for 24 hours

or longer. A prophylactic treatment with plasma-exchange or steroids or both of them can be considered in a woman with a previously affected child, as the risk of recurrent transient neonatal MG is high [16].

1.2.2 Immunopathogenesis

MG is a T-cell dependent antibody-mediated autoimmune neuromuscular disease [11], in which AChR is the main autoantigen and the postsynaptic membrane of the NMJ is the target for antibody-induced damage. In about 85% of patients, MG is caused by pathogenic autoantibodies directed to nicotinic AChR on muscle cells. The amount of Abs in patients serum is variable, but the antibody titer does not correlate with the clinical severity of MG [13].

Structure and function of the NMJ

The neuromuscular junction is formed by the nerve terminals of α–motor neurons that originate from the ventral horns of the spinal cord and brain stem (Figure 1.1). The myelinated axons reach the muscles through peripheral nerves and divide into branches to innervate many individual muscular fibers.

As each branch approaches its target fiber, it loses the myelin sheath and further divides into several pre-synaptic bottons, containing acetylcholine (ACh)-loaded synaptic vesicles and interacts with the surface of the muscle fiber at the NMJ (Figure 1.1). The pre-synaptic botton and the muscle surface are separated by the synaptic cleft, a 20 nm–thick space containing acetylcholinesterase (AChE), enzyme with hydrolytic activity that degrades the ACh, and other proteins, including proteoglycans involved in stabilizing the NMJ structure.

The post-synaptic membrane presents characteristic deep folds, on the top of which the ACh-receptors are densely packed (about 12,000 molecules per μm^2) in highly ordered hexagonal lattices of molecules. Muscle AChRs are transmembrane proteins characterized by 5 subunits: 2 identical α subunits, constituting the important structural elements for the ACh-binding sites, and 3 homologous subunits, β, γ (or ε), and δ. Embryonic muscle expresses AChRs formed by α, β, γ, and δ subunits; after innervation, the adult AChR isoform is generated by the substitution of the γ subunit with the homologous ε subunit (Figure 1.2) [33]. A large amount of the antibodies in the sera of MG patients binds

to the main immunogenic region (MIR), localized between residues 67 and 76 of both α subunits of the receptor (Figure 1.2).

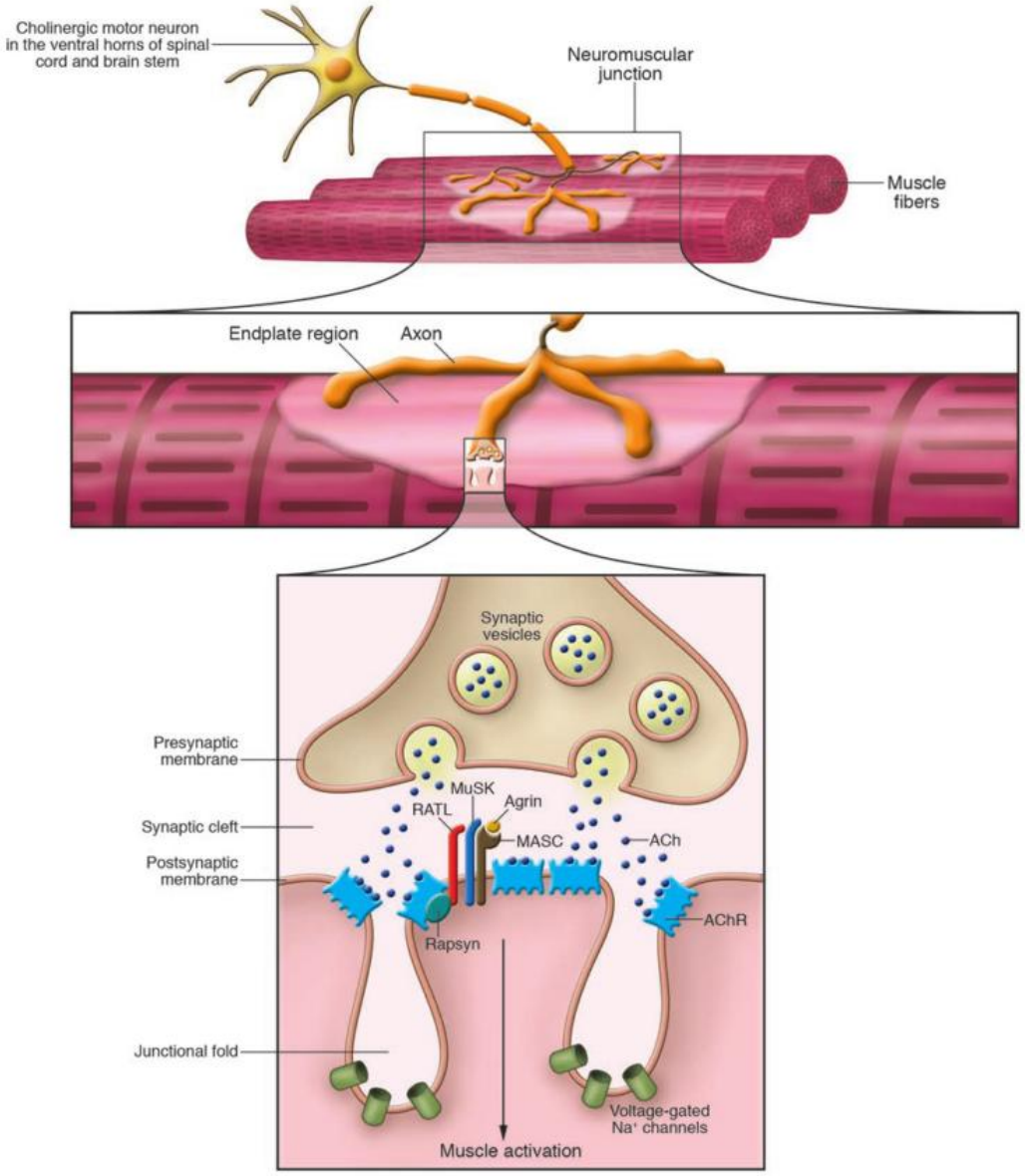

Figure 1.1 Structure of the NMJ, Conti-Fine et al., 2006 [33].

In addition, many patients' Abs bind to the fetal-specific γ subunit. In some cases, these antibodies inhibit the function of the AChR, and can cross the placenta in pregnant mothers, causing fetal muscle paralysis and severe, often fatal, deformities [11].

α-Bungarotoxin (α-BTX), a component of the venom of the snake *Bungarus multicinctus*, it is the most common antagonist of AChR and interacts irreversibly

and competitively with the ACh-binding sites, causing paralysis, respiratory failure and death (Figure 1.2).

When the nerve action potential reaches the synaptic botton, the depolarization opens voltage-gated Ca^{2+} channels on the pre-synaptic membrane. This Ca^{2+} influx triggers fusion between the membrane of the synaptic vesicles and the pre-synaptic membrane, allowing ACh release. The ACh diffuses into the synaptic cleft, reaches and binds its receptor, triggering the opening of cation channel and the consequent influx of Na^{+} into the muscle cell. The resulting endplate potential (EPP) activates voltage-gated Na^{+} channels, leading to further influx of Na^{+} and spreading the action potential along the muscle fiber.

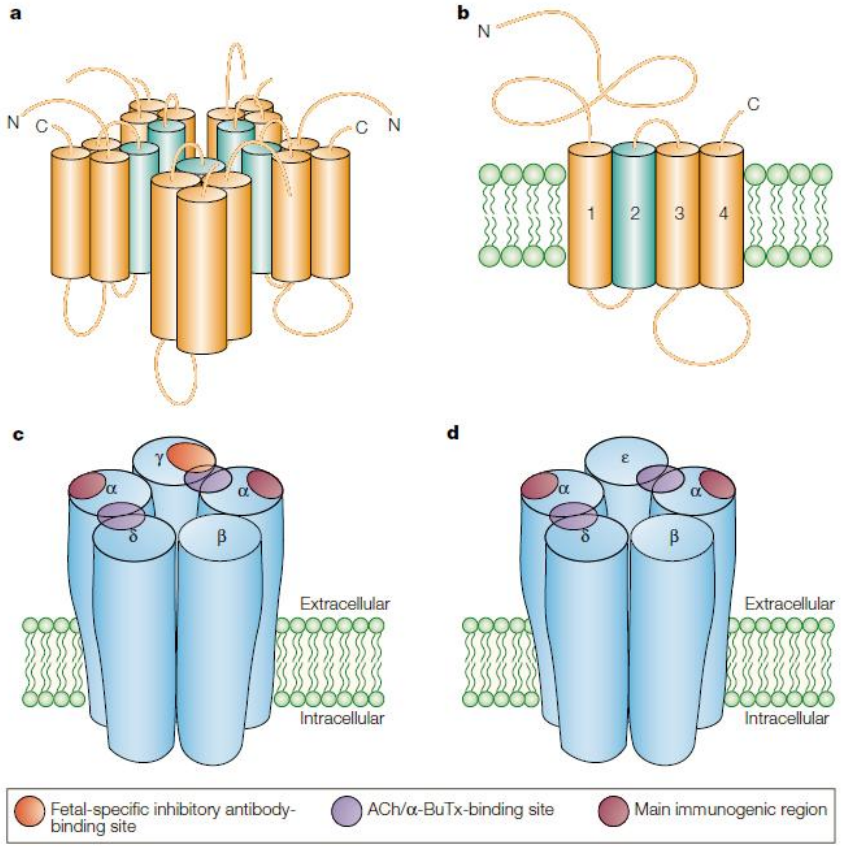

Figure 1.2 Fetal and adult forms of the acetylcholine receptor. (**a**) The acetylcholine receptor is a pentameric membrane protein. (**b**) Each of the subunits consists of an extracellular domain, four transmembrane regions and a cytoplasmic domain. The receptor consists of $(\alpha)_2$, β, γ and δ subunits in the fetal form (**c**), and $(\alpha)_2$, β, δ and ε subunits in the adult form (**d**), Vincent, 2002 [11].

The post-synaptic transmembrane protein, MuSK (Figure 1.1), is the autoantigen in some MG patients [34]. Its expression in both developing and mature muscles is similar to that of AChR. In mature muscle, MuSK is present prominently only at the

NMJ, where seems to be part of the agrin receptor. Agrin is a protein synthesized by motor neurons and secreted into the synaptic basal lamina. The signaling induced by agrin/MuSK interaction triggers and maintains rapsyn-dependent clustering of AChRs and other post-synaptic proteins [35].

Rapsyn, peripheral protein exposed on the cytoplasmic side of the post-synaptic membrane, is necessary for clustering of AChRs. Rapsyn and AChR are physically associated and present in equimolar concentrations at the NMJ. Rapsyn also causes clustering of other NMJ proteins, including MuSK. Mice, lacking agrin or MuSK, fail to form NMJs and die at birth for profound muscle weakness; their AChR and other synaptic proteins are uniformly distributed along the muscle fibers [36].

The quantal content of an impulse, the conduction properties and density of post-synaptic AChR, and the activity of AChE in the synaptic cleft contribute to the EPP [37]. A reduction in the number or activity of the AChR molecules at the NMJ decreases the EPP, despite may still be adequate at rest; however, when the quantal release of ACh is reduced after repetitive activity, the EPP may fall below the threshold needed to trigger the action potential. NMJ properties vary among muscles and may influence muscle susceptibility to MG [37].

Effector mechanisms of anti-AChR Abs

Anti-AChR Abs affect neuromuscular transmission by at least 3 mechanisms: (a) binding and activating the complement cascade at the NMJ; (b) accelerating the degradation of AChR molecules cross-linked by Abs (a process known as antigenic modulation); and (c) blocking functional AChR (Figure 1.3).

The NMJ of MG patients contains activation fragments of complement component 3 (C3), the terminal and lytic complement component 9 (C9), and the membrane attack complex (MAC) [38]. Different evidences suggest that the complement activation at the NMJ may be the primary cause of AChR loss and neuromuscular transmission failure (Figure 1.3 - a).

Studies on MG animal models indicate that the complement depletion protects animals from Experimental Autoimmune MG (EAMG) [39]. For instance, the administration of Abs blocking complement component 6 (anti-C6) [40] or a complement inhibitor (soluble CR1) [41] protects rodents from EAMG; moreover,

mice with a reduced complement function, due to a genetic deficit of complement components, are resistant or less susceptible to EAMG induction than wild type mice [42]. Karachunski and colleagues studied IL-12–deficient mice, characterized by a poorly Abs-generated complement-fixing [43], and demonstrated that IL-12 Knock-Out (KO) mice developed minimal myasthenic symptoms after AChR immunization in spite of elevated anti-AChR Abs synthesis; the NMJs in these mice contained antibodies but not complement, suggesting that, anti-AChR Abs not activate the complement, do not compromise neuromuscular transmission [43]. Cells are protected from activation of autologous complement by intrinsic complement regulators. These include the decay-accelerating factor (DAF or CD55), the membrane co-factor protein (MCP or CD46), and the membrane inhibitor of reactive lysis (MIRL or CD59) [44-46].

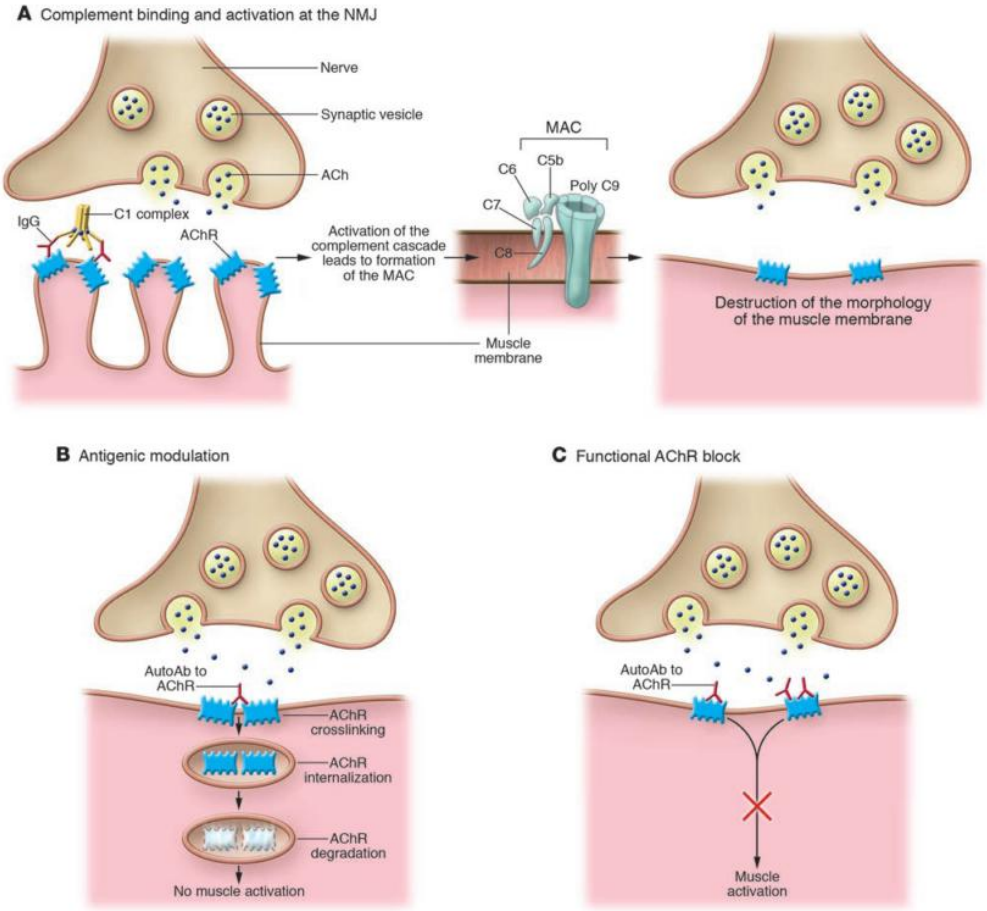

Figure 1.3. Effector mechanisms of anti-AChR Abs. **(a)** Ab binding to the AChR activates the complement cascade, resulting in the formation of membrane attack complex (MAC) and localized destruction of the postsynaptic NMJ membrane. **(b)** Abs cross-link AChR molecules on the NMJ postsynaptic membrane, causing endocytosis of the cross-linked AChR molecules and their degradation (antigenic modulation). **(c)** Ab binding the ACh-binding sites of the AChR causes functional block of the AChR by interfering with binding of ACh released at the NMJ. Conti-Fine et al., 2006 [33].

Antigenic modulation is the ability of an Ab to cross-link two antigen molecules, triggering a cellular signal that causes accelerated endocytosis and degradation of the cross-linked molecules (Figure 1.3 - b). IgG from MG patients causes antigenic modulation of muscle AChRs [47] and this property can be applied as a diagnostic test [48]. if accelerated degradation is not compensated by increased synthesis [48], reduction of the available AChR molecules at the NMJ and myasthenic symptoms will be observed. This feature can be employed as a diagnostic test for MG [48], although, not all anti-AChR Abs cause antigenic modulation: all IgG present two antigen-binding sites, but the epitope location on the AChR surface may inhibits the cross-reaction of an antibody with a second molecule [49].

Functional AChR block, due to the interaction between anti-receptor Abs and ACh-binding site, (Figure 1.3 - c) is an uncommon pathogenic mechanism in MG, yet it may be clinically important. More studies show that the presence of Abs to the ACh-binding site causes acute, severe muscle weakness in rodents without either inflammation or necrosis at the NMJ [50]. Many MG patients develop a low amount of anti-AChR Abs recognizing the ACh-binding site [51]; however, these antibodies may be able to block the receptor function and trigger the acute myasthenic crises [16].

Role of CD4+ T cell subtypes and cytokines in MG and EAMG

Differentiated $CD4^+$ T cells are classified into different subtypes based on the cytokines they produce and secrete. T helper 1 (Th1) and T helper 2 (Th2) cells show different and opposing functions [52] (Figure 1.4). Th1 cells secrete pro-inflammatory cytokines, such as interleukin-2 (IL-2), interferon γ (IFN-γ), and tumor necrosis factor-α (TNF-α), important in cell-mediated immune responses. Th2 cells secrete anti-inflammatory cytokines, such as interleukin-4 (IL-4), interleukin-6 (IL-6), and interleukin-10 (IL-10), important in humoral immune responses. Moreover, IL-4 stimulates the differentiation of T helper 3 (Th3) cells, which are involved in immunosuppressive mechanisms, secreting transforming growth factor-β (TGF-β) [53].

Both Th1 and Th2 cytokines may induce Abs production by B cells. However, they promote the synthesis of different immunoglobulin (Ig) types. Th1 cells induce IgG subclasses that efficiently bind and activate the complement, whereas Th2 cells

induce Ig isotypes and IgG subclasses (IgG4 and IgE) that poorly or not at all fix the complement. MG patients present numerous AChR-specific Th1 cells in blood, that recognize many AChR epitopes and induce synthesis of pathogenic auto-Abs interacting with B cells [54].

MG patients present also AChR-specific Th2 and Th3 cells in blood [55]. Several studies show that Th1 cells and their cytokines are needed for EAMG development, probably for their role in inducing production of complement-binding pathogenic anti-AChR Abs [43, 56]. Moreover, experimental studies on EAMG demonstrate that estrogens promote the disease development in mice increasing interleukin-12 (IL-12) production by AChR-specific Th1 cells, suggesting that estrogens may mediate sex differences in autoimmunity with a Th1-driven mechanism (Figure 1.4) [57].

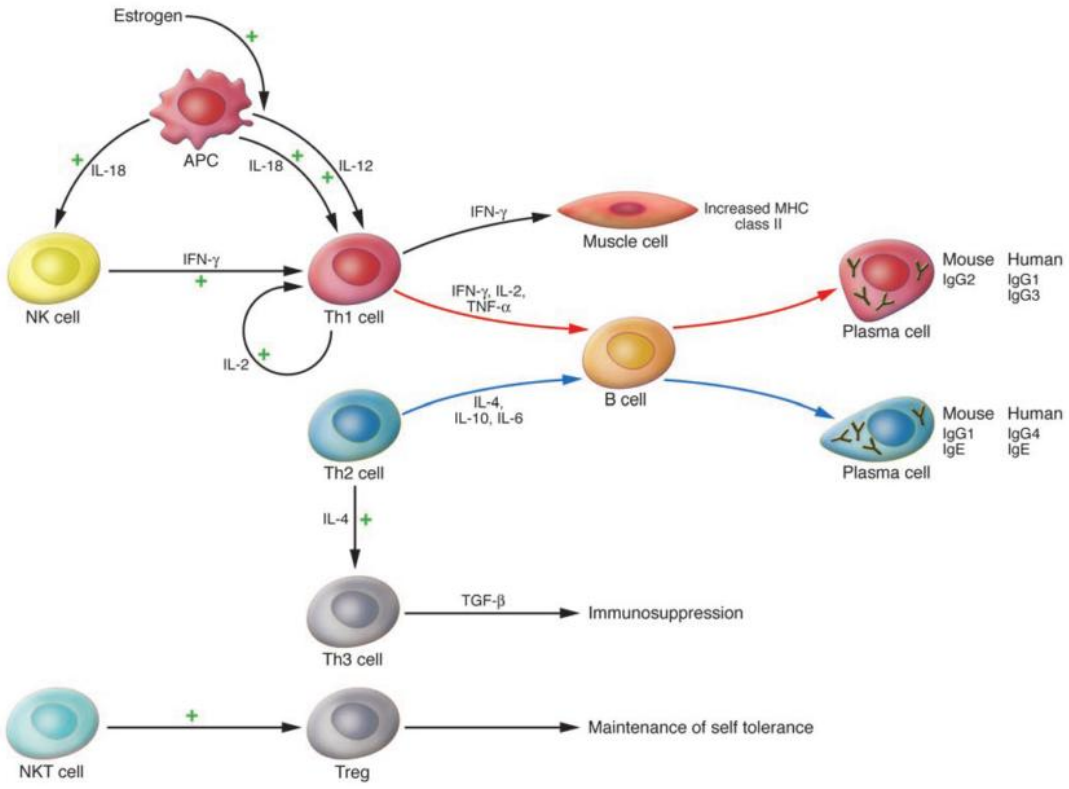

Figure 1.4. Cytokine network and cells involved in the pathogenesis and immune regulation of MG. Th1 cytokines stimulate production of IgG subclasses that bind and activate complement, whereas Th2 cytokines stimulate the production of Ig classes and IgG subclasses that do not involved in MG. The Th2 cytokine IL-4 is also a differentiation factor for Th3 cells, immunosuppressive cells that secrete TGF-β. The Th1 cytokine IFN-γ stimulates expression of MHC class II molecules on the muscle cell membrane, thus facilitating presentation of muscle AChR. The IL-18 secreted by APCs favors the differentiation of Th1 cells both directly and indirectly through the action of NK cells. CD1-d–restricted NKT cells can activate Tregs, thereby inhibiting autoimmune processes, Conti-Fine et al., 2006 [33].

Pro-inflammatory Th1 cytokines induce expression of MHC class II molecules in muscle, promoting the presentation of AChR epitopes and further the expansion of activated AChR-specific CD4+ T cells [58]. Increased IFN-γ production may explain the increased expression of IFN-γ–induced chemokines and monokines and their receptors in muscle, thymus, and lymph nodes in MG patients. A decrease in chemokine expression correlates with a decrease in severity of MG symptoms [59].

Studies performed in EAMG animal models show that AChR-specific Th2 cells present complex and contrasting roles: they can be protective [60, 61], but the cytokines interleukin-5 (IL-5), IL-6, and IL-10 are also able to promote EAMG development [62, 63].

Other helper T cell subtypes may perform a role in MG. CD4+ T cells, that express the CD25 marker and the transcription factor forkhead box P3 (Foxp3) on their surface, are known as Treg cells and are important in maintaining self-tolerance (Figure 1.4). Regulatory T cells in MG patients may be functionally impaired [64]. and the number of circulating Tregs has been shown to increase after thymectomy, according to a symptom improvement [65].

Also Natural Killer (NK) and NKT cells show a role in restoring self-tolerance in MG. NKT cells and Tregs may cooperate in regulating the anti-AChR response. In AChR-immunized mice, the activation of NKT cells, by a synthetic glycolipid agonist, inhibits EAMG development; these therapeutic effects are likely mediated by the increase in number and modulatory functions of induced Tr cells [66]. NK cells can influence the development of EAMG, and possibly MG: indeed, in mice, NK cells are necessary for EAMG induction, promoting IFN-γ secretion and Th1 cells sensitization [67].

Interleukin-18 (IL-18), secreted by APCs is an important growth and differentiation factor for both NK and Th1 cells, especially in cooperation with IL-12 [56]. Thus, IL-18 may be important in the pathogenesis of MG and its experimental model. Indeed, IL-18–deficient mice are resistant to EAMG, as the pharmacologic blockade of IL-18 suppresses the disease [56, 67]. Moreover, MG patients show increased serum levels of this cytokine, which tend to decrease in parallel with clinical improvement, thus supporting a role for IL-18 in human disease [68].

Other autoantigens in MG

Up to 15% of MG patients do not develop anti-AChR Abs and are consequently known as seronegative patients [29]. In 40% of them, [69] antibodies against MuSK are detected. Anti-MuSK-positive MG patients never show anti-AChR Abs [69]. The agrin/MuSK signaling pathway likely maintains the structural and functional integrity of the post-synaptic NMJ apparatus also in adult muscle. IgG from anti-MuSK-positive MG patients disrupt AChR aggregation in myotubes from MuSK-immunized animals and block agrin-induced AChR clustering [34, 70]. Several studies confirm the effect of anti-MuSK Abs on the agrin-dependent maintenance of AChR clusters at the NMJ, leading to reduced AChR number [70]. Complement-mediated damage may also be responsible for loss of AChRs and NMJ impairment. However, some MG patients with anti-MuSK Abs do not present AChR loss at the NMJ [71], because they predominantly belong to IgG4 subtype and do not efficiently bind the complement [26]. Moreover, some studies show that anti-MuSK Abs do not cause significant AChR loss, complement deposition, or morphologic damage at the NMJ [71].

Anti-MuSK Abs may perform other pathogenic mechanisms; in a recent study effects of sera from anti-MuSK-positive MG patients on muscle cell cultures has been investigated [72]. Authors shown that some sera inhibit cell proliferation, by causing cell cycle arrest, and down-regulate the expression of AChR subunits, rapsyn, and other muscle proteins [72].

Some anti-AChR/MuSK-negative patients may develop a plasma factor that activates an alternative pathway in the muscle, resulting in phosphorylation and inactivation of the AChR [73].

MG patients may also synthesize Abs against non-muscle-specific proteins, such as myofibrillar proteins [22]; some of those antibodies, especially anti-myosin Abs and anti-fast troponin Abs, may cross-react with the AChR [74]. Moreover, MG patients with thymoma can develop Abs against titin [75] and ryanodine receptor [76].

The role of the thymus

The role of the thymus in MG is still unknown, even if its involvement has been reported since the beginning of the 1900s [77]. Marked pathological alterations of

thymus occur in over 80% of anti-AChR-positive patients [11]: thymic hyperplasia is observed in 50–60% of seropositive cases and in a variable proportion of seronegative cases, thymoma is present in 10–15% of cases [78]. A hyperplastic thymus typically contains B-cell infiltrates that can organize into ectopic germinal centers (GCs - structures specialized for the expansion and affinity maturation of B-cell clones), forming B-cell follicles (follicular hyperplasia) or being distributed throughout thymic medulla (diffuse hyperplasia, also known as thymitis) [78]. 10–20% of anti-AChR-positive patients show an atrophic thymus very similar to that of age-matched controls for the amount of adipose tissue and epithelial space; it is characterized by the presence of infiltrating B cells, being indicative of thymic hyperplasia and immune activation. The thymus of anti-AChR-positive MG patients contains all the components required to initiate and sustain the autoimmune response: autoantigen, expressed on muscle-like myoid cells [79] and thymic epithelial cells (TECs) [80], professional APCs [81], AChR-specific T cells [82], and plasma cells producing anti-AChR antibodies [83].

Furthermore, in 2003 Levinson and colleagues developed a new model of intra-thymic inflammation and speculated a feasible mechanism explaining thymus involvement. The hypothesis, still to be tested, was that an inflammatory reaction against an unrelated antigen within the thymic medulla might promote the entry of peripheral AChR α-reactive CD4+ T cells that escaped central deletion (Figure 1.5) [84].

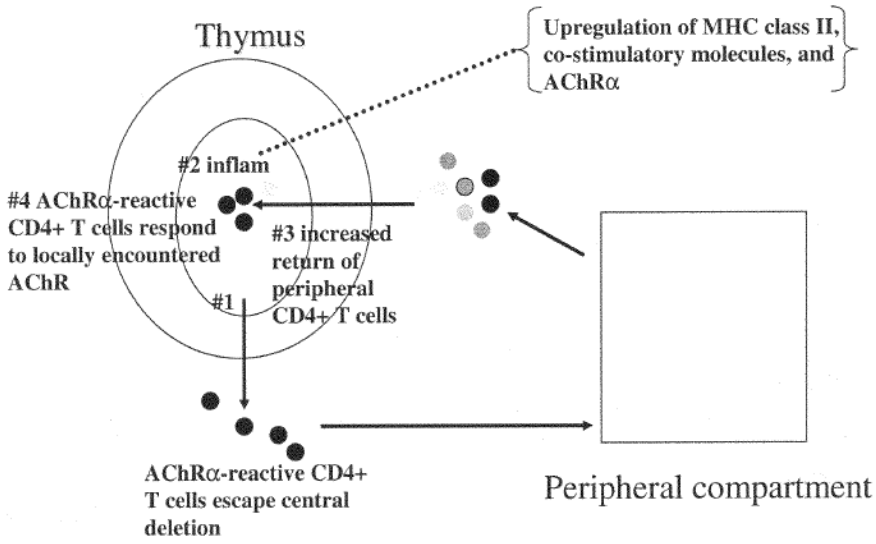

Figure 1.5 Intra-thymic pathogenesis of MG, Levison et al., 2003 [84].

These cells entered the thymic medullary compartment, where they encountered AChR α expressed on APCs. The concomitant intra-thymic inflammatory reaction created a milieu that favored the activation of these cells, with up-regulation of MHC class II and co-stimulatory molecules on APCs and, maybe, with up-regulation of AChR expression. The presentation of AChR α epitopes to migrated-thymus CD4+ T cells might lead to Th1 cell activation, increased local stimulation of AChR α-reactive B cells/plasmacells with consequent secretion of anti-AChR Abs and production of germinal centers (GCs), finally promoting MG development [84].

Several evidences demonstrate the association between MG and thymic tumor; however, although the thymectomy benefits younger MG patients with thymoma, no clinical improvement are often observed, requiring additional immunosuppressive therapies. MG patients with thymoma can develop also anti-titin and anti-ryanodine receptor antibodies [85].

Molecular mimicry

Some studies suggest that environmental factors are involved in the etiology of MG [86, 87]. Among them, viral infections are suspected to play a role in the development of autoimmunity by mechanisms including general activation of the host immune system and molecular mimicry [88]. The pathogens act as promoters of an auto-sensitization process mainly by initiating an innate immune response that stimulates inflammation and activates the host immune system [88].

Furthermore, evidences of chronic thymic inflammation in most MG patients [89] makes plausible the hypothesis that persistent viruses or other microbial agents may contribute to intra-thymic pathological mechanisms of the disease. The high affinity and specificity of anti-AChR antibodies in MG patients [12] imply that the human AChR is the antigen, even if another antibody or a microbial antigen that contains epitopes mimicking the AChR, could be involved in the etiology of MG. Indeed, thymic expression of antigenic fragments, or some idiotypic perturbation or microbial trigger, might initiate the production of low-affinity antibodies specific for AChR, which ‘spread’ to induce the high-affinity and heterogeneous antibodies that cause disease symptoms [11].

Cavalcante and colleagues have found indication about a viral contribution to onset or maintenance of the intra-thymic autoimmune response in MG patients [86, 87, 90]. In these studies, they have discovered evidences of chronic poliovirus infection in the thymus of some MG patients, suggesting that persisting viruses, which stimulate innate immune responses and chronic inflammation, may be responsible for immunological alterations and auto-sensitization in this organ [86, 90].

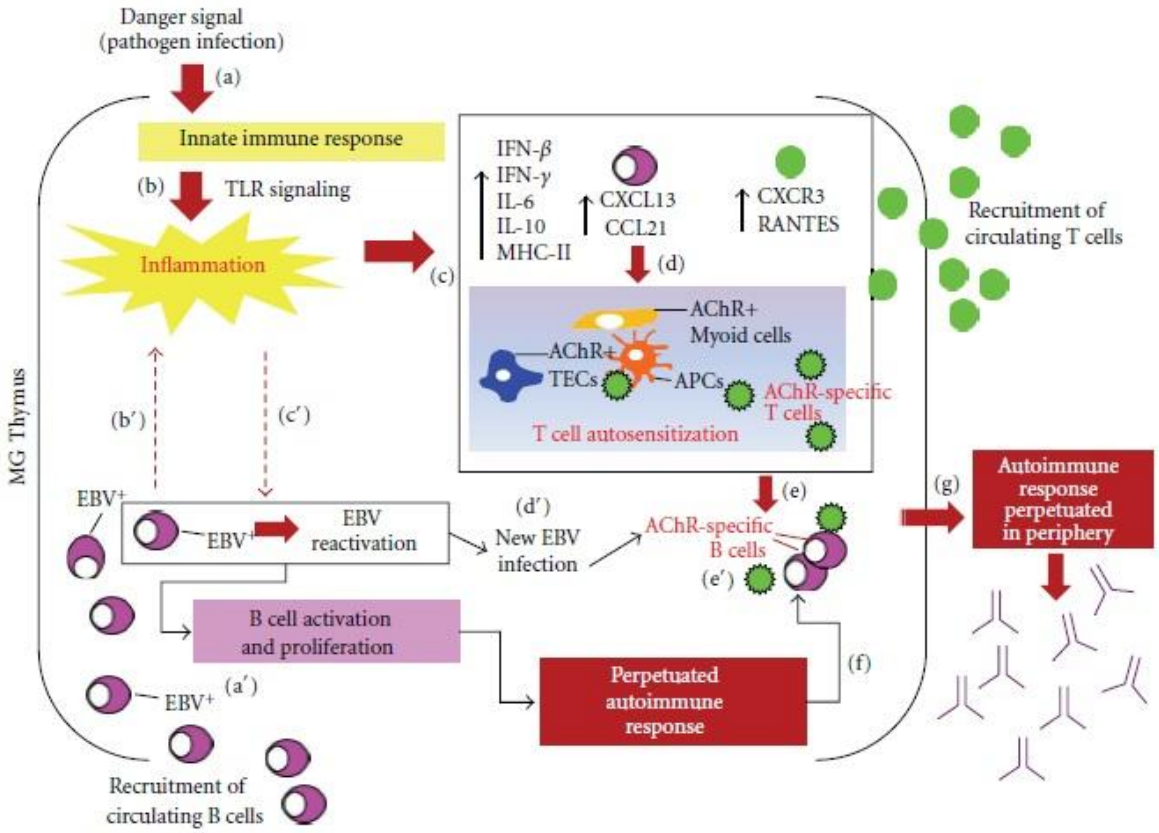

Figure 1.6 Proposed model of virus-induced autoimmunity in MG. A "danger signal" (e.g., pathogen infection) stimulates Toll-like receptor- (TLR-) mediated innate immune responses (a), whose dysregulated or persistent activation leads to the chronic inflammation characteristic of MG thymus (b). The chronically established thymic inflammatory state (c), characterized by overexpression of proinflammatory cytokines (e.g., IL-6, IL-10), type I and II IFNs, and T- and B-lymphocyte-attracting chemokines (e.g., CXCR3, RANTES, CXCL13, CCL21), is essential, in the context of a genetically predisposing background, for the establishment of mechanisms (d) contributing to T-cell autosensitization, including presentation or cross-presentation of "self-epitopes" by TECs or myoid cells expressing the autoantigen; upregulation of MHC genes; activation of APCs; as well as the constant priming of autoreactive T cells, which in turn promote autoimmune response by autoreactive B cells (e). B cell attractants CXCL13 and CCL21 recruit circulating B cells to thymus, including those harboring EBV (a'). EBV infection itself contributes to thymic inflammation (b'). EBV reactivation, influenced by the inflammatory state (c'), results in EBV propagation to uninfected B cells (d') including AChR-specific B cells (e'). The chronically established inflammation and EBV infection promote the maintenance within the thymus of the autoimmune response (f), which may be thus perpetuated in periphery (g), Cavalcante et al., 2011 [87].

Moreover, they have identified an abnormal accumulation of Epstein-Barr virus-(EBV-) infected B cells and plasma cells in MG thymuses, but not in normal control [86]. Since EBV has the unique ability to disrupt B-cell regulatory check-points and

to interfere with the B-cell differentiation program [91], their findings have suggested that EBV infection may contribute to chronic B-cell activation and persistent autoimmune response in this organ in MG patients [86].

Finally, the authors have proposed a mechanism to explain the etiology of MG: the inflammation, triggered by an endogenous or exogenous (e.g., microbial infection) danger signal, might drive the colonization of thymus by EBV-harbouring B cells and the subsequent EBV re-activation (Figure 1.6). Persistent EBV infection itself might contribute to maintain a chronically inflamed thymic microenvironment, where the virus might promote disruption of B-cell tolerance check-points, resulting in expansion of auto-reactive B-cell clones (Figure 1.6). EBV infection thus could explain how the autoimmune response has appeared perpetuated in MG thymus, for the potential capability of the virus to immortalize B cells producing AChR antibodies [87].

Immunogenetics in MG

The biological and clinical heterogeneity of autoimmune MG seems to correlate with genetic markers, most notably the Human Leukocyte Antigen (HLA) genes [92]. The HLA genes encode for the MHC region, i.e. the antigen-presenting proteins on the cell surface. The most intensely studied HLA genes are the nine so-called “classical MHC genes”: HLA-A, HLA-B, HLA-C, HLA-DPA1, HLA-DPB1, HLA-DQA1, HLA-DQB1, HLA-DRA, and HLA-DRB1. In humans, the MHC is divided into three regions: Class I, II, and III. The A, B, C, E, F, and G genes belong to MHC class I, whereas the six D genes belong to class II. MHC genes are expressed in co-dominant fashion: the alleles (variants) inherited from both progenitors are expressed in equivalent way:

- each person inherits a set of genes, belonged to Class I locus (named in humans HLA-A, HLA-B and HLA-C) from each progenitor, thus any cell in each individual can express 6 different types of MHC-I molecules;
- in the Class II locus, each person inherits a couple of genes HLA-DP (DPA1 and DPA2, encoding α and β chains), a couple of genes HLA-DQ (DQA1 and DQA2, encoding α and β chains), one gene HLA-DRα (DRA1) and one or two genes HLA-DRβ (DRB1 and DRB3, -4 or -5). Thus, a heterozygous individual can inherit 6 or 8 Class II alleles, three or four from each progenitor.

The set of alleles, present in each chromosome, is called MHC haplotype. In humans, each HLA allele is identified by a number (e.g. HLA-A2, HLA-B5, HLA-DR3). Each heterozygous individual will have two MHC haplotypes, one for each chromosome (one of paternal origin and the other of maternal origin).

The most consistent finding in MG patients is the association of HLA-DR3 and B8 alleles with early-onset MG and thymic hyperplasia [92]. Late-onset MG is less strongly associated with HLA-DR2 and B7 [93]. HLA-DR3 and DR7 seem to have opposing effects on MG phenotype: DR3 show a positive association with early-onset MG and a negative association with late-onset anti-titin-positive MG; DR7 show opposite effects [94]. No clear genetic links have been found for MG with thymoma, but patients with particular genetic profiles present a higher risk of developing MG [95].

Recently, an association with DR14-DQ5 has been reported in patients with anti-MuSK antibodies [96]. Anti-MuSK-positive MG is less frequent in some ethnic groups or geographical locations (e.g. China, Netherlands), suggesting genetic as well as possible environmental influences [97].

It has been also demonstrates that several non-HLA genes, such as the protein tyrosine phosphatase, non-receptor type 22 (*PTPN22* - lymphoid*)* and the cholinergic receptor nicotinic alpha polypeptide 1 (*CHRNA1* - muscle), are associated with MG [93]; some of these genes are involved in other autoimmune diseases, suggesting a non-specific susceptibility to autoimmunity. An exception is represented by *CHRNA1* gene, which encodes for the α-subunit of the AChR and might provide MG-specific pathogenetic clues [16].

1.2.3 Clinical Symptoms

The main MG clinical symptoms ~~a~~re muscular weakness and fatigability, usually involving specifically susceptible muscle groups. Patients often show weakness fluctuating from day to day or even from hour to hour, worsening with activity, and improving with rest. Patients can be affected by varying degrees of ptosis, diplopia, dysarthria, dysphagia, dyspnea, facial weakness, or fatigable limb or axial weakness [16].

Ocular weakness, as fluctuating ptosis and/or diplopia, is most commonly the first presentation of MG, occurring in approximately 85% of patients [17]. Disease

progression to generalized weakness usually occurs within 2 years of the onset and weakness of facial muscles is common [16].

Bulbar weakness, characterized by painless, dysphagia, dysarthria, or chewing difficulties, is the first symptom in up to 15% of patients [98]. Weakness involving respiratory muscles rarely occurs, but it can be life-threatening and need an immediate therapeutic action [16].

1.2.4 Diagnosis

Available tests, applied to confirm the clinical diagnosis of MG, include bedside tests, such as the edrophonium or ice-pack test, electrophysiological analysis, measure of the concentrations of serum autoantibodies and scan tests [16].

Bedside tests

Edrophonium chloride is a short-acting AChE inhibitor that increases availability of ACh and thus prolongs its action at the NMJ, increasing the amplitude and duration of the EPP. The test consists in edrophonium intravenous administration and observation of the patient for an immediate improvement in muscle strength [99]. Some studies indicate that its sensitivity in the diagnosis of MG is 71.5–95% for generalized disease [99], but today this test is not commonly used.

The ice-pack test is a non-pharmacological test that is performed by applying an ice pack over the eye for 2–5 minutes and assessing for an improvement in ptosis [100]. Its use should be mainly considered in patients with ptosis, in whom the edrophonium test is contraindicated.

Electrophysiological tests

Repetitive nerve stimulation is the most commonly employed electrophysiological test for evaluating the neuromuscular transmission. In disorders of the NMJ, low rates of nerve stimulation (2–5 Hz) produce a progressive decrement in amplitude of the compound muscle action potential. The result of this test is abnormal in approximately 75% of patients with generalized MG (<50% of ocular MG) [100]. The single-fiber electromyography test (SFEMG) measures the neuromuscular jitter resulted from fluctuations in the time taken for the EPP to reach the threshold for muscle fiber action potential, generated from an individual muscle fiber.

SFEMG reveals abnormal jitter in 95–99% of MG patients [101]. The jitter can also be assessed by using conventional electromyography electrodes, less sensitive than SFEMG [102]. Although highly sensitive, increased jitter is not specific for NMJ disease, but it might be found in nerve or even muscle disease [100].

Immunological tests

The immunological tests are most commonly applied for MG diagnosis. They measure the amount of serum antibody that bind muscle AChR, as detected by radio-immuno assay with ^{125}I-labelled α-Bungarotoxin [13]. The presence of anti-AChR Abs is estimated around 85% for generalized MG and 50% for ocular MG [13]. Anti-AChR antibody titer widely varies among patients with similar muscular weakness, thus they can not predict the severity of disease. Patients might result falsely seronegative if they are following an immunosuppression therapy or if the test is performed too early in the disease [103].

Other immunological assays measure in patients' sera anti-striated muscle (striational) Abs recognizing muscle cytoplasmic proteins (titin, myosin, actin, and ryanodine receptors). Anti-striational Abs are detected in 75–85% of MG patients with thymoma and in some subjects affect by the tumor without MG [22, 104]. Thus, the presence of anti-striational Abs in early-onset MG raises the suspicion of thymoma. Nevertheless, antibodies reactive against titin and other anti-striational Abs are also found in up to 50% of patients with late-onset MG, without thymoma; therefore these Abs cannot be considered predictors of the tumor in patients over 50 years aged [21, 105].

Patients with anti-AChR-negative generalized MG are tested for the presence of anti-MuSK Abs, found in approximately 40% of them [26]. Low affinity anti-AChR Abs, binding to clustered AChRs, have been found in 66% of seronegative generalized MG patients sera [106], but this test is not currently commercially available.

Scan Exams

Chest X ray computed tomography or Magnetic Resonance Imaging (MRI) are routinely performed in all patients with confirmed MG to exclude the presence of thymoma. However, contrast agents should be used with caution because they

might exacerbate myasthenic weakness [107]. MG often coexists with thyroid disease, so baseline testing of thyroid function should be obtained at the time of diagnosis [16].

1.2.5 Therapeutic Treatment

The common therapeutic approach for the treatment of MG includes the use of cholinesterase inhibitors, short-term immune and long-term immune therapies. MG patients refractory to common therapies can try treatments with new immunosuppressive drugs such as cyclophosphamide and rituximab. In the case of thymoma or thymic hyperplasia, the patients undergo thymectomy surgery.

Cholinesterase inhibitors

Oral cholinesterase inhibitors increase the amount of AChR available for binding at the NMJ, and are the first-line treatment in patients with MG [108]. Pyridostigmine bromide is the most commonly used reversible cholinesterase inhibitor. The initial oral dose in adults is 15–30 mg every 4–6 h, which is increased and adjusted to maximise benefit and minimize side effects (diarrhoea, stomach cramps). Cholinesterase inhibitors rarely induce complete or sustained relief of MG symptoms and do not affect disease progression, but might be sufficient for adequate management in certain patients with non-progressive mild or purely ocular disease. Doses of pyridostigmine over 450 mg daily can induce worsening muscle weakness due to a depolarisation block of neuromuscular transmission. Cholinergic overdose is often accompanied by the muscarinic symptoms of hypersalivation, bradycardia, hyperhidrosis, lacrimation, and miosis [16].

Short-term immune therapies

Plasmapheresis and intravenous immunoglobulin infusion are used for short-term treatment of MG exacerbations and in cases of a rapid clinical response. Plasmapheresis temporarily reduces the concentrations of circulating anti-AChR antibodies [109]. Typically one exchange, removing one to two plasma volumes, is done every other day, up to a total of four to six times. Published reports indicate that plasma exchange effectively improves strength in most patients with severe MG [109]. Common side-effects include hypotension, paresthesias, infections and

thrombotic complications related to venous access [110]. Plasma exchange can also reduce coagulation factors, particularly in repeated treatments [111].

Circulating anti-AChR pathogenic factors can also be removed using immunoadsorption columns, some of which use immobilized AChR as an immunoadsorbent [112]. Intravenous immunoglobulin infusion is widely used for patients with exacerbating MG. The exact mechanism by which intravenous immunoglobulins ameliorate the disease is not clear, but it likely involves competition with autoantibodies and Fc (Fragment, crystallisable region)-receptor binding on immunoglobulin [113]. The standard dosing regimen for intravenous immunoglobulin (1–2 g/kg) involves the infusion of large volumes and is very expensive. Although rare, severe complications do occur, some of which are related to the large volume and high viscosity of the infused preparation [114].

Long-term immune therapies

The long-term immune therapies are the most commonly used treatments in MG patients. Three main classes of drugs belonged to this category: corticosteroids, non-steroidal immunosuppressive agents and other immunosuppressive agents.

Corticosteroids — Corticosteroids were the first immunosuppressant medications used in MG, and still remain the most commonly used immune-directed therapy [115]. Several studies demonstrated that steroid treatment, administered at various doses, had a good efficacy in more than 73% of the patients with generalized MG with an improvement or remission [116, 117]. Prednisone is a synthetic corticosteroid drug generally used in MG patients, who do not respond adequately to cholinesterase inhibitors [115]. Prednisone irreversibly binds to α and β glucocorticoid receptors (GR – present in all tissues) with high affinity. The steroid receptor complexes dimerize and interact with cellular DNA in the nucleus, binding to steroid-response elements and modifying gene transcription. The immunosuppressive actions of the drug involve the inhibition of gene transcription for COX-2, cytokines, cell adhesion molecules and inducible Nitric Oxide synthetase, determine blockage of Vitamin D3-mediated induction of osteocalcin gene in osteoblasts, modification of collegenase gene transcription and increased synthesis of annexin-1. Initially, prednisone can be administered at high doses

(0.75–1.0 mg/kg daily) and then gradually reduced or continued at low doses for many years. Approximately a third of patients have a temporary exacerbation after starting prednisone; this usually begins within the first 7–10 days with high prednisone doses and lasts for several days [117]. In patients with oropharyngeal or respiratory involvement, plasma exchange or intravenous immunoglobulin can be given before beginning prednisone treatment to prevent or reduce the severity of corticosteroid-induced exacerbations and to induce a more rapid response [16]. Some neurologists prefer to appoint prednisone with a low dose (10–25 mg) and gradually increase to 60–100 mg on alternate days [118]. Whereas corticosteroids are highly effective in MG, they usually must be given chronically, with significant risk for adverse events [119]. Prednisone should therefore be considered in all patients with ocular MG who do not achieve full control of symptoms with anticholinesterase medications. Although not definitive, evidence suggests that corticosteroid treatment might delay or reduce the frequency of progression of ocular MG to generalised disease [30].

Non-steroid immunosuppressive agents — Azathioprine is a pro-drug that, following oral administration, it is metabolized by the purine methyl transferase into the active 6-mercaptopurine, itself a purine synthesis inhibitor. 6-Mercaptopurine blocks DNA synthesis and thus inhibits T and B cell proliferation [16]. More studies indicate that azathioprine is effective in 70–90% of patients with MG, but the onset of benefit might be delayed for as long as 12 months [120, 121]. Azathioprine (50 mg daily) can be used alone or as a steroid-sparing agent in MG. When these drugs are used in combination resulted more effective and better tolerated than administered alone [122]. In the absence of systemic side effects, the dose is then gradually increased by 50 mg per week to a daily dose of 2–3 mg/kg. Hepatotoxicity and leukopenia are important adverse effects [123], which can be reverted, if detected early, administrating reduced or discontinued doses of drug. Patients with thiopurine methyl transferase deficiency cannot completely metabolise azathioprine and a normal dose might lead to dangerous leukopenia [124]. Therefore, measurement of thiopurine methyl transferase concentrations is recommended before initiating azathioprine therapy.

Mycophenolate mofetil (MMF) is a pro-drug of mycophenolic acid and a reversible inhibitor of inosine monophosphate dehydrogenase (IMPDH) [125] in purine biosynthesis, thereby suppressing T and B cell proliferation [16]. MMF is selective for *de novo* pathways critical to lymphocytic proliferation and activation. Other cells are able to recover purines via a separate, scavenger pathway and are thus able to escape the effect. MMF represents an useful alternative to the highly toxic azathioprine. The standard dose used in MG is 1 g twice daily, but doses up to 3 g can be used. Higher doses are associated with myelosuppression, therefore MG patients should be constantly monitored with blood test.

Ciclosporin inhibits T-cell proliferation via disruption of calcineurin signalling, binding the cytosolic protein cyclophilin of T-lymphocytes. This complex of ciclosporin and cyclophilin inhibits calcineurin, which is responsible for activating the transcription of IL-2, cytokine that interacts with its specific receptor and stimulate the growth, differentiation and survival of T cells via the activation of the expression of specific genes. In T-cells, activation of the T-cell receptor normally increases intracellular calcium, which acts via calmodulin to activate calcineurin. Calcineurin then dephosphorylates the transcriptional nuclear factor of activated T-cells (NF-AT), which translocates from cytosol to the nucleus of the T-cell and sustains the expression of genes such as IL-2 and other pro-inflammatory cytokines. Ciclosporin prevents the dephosphorlyation of NF-AT by binding to cyclophilin inhibiting lymphocytes production and IL-2 release. Ciclosporin is used mainly in patients in which azathioprine is either ineffective or not tolerated. The recommended initial daily dose is 4–6 mg/kg nevertheless, daily doses of 3–4 mg/kg or less are often adequate. Common side effects are hirsutism, tremor, gum hyperplasia, and anaemia, but hypertension and nephrotoxicity are the main treatment-limiting adverse reactions [126].

Tacrolimus (FK506) has a mechanism of action similar to ciclosporin, with potential benefits for MG [127, 128]. Sustained benefit has been reported in anti-ryanodine-receptor- positive patients, probably due to enhancement of ryanodine-receptor-related sarcoplasmic calcium release [129]. Daily doses are of 3–5 mg, with a less nephrotoxicity compared to ciclosporin as side effect [16].

Other immunosuppressive agents — A small percentage of patients with MG are refractory or develop intolerable side effects to treatment with corticosteroids in combination with one or more of the immunosuppressive agents. These patients can therefore be considered in treatments with cyclophosphamide and rituximab.

Cyclophosphamide is a nitrogen mustard alkylating agent, which forms DNA crosslinks between (interstrand crosslinkages) and within (intrastrand crosslinkages) DNA strands at guanine N-7 positions. This reactions are irreversible and lead to cell death. Recent studies demonstrated that different doses of intravenous cyclophosphamide given to patients with refractory MG improved muscle strength and reduced steroid requirement [130-132]. Side effects of cyclophosphamide include myelosuppression, haemorrhagic cystitis, and an increased risk for infection and malignancy [133].

Rituximab is a chimeric monoclonal antibody directed against the B-cell surface marker CD20 (an activated-glycosylated phosphoprotein expressed on the surface of all B-cells, increasing with the maturation of cells). It reduces circulating B-cell counts, and on the basis of its potential for targeting autoreactive B-cell clones, might have a therapeutic role in antibody-mediated autoimmune diseases. Refractory and MuSK MG patients have reported benefits with this treatment [134].

Thymectomy

The use of thymectomy in MG was initially based on empirical observations that patients with MG improved after removal of the thymus. The presumed role of the thymus in MG has provided theoretical justification for the procedure, and thymectomy has been used as a treatment for non-thymomatous MG for nearly 70 years. The role of thymectomy in non-thymomatous MG patients is still not clear [16] even if some benefits arise from thymectomy and therefore it should be considered as a treatment option in selected patients with MG [135]. Most experts consider thymectomy as a therapeutic option in anti-AChR-positive, generalised MG with disease onset before the age of 50 years; some neurologists recommend it also in patients who lack anti-AChR antibodies [16]. Nowadays, the only absolute indication for thymectomy is the presence of thymoma. The role of thymectomy in anti-MuSK MG is not yet clear [27].

1.3 EAMG

Experimental Autoimmune Myasthenia Gravis is the animal model for human MG. It can be induced in vertebrates by active immunization with *Torpedo Californica Electroplax* AChR (TAChR) in Complete Freund's Adjuvant (CFA) as well as by passive transfer of MG sera or anti-AChR Abs [136, 137]. EAMG has been induced in a wide variety of experimental animals: rabbits [137], rats (Lewis strain is preferred, but Brown-Norway or Fisher might also be used) [138], guinea pigs [139], mice [140], and rhesus monkeys [141]. The most detailed models and studies have been performed in rats (65%) and mice (35%); the incidence of clinical EAMG in rats is higher (about 90%) than in mice (70-85%), thus indirectly indicating a superiority of the rat EAMG model [136]. Susceptibility to EAMG is influenced by age, sex and genetic background of different rat and mice strains [142].

In particular experimental MG is induced in young (6-8 weeks aged) Lewis rats with a single administration of TAChR in CFA. Clinical signs of the disease are due to anti-TAChR antibodies cross-reacting with the self (rat) AChR on the NMJ. EAMG in Lewis rats is clinically characterized by two distinct phases: (1) a transient acute phase with a mild muscular weakness, beginning approximately 8-10 days post immunization (p.i.) and recovering after 3-4 days; (2) a severe, progressive chronic phase starting approximately 25-30 days p.i., ending often in death [139].

The immunization with TAChR in CFA results in activation of specific-CD4+ T cells responding to the antigen of immunization, with consequent production of anti-TAChR Abs cross-reacting with self-AChR and generation of anti-rat AChR Abs; this finding induces complement system activation and receptor and end-plate degradation, with consequent impairment of the nervous transmission from motor neuron to muscle at the NMJ. In particular, IgM directed to TAChR are readily detectable in rat serum during the acute phase of EAMG, while more than half of the antibody repertoire belong to IgG subtype, 10 days p.i; this suggests that a switch from IgM to IgG production has been very early occurred. During the chronic phase, 35 days p.i., all detectable anti-TAChR antibodies are IgGs. These late-IgGs cross-react with rat muscle AChR and, in this phase, the high affinity antibody populations may be represented by both Torpedo and Rat Abs [137]. As

EAMG progresses into its chronic phase, the titre of cross-reacting antibodies continues to increase [137], and the amount of AChR in muscle results greatly reduced. The initial AChR decrease during the early acute phase is due to an extensive phagocytosis of the post-synaptic membrane by macrophages. Indeed, during this phase muscle end-plates are invaded by phagocytic cells that intrude between nerve and muscle and phagocytize the post-synaptic membrane, thus resulting in functional denervation of up to 90% of the fibers. Around 10-12 days p.i., phagocytic cells disappear from the end-plate zone and the inflammatory response at the motor end-plate subsides. Thus, there is an increased yield of AChR that is presumably due to synthesis of extra-junctional AChR, occurring as consequence of denervation and to repair synthesis of junctional AChR. These processes probably occur for the rapid return of AChR yields to normal levels around 13 day p.i.. After the acute phase a continued gradual loss of AChR is observed. The development of the chronic phase is associated with dramatic increase in titre of serum antibody to muscle AChR, decrease in the area of post-synaptic membrane and simplification of its folded structure. The impairment of neuromuscular transmission could result from decreased muscle AChR content, as the amount of receptor extracted from myasthenic muscles is greatly reduced, of about 30% compared to normal muscles. Furthermore, in chronic EAMG a large portion of the remaining AChR is complexed with antibodies, so that the function of these receptors *in vivo* may be partially or completely impaired [143].

The severity of EAMG in Lewis rats is monitored by evaluation of muscular weakness. Clinical score is based on the presence of tremor, hunched posture, reduced muscle strength and fatigability. The latter manifestation can be assessed after exercise (grip strength test: repetitive paw grips on the cage grid) for 30 seconds. Disease severity is expressed as follows [139]:

- *grade 0:* normal strength and no abnormalities;
- *grade 1:* mildly decreased activity and weak grip or cry, more evident at the end of testing;
- *grade 2:* clinical signs present before exercise (tremor, head down, hunched posture, weak grip);
- *grade 3:* severe clinical signs present before exercise, no grip, moribund;
- *grade 4:* dead.

EAMG rat models mimic many aspects of the pathophysiology of human MG, sharing various features with it. Thus, these animals provide an opportunity to explore therapeutic strategies of possible use in humans [136].

1.4 Role of the peptide R97-116 in EAMG

The native AChR contains multiple antigenic determinants, both conformational (B cell epitopes) and linear (T cell epitopes), that are highly effective in activating the host immune system. Similarly to the human disease, Abs production in EAMG is thought to be under the control of AChR-specific T cells. The identification of immunodominant AChR sequences, relevant for T or B cell compartments that are effective in inducing EAMG, may be helpful to understand the key immunological processes leading to the disease and hence improve the knowledge of MG pathogenesis [138]. In some experimental models, myasthenogenic sequences were identified among B cell epitopes, located within the MIR of AChR α-subunit (α67–76) [144], or near the αBTX-binding site or acetylcholine-binding site, α125–147 [145] and α183–200 [146] respectively. Anti-peptide Abs, able to bind to conformational extracellular domains of the AChR, can directly attack the receptor or interfere with the ACh-binding site. Indeed, rats immunized with α125–147 sequence derived from Torpedo produce anti-peptide Abs that bind to the native receptor [147].

Baggi and colleagues have shown that EAMG can be induced in Lewis rats by a single injection of the peptide corresponding to the 97-116 sequence (R97-116) present on the rat AChR α-subunit and demonstrated that it plays an important role in the pathogenesis of the disease for its immunodominant properties [138]. As one single immunization with R97-116 has not been sufficient in inducing EAMG clinical manifestations, the immunization protocol have comprised one administration with peptide in CFA on day 0, followed by a booster injection of peptide in Incomplete Freund's Adjuvant (IFA) one month later. By this protocol, EAMG manifestations have become evident starting 7 weeks after the first immunization. When disease onset and progression have been compared to those observed in TAChR-induced EAMG, a slower progression has been observed in peptide-immunized rats. High incidence of the disease has been demonstrated in rats immunized with the self-peptide: more than 70% of animals have developed

EAMG, as confirmed by loss of body weight and clinical score,. elevated anti-rat AChR Abs and reduced muscle AChR content. In this model, Abs and T cells both specific for the peptide have been induced. R97-116-specific Abs have not exerted any myasthenogenic activity, because this sequence is hidden in AChR structure, but R97-116-specific T cells might recognize the self-peptide presented by muscle cells. Activated T cells might then exert cytotoxic activity toward the APCs, resulting in the release of native AChR, hence in breakdown of tolerance to AChR and in EAMG development [138].

1.5 Pixantrone

The majority of MG patients show a positive response to standard treatments, nevertheless some require long-term immunosuppression with corticosteroids and/or immunosuppressive drugs. The importance of drug-related side effects (e.g. as a consequence of long-term treatment with corticosteroids) has been underlined in MG, as side effects might even lead to a degree of disability greater than that caused by MG itself. In this context, new immunosuppressants are needed for patients with intolerable side effects or with major contraindications to prolonged high-dose corticosteroid treatment.

Pixantrone (PIX), also named BBR2778, is a novel aza-anthracenedione molecule with antitumor properties, developed to reduce the cardiotoxic effect of its analogue Mitoxantrone (MTX). The anti-tumor activity of PIX was evaluated in various hematologic and solid tumor animal models. Compared to MTX, PIX showed better anti-tumor activity in advanced leukemias and in human prostate carcinoma, and comparable anti-tumor activity in most solid tumor models [148]. Furthermore, while MTX is mostly effective at its maximum tolerated doses (MTD), PIX showed a high level of efficacy at approximately one third of its MTD [148].

Now PIX is in trial in patients with solid tumors, leukemias and relapsed aggressive non-Hodgking’s lymphoma, in which, given as a single agent, PIX demonstrated good activity [149, 150]. PIX, as MTX, is classified as immunosuppressant drug by the World Health Organization (WHO - Table 1.1), in the category of intracellular ligands for its antiproliferative action [151, 152]. Finally, preclinical studies in the experimental model of Multiple Sclerosis (MS) confirmed the immunosuppressive effects of PIX [153].

Category	Drugs
Intracellular ligands	
Antimetabolites	Azathioprine
Calcineurin Inhibitors	Cyclosporine A
Antiproliferative	Mitoxantrone
	Pixantrone
Cell surface ligands	Rituximab
	Natalizumab
Anticytokine	Anti TNFα
	Daclizumab

Table 1.1 Immunosuppressant Classification by WHO [152].

1.5.1 Structure and mechanism of action

Anthracyclines have been shown to be clinically very effective in some of tumor types, such as lymphoma, leukemia and breast cancer. For these diseases, anthracycline-containing chemotherapeutic regimens are effective as first-line initial treatment. However, they may cause cumulative heart damage that limits lifetime dosage and does not allow further re-treatment. PIX has been designed to reduce the heart damage compared to currently available anthracyclines or anthracenediones without a loss in anti-tumor activities.

PIX contains an aza-anthracenedione molecular structure and, such MTX, shares some general similarities with anthracyclines, as the central quinoid ring (Ring-b in Figure 1.7).

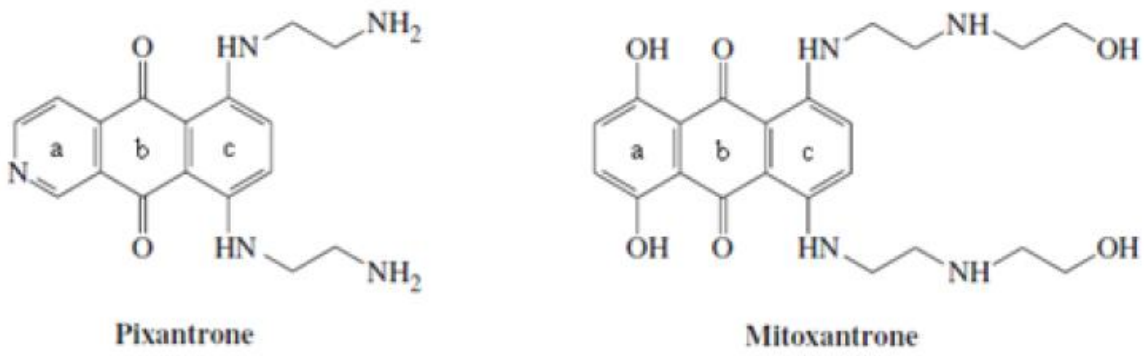

Figure 1.7 Chemical Structure of PIX and MTX, adapted from Baggi et al., 2009 [154].

The main differences between the two compounds reside in Ring-a, where the typical 5,8-dihydroxy-substitution pattern of MTX is substituted with pyridin ring, and in Ring-c, where both side-chains have a primary amino group more susceptible to formaldehyde activation and consequently has a greater potential to form complexes with DNA [155]. As a result of these structural differences, PIX is not subject to oxidative metabolism with formation of free radicals, allowing less severe toxic effects and decreased cardiotoxicity compared to MTX [156].

In vitro and *in vivo* studies demonstrated the effects of PIX in murine and human tumor cell lines and in tumor animal models, showing that PIX maintain a high level of activity in a wide range of doses despite of its significantly reduced cytotoxicity in comparison to MTX [156]. However, the mechanism of PIX cytotoxicity has not yet been well elucidated: although PIX intercalates DNA (due to its anthracenedionic structure) and inhibits topoisomerase II, DNA damage seems to be not correlated with cytotoxicity [157]. DNA topoisomerase II is associated with the nuclear matrix of interphase cells and is a major constituent of the chromosome scaffold. The latter plays essential roles in chromosome condensation and separation of daughter DNA molecules at the end of DNA replication (decatenation). Topoisomerase II is a DNA-relaxing enzyme, which removes DNA supercoiling and torsional tension as they arise during transcription and DNA replication [158]. DNA topoisomerase II has been identified as the target of a number of DNA intercalators, including anthracyclines (doxorubicina), anthracenediones (MTX) and aza-anthracenediones (PIX) [158]. DNA-damage induced by PIX may result from an alternative mechanism, i.e. “poisoning” of the topoisomerase-DNA complex, that in turn prevents the formation of a cleavable complex.

1.5.2 Pharmacokinetic

Pharmacokinetic data for PIX in rodents indicate a multi-exponential clearance with a rapid decline in drug concentration during the distribution phase, followed by a slow and prolonged elimination phase lasting up to 192 h (equal to 8 days – Figure 1.8) [159].

Since it is poorly absorbed orally, PIX is usually administrated intravenously (i.v.), allowing a rapid distribution throughout the body with accumulation in some tissue

compartments. In studies with radiolabelled PIX, the highest percentages of the administered dose were found in the skeletal muscles, liver and kidneys of mice and rats [159]. Less than 0.05% of the administered dose was found in the brain, suggesting that the drug and its metabolites do not cross the blood brain barrier. The efficiency of unchanged drug elimination from the systemic circulation was also shown to be high, but the metabolites (derived primarily from acetylation metabolism) were cleared more slowly with a greater volume of distribution. The terminal half-lives of PIX and total radioactivity derived from the plasma data were 2.9 h and 78.6 h in mice and 7.1 h and 81.9 h in rats, which suggested that PIX has a large volume of distribution with a relatively slow clearance of the drug and its metabolites [159].

PIX pharmacokinetics in human determined by HPLC assessment of PIX concentration in plasma and urine (treatment dosage of 180 mg/m^2), showed that the drug has a large volume of distribution (21.4 l/kg) and a rapid clearance (1.15 l/h/kg): its terminal half-life was 14.1 h, and PIX urinary elimination as unchanged drug was less than 10% [159].

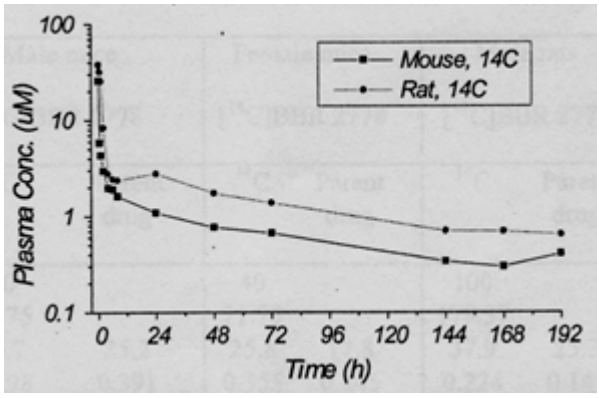

Figure 1.8 PIX pharmacokinetic in rodents, Cell Therapeutics, Investigator's Brochure, 2002 [159].

1.5.3 Toxicity

The acute toxicity tests of PIX in rodents after a single i.v. injection are used to define the lethal dose in 10% of animals - LD_{10} (65.4-77.8 mg/Kg for mouse males and females, respectively) and lethal dose in 50% of animals - LD_{50} (77.8-84.9 mg/Kg for mouse males and females, respectively). In rats, the corresponding values are: LD_{10} 98.1-65.2 mg/Kg and LD_{50} 119.5-111.5 mg/Kg (table 1.2) [159]. The main symptoms in the acute toxicity tests after i.v. injection are: dyspnea,

piloerection, reduced motility, swollen snout and blue pigmentation of the skin. These symptoms last no longer than four days with the exception of blue pigmentation that persists for the whole study period. Moreover the body-weight of the animals decrease in a dose-dependent manner (more evident in males) and a complete recovery is observed in females at the end of the study [159]. The histopathological examinations of the organs of rats treated with high dose of PIX show degenerative atrophy of spleen, bone marrow, gastrointestinal mucosa and kidney [160]. Animals receiving single or repeated toxic dosages of PIX do not present myocardial lesions typical of treatments with anthracyclines and anthracenediones (MTX) [159]; PIX results also less myelosuppressive than MTX [69, 161]. These studies confirm the low cardiotoxicity of PIX as one of its most important features.

Rat's Gender	Lethal dose	i.v. (mg/Kg)
Male	LD_{10}	98.1 (74.6 - 108.0)
	LD_{50}	119.0 (109.0 - 131.7)
Female	LD_{10}	65.2 (58.1 - 72.2)
	LD_{50}	111.5 (103.8 - 119.2)

Table 1.2 Acute toxicity of PIX administrated i.v. in rat [159].

1.5.4 Immunosuppresant potential of Pixantrone

Immunosuppressive drugs are effective in the treatment of various autoimmune diseases; however, their prolonged use is hampered in most cases by severe side effects [162]. Several lines of evidence link immunosuppression to inflammation in patients with MS and provide a rationale for the increasing use of immunosuppressive drugs in the treatment of MS [153]. Various immunosuppressive agents have been tested in pre-clinical studies, such as on Experimental Autoimmune Encephalomyelitis (EAE), the animal model of demyelinating MS disease, in which axonal loss is the consequence of inflammation and T-cell infiltration in the nervous system, resulting effective in preventing and treating the disease [151]. Topoisomerase II inhibitors (initially MTX) were also used as immunosuppressant agents in the experimental model of arthritis [163], and pre-clinical *in vitro* and *in vivo* studies in EAE models have

demonstrated that MTX is effective in reducing T- and B-cell numbers, in suppressing humoral immunity and T-helper cells [164]. Moreover, various clinical trials in MS patients confirmed its immunosuppressive effect [165]. Compared with other immunosuppressant drugs, MTX show two important benefits: the immediate tolerance and a more comfortable treatment regimen for the patients [151]. Unlike other immunosuppressants, MTX induces apoptosis of B lymphocytes in peripheral blood [166] and *ex vivo* analysis of the cytokine profile of immune cells from MS patients treated with MTX, has revealed a long-lasting decrease of IL-10 expressed in monocytes and of IL-2R1 expressed in T cells [164]. This drug has, therefore, been approved for MS treatment in the USA and Europe [165]. Nevertheless, severe long-term side effects may be associated with its prolonged use: cardiotoxicity is the major side effect, and it is strictly dependent on the total cumulative dose. Moreover, cases of therapy-related acute leukaemia have been reported after MTX therapy for MS [167].

Due to its close chemical relationship with MTX, PIX has been also studied and evaluated as immunosuppressive/antiproliferative drug, thanks to its ability to interfere with DNA structure during cell cycle and cell duplication, events that play a crucial role during immune activation processes in autoimmune diseases [163]. Several studies evaluated the effect of PIX *in vivo* in acute and chronic EAE models, and *in vitro* on antigen- and mitogen-induced T-cell proliferation. In 2004, Cavaletti and colleagues demonstrated that PIX modified the course of acute and relapsing-remitting EAE in rats [148]. The reduced EAE severity in PIX-treated rats was due to the marked and long-lasting effect of the drug on lymphocyte subpopulations and to the suppression of antigen-specific responses dose-dependently: circulating $CD4^+$ and $CD8^+$ cells were markedly reduced until the end of the experiment; a very evident effect was also observed on $CD45RA^+$ B cells. The antigen-specific autoantibody response to myelin basic protein was also abolished by the highest dose of PIX and MTX. Moreover, PIX was similarly active on *in vitro* human peripheral blood cells and rat cells [148]. Experimental data indicate that PIX is as potent as MTX to prevent EAE development as well as relapses in established disease [151]; nevertheless, PIX has a higher safety profile compared to MTX, and can represent a potential immunosuppressant agent in clinical trial in MS [148].

1.5.5 Pixantrone in EAMG

MG is probably the most thoroughly understood autoimmune disease of the nervous system [168]. Current therapies aim to restore the functional pool of AChR at the NMJ, by depleting anti-AChR specific Abs and by suppressing the autoimmune response at T cell level [169]. However, prolonged drug treatments (i.e. corticosteroids) can be required with high risk of severe side effects [168] and some subjects do not respond to conventional therapies. In this context the investigation of new immunosuppressive drugs is needed and the availability of experimental models for MG allows to study innovative therapies aiming the suppression/modulation of disease manifestations [170].

Evaluating Pixantrone efficacy in MG is important, since PIX (as MTX) mediates a complex immune suppression involving both B and T lymphocytes [171]. In 2008, Ubiali and colleagues have investigated the effects of PIX in EAMG Lewis rats [1]. Firstly, PIX has been tested *in vitro* on R97–116-specific T cell lines, grown from R97-116-immunized rats, [138]. T cells have been challenged with their specific peptide (R97-116 5 µg/ml) or with Concanavalin A (ConA 2 µg/ml – aspecific mytogen as positive control) in the presence of PIX (range 0.01–10 µM). Results have showed that, already at the lowest concentration ([PIX] = 0.01 µM), a significant reduction of Ag-specific as well as ConA-induced T cell responses has been observed. Micromolar concentrations of PIX almost completely abrogated T cell responses. PIX efficacy on *ex vivo* lymphocytes proliferation has been tested by intravenously (i.v.) administration, at a dosage of 16.25 mg/kg, into TAChR-immunized Lewis rats, 28 days p.i.; the treatment has been performed once weekly for three times (q7dx3). This dosage has been equal to 1/4 of the LD_{10} in female rats (65.2 mg/Kg of PIX administrated i.v. [159]). Both spleens and lymph nodes (LN) have been aseptically removed one week after the last treatment, total cell count and cell viability have been evaluated. Total splenic cell count have been found decreased in the PIX-treated animals compared to the vehicle-treated ones and PIX effect has been even more dramatic in draining LNs. The expression of CD3, CD4, and CD8 molecules, as T-cell markers, has been studied by FACS analysis on both spleen and LN cells (LNCs) populations; results have not shown any effects of the drug on the percentage of T-cell subsets, among living cells. Ex vivo proliferative responses from LNCs, challenged with TAChR (1.25 and 0.25

μg/ml), Torpedo and Rat AChR 97–116 peptides (10 μg/ml), have been found reduced compared to the vehicle-treated animals [1]. Secondly, PIX efficacy has been assessed in EAMG Lewis rat model, by a preventive treatment protocol (i.e. starting before the onset of clinical manifestations) and by a therapeutic protocol (i.e. starting after the onset of symptoms), in order to observe effects in delaying or modulating ongoing EAMG, respectively. The preventive treatment have started 4 days after TAChR immunization, while the therapeutic treatment have started 4 weeks after immunization; PIX has been administrated i.v. at the dosage of 16.25 mg/kg, once a week for three times (q7dx3 – representative of 1/4 of PIX LD_{10} in female rats, cumulative dosage 48.75 mg/Kg) for both treatment schedules. Animals have been scored every other day by assessing their body weight and muscular strength [139]. Results have indicated that both therapies have modified the course of the disease, according to increased body weight and decreased EAMG score, in PIX-treated rats compared to the vehicle. Biochemical and immunological assays have confirmed PIX efficacy: treated animals have significantly shown an increased muscle AChR content and a reduced anti-Rat AChR Abs titer. *Ex vivo* proliferative LNCs responses to TAChR have been affected by both PIX protocols, whereas to ConA-induced have been unaltered. The pathological examination of hearts collected from PIX- and vehicle-treated rats have not shown signs of cardiotoxic damage, confirming the drug safe profile [1]. Therefore, PIX has able to modulate EAMG clinical symptoms, proving effective ameliorating actions, both in preventive and therapeutic treatments, and showing *ex vivo* effects on immune cell count, similar to those reported on circulating lymphocytes in PIX- treated EAE [148].

These data indicate that PIX might be a promising immunomodulatory agent for clinical investigation in MG [154], but more studies are necessary in order to better investigate the use of alternative schedules and PIX related toxicity.

Chapter 2 – Aim of the study

As mentioned above, some MG patients are non-responsive to the commonly used immunotherapies, or develop severe side effects. In this regard, new potent but well-tolerated immunosuppressive drugs are needed for treatment-resistant MG, for patients with intolerable side effects or with major contraindications to high-dose corticosteroid treatment. Pixantrone can play an important role due to its anti-proliferative and immunosuppressive properties. Indeed, PIX has been tested in MS animal model [148, 171], resulting effective in the modulation of EAE; hence a clinical study (phase 1) has been proposed in MS patients [152]. Recently, Ubiali and colleagues have demonstrated the effect of PIX in the preventive and therapeutic treatments of EAMG; the drug has been able to ameliorate clinical symptoms and myasthenic typical *ex vivo* parameters when administrated i.v. at the dose 16.25 mg/kg (1/4 of PIX LD_{10} q7dx3), with a cumulative dosage of 48.75 mg/Kg [1].

In the current research project we have focused our attention to the therapeutic treatment of EAMG; in particular, the aim of our study has been to evaluate different PIX treatment schedules, in order to identify the lowest dosage able to ameliorate ongoing EAMG, thus further lowering the risk of toxic effects.

For this purpose, we have analyzed two aspects:

1. *In vitro* studies:
   - We have evaluated the lowest concentration of PIX able to reduce lymphoid cells proliferation, to determine its pharmacological effects on distinct cell subpopulations, each with different proliferative responses. For this purpose, we have tested the drug, in a logarithmic range of concentrations (from 0.1 pM to 1 µM), on various cell populations: a) CD4+ T cell lines specific for R97-116 peptide (≥ 90% of cells in active proliferation); b) lymphocytes and splenocytes isolated from R97-116-immunized rats, sacrificed 10 days p.i. (20-40% of cells in active proliferation); c) splenocytes from healthy rats and peripheral blood mononuclear cells (PBMCs) from healthy human donors (5-10% of cells in active proliferation).

- We have studied PIX toxic effects on differentiation of myeloid precursor cells, derived from bone marrow of healthy rats. Myeloid precursors have been differentiated in immature dendritic cells (iDCs) and exposed at day 0 and day 3 to various PIX concentrations (0.1nM, 1nM, 10nM, 10µM), during their differentiation process. At the end of the exposure, we have analyzed iDCs viability.

These studies allowed us to evaluate the lowest concentration of PIX able to generate anti-proliferative responses and correlate its pharmacological effects to toxic effects *in vitro*. Both these aspects have been essential for the assessment of new therapeutic schedules in EAMG rats.

2. *In vivo* studies:
   - Firstly, we have tested the effect of a single administration of PIX in TAChR-primed rats: animals were immunized with TAChR and i.v. treated 24 hours later with different concentrations of the drug: 16.25 mg/Kg (1/4 of PIX LD_{10}), 8.12 mg/Kg (1/8 of PIX LD_{10}), 4.06 mg/Kg (1/16 of PIX LD_{10});
   - Secondly, we have modified the schedule of administration, but not the PIX cumulative dosage previously studied (48.75 mg/Kg) [1]. TAChR-immunized female Lewis rats have been treated, starting 4 weeks p.i., with different schedules: PIX 8.12 mg/Kg, q7dx6; PIX 16.25 mg/Kg, q14dx3; vehicle, q7dx6.
   - Finally, we have decreased the PIX cumulative dosage to 24.36 mg/Kg (50% of the studied dosage), with two different PIX treatments: PIX 4.06 mg/Kg, q7dx6; 8.12 mg/Kg, q14dx3; vehicle, q7dx6. TAChR-immunized female Lewis rats have been treated with PIX starting at week 4 p.i.

During all the experiments, EAMG rats have been monitored for body weight and clinical score. At the end of the experiment (11 weeks p.i.), we have performed *ex vivo* analysis (e.g. proliferation assay, anti-rat AChR Abs titer) to evaluate the efficacy of PIX treatments.

The ultimate goal of our study is to further evaluate the potential of Pixantrone-based therapeutic approach in the experimental model of Myasthenia Gravis by optimizing/refining the published schedule of treatment [1]. Our data will be the basis for the design of a clinical study in MG patients non-responsive to common therapies.

Chapter 3 – Materials and Methods

3.1 Pixantrone

BBR2778 (Pixantrone, 6,9-bis {([(2-amino) ethyl] amino) benzo[g]isoquinoline-5,10-dione dimaleate}) is a novel aza-anthracenedionic derivate with immunosuppressive properties, developed by Cell Therapeutics, Inc. (Seattle, USA). Pixantrone is a blue lyophilized powder (PM=557.52), reconstituted with sterile saline solution (0.9% NaCl, B. Braun, Milan, Italy) and added *in vitro* in cell cultures or administered intravenous (i.v.) in healthy and EAMG rats [159].

3.2 Animals

Female Lewis rats, 6–8 weeks of age, have been purchased from Charles River Laboratories (Calco, Italy) and housed at the animal facility of the Neurological Institute Foundation Carlo Besta (Milan, Italy). Experiments have been approved by the Ethic Committee of the Institute and performed in accordance with the Principles of Laboratory Animal Care (European Communities Council Directive 86/609/EEC). Animals have been sacrificed after deep anesthesia obtained by carbon dioxide; low-grade anesthesia with 4% chloral hydrate (Sigma-Aldrich, St. Louis, MO) administered intra peritoneal (i.p.) has been used for immunizations and treatments.

3.3 Antigens

AChR has been purified from *Torpedo Californica Electroplax* tissue (Aquatic Research Consultants, San Pedro, CA) by affinity chromatography on Naja-naja siamensis toxin coupled to Sepharose 4B (GE Healthcare Life Sciences, Piscataway, NJ) [172]. The purified receptor has been analyzed on SDS PAGE. TAChR preparations have been dialyzed extensively against 0.01 M Tris-HCl (pH 7.5), 0.001 M EDTA, 0.1 M NaCl (Tris-HCl buffer), and 0.1% Triton X-100 (all from Sigma-Aldrich, Milan, Italy). TAChR, used for T cell proliferations, has been further dialyzed against Tris-HCl buffer plus 0.025% Triton X-100 and filter-sterilized (0.2 mm).

The peptide R97–116, corresponding to region 97–116 of the rat AChR α-subunit, has been synthesized by Dr. Longhi (C.N.R., Milan, Italy), according to sequences already published in GenBank (http://www.ncbi.nlm.nih.gov/genbank/; accession number X74832 for rat AChR, sequence DGDFAIVKFTKVLLDYTGHI). R97-116 has been synthesized using F-moc chemistry on a 431A automated peptide synthesizer (PE Applied Biosystems, Foster City, CA). The peptide has been purified by reverse-phase HPLC and the synthesis has been confirmed by mass spectroscopy.

3.4 *In vitro* studies

3.4.1 Evaluation of PIX IC_{50} in different cell subpopulations

R97-116 specific T cell lines

Female Lewis rats (n=3) have been immunized with 50 µg of R97–116 of the rat AChR α-subunit in CFA (Difco Laboratories Inc, Detroit, MI), added with Mycobacterium tuberculosis H37Ra (inactivated strain, Difco Laboratories). Lymph nodes have been aseptically removed 10 days post immunization and processed into a single-cell suspension. LNCs have been cultured, at 37°C with 5% CO_2, in Roswell Park Memorial Institute Medium (RPMI) 1640 medium, supplemented with, 1% Na-pyruvate, 1% non-essential amino acids, 1% L-glutamine, 1% penicillin-streptomycin (all from Euroclone Spa, Milan, Italy), and $5x10^{-5}$ M 2-mercaptoethanol (2-ME, BDH, Milan, Italy), defined completed RPMI medium, plus 10% Fetal Bovine Serum (FBS, Gibco, Monza, Italy) and stimulated with 5 µg/ml of R97–116 peptide. Every 15 days, cells ($3x10^5$ cells/well) have been re-stimulated in co-culture with irradiated (3000 cGy) splenic cells (SPNs, $2x10^6$ cells/well) from healthy donor (HD) rats, used as APCs source, in presence of the specific peptide. Every 3–4 days, R97-116-specific T cells have been expanded with IL-2 [173]. The cell number has been counted by trypan blue exclusion dye technique.

The proliferative capability of R97-116-specific T cells ($3x10^4$ cells/well) has been evaluated (in triplicates) in co-culture with irradiated SPNs ($2x10^5$ cells/well) and in presence of the peptide (5 µg/ml), in completed RPMI medium added with 2% normal rat serum. 2 µg/ml ConA (Sigma-Aldrich) has been used as positive control.

Cells have been exposed to different concentrations of PIX or to Phosphate Buffer Saline (PBS, Euroclone Spa, Milan, Italy), as vehicle. After 72 h of culture, T cells have been tested in proliferation assays.

LN and SPN cells from healthy and primed rats

LNCs and SPNs have been aseptically removed from healthy (n=3) and R97-116-immunized (n=3) female Lewis rats (10 days p.i.) and processed into a single-cell suspension. Cell number has been counted by trypan blue exclusion dye technique. LNCs and SPNs ($2x10^5$ cells/well) have been cultured, at 37°C with 5% CO_2, in presence of R97–116 peptide (5 µg/ml) or ConA (2 µg/ml) in completed RPMI medium plus 2% normal rat serum and exposed to different concentrations of PIX or PBS, as vehicle. After 72 h of culture, cells have been tested in proliferation assays [173].

Peripheral blood mononuclear cells from human healthy donors

Peripheral blood mononuclear cells (PBMCs) have been isolated from blood of unrelated human HD by centrifugation over a Ficoll-Paque^{TM} Plus (Amersham Biosciences, Milan, Italy) gradient and re-suspended in RPMI 1640 medium, supplemented with 5% human male serum type AB (Sigma–Aldrich, Milan, Italy), 1% Na-pyruvate, 1% non-essential amino acids, 1% L-glutamine, 1% penicillin–streptomycin and $5x10^{-5}$ M 2-ME [174]. Cell number has been counted by trypan blue exclusion dye technique. PBMCs ($2x10^5$ cells/well) have been challenged with 10 µg/ml phytohaemagglutinin (PHA, aspecific mitogen, Sigma-Aldrich) and exposed to different concentrations of PIX or PBS, as vehicle. After 72 h of culture, at 37°C with 5% CO_2, cells have been tested in proliferation assays.

Proliferation Assay

The proliferation assay allows determining the number of cells that are growing in presence or absence of certain proliferation-affecting agents. In this study, logaritmic concentrations of PIX (0.1 pM – 1 µM) or vehicle (PBS) have been added to stimulated cells (see above), in 96-well culture plates (Corning Life Science, Chorges, France). After 72 h, at 37°C with 5% CO_2, cells have been incubated with [^{3}H]-thymidine (0.5 µCi/well, Perkin Elmer, Burnsville, MN). After further 16-18 h of culture, plates have been harvested and counted on a Wallac MicroBeta TriLux Counter (PerkinElmer) [173]. Results from *in vitro* PBS treated

cells, obtained as counts per minute (cpm), have been expressed as mean cpm ± SE proliferation response for each conditions, from three independent experiments.

Determination of PIX IC_{50}

The half maximal inhibitory concentration (IC_{50}) is a measure of the efficacy of a compound in inhibiting cellular biological functions. In this study, quantitative measure indicates the amount of PIX necessary to halve the proliferation of different cell populations, as pharmacological effect.

The IC_{50} of the drug has been determined evaluating the proliferation values % normalized to vehicle of each PIX-treated subpopulation and constructing a dose-response curves (GraphPad Prism, CA), to examin the effect of different concentrations of the drug on cells stimulated with aspecific or specific antigens (see above). Results have been expressed as mean of IC_{50} ± SE of three independent experiments.

3.4.2 PIX toxicity on differentiation of Myeloid Precursor Cells

Tibiae and femurs have been aseptically removed from healthy female Lewis rats (6-8 weeks of age; n=3) and after the complete removal of the muscle; bones have been washed in 70% ethanol, for 2 minutes, and twice rinsed in sterile PBS. After removal of epiphysis, bone marrow has been pushed through a syringe, into a sterile petri-dish (Corning Life Science, Chorges, France) containing RPMI 1640 medium and processed in a single-cell suspension. After osmotic lysis, in sterile H_2O at 4°C for 30 seconds, in order to completely eliminate erytrocytes, Myeloid Precursor Cells (MPCs) have been re-suspended in the same medium and counted by trypan blue exclusion tecnique. At day 0 (t=0), MPCs ($1x10^6$ cells/ml) have been cultured, at 37°C with 5% CO_2, in completed RPMI medium for 2 h. Then non-adherent cells have been removed and myeloid precursors have been cultured (t=0) in completed RPMI medium plus 10% FBS, in presence of 5 ng/ml rat Granulocyte-Macrophage Colony Stimulating Factor (rGM-CSF – Pepro Tech, London, UK) and 5 ng/ml rat Interleukine 4 (rIL-4 – Pepro Tech): these cytokines are involved in the differentiation of MPCs into immature Dendritic Cells (iDCs). After 3 days of culture (t=3), fresh medium containing rGM-CSF (5 ng/ml) and rIL-4

(5 ng/ml) has been added. The differentiation of MPCs in iDCs occurs in 7 days of culture (t=7) [175].

At t=0 or t=3 of this differentiation process, cells have been exposed to PIX at the following concentrations: 0.1 nM, 1 nM, 10 nM, 10 µM. At the end of the experiment (t=7), cells have been counted by trypan blue exclusion technique and their viability has been evaluated as the ratio between living and total cell counts (living and dead cells). These data have been expressed as mean ± SE of three independent experiments.

3.5 *In vivo* evaluation of PIX efficacy on EAMG rats

3.5.1 Immunization and treatment protocols

EAMG has been induced in female Lewis rats (6–8 weeks of age) by immunization into hind footpads with 50 µg purified TAChR in CFA (Difco Laboratories Inc.) supplemented with 1 mg/rat H37Ra.

The effect of different PIX dosages has been firstly evaluated on TAChR-immunized rats (n=4, for each treatment group), receiving i.v. (via tail vein) a single dose of the drug, 24 h post immunization (16.25 / 8.12 / 4.06 mg/Kg PIX), and sacrificed 10 days after. Single-cell suspensions, from draining lymph nodes of these animals, have been challenged *in vitro* with TAChR (0.25 and 1.25 µg/ml) or ConA (2 µg/ml), as positive control. In parallel, we have been evaluated the effect of the highest concentration of PIX (16.25 mg/Kg) in a single dose on healthy rats (n=3), defined HD/PIX, to assess the drug toxicity in physiological conditions.

Secondly, we have evaluated the efficacy of PIX treatments in a therapeutic schedule. PIX has been administered i.v. at the onset of the typical EAMG clinical signs (4 weeks p.i.) by two different cumulative dosages: 48.75 mg/Kg (1/4 of the LD_{10} in rats [1]) and 24.36 mg/Kg (1/8 of the LD_{10}). AChR-immunized rats have been randomly assigned to different experimental groups and administered as follow: 1) 16.25 mg/Kg, q14dx3 (16.25x3 group), for a cumulative dosage of 48.75 mg/Kg (n=3), as positive control; 2) 8.12 mg/Kg, q7dx6 (8.12x6 group), for the same cumulative dosage (n=6); 3) 8.12 mg/Kg, q14dx3 (8.12x3 group), for the cumulative dosage halved compared to the former (n=6); 4) 4.06 mg/Kg, q7dx6 (4.06x6 group) for the cumulative dosage of 24.36 mg/Kg (n=6); 5) sterile saline

solution (0.9% NaCl), q7dx6 (vehicle group), as negative control (n=7). In parallel, we have evaluated the effect of the highest concentration of PIX (16.25 mg/Kg) in three different injections (q14dx3, cumulative dosage of 48.75 mg/Kg) on healthy rats (n=3); this investigation has assessed a potential toxicity of repetitive drug administrations on physiological conditions.

3.5.2 EAMG clinical evaluation

Disease manifestations in EAMG rats have been evaluated by measuring body weight and testing muscular weakness. Clinical score has been based on the presence of tremor, hunched posture, low muscle strength and fatigability, and assessed after exercise (repetitive paw grips on the cage grid) for 30 seconds, using the grip strength test.

Disease severity has been commonly graded as follows: grade 0, normal strength and no abnormalities; grade 1, mildly decreased activity and weak grip or cry, more evident at the end of testing; grade 2, clinical signs present before exercise (tremor, head down, hunched posture, weak grip); grade 3, severe clinical signs before exercise, no grip, moribund; and grade 4, dead [139].

Each animal has been weighed and scored twice weekly, until 4 weeks p.i., and 3 times a week, until the end of the experiment. EAMG has been confirmed by edrophonium chloride (Sigma-Aldrich) test. Results have been expressed as mean ± SE of body weight and clinical score for each animal at each time point. Data have been derived from three independent experiments.

3.5.3 Effects of PIX on *ex vivo* proliferative responses to TAChR

Popliteal and inguinal lymph nodes have been aseptically removed from PIX- or vehicle-treated female Lewis rats (11 weeks after TAChR-immunization), processed into single-cell suspensions and counted by trypan blue exclusion tecnique. LNCs ($2x10^5$ cells/well) have been seeded in triplicates, in 96-well culture plates (Corning Life Science, Chorges, France), in presence of TAChR (0.25 and 1.25 µg/ml) or ConA (2 µg/ml), as positive control; these stimuli have been previously diluted in completed RPMI medium plus 2% normal rat serum. After 72 h of culture, at 37°C with 5% CO_2, 0.5 µCi of [^{3}H]-thymidine has been added to each well and cells have been harvested after further 16–18 h. Results from three independent experiments have been expressed as proliferation values

% normalized to vehicle of each PIX-treated group ± SE. The data from control wells (medium alone) were reported as mean cpm ± SE of proliferative responses.

3.5.4 PIX myelotoxicity *in vivo*

Tibiae and femurs have been aseptically removed from PIX- and vehicle-treated female Lewis rats, 11 weeks after TAChR-immunization.
Following the procedure previously described (paragraph 3.4.2), single-cell suspensions have been obtained and cells counted by trypan blue exclusion [175]. The myelotoxicity of PIX treatments has been evaluated as the ratio between living and total cell counts (living and dead cells). Data from three independent experiments have been expressed as mean ± SE.

3.5.5 Real Time qPCR

Total RNA has been extracted from SPNs of EAMG and HD rats, treated with PIX or vehicle, using Trizol reagent (Invitrogen, Carlsbad, CA). cDNA has been synthesized from RNA using random hexamers (GE Healthcare Life Sciences Europe GmbH, Milan, Italy) and reverse transcriptase (Moloney murine leukemia virus; Life Technologies, Carlsbad, CA).
Real-time quantitative PCR (TaqMan-qPCR, Applied Biosystems, Foster City, California) for IFNγ (http://www.ncbi.nlm.nih.gov/nuccore/, Reference Sequence NM_138880) has been performed using Assay-on Demand Gene Expression Products (Applied Biosystems, Foster City, CA). β Actin (Applied Biosystems, http://www.ncbi.nlm.nih.gov/nuccore/, Refarence Sequence NM_031144) has been used as endogenous control. Real-time PCR reactions of each gene have been performed in triplicates with an ABI Prism 7500 FAST Real-Time PCR System (Applied Biosystems). Levels of mRNA expression for each gene have been calculated using the $2^{-\Delta Ct}$ method [176], in which ΔCt represents the difference between cycle threshold (Ct) of the target gene and Ct of the house-keeping gene. Data, from three independent experiments, have been represented as $2^{-\Delta Ct}$ x100 and expressed as mean (%) ± SE.

3.5.6 Anti Rat-AChR Antibodies Assay

Anti-rat AChR Abs have been assayed in individual sera by conventional radio-immunoprecipitation method [177]. Rat AChR has been extracted from healthy rat

muscle and labeled with $2x10^{-9}$ M [^{125}I]-αBTX (Perkin Elmer). Sera from HD (negative control), PIX- and vehicle-treated rats have been incubated over-night (ON) with radio-labeled rat AChR. Ab–AChR complexes have been precipitated by adding rabbit anti-rat IgG (Sigma-Aldrich, Milan, Italy). Immuno-precipitates have been washed twice with 1 ml cold PBS 0.5% Triton X-100 (Carlo Erba, Milan, Italy) and [^{125}I]-αBTX labeling has been evaluated by γ-counter (Perkin Elmer). A parallel assay has been performed on radio-labeled rat AChR co-incubated with unlabeled α-BTX (Invitrogen, Milan, Italy) to eliminate aspecific binding rate. Anti-AChR Ab titers have been expressed as picomoles of [^{125}I]-αBTX binding sites precipitated per milliliter of serum ± SE. Data have been the result of three independent experiments.

3.6 Statistical analysis

All values have been expressed as means ± SE. Statistical analysis has been performed using Student's t test for two-group analysis or one-way ANOVA for multiple group comparisons (GraphPad Prism, CA). Statistical significance has been set at $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***).

Chapter 4 – Results

4.1 Effect of PIX on active cell proliferation *in vitro*

We studied the effect of PIX on cell proliferation *in vitro* to evaluate the pharmacologic profile of the drug. For this purpose, we selected different cell subpopulations, characterized by different proliferative responses, stimulated with specific or aspecific antigen and concurrently exposed to a logarithmic range of PIX (0.1 pM - 1 µM) or vehicle (PBS), obtaining dose-response curves (Figure 4.1 and 4.2).

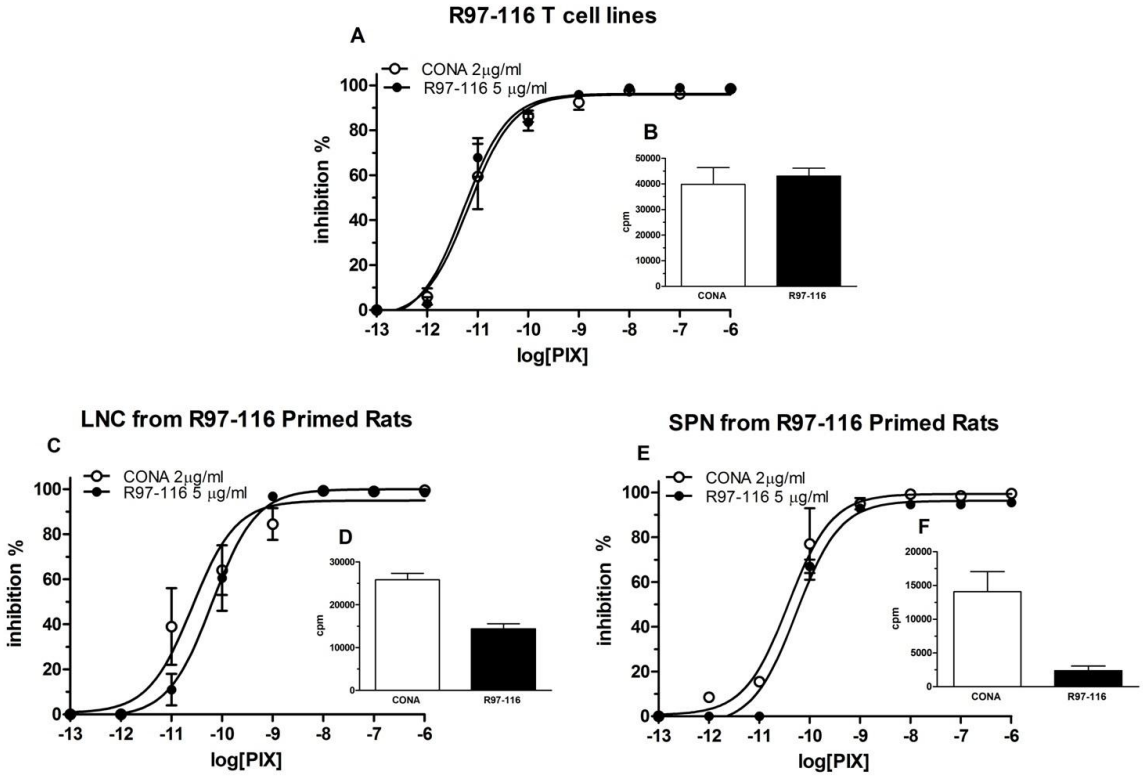

Figure 4.1 *In vitro* evaluation of PIX dose-response curves, on a R97-116 specific T cell line (Panel A), LNCs (Panel C) and SPNs (Panel E) from R97-116 immunized primed rats, stimulated with R97-116 (5 µg/ml, black circle) and ConA (2 µg/ml, white circle) in presence of a logarithmic range of PIX (0.1 pM-1 µM). Panel B, D and F represented the basal proliferation of cells in presence of R97-116 (5 µg/ml, black bar) and ConA (2 µg/ml, white bar), exposed to vehicle (PBS). The lymphoid cells were stimulated with specific and aspecific antigens and exposed to PIX or vehicle for three days of culture, then 0.5 µCi of [^{3}H] thymidine was added for a further 18h. Data were expressed as a PIX mean IC_{50} pM ± SE, as a result of at least three independent experiments. Data for basal proliferations were expressed as mean cpm ± SE, from triplicate wells for each conditions.

As shown in Figure 4.1 – Panel A, PIX mean IC_{50} ± SE pM was 5.4 ± 1.4 pM and 6.7 ± 1.2 pM in a rat CD4+ T cell line specific for the immunodominant peptide

R97–116 stimulated with ConA (2 µg/ml, aspecific mitogen) or R97-116 (5 µg/ml, specific antigen) respectively and exposed to PIX (0.1 pM – 1 µM). The Panel B showed the proliferation of the T cell line exposed to PBS and stimulated with the same antigens (mean cpm ± SE: CONA 39808 ± 6590 cpm; R97-116 43105 ± 3043 cpm).

Then we investigated the effect of PIX on mixed cell populations from immunized rats, HD rats and healthy human donors, with different percentage of cells in active proliferations, to delineate a diverse pharmacologic profile of the drug.

LNCs and SPNs, derived from primed rats immunized with R97-116 (Figure 4.1 – Panel C and E), were stimulated with ConA (2 µg/ml) or antigen of immunization (R97-116, 5 µg/ml) and exposed simultaneously to PIX at the same concentrations previously described. The LNCs PIX mean IC_{50} ± SE pM resulted: ConA 25.7 ± 1.4 pM, R97-116 63.3 ± 1.2 pM (Figure 4.1 - Panel C), while the SPNs PIX mean IC_{50} ± SE pM was: ConA 37.4 ± 1.2 pM; R97-116 54.3 ± 1.2 pM (Figure 4.1 - Panel E). The proliferation of both LNCs and SPNs (Figure 4.1 - Panel D and F) after exposure to the antigens and vehicle was: ConA 25808 ± 1505 cpm and R97-116 14365 ±1209 cpm for LNCs, ConA 14055 ± 2986 cpm and R97-116 2376 ± 675 cpm for SPNs.

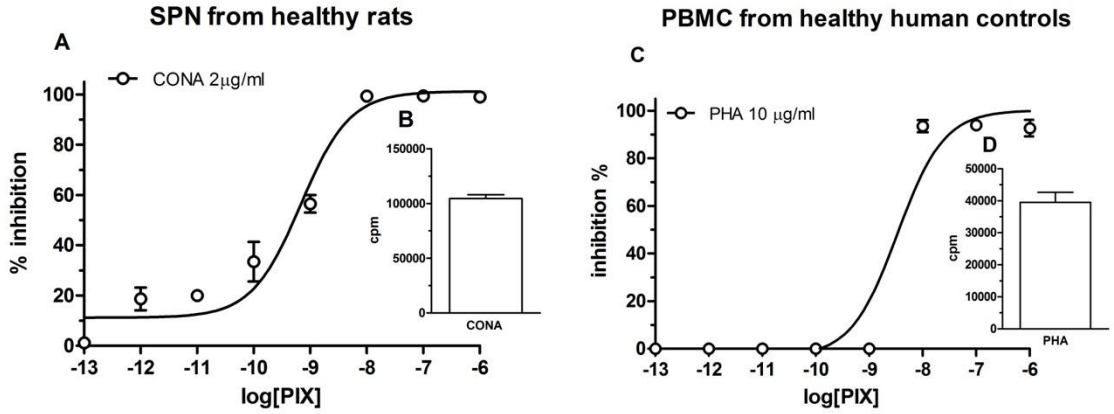

Figure 4.2 *In vitro* evaluation of PIX dose-response curves, on SPNs from HD rats (Panel A) and on PBMCs from healthy human donors (Panel C) stimulated with ConA (2 µg/ml) and PHA (10 µg/ml) respectively, in presence of a logarithmic range of PIX (0.1 pM-1 µM). Panel B and D represent the basal proliferation of cells in presence of ConA (2 µg/ml) and PHA (10 µg/ml) respectively, in presence of vehicle (PBS). The lymphoid cells were stimulated with aspecific antigens and exposed to PIX or vehicle for three days of culture, then 0.5 µCi of [^{3}H] thymidine was added for a further 18h. Data were expressed as a PIX mean IC_{50} pM ± SE, as a result of at least three independent experiments. Data for basal proliferations were expressed as mean cpm ± SE, from triplicate wells for each conditions.

We considered the effect of the drug on lymphoid cells of HD rats (SPNs) and HD human donors (PBMCs), stimulated with aspecific mytogens (ConA, 2 µg/ml and

PHA, 10 µg/ml respectively) and exposed to PIX (0.1 pM - 1 µM, Figure 4.2 – Panel A and C). PIX effect resulted decreased compared to the previous experiments, since SPNs PIX mean IC_{50} ± SE pM resulted 679.5 ± 2.6 pM, and PBMCs PIX mean IC_{50} ± SE pM was equal to 3362.0 ± 1.5 pM. The proliferation of both cell subpopulations after exposure of aspecific mytogen and vehicle was equal to: mean cpm ± SE, 104623 ± 3595 cpm and 39572 ± 3094 cpm respectively (Figure 4.2 – Panel B and D).

Furthermore for all the conditions studied we observed the complete suppression of proliferative response at the nM range, except for SPNs from HD rats and PBMCs from human healthy donors in which this was reached in µM range.

4.2 PIX toxicity on MPCs differentiation *in vitro*

We evaluated the *in vitro* toxic effect of PIX on the differentiations of bone marrow derived myeloid precursors cells (MPCs) from HD rats in iDCs.

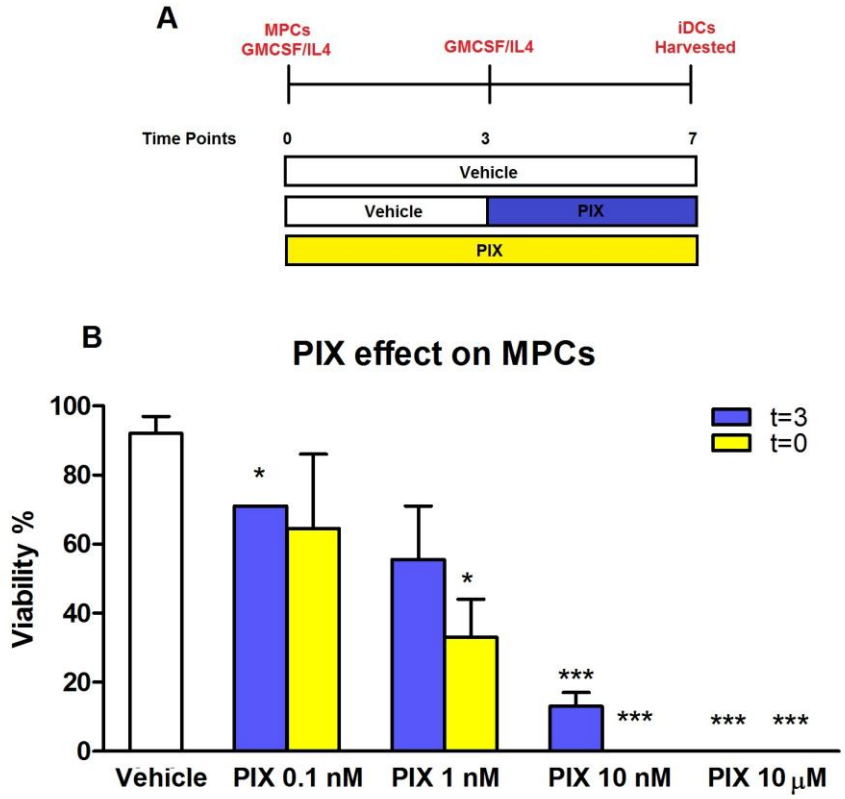

Figure 4.3 Panel A – Time course of *in vitro* toxic effect: MPCs were differentiated in iDCs, by adding GMCFS (5 ng/ml) and IL4 (5 ng/ml) to the growth medium and then exposed to vehicle (PBS) or PIX (0.1nM, 1nM, 10nM, 10 µM) at the time points 0 and 3 of cell differentiation. At the end of the experiments (t=7) cell numbers were counted by trypan blue exclusion. Panel B – Evaluation of iDCs viability at t=7 as the ratio between living cells and total cell counts (living and dead cells). The data were expressed as means viability % ± SE of three independent experiments. * $p<0.05$; *** $p<0.001$.

Rat MPCs were treated with PBS or PIX (0.1nM, 1nM, 10nM, 10 µM) at the time points 0 (t=0) and 3 (t=3) of differentiation in iDC (Figure 4.3 – Panel A). At the end of the experiments (t=7) cell numbers were counted by trypan blue exclusion and cell viability was evaluated as the ratio between living cells and total cell counts (living and dead cells). We considered as toxic effect a reduction in the cell viability ≤ 50%. The iDCs viability % ± SE at time point 7 resulted: PIX 0.1nM t=3 71.0 ± 0.5 %; t=0 64.5 ± 21.5 %; PIX 1 nM t=3 55.5 ± 15.5 %; t=0 33.0 ± 11.0 %; PIX 10 nM t=3 13.0 ± 4.0 %; t=0 ALL DEAD %; PIX 10 µM t=3 ALL DEAD %; t=0 ALL DEAD %; vehicle t=7 92.0 ± 4.9 % (Figure 4.3 – Panel B).
The toxic effect of PIX can be observed at the concentration 1 nM at day 7, moreover the total cell death was observed at PIX concentrations ≥ 10nM.

4.3 PIX in TAChR-immunized primed rats

Since PIX demonstrated *in vitro* a pharmacologic effect at picomolar concentrations and a significant toxic effect at nanomolar range, we investigated *in vivo* the effect of lower PIX dosage compared to that previously described [1].
We treated the TAChR-immunized rats 24 h p.i. with three different PIX dosages, chosen on the basis of the LD_{10}: 16.25 mg/Kg (1/4 of PIX LD_{10}; 16.25x1) as a positive control [1], 8.12 mg/Kg (8.12x1) equal to 1/8 of PIX LD_{10}, and 4.06 mg/Kg (4.06x1) equal to 1/16 of PIX LD_{10}. Negative control was represented by injection i.v. of a sterile saline solution (0.9% NaCl; vehicle). Healthy rats were also treated with 16.25 mg/Kg of PIX (HD/PIX) or vehicle (0.9% NaCl; HD) to evaluate the effect of PIX on LNCs in physiological conditions (Figure 4.4 – Panel A).
Ten days p.i. LNCs were aseptically removed from all the treated rats, processed in a single cell suspensions and counted by trypan blue exclusion. As shown in Figure 4.4 – Panel B, the LNCs number from TAChR-immunized PIX treated rats was significantly decreased compared to vehicle treated rats. The number of total live cell count ± SE for each treatment was: 16.25x1 $35.7x10^6$ ± 3.6 live cells, 8.12x1 $56.7x10^6$ ± 5.8 live cells, 4.06x1 $45.3x10^6$ ± 18.9 live cells; vehicle $176.7x10^6$ ± 29.9 live cells; $p<0.05$; while there was not any effect on the number of total live cell count in HD/PIX ($6.1x10^6$ ± 1.3 live cells) compared to HD controls ($6.9x10^6$ ± 0.9 live cells).

LNCs from TAChR-immunized rats were stimulated *ex vivo* with the antigen of immunization (TAChR 0.25 and 1.25 µg/ml) and ConA (2 µg/ml), as positive control. We evaluated the LNCs proliferation responses, normalizing the cpm data obtained for each PIX group versus vehicle and expressing the values as percentage: TAChR 0.25 µg/ml (proliferation responses % ± SE: 16.25x1 1.6 ± 0.1%, 8.12x1 9.9 ± 1.4%, 4.06x1 5.1 ± 1.8%), TAChR 1.25 µg/ml (proliferation responses % ± SE: 16.25x1 5.6 ± 2.5%, 8.12x1 6.7 ± 2.0%, 4.06x1 7.0 ±2.4%) (Figure 4.4 – Panel D and E). ConA proliferative responses were not affected by PIX treatments (Figure 4.4 – Panel C).

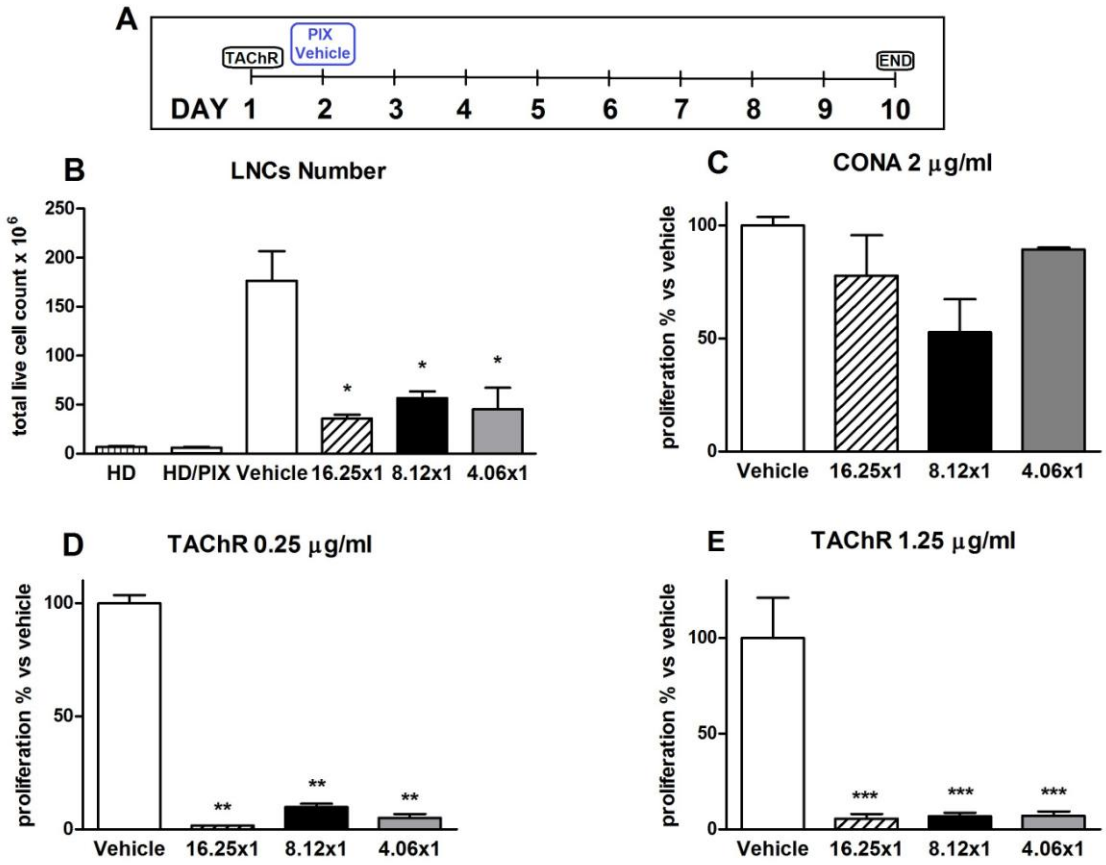

Figure 4.4 Effect of PIX on proliferative responses of LNCs from primed TAChR-immunized Lewis rats, treated i.v. with a single dose of PIX (16.25x1, 8.12x1, 4.06x1, n = 4 for each group) and vehicle-treated rats (n = 4) 24 hours p.i. (Panel A). Ten days p.i. popliteal and inguinal LNCs were aseptically removed and processed into a single-cell suspension. LNCs number were counted by trypan blue exclusion (Panel B). LNCs were stimulated *ex vivo* with ConA (2 µg/ml – Panel C) and TAChR (0.25µ g/ml and 1.25 µg/ml- Panel D and E). After 3 days of culture, 0.5 µCi of [^{3}H]thymidine was added for a further 18 h. Data were expressed as mean proliferation percentage normalized to vehicle ± SE (triplicate wells from two independent experiments). The cpm values ± SE for control wells (medium alone) were: 16.25x1 273 ± 65 cpm, 8.12x1 430 ± 121 cpm, 4.06x1 449 ± 172 cpm; for vehicle group 626 ± 60 cpm. *, p<0.05; **, p<0.01; ***, p<0.001.

All dosages of PIX in TAChR-immunized rats led to a decreased LNCs antigen specific proliferation after exposure to both concentrations of TAChR, correlating with a decrease in total live cell count in LNCs.

4.4 Comparison of PIX therapeutic schedules in EAMG

Previous results on TAChR-immunized primed rats demonstrated the effect of lower PIX dosages on antigen specific T cell proliferation responses, thus we focalized on EAMG therapeutic treatments with different PIX administrations (Table 4.1), decreasing the cumulative dosage at 24.36 mg/Kg or preserving it (48.75 mg/Kg) [1].

As shown in Figure 4.5 (Panel A and B) two PIX treatments were used for a cumulative dosage of 48.75 mg/Kg: 1) PIX 16.25 mg/Kg (16.25x3) q14dx3 as positive control [1]; 2) PIX 8.12 mg/Kg (8.12x6), q7dx6. Negative controls were represented to EAMG rats treated with sterile saline solution (0.9% NaCl, vehicle). EAMG clinical evaluations in PIX treated rats were significantly ameliorate compared to vehicle: mean of body weight ± SE 8.12x6 215 ± 8.5 g; 16.25x3 215 ± 9.9 g; vehicle 164 ± 4.2 g; $p<0.05$; mean of clinical score (arbitrary units, a.u.) ± SE 8.12x6 0 ± 0.1 a.u.; 16.25x3 1 ± 0.2 a.u.; vehicle 3 ± 0.2 a.u.

Both of PIX treatments demonstrated an effective modulation of symptoms in EAMG model, but the posology equal to 8.12x6 determined an improved clinical score at the end of experiments (11 weeks p.i.) with a significative decrease compared to the positive control 16.25x3.

Group	#	Posology	Administration	Cumulative Dosage
16.25x3	3	q14dx3	16.25 mg/Kg	48.75 mg/Kg
8.12x6	6	q7dx6	8.12 mg/Kg	48.75 mg/Kg
8.12x3	6	q14dx3	8.12 mg/Kg	24.36 mg/Kg
4.06x6	6	q7dx6	4.06 mg/Kg	24.36 mg/Kg
Vehicle	7	q7dx6	0.9% NaCl	-
HD/PIX	3	q14dx3	16.25 mg/Kg	48.75 mg/Kg
HD	3	q14dx3	0.9% NaCl	-

Table 4.1 Schedule of *in vivo* therapeutic treatments. Female Lewis rats, immunized with TAChR/CFA, were treated 4 weeks p.i with PIX (i.v.) according different administrations: a cumulative dosage of 48.75 mg/Kg and a decreased dosage of 24.36 mg/Kg, both with three times every 14 days (q14dx3) and once a week for six times (q7dx6). Vehicle and PIX 16.25 mg/Kg q14dx3 (16.25x3) represented negative and positive control respectively. HD rats were administered with PIX (16.25 mg/Kg) or vehicle at the posology q14dx3.

Concurrently we performed tests on body weight of healthy rats treated with PIX 16.25mg/Kg (q14dx3; HD/PIX), compared with HD controls, in order to assess a decrease of this parameter as possible side effect. The data indicated that PIX

treatment did not affect the body weight of HD/PIX compared to HD controls (mean body weight ± SE: HD/PIX 243 ± 8.7 g, HD 232 ± 3.1 g).

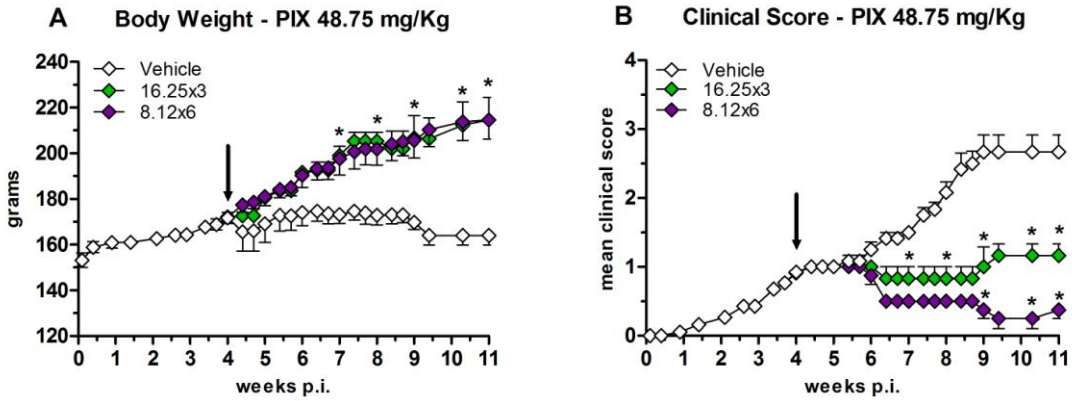

Figure 4.5 Comparison of therapeutic PIX treatments with a cumulative dosage of drug equal to 48.75mg/Kg on EAMG rats. Body weight (Panel A) and clinical score (Panel B) were recorded three times a week for 11 weeks. Rats were randomly assigned to PIX group (n=6, filled diamonds) or remained in vehicle group (n=7, open diamonds) 4 weeks p.i. with TAChR/CFA. The protocol 16.25x3 was used as positive control (n=3) [1]. Vehicle group received sterile saline solution (n=7, negative control). The arrow indicates the first PIX injection (i.v.) for each protocol. Data were the result of two different experiments. Each PIX schedule was followed as indicated in Table 4.1. * $p < 0.05$ compared to vehicle.

Since the cumulative dosage of 48.75 mg/Kg was effective on EMAG model, we investigated *in vivo* also the effect of a lower dosage of PIX and therefore we decreased the cumulative dosage of PIX to 24.36 mg/Kg, preserving PIX treatment 8.12 mg/Kg (8.12x6, q7dx6) as positive control and vehicle as negative control (Table 4.1).

The two administrations of PIX 4.06 mg/Kg (4.06x6), q7dx6 and PIX 8.12 mg/Kg (8.12x3), q14dx3, both characterized by a cumulative dosage of 24.36 mg/Kg, resulted ineffective on improving EAMG, as at the end of the treatments (11 wk p.i.) the values related to body weight and clinical score were not modified compared to vehicle: mean of body weight ± SE: 4.06x6 164 ± 17.5 g; 8.12x3 133 ± 1.2 g; 8.12x6 215 ± 8.5 g; vehicle 164 ± 4.2 g; mean clinical score ± SE: 4.06x6 3 ± 0.7 a.u.; 8.12x3 3.0 ± 0.9 a.u.; 8.12x6 0 ± 0.1 a.u.; vehicle 3 ± 0.2 a.u. (Figure 4.6 - Panel A and B).

Therefore only the schedules with PIX cumulative dosage 48.75 mg/Kg modulated the classical monitoring parameters of EAMG, resulting able to ameliorate the disease. Then these data underlined the importance of PIX cumulative dosage for a better efficacy of the drug in EAMG.

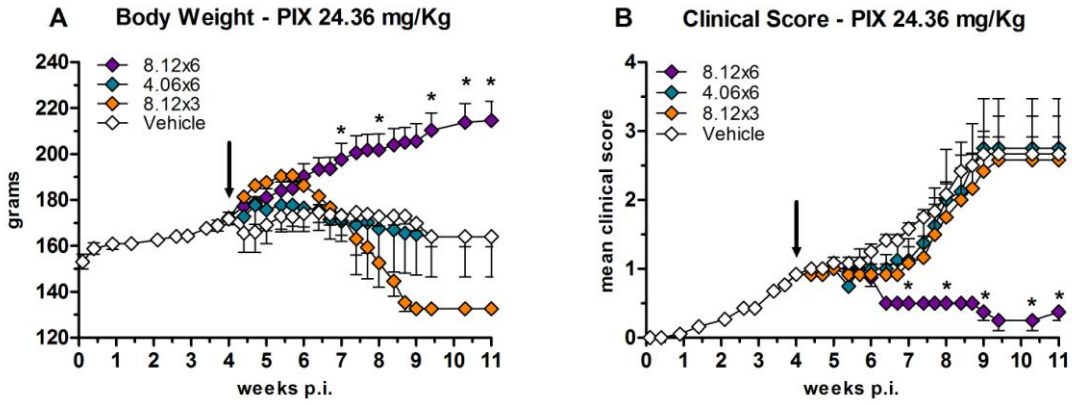

Figure 4.6 Comparison of therapeutic PIX treatments with a cumulative dosage of drug equal to 24.36 mg/Kg on EAMG rats. Body weight (Panel A) and clinical score (Panel B) were recorded three times a week for 11 weeks. Rats were randomly assigned to PIX group (n=6, filled diamonds) or remained in vehicle group (n=7, open diamonds) 4 weeks p.i. with TAChR/CFA. The protocol 8.12x6 was used as positive control (n=6). Vehicle group received sterile saline solution, as negative control. The arrow indicate the first PIX injection (i.v.) for each protocol. Data were the result of two different experiments. Each PIX schedule was followed as indicated in Table 4.1. * $p < 0.05$ compared to vehicle.

4.5 Effect of PIX treatments on lymphoid cells

At the end of the experiments (11 weeks p.i.), LNCs and MPCs were aseptically removed from vehicle, PIX-treated and HD rats, processed in a single cell suspension, and counted by trypan blue exclusion, evaluating the effect of PIX on LNCs and MPCs, two cell subpopulations with pathological and physiological implications respectively (Figure 4.7 - Panel A and B).

PIX treatments at both cumulative dosage (48.75-24.36 mg/Kg) were able to affect the LNCs number: total live cell count ± SE: 16.25x3 $13.0x10^6$ ± 2.3 live cells, 8.12x6 $14.0x10^6$ ± 3.8 live cells, 4.06x6 $28.2x10^6$ ± 3.0 live cells; 8.12x3 $33.5x10^6$ ± 8.9 live cells, vehicle $168.2x10^6$ ± 19.7 live cells; $p<0.05$). LNCs cell count from HD/PIX ($7.1x10^6$ ± 4.1 live cells) and HD controls ($6.9x10^6$ ± 0.9 live cells) were not significantly different (Figure 4.7 - Panel A).

Data on *in vivo* PIX myelotoxic effect (Figure 4.7 - Panel B) demonstrated that the number of MPCs from bone marrow were not impaired by TAChR-immunization compared to HD rats (total live cell count ± SE: vehicle 27.4 $x10^6$ ± 4.3 live cells; HD $32.9x10^6$ ± 2.3 live cells) while the administration of PIX at the cumulative dosage 48.74 mg/Kg caused a significative decrease in this cell compartment compared to HD rats: total live cell count ± SE: 16.25x3 11.7 $x10^6$ ± 0.7 live cells; 8.12x6 $15.6x10^6$ ± 1.5 live cells; HD/PIX $9.6x10^6$ ± 2.6 live cells). Conversely the cumulative dosage of 24.36mg/Kg kept the level of cell subset unchanged

compared to HD controls (total live cell count ± SE: 4.06x3 31.2 x10^6 ± 10.6 live cells; 8.12x3 23.5x10^6 ± 3.1live cells).

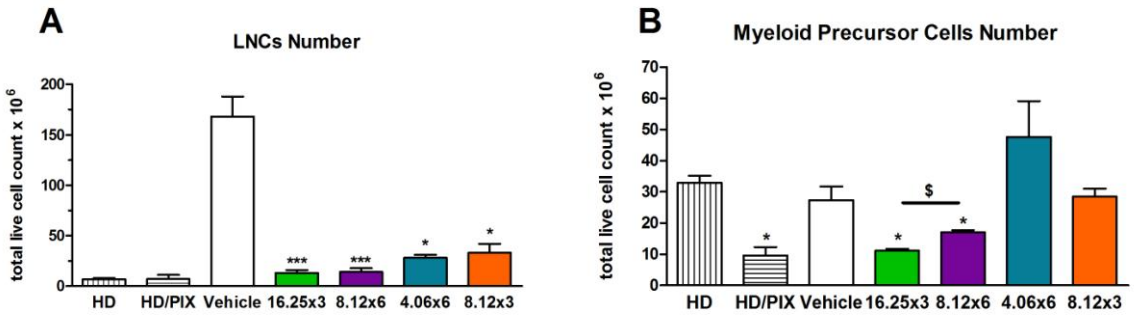

Figure 4.7 Popliteal/inguinal lymph nodes, such as myeloid precursor cells from bone marrow, were aseptically removed at the end of experiments from HD, vehicle and PIX treated rats and processed into single-cell suspensions. Cells were counted by trypan blue staining. Total live cell numbers of LNCs (Panel A) and MPCs (Panel B) were reported as mean ± SE from HD/PIX (n=3), HD controls (n= 3), vehicle-treated EAMG rats (n=7), and PIX treated EAMG rats (n=6 for each group), 16.25x3 (n=3), 8.12x6, 4.06x6 and 8.12x3. Data were the result of two different independent experiments. * $p<0.05$; *** $p<0.001$ compared to HD controls. $ $p<0.05$ indicate the significant difference between the protocols 16.25x3 and 8.12x6

Thus we evaluated the effect of *in vivo* PIX treatments in EAMG rats on LNCs proliferative responses. LNCs from TAChR-immunized rats were stimulated *ex vivo* with the antigen of immunization (TAChR 0.25 and 1.25 µg/ml). We evaluated the LNCs proliferation responses, normalizing the cpm data obtained for each PIX group versus vehicle and expressing the values as percentage: TAChR 0.25 µg/ml (proliferative response % ± SE: 16.25x3 0.2 ± 0.01 % , 8.12x6 4 ± 2.2 %, 4.06x6 3.4 ± 0.9 %, 8.12x3 16.4 ± 10.3 %), TAChR 1.25 µg/ml (proliferative response ± SE: 16.25x3 0.1 ± 0.01 % , 8.12x6 5.7 ± 3.4 %, 4.06x6 3.7 ± 2.3 %, 8.12x3 10.7 ± 6.4 %), compared with the proliferative response observed in vehicle (TAChR 0.25 µg/ml, proliferative response ± SE: 100.0 ± 4.3 %; TAChR 1.25 µg/ml, proliferative response ± SE: 100.0 ± 20.2 %, Figure 4.8, Panel A and B). PIX affected also ConA (2 g/ml – positive control) induced LNCs responses: proliferative response ± SE: 16.25x3 16.6 ± 0.8 % , 8.12x6 14.8 ± 1.4 %, 4.06x6 12.5 ± 0.9 %, 8.12x3 21.2 ± 4.3 %; vehicle 100.0 ± 4.5 %, $p<0.01$ (data not shown).

All the treatments tested resulted able to interfere with specific TAChR proliferation responses, accordingly with a decrease in total live cell count on LNCs, demonstrating a possible pharmacological effect *in vivo*. The PIX myelotoxic effect instead was evident only for the cumulative dosage 48.75 mg/Kg, also in HD/PIX, but the schedule 8.12x6 resulted fewer toxic compared to the protocols 16.25x3 administered both in EAMG and HD rats.

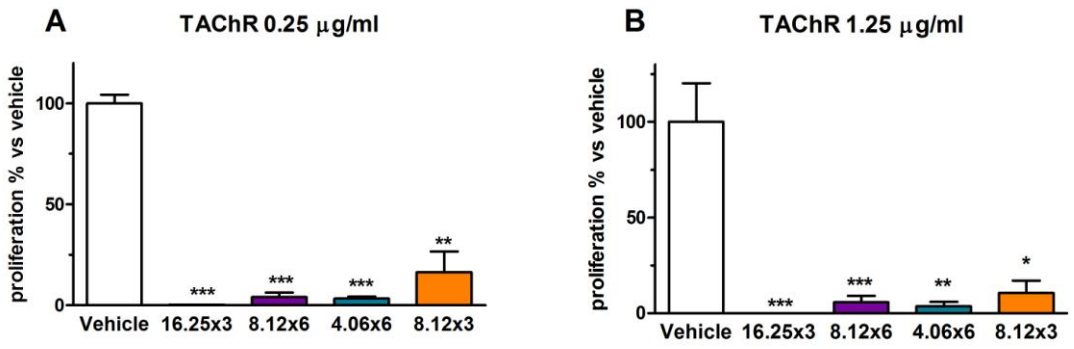

Figure 4.8 LNCs were processed into a single-cell suspension and challenged *in vitro* with TAChR (0.25 – 1.25 µg/ml) for 3 days, followed by [^{3}H] thymidine for 18h. Data were expressed as mean proliferation percentage normalized to vehicle ± SE from triplicate wells. PIX treatments: 16.25x3 (n=3), 8.12x6 (n=6); 4.06x6 (n=6); 8.06x3 (n=6). Vehicle group (n=7) was treated with sterile saline solution as placebo. Mean cpm ± SE for control wells (medium alone) were: 16.25x3 176 ± 57; 8.12x6 149 ± 31; 4.06x6 111 ± 23; 8.06x3 76 ± 12; vehicle 105±11. Data were the result of two different independent experiments. *, p<0.05; **, p<0.01; ***, p<0.001.

4.6 Effect of PIX on IFNγ mRNA transcription level and Anti Rat-Abs titer

We then tested the effect of PIX on mRNA IFNγ transcription levels, analyzing a population of lymphoid cells from spleen (SPNs). The spleen is a secondary lymphoid organ, such as lymph nodes, involved in production of Th1 cells in response to the antigen and involved in the autoimmune process. Total mRNA was extracted from SPNs of EAMG PIX treated rats with both cumulative dosages and vehicle. In parallel we evaluated also mRNA transcription level on SPNs from HD rats (n=3) in order to assess the IFNγ physiological level and correlate it with the effect of the PIX treatments.

As shown in Figure 4.9 only the treatments with a cumulative dosage equal to 48.75 mg/Kg were able to reduce IFNγ mRNA transcription levels compared to vehicle, with values close to HD rats (not significant difference when it was compared with HD controls). Instead IFNγ mRNA transcription levels in the PIX treatments with a cumulative dosage of 24.36 mg/Kg were unchanged compared to vehicle. IFNγ mRNA transcription levels for each group of treatments and HD controls were expressed as mean $2^{-\Delta Ct}$x100 ± SE: HD rats 0.0063 ± 0.0004%; vehicle 0.406 ± 0.099%; 16.25x3 0.018 ± 0.006% (p<0.001); 8.12x6 0.0215 ± 0.005% (p<0.001); 4.06x6 0.0915 ± 0.0665%; 8.12x3 0.9690 ± 0.1060%.

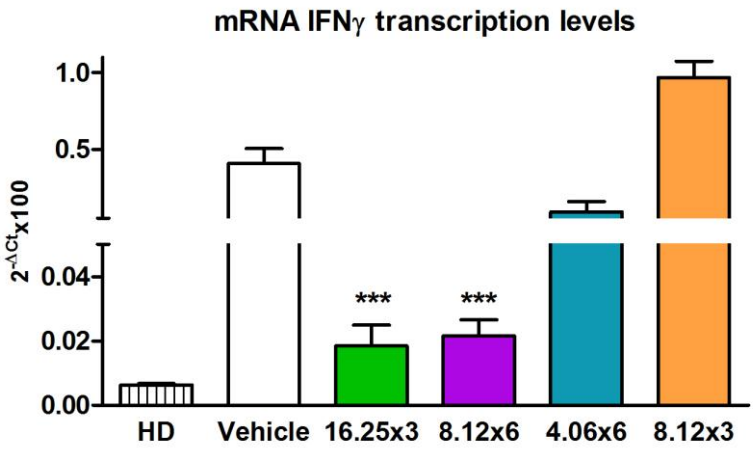

Figure 4.9 IFNγ mRNA transcription levels evaluation by *ex vivo* Real-Time qPCR analysis of SPNs from HD (n=3), EAMG rats treated with PIX (n = 6 for each group) or vehicle(n=7). Transcript levels were calculated according to the $2^{-\Delta Ct}$ method; β actin was used as endogenous control to normalize mRNA amount. Bars represented means $2^{-\Delta Ct}$x100 ± SE. Data were the result of three independent experiments. *** $p < 0.001$ compared to vehicle.

We then evaluated the effect of PIX treatments on the level of pathogenic Abs (IgG type) against Rat-AChR in the sera of PIX and vehicle treated rats. Also in this case the effect of PIX resulted cumulative dosage dependent (Figure 4.10).

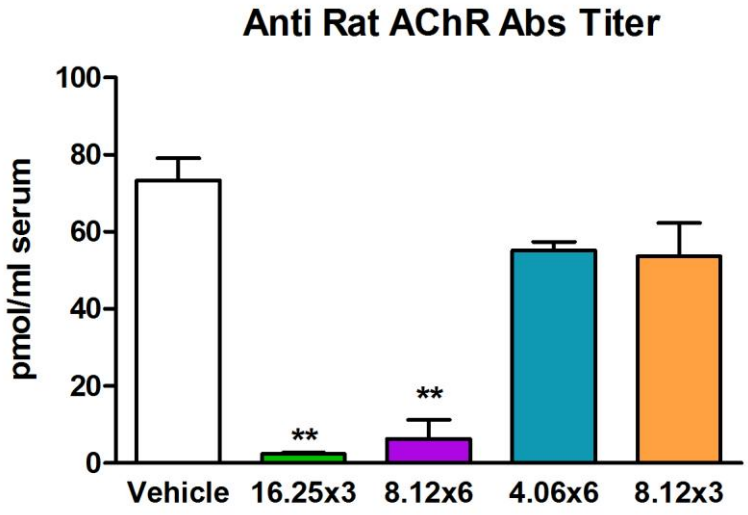

Figure 4.10 Anti-Rat AChR Abs titer were measured by conventional radioimmunoprecipitation assay in serum samples collected at the end of the experiments. Circulating anti-rat AChR Abs (IgG type) have been evaluated in individual samples in duplicate and were expressed as mean ± SE (pmol/ml) for treatment groups: vehicle treated EAMG rats (n=7) and therapeutic PIX schedules in EAMG rats, 16.25x3 (n=3) 8.12x6 (n=6), 4.06x6 (n=6) and 8.12x3(n=6). PIX 16.25x3 was used as positive control, while HD rats as negative control: mean pmol serum/ml ± SE was equal to 0.31 ± 0.005 pmol/ml. Data were the result of two independent experiments. ** $p < 0.01$.

EAMG rats treated with PIX at the cumulative dosage of 48.75 mg/Kg showed a significative decrease in the Abs titer (mean titer pmol/ml sera ± SE: 16.25x3 2.4 ± 0.3 pmol/ml, 8.12x6 6.3 ± 4.9 pmol/ml) compared to vehicle group (mean titer

pmol/ml sera ± SE: 73.4 ± 5.7 pmol/ml). Whereas animals treated with PIX at the cumulative dosage 24.36 mg/Kg showed no improvement (mean titer pmol/ml sera ± SE: 4.06x6 55.1 ± 0.7 pmol/ml, 8.12x3 53.6 ± 8.7 pmol/ml). The reduction percentage of Rat AChR Abs titer for each group compared to vehicle was: 16.25x6 96.8 %; 8.12x3 91.5 %; 4.06x6 24.9 %; 8.12x3 26.9 %.

Only the treatments with the cumulative dosage 48.75 mg/Kg were able to reduced mRNA IFNγ transcription levels on SPNs, accordingly with a decrease on Anti-Rat Abs titer in the sera of treated rats, that correlated at the end with an improved on the clinical parameters of EAMG. On the contrary the cumulative dosage 24.36 mg/Kg resulted not sufficient to interfere with levels of IFNγ with a consequent production of Anti-Rat Abs and an exacerbation of the disease.

Chapter 5 – Discussion

Therapeutic options for MG include corticosteroids and immunosuppressive drugs, pharmacological agents exerting a nonspecific generalized immune dysfunction [33]. These drugs are effective in a large proportion of patients. However, the clinical response to conventional treatments is unsatisfactory in some patients, or the severity of side effects may represent a limiting factor to the prolonged drug-administration. In this regard, new potent but well-tolerated immunosuppressive drugs are needed for MG [1].

Indeed, new immunosuppressants are under investigation in clinical trials to assess their efficacy on human autoimmune diseases. Mitoxantrone is a synthetic anthracenedione antineoplastic compound, initially used for the treatment of adult acute myeloid leukemia; more recently this compound has been approved by FDA for use in specific forms of multiple sclerosis [178]. MTX has a broad range of effects on the immune system because it is able to suppress proliferation of T cells, B cells, macrophages, and impairs antigen presentation mainly by the induction of APCs apoptosis. Moreover, experimental studies showed that MTX suppresses B cell function, Ab production, and reduces the release of proinflammatory cytokines.

Such a wide range of effects might be useful to control the immune attack in patients with MG, a T cell-dependent, B cell-mediated autoimmune disease. However, the major limitation to the use of MTX in MG as well as in other autoimmune disorders is its cardiotoxicity, resulting in congestive heart failure, with an increased risk with cumulative doses above 100 mg/m^2 [179].

PIX, structurally related to MTX, has similar mechanism of actions, same antineoplastic activity but presents a lower cardiotoxicity. The effect of PIX has been evaluated in the animal model of multiple sclerosis (EAE). Experimental studies showed that PIX can prevent the onset of EAE in SJL mice but is also able to modify the course of the disease in rats once the disease is established [148, 171]. These effects have been attributed to the long-lasting effect of PIX on several lymphocyte subpopulations and on Ag presentation. The observed effects were dose-dependent, and no signs of cardiotoxicity compared with MTX were

observed. Interestingly, the B cell response to myelin basic protein was also inhibited, providing a further mechanism of action to explain the reduced demyelination observed in treated animals.

The effect of PIX has been also investigated in EAMG, the animal model of MG. Ubiali et al. demonstrated that PIX was able to ameliorate EAMG both in the preventive and therapeutic treatments, when administered i.v. at the dose 16.25 mg/Kg (1/4 of the LD_{10} q7dx3), with a cumulative dosage of 48.75 mg/Kg [1].

In the present study we investigated whether reduced PIX cumulative dosages or modified schedules of treatments were still able to ameliorate EAMG.

We first addressed the issue of PIX effect on the *in vitro* proliferative response of lymphoid cells, in order to determine the pharmacologic properties of the drug in presence of different cells subpopulations, characterized by different proliferative responses. For this purpose, by dose-response curves we demonstrated that PIX (0.1 pM - 1 µM) inhibits lymphoid cell responses in a dose dependent manner. As shown in Figure 4.1 and 4.2, the calculated IC_{50} values were specific for the different cell subpopulations studied: IC_{50} was 5.4 pM for ConA-stimulated CD4+ Tcell line, 6.7 pM for R97-116-stimulated CD4+ Tcell line; 25.7 pM for ConA-stimulated LNCs, 63.3 pM for AChR (R97-116) stimulated LNCs; 37.4 pM ConA-stimulated SPNs and 54.3 pM for AChR (R97-116) stimulated SPNs. Complete suppression of proliferative responses was achieved with nM PIX concentrations for cells in active proliferation such as the R97-116 T cell line or LNCs and SPNs from R97-116 primed rats (Figure 4.1), and with µM PIX concentrations for SPNs from HD rats and PBMCs from healthy human donors (Figure 4.2).

Our data demonstrated that a lower concentration of PIX was sufficient to inhibit 50% of maximal response of an antigen specific T cell line, represented by an homogeneous pool of cells (more than 90%) capable of producing a robust cell proliferation in response of the specific antigen, i.e. the R97-116 peptide. In mixed populations, such as LNCs and SPNs isolated from R97-116 primed rats, in which we estimated to have 20-40% of antigen-specific cells, or even in SPNs from HD rats and PBMCs from healthy human donors, the IC_{50} for PIX is found to be increased. Our *in vitro* experiments suggested that PIX efficacy is higher for T cells that are able to actively proliferate.

We also evaluated whether PIX might alter differentiation of bone marrow MPCs from HD rats (Figure 4.3) in immature dendritic cells. Our data demonstrated that PIX 1 nM was able to reduce cell viability below 50% at day 7 of the differentiation protocol when the drug is added to the cell culture medium at day=0. When PIX was used at 10 nM and 10 µM, the observed vitality was dramatically dropped down to 0%.

Altogether our data on the *in vitro* pharmacological and toxicological effect of PIX demonstrated that PIX was able to exert a pharmacological effect when used in the picomolar range, compared with a toxic effect observed in the nanomolar range. Both of these *in vitro* aspects were relevant for the design of a different therapeutic schedules of *in vivo* treatments with PIX in the EAMG model.

Next, we studied the *ex vivo* response of LNCs from TAChR immunized rats (Figure 4.4) treated 24 h p.i. with a single dose of PIX (16.25 mg/Kg x1, 8.12 mg/Kg x1, 4.06 mg/Kg x1) or vehicle, and sacrificed after 10 days. All the conditions tested demonstrated that PIX was able to interfere with the immunocompetent cells, as assessed by a 70% reduction in total live cell count in PIX-treated TAChR immunized rats. These results correlated with a decrease (90%) of TAChR-specific proliferation from LNCs obtained from PIX treated rats compared to vehicle treated animals. In parallel we tested the effect of the higher PIX treatment (16.25 mg/Kg x1) in HD rats, in order to assess if PIX was able to interact with lymphoid cell compartment in physiological conditions: our data indicated that the drug had no effect on the number of total living cells. We speculated that PIX did not interfere *in vivo* with the pool of naïve T cells, physiologically present in LNCs, compared to the strong effect of the drug in AChR-primed rats, in which the immunization stimulated the active proliferation of pathogenic T CD4+ cells against TAChR. Furthermore we analyzed the effect of PIX treatment (16.25 mg/Kg x1) on HD rats immunized only with CFA, to investigate the eventual influence of PIX on antigens present in CFA, but the drug had no effect on total living cell count in this condition (data not shown).

Our data demonstrated that also the lower PIX dosage (4.06 mg/Kg x1) was able to exert immunomodulating properties *in vivo* and allowed us to test new therapeutic schedules for EAMG treatment.

Furthermore, we investigated *in vivo* the effect of long term treatments with PIX in EAMG rats, with two cumulative dosage (48.75 mg/Kg, Figure 4.5, and 24.36 mg/Kg, Figure 4.6), and by comparing different treatment protocols described in Table 4.1. The effect of a chronic administration of the drug has been also assessed in HD rats, by extending the treatment period to 6 weeks compared to the previous study (4 weeks) [1]. We initially assessed whether a difference can occur between the protocol 16.25 mg/Kg q14dx3 and q7dx6 [1]. Our preliminary data shown that both protocols were efficacy in EAMG modulation. According to these results, we selected the schedule 16.25x3 q14dx3 as positive control.

The PIX cumulative dosage of 48.75 mg/Kg was able to modify the course of the disease, as shown by the positive trend of clinical scores in treated rats, and by the assessment of body weight. At week 7 a difference in the mean clinical score of PIX-treated rats compared to EAMG controls can be observed. At the end of experiment (week 11), animals that received PIX 8.12 mg/Kg x6 had a statistically different mean clinical score compared with the protocol 16.25 mg/Kg x3 ($p<0.05$), with EAMG clinical score similar to normal control animals. We speculated that this effect could be related to the PIX pharmacokinetic profile in the schedule 8.12x6. In this protocol, PIX administration overlaps well with its clearance (8 days), thus allowing the achievement of a systemic steady-state, with the consequent improvement of the efficacy of this treatment compared to 16.25x3 schedule.

As regard to the PIX cumulative dosage corresponding to 24.36 mg/Kg, both of schedules (8.12x3 mg/Kg and 4.06x6 mg/Kg) compared to 8.12x6, as it proved to be the most effective, did not present any modifications in EAMG clinical manifestations (clinical score and body weight) compared to vehicle, but caused a marked inhibition of TAChR-specific proliferation from LNCs obtained from PIX treated rats compared to vehicle treated animals (Figure 4.8, Panel A and B). In parallel we demonstrated that these data were correlated to a reduction of 80% in the number of total live cell counts in the same organ (Figure 4.7, Panel A).

We also evaluated the myelotoxicity of drug in myeloid precursors cells, derived from PIX-treated rats with different schedules, counting total live cell numbers (Table 4.1). We observed that TAChR-immunization did not affect the viability of myeloid precursor cells compared to HD controls and that PIX cumulative dosage of 48.75 mg/Kg (16.25x3 mg/Kg and 8.12x6 mg/Kg) caused myelotoxicity both in

EAMG/PIX and HD/PIX rats compared to HD controls (Figure 4.7, Panel B). Indeed treatment of PIX 8.12x6 mg/Kg resulted less toxic (48%) than the protocol of 16.25x3 mg/Kg (60%). On the contrary the cumulative dosage of 24.75 mg/Kg did not interfere on cell viability compared to HD controls.

Next we evaluated in SPNs the mRNA transcription level of IFNγ, an important cytokine involved in the development of EAMG, produced by Th1 cells and activating Abs production from B cells (Figure 4.9). We studied mRNA transcription level in splenocytes, since spleen is a secondary lymphoid organ, involved in production of Th1 cells in response to the antigen and involved in the autoimmune process. PIX cumulative dosage of 48.75 mg/Kg was able to significantly decrease IFNγ mRNA transcription level in both schedules compared to the vehicle. On the contrary, treatments with cumulative dosage of 24.36 mg/Kg were not effective in decreasing these values.

These data were correlated with the specific Anti Rat AChR Abs titers detected in the sera of PIX and vehicle EAMG rats (Figure 4.10). In fact the decrease of IFNγ mRNA transcription level, found in PIX treated EAMG rats (cumulative dosage of 48.75 mg/Kg), corresponded to a reduction of 90% in Abs titer compared to vehicle. Although we observed an effect on TAChR specific proliferation of LNCs, treatments with cumulative dosage of 24.36 mg/Kg resulted ineffective to inhibit the production of pathogenic Anti Rat AChR Abs (Figure 4.10).

In conclusion, our data demonstrated that only PIX schedules with cumulative dosage of 48.75 mg/Kg, were able to ameliorate ongoing EAMG; in particular, the treatment with 8.12x6 mg/Kg resulted more effective due to its pharmacokinetic profile. The cumulative dosage of 24.36 mg/Kg resulted inefficacious to modulate EAMG because it didn’t reach the systemic effective dose in rats.

A further important feature of PIX treatment is its administration schedule once a week, thus limiting the severity of side effects. On the basis of our results we propose PIX as a promising immunomodulatory drug for the design of a clinical study in MG patients non-responsive to common therapies.

Chapter 6 – Reference

1. Ubiali, F., et al., *Pixantrone (BBR2778) reduces the severity of experimental autoimmune myasthenia gravis in Lewis rats.* J Immunol, 2008. **180**(4): p. 2696-703.
2. Sakaguchi, S., *Naturally arising Foxp3-expressing CD25+CD4+ regulatory T cells in immunological tolerance to self and non-self.* Nat Immunol, 2005. **6**(4): p. 345-52.
3. Roncarolo, M.G. and M.K. Levings, *The role of different subsets of T regulatory cells in controlling autoimmunity.* Curr Opin Immunol, 2000. **12**(6): p. 676-83.
4. Romagnani, S., *Immunological tolerance and autoimmunity.* Intern Emerg Med, 2006. **1**(3): p. 187-96.
5. Bacchetta, R., S. Gregori, and M.G. Roncarolo, *CD4+ regulatory T cells: mechanisms of induction and effector function.* Autoimmun Rev, 2005. **4**(8): p. 491-6.
6. Alam, S.M., et al., *T-cell-receptor affinity and thymocyte positive selection.* Nature, 1996. **381**(6583): p. 616-20.
7. Schwartz, R.H., *Natural regulatory T cells and self-tolerance.* Nat Immunol, 2005. **6**(4): p. 327-30.
8. Jiang, H. and L. Chess, *Regulation of immune responses by T cells.* N Engl J Med, 2006. **354**(11): p. 1166-76.
9. Sykes, M., *Immune tolerance: mechanisms and application in clinical transplantation.* J Intern Med, 2007. **262**(3): p. 288-310.
10. Piccirillo, C.A. and E.M. Shevach, *Naturally-occurring CD4+CD25+ immunoregulatory T cells: central players in the arena of peripheral tolerance.* Semin Immunol, 2004. **16**(2): p. 81-8.
11. Vincent, A., *Unravelling the pathogenesis of myasthenia gravis.* Nat Rev Immunol, 2002. **2**(10): p. 797-804.
12. Tzartos, S.J., et al., *Anatomy of the antigenic structure of a large membrane autoantigen, the muscle-type nicotinic acetylcholine receptor.* Immunol Rev, 1998. **163**: p. 89-120.
13. Lindstrom, J.M., et al., *Antibody to acetylcholine receptor in myasthenia gravis. Prevalence, clinical correlates, and diagnostic value.* Neurology, 1976. **26**(11): p. 1054-9.
14. Mantegazza, R., et al., *Myasthenia gravis (MG): epidemiological data and prognostic factors.* Ann N Y Acad Sci, 2003. **998**: p. 413-23.
15. Phillips, L.H., 2nd, *The epidemiology of myasthenia gravis.* Ann N Y Acad Sci, 2003. **998**: p. 407-12.
16. Meriggioli, M.N. and D.B. Sanders, *Autoimmune myasthenia gravis: emerging clinical and biological heterogeneity.* Lancet Neurol, 2009. **8**(5): p. 475-90.
17. Grob, D., et al., *Lifetime course of myasthenia gravis.* Muscle Nerve, 2008. **37**(2): p. 141-9.
18. Zhang, X., et al., *Clinical and serological study of myasthenia gravis in HuBei Province, China.* J Neurol Neurosurg Psychiatry, 2007. **78**(4): p. 386-90.
19. Compston, D.A., et al., *Clinical, pathological, HLA antigen and immunological evidence for disease heterogeneity in myasthenia gravis.* Brain, 1980. **103**(3): p. 579-601.
20. Christensen, P.B., et al., *Associated autoimmune diseases in myasthenia gravis. A population-based study.* Acta Neurol Scand, 1995. **91**(3): p. 192-5.
21. Aarli, J.A., *Late-onset myasthenia gravis: a changing scene.* Arch Neurol, 1999. **56**(1): p. 25-7.

22. Romi, F., et al., *Striational antibodies in myasthenia gravis: reactivity and possible clinical significance.* Arch Neurol, 2005. **62**(3): p. 442-6.
23. Romi, F., et al., *The severity of myasthenia gravis correlates with the serum concentration of titin and ryanodine receptor antibodies.* Arch Neurol, 2000. **57**(11): p. 1596-600.
24. Evoli, A., et al., *Thymoma in patients with MG: characteristics and long-term outcome.* Neurology, 2002. **59**(12): p. 1844-50.
25. Skeie, G.O. and F. Romi, *Paraneoplastic myasthenia gravis: immunological and clinical aspects.* Eur J Neurol, 2008. **15**(10): p. 1029-33.
26. McConville, J., et al., *Detection and characterization of MuSK antibodies in seronegative myasthenia gravis.* Ann Neurol, 2004. **55**(4): p. 580-4.
27. Sanders, D.B., et al., *Clinical aspects of MuSK antibody positive seronegative MG.* Neurology, 2003. **60**(12): p. 1978-80.
28. Leite, M.I., et al., *Fewer thymic changes in MuSK antibody-positive than in MuSK antibody-negative MG.* Ann Neurol, 2005. **57**(3): p. 444-8.
29. Vincent, A., et al., *Seronegative myasthenia gravis.* Semin Neurol, 2004. **24**(1): p. 125-33.
30. Kupersmith, M.J., R. Latkany, and P. Homel, *Development of generalized disease at 2 years in patients with ocular myasthenia gravis.* Arch Neurol, 2003. **60**(2): p. 243-8.
31. Bennett, D.L., et al., *Anti-MuSK antibodies in a case of ocular myasthenia gravis.* J Neurol Neurosurg Psychiatry, 2006. **77**(4): p. 564-5.
32. Oosterhuis, H.J., *The natural course of myasthenia gravis: a long term follow up study.* J Neurol Neurosurg Psychiatry, 1989. **52**(10): p. 1121-7.
33. Conti-Fine, B.M., M. Milani, and H.J. Kaminski, *Myasthenia gravis: past, present, and future.* J Clin Invest, 2006. **116**(11): p. 2843-54.
34. Hoch, W., et al., *Auto-antibodies to the receptor tyrosine kinase MuSK in patients with myasthenia gravis without acetylcholine receptor antibodies.* Nat Med, 2001. **7**(3): p. 365-8.
35. Ruegg, M.A. and J.L. Bixby, *Agrin orchestrates synaptic differentiation at the vertebrate neuromuscular junction.* Trends Neurosci, 1998. **21**(1): p. 22-7.
36. Glass, D.J., et al., *The receptor tyrosine kinase MuSK is required for neuromuscular junction formation and is a functional receptor for agrin.* Cold Spring Harb Symp Quant Biol, 1996. **61**: p. 435-44.
37. Hughes, B.W., L.L. Kusner, and H.J. Kaminski, *Molecular architecture of the neuromuscular junction.* Muscle Nerve, 2006. **33**(4): p. 445-61.
38. Sahashi, K., et al., *Ultrastructural localization of the terminal and lytic ninth complement component (C9) at the motor end-plate in myasthenia gravis.* J Neuropathol Exp Neurol, 1980. **39**(2): p. 160-72.
39. Lennon, V.A., et al., *Role of complement in the pathogenesis of experimental autoimmune myasthenia gravis.* J Exp Med, 1978. **147**(4): p. 973-83.
40. Biesecker, G. and C.M. Gomez, *Inhibition of acute passive transfer experimental autoimmune myasthenia gravis with Fab antibody to complement C6.* J Immunol, 1989. **142**(8): p. 2654-9.
41. Piddlesden, S.J., et al., *Soluble complement receptor 1 (sCR1) protects against experimental autoimmune myasthenia gravis.* J Neuroimmunol, 1996. **71**(1-2): p. 173-7.
42. Christadoss, P., *C5 gene influences the development of murine myasthenia gravis.* J Immunol, 1988. **140**(8): p. 2589-92.
43. Karachunski, P.I., et al., *Absence of IFN-gamma or IL-12 has different effects on experimental myasthenia gravis in C57BL/6 mice.* J Immunol, 2000. **164**(10): p. 5236-44.

44. Medof, M.E., et al., *Identification of the complement decay-accelerating factor (DAF) on epithelium and glandular cells and in body fluids.* J Exp Med, 1987. **165**(3): p. 848-64.
45. McNearney, T., et al., *Membrane cofactor protein of complement is present on human fibroblast, epithelial, and endothelial cells.* J Clin Invest, 1989. **84**(2): p. 538-45.
46. Meri, S., H. Waldmann, and P.J. Lachmann, *Distribution of protectin (CD59), a complement membrane attack inhibitor, in normal human tissues.* Lab Invest, 1991. **65**(5): p. 532-7.
47. Drachman, D.B., et al., *Myasthenic antibodies cross-link acetylcholine receptors to accelerate degradation.* N Engl J Med, 1978. **298**(20): p. 1116-22.
48. Engel, A.G. and G. Fumagalli, *Mechanisms of acetylcholine receptor loss from the neuromuscular junction.* Ciba Found Symp, 1982(90): p. 197-224.
49. Conti-Tronconi, B., S. Tzartos, and J. Lindstrom, *Monoclonal antibodies as probes of acetylcholine receptor structure. 2. Binding to native receptor.* Biochemistry, 1981. **20**(8): p. 2181-91.
50. Gomez, C.M. and D.P. Richman, *Anti-acetylcholine receptor antibodies directed against the alpha-bungarotoxin binding site induce a unique form of experimental myasthenia.* Proc Natl Acad Sci U S A, 1983. **80**(13): p. 4089-93.
51. Whiting, P.J., A. Vincent, and J. Newsom-Davis, *Acetylcholine receptor antibody characteristics in myasthenia gravis. Fractionation of alpha-bungarotoxin binding site antibodies and their relationship to IgG subclass.* J Neuroimmunol, 1983. **5**(1): p. 1-9.
52. Yates, A., et al., *Cytokine-modulated regulation of helper T cell populations.* J Theor Biol, 2000. **206**(4): p. 539-60.
53. Weiner, H.L., *Induction and mechanism of action of transforming growth factor-beta-secreting Th3 regulatory cells.* Immunol Rev, 2001. **182**: p. 207-14.
54. Moiola, L., et al., *Epitopes on the beta subunit of human muscle acetylcholine receptor recognized by CD4+ cells of myasthenia gravis patients and healthy subjects.* J Clin Invest, 1994. **93**(3): p. 1020-8.
55. Yi, Q., et al., *Acetylcholine receptor-reactive T cells in myasthenia gravis: evidence for the involvement of different subpopulations of T helper cells.* J Neuroimmunol, 1994. **50**(2): p. 177-86.
56. Im, S.H., et al., *Suppression of experimental myasthenia gravis, a B cell-mediated autoimmune disease, by blockade of IL-18.* FASEB J, 2001. **15**(12): p. 2140-8.
57. Delpy, L., et al., *Estrogen enhances susceptibility to experimental autoimmune myasthenia gravis by promoting type 1-polarized immune responses.* J Immunol, 2005. **175**(8): p. 5050-7.
58. Hohlfeld, R. and A.G. Engel, *Induction of HLA-DR expression on human myoblasts with interferon-gamma.* Am J Pathol, 1990. **136**(3): p. 503-8.
59. Feferman, T., et al., *Overexpression of IFN-induced protein 10 and its receptor CXCR3 in myasthenia gravis.* J Immunol, 2005. **174**(9): p. 5324-31.
60. Ostlie, N., et al., *Absence of IL-4 facilitates the development of chronic autoimmune myasthenia gravis in C57BL/6 mice.* J Immunol, 2003. **170**(1): p. 604-12.
61. Balasa, B., et al., *The Th2 cytokine IL-4 is not required for the progression of antibody-dependent autoimmune myasthenia gravis.* J Immunol, 1998. **161**(6): p. 2856-62.
62. Poussin, M.A., et al., *Suppression of experimental autoimmune myasthenia gravis in IL-10 gene-disrupted mice is associated with reduced B cells and serum cytotoxicity on mouse cell line expressing AChR.* J Neuroimmunol, 2000. **111**(1-2): p. 152-60.

63. Deng, C., et al., *Resistance to experimental autoimmune myasthenia gravis in IL-6-deficient mice is associated with reduced germinal center formation and C3 production.* J Immunol, 2002. **169**(2): p. 1077-83.
64. Balandina, A., et al., *Functional defect of regulatory CD4(+)CD25+ T cells in the thymus of patients with autoimmune myasthenia gravis.* Blood, 2005. **105**(2): p. 735-41.
65. Sun, Y., et al., *Increase of circulating CD4+CD25+ T cells in myasthenia gravis patients with stability and thymectomy.* Clin Immunol, 2004. **112**(3): p. 284-9.
66. Liu, R., et al., *Cooperation of invariant NKT cells and CD4+CD25+ T regulatory cells in the prevention of autoimmune myasthenia.* J Immunol, 2005. **175**(12): p. 7898-904.
67. Shi, F.D., et al., *Natural killer cells determine the outcome of B cell-mediated autoimmunity.* Nat Immunol, 2000. **1**(3): p. 245-51.
68. Jander, S. and G. Stoll, *Increased serum levels of the interferon-gamma-inducing cytokine interleukin-18 in myasthenia gravis.* Neurology, 2002. **59**(2): p. 287-9.
69. Vincent, A. and M.I. Leite, *Neuromuscular junction autoimmune disease: muscle specific kinase antibodies and treatments for myasthenia gravis.* Curr Opin Neurol, 2005. **18**(5): p. 519-25.
70. Shigemoto, K., et al., *Induction of myasthenia by immunization against muscle-specific kinase.* J Clin Invest, 2006. **116**(4): p. 1016-24.
71. Shiraishi, H., et al., *Acetylcholine receptors loss and postsynaptic damage in MuSK antibody-positive myasthenia gravis.* Ann Neurol, 2005. **57**(2): p. 289-93.
72. Boneva, N., et al., *Major pathogenic effects of anti-MuSK antibodies in myasthenia gravis.* J Neuroimmunol, 2006. **177**(1-2): p. 119-31.
73. Plested, C.P., et al., *AChR phosphorylation and indirect inhibition of AChR function in seronegative MG.* Neurology, 2002. **59**(11): p. 1682-8.
74. Mohan, S., et al., *Evaluation of myosin-reactive antibodies from a panel of myasthenia gravis patients.* Clin Immunol Immunopathol, 1994. **70**(3): p. 266-73.
75. Aarli, J.A., *Titin, thymoma, and myasthenia gravis.* Arch Neurol, 2001. **58**(6): p. 869-70.
76. Baggi, F., et al., *Anti-titin and antiryanodine receptor antibodies in myasthenia gravis patients with thymoma.* Ann N Y Acad Sci, 1998. **841**: p. 538-41.
77. Buzzard, E.F., *Myasthenia Gravis.* Proc R Soc Med, 1912. **5**(Neurol Sect): p. 138-40.
78. Marx, A., et al., *Pathogenesis of myasthenia gravis.* Virchows Arch, 1997. **430**(5): p. 355-64.
79. Schluep, M., et al., *Acetylcholine receptors in human thymic myoid cells in situ: an immunohistological study.* Ann Neurol, 1987. **22**(2): p. 212-22.
80. Wakkach, A., et al., *Expression of acetylcholine receptor genes in human thymic epithelial cells: implications for myasthenia gravis.* J Immunol, 1996. **157**(8): p. 3752-60.
81. Roxanis, I., et al., *Thymic myoid cells and germinal center formation in myasthenia gravis; possible roles in pathogenesis.* J Neuroimmunol, 2002. **125**(1-2): p. 185-97.
82. Sommer, N., et al., *Myasthenic thymus and thymoma are selectively enriched in acetylcholine receptor-reactive T cells.* Ann Neurol, 1990. **28**(3): p. 312-9.
83. Hill, M.E., et al., *The myasthenia gravis thymus: a rare source of human autoantibody-secreting plasma cells for testing potential therapeutics.* J Neuroimmunol, 2008. **201-202**: p. 50-6.
84. Levinson, A.I., et al., *A new model linking intrathymic acetylcholine receptor expression and the pathogenesis of myasthenia gravis.* Ann N Y Acad Sci, 2003. **998**: p. 257-65.
85. Aarli, J.A., et al., *Patients with myasthenia gravis and thymoma have in their sera IgG autoantibodies against titin.* Clin Exp Immunol, 1990. **82**(2): p. 284-8.

86. Cavalcante, P., et al., *Epstein-Barr virus persistence and reactivation in myasthenia gravis thymus.* Ann Neurol, 2010. **67**(6): p. 726-38.
87. Cavalcante, P., et al., *Inflammation and epstein-barr virus infection are common features of myasthenia gravis thymus: possible roles in pathogenesis.* Autoimmune Dis, 2011. **2011**: p. 213092.
88. Munz, C., et al., *Antiviral immune responses: triggers of or triggered by autoimmunity?* Nat Rev Immunol, 2009. **9**(4): p. 246-58.
89. Poea-Guyon, S., et al., *Effects of cytokines on acetylcholine receptor expression: implications for myasthenia gravis.* J Immunol, 2005. **174**(10): p. 5941-9.
90. Cavalcante, P., et al., *Detection of poliovirus-infected macrophages in thymus of patients with myasthenia gravis.* Neurology, 2010. **74**(14): p. 1118-26.
91. Kuppers, R., *B cells under influence: transformation of B cells by Epstein-Barr virus.* Nat Rev Immunol, 2003. **3**(10): p. 801-12.
92. Janer, M., et al., *A susceptibility region for myasthenia gravis extending into the HLA-class I sector telomeric to HLA-C.* Hum Immunol, 1999. **60**(9): p. 909-17.
93. Giraud, M., C. Vandiedonck, and H.J. Garchon, *Genetic factors in autoimmune myasthenia gravis.* Ann N Y Acad Sci, 2008. **1132**: p. 180-92.
94. Giraud, M., et al., *Linkage of HLA to myasthenia gravis and genetic heterogeneity depending on anti-titin antibodies.* Neurology, 2001. **57**(9): p. 1555-60.
95. Amdahl, C., et al., *Polygenic disease associations in thymomatous myasthenia gravis.* Arch Neurol, 2007. **64**(12): p. 1729-33.
96. Niks, E.H., et al., *Strong association of MuSK antibody-positive myasthenia gravis and HLA-DR14-DQ5.* Neurology, 2006. **66**(11): p. 1772-4.
97. Niks, E.H., J.B. Kuks, and J.J. Verschuuren, *Epidemiology of myasthenia gravis with anti-muscle specific kinase antibodies in The Netherlands.* J Neurol Neurosurg Psychiatry, 2007. **78**(4): p. 417-8.
98. Grob, D., *Course and management of myasthenia gravis.* J Am Med Assoc, 1953. **153**(6): p. 529-32.
99. Pascuzzi, R.M., *The edrophonium test.* Semin Neurol, 2003. **23**(1): p. 83-8.
100. Meriggioli, M.N. and D.B. Sanders, *Advances in the diagnosis of neuromuscular junction disorders.* Am J Phys Med Rehabil, 2005. **84**(8): p. 627-38.
101. Sanders, D.B., J.F. Howard, Jr., and T.R. Johns, *Single-fiber electromyography in myasthenia gravis.* Neurology, 1979. **29**(1): p. 68-76.
102. Oh, S.J., et al., *Diagnostic sensitivity of the laboratory tests in myasthenia gravis.* Muscle Nerve, 1992. **15**(6): p. 720-4.
103. Chan, K.H., et al., *Frequency of seronegativity in adult-acquired generalized myasthenia gravis.* Muscle Nerve, 2007. **36**(5): p. 651-8.
104. Cikes, N., et al., *Striational autoantibodies: quantitative detection by enzyme immunoassay in myasthenia gravis, thymoma, and recipients of D-penicillamine or allogeneic bone marrow.* Mayo Clin Proc, 1988. **63**(5): p. 474-81.
105. Grob, D. and A.M. Harvey, *Abnormalities in neuromuscular transmission, with special reference to myasthenia gravis.* Am J Med, 1953. **15**(5): p. 695-709.
106. Leite, M.I., et al., *IgG1 antibodies to acetylcholine receptors in 'seronegative' myasthenia gravis.* Brain, 2008. **131**(Pt 7): p. 1940-52.
107. Chagnac, Y., M. Hadani, and Y. Goldhammer, *Myasthenic crisis after intravenous administration of iodinated contrast agent.* Neurology, 1985. **35**(8): p. 1219-20.
108. Drachman, D.B., *Myasthenia gravis.* N Engl J Med, 1994. **330**(25): p. 1797-810.
109. Batocchi, A.P., et al., *Therapeutic apheresis in myasthenia gravis.* Ther Apher, 2000. **4**(4): p. 275-9.
110. Yeh, J.H. and H.C. Chiu, *Double filtration plasmapheresis in myasthenia gravis--analysis of clinical efficacy and prognostic parameters.* Acta Neurol Scand, 1999. **100**(5): p. 305-9.
111. Seybold, M.E., *Plasmapheresis in myasthenia gravis.* Ann N Y Acad Sci, 1987. **505**: p. 584-7.

112. Takamori, M. and T. Maruta, *Immunoadsorption in myasthenia gravis based on specific ligands mimicking the immunogenic sites of the acetylcholine receptor.* Ther Apher, 2001. **5**(5): p. 340-50.
113. Samuelsson, A., T.L. Towers, and J.V. Ravetch, *Anti-inflammatory activity of IVIG mediated through the inhibitory Fc receptor.* Science, 2001. **291**(5503): p. 484-6.
114. Brannagan, T.H., 3rd, et al., *Complications of intravenous immune globulin treatment in neurologic disease.* Neurology, 1996. **47**(3): p. 674-7.
115. Vincent, A. and D.B. Drachman, *Myasthenia gravis.* Adv Neurol, 2002. **88**: p. 159-88.
116. Evoli, A., et al., *Long-term results of corticosteroid therapy in patients with myasthenia gravis.* Eur Neurol, 1992. **32**(1): p. 37-43.
117. Pascuzzi, R.M., H.B. Coslett, and T.R. Johns, *Long-term corticosteroid treatment of myasthenia gravis: report of 116 patients.* Ann Neurol, 1984. **15**(3): p. 291-8.
118. Engel, W.K. and J.R. Warmolts, *Myasthenia gravis: a new hypothesis of the pathogenesis and a new form of treatment.* Ann N Y Acad Sci, 1971. **183**: p. 72-87.
119. Frauman, A.G., *An overview of the adverse reactions to adrenal corticosteroids.* Adverse Drug React Toxicol Rev, 1996. **15**(4): p. 203-6.
120. Witte, A.S., et al., *Azathioprine in the treatment of myasthenia gravis.* Ann Neurol, 1984. **15**(6): p. 602-5.
121. Mantegazza, R., et al., *Azathioprine as a single drug or in combination with steroids in the treatment of myasthenia gravis.* J Neurol, 1988. **235**(8): p. 449-53.
122. Palace, J., J. Newsom-Davis, and B. Lecky, *A randomized double-blind trial of prednisolone alone or with azathioprine in myasthenia gravis. Myasthenia Gravis Study Group.* Neurology, 1998. **50**(6): p. 1778-83.
123. Kissel, J.T., et al., *Azathioprine toxicity in neuromuscular disease.* Neurology, 1986. **36**(1): p. 35-9.
124. Slanar, O., et al., *Fatal myelotoxicity after azathioprine treatment.* Nucleosides Nucleotides Nucleic Acids, 2008. **27**(6): p. 661-5.
125. Fulton, B. and A. Markham, *Mycophenolate mofetil. A review of its pharmacodynamic and pharmacokinetic properties and clinical efficacy in renal transplantation.* Drugs, 1996. **51**(2): p. 278-98.
126. Ciafaloni, E., et al., *Retrospective analysis of the use of cyclosporine in myasthenia gravis.* Neurology, 2000. **55**(3): p. 448-50.
127. Ponseti, J.M., et al., *Tacrolimus for myasthenia gravis: a clinical study of 212 patients.* Ann N Y Acad Sci, 2008. **1132**: p. 254-63.
128. Nagane, Y., et al., *Efficacy of low-dose FK506 in the treatment of Myasthenia gravis--a randomized pilot study.* Eur Neurol, 2005. **53**(3): p. 146-50.
129. Takamori, M., et al., *Anti-ryanodine receptor antibodies and FK506 in myasthenia gravis.* Neurology, 2004. **62**(10): p. 1894-6.
130. De Feo, L.G., et al., *Use of intravenous pulsed cyclophosphamide in severe, generalized myasthenia gravis.* Muscle Nerve, 2002. **26**(1): p. 31-6.
131. Drachman, D.B., et al., *Rebooting the immune system with high-dose cyclophosphamide for treatment of refractory myasthenia gravis.* Ann N Y Acad Sci, 2008. **1132**: p. 305-14.
132. Drachman, D.B., R.J. Jones, and R.A. Brodsky, *Treatment of refractory myasthenia: "rebooting" with high-dose cyclophosphamide.* Ann Neurol, 2003. **53**(1): p. 29-34.
133. Martin, F., et al., *Side-effects of intravenous cyclophosphamide pulse therapy.* Lupus, 1997. **6**(3): p. 254-7.
134. Illa, I., et al., *Sustained response to Rituximab in anti-AChR and anti-MuSK positive Myasthenia Gravis patients.* J Neuroimmunol, 2008. **201-202**: p. 90-4.
135. Gronseth, G.S. and R.J. Barohn, *Practice parameter: thymectomy for autoimmune myasthenia gravis (an evidence-based review): report of the Quality Standards*

Subcommittee of the American Academy of Neurology. Neurology, 2000. **55**(1): p. 7-15.
136. Link, H. and B.G. Xiao, *Rat models as tool to develop new immunotherapies.* Immunol Rev, 2001. **184**: p. 117-28.
137. Lindstrom, J.M., et al., *Experimental autoimmune myasthenia gravis and myasthenia gravis: biochemical and immunochemical aspects.* Ann N Y Acad Sci, 1976. **274**: p. 254-74.
138. Baggi, F., et al., *Breakdown of tolerance to a self-peptide of acetylcholine receptor alpha-subunit induces experimental myasthenia gravis in rats.* J Immunol, 2004. **172**(4): p. 2697-703.
139. Lennon, V.A., J.M. Lindstrom, and M.E. Seybold, *Experimental autoimmune myasthenia gravis: cellular and humoral immune responses.* Ann N Y Acad Sci, 1976. **274**: p. 283-99.
140. Stassen, M.H., et al., *Experimental autoimmune myasthenia gravis in mice expressing human immunoglobulin loci.* J Neuroimmunol, 2003. **135**(1-2): p. 56-61.
141. Toro-Goyco, E., et al., *Induction of experimental myasthenia gravis in rhesus monkeys: a model for the study of the human disease.* P R Health Sci J, 1986. **5**(1): p. 13-8.
142. Hoedemaekers, A.C., et al., *Age-related susceptibility to experimental autoimmune myasthenia gravis: immunological and electrophysiological aspects.* Muscle Nerve, 1997. **20**(9): p. 1091-101.
143. Lindstrom, J.M., *Acetylcholine receptors and myasthenia.* Muscle Nerve, 2000. **23**(4): p. 453-77.
144. Tzartos, S.J., et al., *Localization of the main immunogenic region of human muscle acetylcholine receptor to residues 67-76 of the alpha subunit.* Proc Natl Acad Sci U S A, 1988. **85**(9): p. 2899-903.
145. McCormick, D.J. and M.Z. Atassi, *Localization and synthesis of the acetylcholine-binding site in the alpha-chain of the Torpedo californica acetylcholine receptor.* Biochem J, 1984. **224**(3): p. 995-1000.
146. Takamori, M., et al., *Myasthenogenic significance of synthetic alpha-subunit peptide 183-200 of Torpedo californica and human acetylcholine receptor.* J Neurol Sci, 1988. **85**(2): p. 121-9.
147. Lennon, V.A., et al., *Region of peptide 125-147 of acetylcholine receptor alpha subunit is exposed at neuromuscular junction and induces experimental autoimmune myasthenia gravis, T-cell immunity, and modulating autoantibodies.* Proc Natl Acad Sci U S A, 1985. **82**(24): p. 8805-9.
148. Cavaletti, G., et al., *Pixantrone (BBR2778) reduces the severity of experimental allergic encephalomyelitis.* J Neuroimmunol, 2004. **151**(1-2): p. 55-65.
149. Engert, A., et al., *EXTEND PIX301: a phase III randomized trial of pixantrone versus other chemotherapeutic agents as third-line monotherapy in patients with relapsed, aggressive non-Hodgkin's lymphoma.* Clin Lymphoma Myeloma, 2006. **7**(2): p. 152-4.
150. Lim, S.T., et al., *A phase I/II trial of pixantrone (BBR2778), methylprednisolone, cisplatin, and cytosine arabinoside (PSHAP) in relapsed/refractory aggressive non-Hodgkin's lymphoma.* Leuk Lymphoma, 2007. **48**(2): p. 374-80.
151. Fox, E.J., *Mechanism of action of mitoxantrone.* Neurology, 2004. **63**(12 Suppl 6): p. S15-8.
152. Gonsette, R.E. and B. Dubois, *Pixantrone (BBR2778): a new immunosuppressant in multiple sclerosis with a low cardiotoxicity.* J Neurol Sci, 2004. **223**(1): p. 81-6.
153. Gonsette, R.E., *New immunosuppressants with potential implication in multiple sclerosis.* J Neurol Sci, 2004. **223**(1): p. 87-93.

154. Baggi F, N.S., Ruocco C., *Pixantrone: Preclinical Studies in EAMG.* Linus Publications Inc., 2009. **Chapter 22** - Myasthenia Gravis Disease Mechanisms and Immune Intervention.
155. Evison, B.J., et al., *Pixantrone can be activated by formaldehyde to generate a potent DNA adduct forming agent.* Nucleic Acids Res, 2007. **35**(11): p. 3581-9.
156. Beggiolin, G., et al., *Bbr 2778, an aza-anthracenedione endowed with preclinical anticancer activity and lack of delayed cardiotoxicity.* Tumori, 2001. **87**(6): p. 407-16.
157. Borchmann, P. and M. Reiser, *Pixantrone (Novuspharma).* IDrugs, 2003. **6**(5): p. 486-90.
158. Pommier, Y., *DNA topoisomerase I and II in cancer chemotherapy: update and perspectives.* Cancer Chemother Pharmacol, 1993. **32**(2): p. 103-8.
159. CellTherapeutics, *Investigator's Brochure for BBR2778.* 2002. **Edition no. 6**.
160. Dawson, L.K., et al., *A clinical phase I and pharmacokinetic study of BBR 2778, a novel anthracenedione analogue, administered intravenously, 3 weekly.* Eur J Cancer, 2000. **36**(18): p. 2353-9.
161. Borchmann, P., et al., *Phase-II study of the new aza-anthracenedione, BBR 2778, in patients with relapsed aggressive non-Hodgkin's lymphomas.* Haematologica, 2003. **88**(8): p. 888-94.
162. Chan, A., O. Stuve, and N. von Ahsen, *Immunosuppression in clinical practice: approaches to individualized therapy.* J Neurol, 2008. **255 Suppl 6**: p. 22-7.
163. Verdrengh, M., O. Isaksson, and A. Tarkowski, *Topoisomerase II inhibitors, irrespective of their chemical composition, ameliorate experimental arthritis.* Rheumatology (Oxford), 2005. **44**(2): p. 183-6.
164. Gbadamosi, J., et al., *Effects of mitoxantrone on multiple sclerosis patients' lymphocyte subpopulations and production of immunoglobulin, TNF-alpha and IL-10.* Eur Neurol, 2003. **49**(3): p. 137-41.
165. Martinelli Boneschi, F., et al., *Mitoxantrone for multiple sclerosis.* Cochrane Database Syst Rev, 2005(4): p. CD002127.
166. Chan, A., et al., *Mitoxantrone induces cell death in peripheral blood leucocytes of multiple sclerosis patients.* Clin Exp Immunol, 2005. **139**(1): p. 152-8.
167. Ghalie, R.G., et al., *A study of therapy-related acute leukaemia after mitoxantrone therapy for multiple sclerosis.* Mult Scler, 2002. **8**(5): p. 441-5.
168. Sieb, J.P., *Myasthenia gravis: emerging new therapy options.* Curr Opin Pharmacol, 2005. **5**(3): p. 303-7.
169. Drachman, D.B., K.R. McIntosh, and B. Yang, *Factors that determine the severity of experimental myasthenia gravis.* Ann N Y Acad Sci, 1998. **841**: p. 262-82.
170. Fuchs, S., et al., *Immune regulation of experimental myasthenia.* J Neurol Neurosurg Psychiatry, 1980. **43**(7): p. 634-43.
171. Mazzanti, B., et al., *Effects of pixantrone on immune-cell function in the course of acute rat experimental allergic encephalomyelitis.* J Neuroimmunol, 2005. **168**(1-2): p. 111-7.
172. Aharonov, A., et al., *Immunochemical studies on acetylcholine receptor from Torpedo californica.* Immunochemistry, 1977. **14**(2): p. 129-37.
173. Nessi, V., et al., *Naturally occurring CD4+CD25+ regulatory T cells prevent but do not improve experimental myasthenia gravis.* J Immunol, 2010. **185**(9): p. 5656-67.
174. Ubiali, F., et al., *Allorecognition of human neural stem cells by peripheral blood lymphocytes despite low expression of MHC molecules: role of TGF-beta in modulating proliferation.* Int Immunol, 2007. **19**(9): p. 1063-74.
175. Liu, X., et al., *CD24 and myosin light polypeptide 2 are involved in prevention of experimental autoimmune encephalomyelitis by myelin basic protein-pulsed dendritic cells.* J Neuroimmunol, 2006. **172**(1-2): p. 137-44.

176. Ludwig, A., et al., *Enhanced expression and shedding of the transmembrane chemokine CXCL16 by reactive astrocytes and glioma cells.* J Neurochem, 2005. **93**(5): p. 1293-303.
177. Lindstrom, J., B. Einarson, and S. Tzartos, *Production and assay of antibodies to acetylcholine receptors.* Methods Enzymol, 1981. **74 Pt C**: p. 432-60.
178. Gonsette, R.E., *Compared benefit of approved and experimental immunosuppressive therapeutic approaches in multiple sclerosis.* Expert Opin Pharmacother, 2007. **8**(8): p. 1103-16.
179. Cohen, B.A. and D.D. Mikol, *Mitoxantrone treatment of multiple sclerosis: safety considerations.* Neurology, 2004. **63**(12 Suppl 6): p. S28-32.