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Review

Experimental Autoimmune Myasthenia Gravis (EAMG): From immunochemical characterization to therapeutic approaches

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ABSTRACT

Myasthenia Gravis (MG) is an organ-specific autoimmune disease. In high percentage of patients there are autoantibodies to the nicotinic acetylcholine receptor (AChR) that attack AChR on muscle cells at the neuromuscular junction, resulting in muscle weakness. Experimental Autoimmune Myasthenia Gravis (EAMG) is an experimental model disease for MG. EAMG is induced in several animal species by immunization with acetylcholine receptor (AChR), usually isolated from the electric organ of electric fish, which is a rich source for this antigen. Our lab has been involved for several decades in research of AChR and of EAMG. The availability of an experimental autoimmune disease that mimics in many aspects the human disease, provides an excellent model system for elucidating the immunological nature and origin of MG, for studying various existing treatment modalities and for attempting the development of novel treatment approaches. In this review in honor of Michael Sela and Ruth Arnon, we report first on our early pioneering contributions to research on EAMG. These include the induction of EAMG in several animal species, early attempts for antigen-specific treatment for EAMG, elicitation and characterization of monoclonal antibodies and anti-idiotypic antibodies, measuring humoral and cellular AChR-specific immune responses in MG patient and more. In the second part of the review we discuss more recent studies from our lab towards developing and testing novel treatment approaches for myasthenia. These include antigen-dependent treatments aimed at specifically abrogating the humoral and cellular anti-AChR responses, as well as immunomodulatory approaches that could be used either alone, or in conjunction with antigen-specific treatments, or alternatively, serve as steroid-sparing agents.

1. Introduction: from synthetic antigens to Experimental Autoimmune Myasthenia Gravis (EAMG). How did we get there?

One of us (S. F) has been Michael Sela's second Ph.D student. The first one was Ruth Arnon. The exciting climax of her thesis work has been the preparation and identification of the first completely synthetic antigen (T, G)-A–L [1]. Ruth (Ruthie, as she is known to all of us) went overseas for her post doctoral training and I continued her project from that point, to a detailed analysis towards the elucidation of the chemical basis of antigenicity and immunogenicity [2,3]. Working with Michael as a teacher, a mentor and a colleague was a real challenge and with his superb and friendly leadership, we made many contributions to the growing field of molecular immunology. Following my post doc in protein chemistry at the prestigious laboratory of Christian Anfinsen at the NIH, I returned back to Sela's Chemical Immunology department and started to develop my own projects. Among other projects I have touched, I started to work on proteins from the excitable membrane, primarily on the acetylcholine receptor (AChR) that had just been purified [4]. That was when I experienced 'serendipity' in my own practice, which in a way determined my future career. We immunized rabbits to elicit antibodies for immunochemical studies of the newly purified AChR protein but frustratingly, our rabbits got sick and died before we collected their antibodies. This happened over and again until Professor Ian Mackay from Australia visited us in Israel, being interested in our work on the autoimmunity of collagen [5]. During our discussion, I also told him about our AChR-immunized rabbits. It was he who told us about the inside information he had, saying that our rabbits had a Myasthenia Gravis-like autoimmune disease (Experimental Autoimmune Myasthenia

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[1] Deceased. While working on this review our beloved colleague Professor Miriam C. Souroujon (Miry) passed away. It is a great loss to all of us. Miry was an excellent scientist, an outstanding and unique human being and a great friend. We miss her a lot and cherish her memory.

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Gravis, EAMG [6]). This had been the first time I heard the term Myasthenia Gravis. That discussion with Prof. McKay had a very significant impact on my scientific career and ever since, Experimental Autoimmune Myasthenia Gravis (EAMG) and its major autoantigen, the acetylcholine receptor (AChR), have been the main research topic of my lab.

In the following we will report first on our early pioneering studies on EAMG after its establishment, and will then focus on more recent studies towards the development of new treatment approaches for myasthenia, based on our research in EAMG.

2. The early days

Once EAMG has been discovered and reported [6], an exciting and challenging immunological aspect has been added to research of the acetylcholine receptor. EAMG has become an excellent experimental model for an antibody-mediated human autoimmune disease, myasthenia gravis in particular. Both immunologists and neurologists became interested and involved in this challenging new topic. The department of Chemical Immunology provided the optimal environment for carrying out immunological and immunochemical studies on EAMG, along with structural investigations on the AChR itself [7].

During the early years EAMG has been induced in several animal species. It was first induced in rabbits [6,8]. However, the rabbit model is rather severe and additional models, which mimic better the human disease and were more suitable for immunological manipulations, were needed. EAMG has been induced in additional species such as rats and guinea pigs [9,10], monkeys [11], mice [12] and chicken [13,14]. Our early study in mouse EAMG revealed strain differences in the susceptibility to the disease [12], thus enabling later detailed studies in various inbred mouse strains and other genetically manipulated animals [15,16].

The identification of AChR as the main autoantigen in myasthenia led quite early to the development of procedures to measure cellular [17] and humoral [18,19] anti-AChR activities in myasthenia gravis (MG) patients. Nowadays, testing for AChR-specific antibodies by sensitive radio-immunoassays is probably the most reliable diagnostic assay for MG.

The development of monoclonal antibodies to AChR [20–22] and the identification of monoclonal antibodies directed to biologically specific sites as *e.g.* the binding site [23], the main immunogenic region (MIR) [24] and other sites and regions of the AChR molecule, resulted in many studies and applications of biochemical, immunological, physiological and structural nature.

The availability of an experimental autoimmune disease induced by a well-defined antigen (AChR) provided a valuable tool for attempting to regulate and treat the disease. Experiments were made to study the immunosuppressive effects of the nonspecific drugs, hydrocortisone and the anti-metabolite azathioprine on EAMG [25,26]. These studies contributed to the elucidation of some aspects of the mechanism of action of nonspecific immunosuppressive drugs in the therapy of autoimmune diseases in general and MG in particular.

Our first approach toward antigen-specific treatment for EAMG has been the application of a denatured AChR preparation (reduced and carboxymethylated AChR (RCM-AChR)) for immunosuppression of EAMG [7,27]. In this study we have demonstrated that RCM-AChR, which by itself does not induce EAMG can prevent its onset and even immunosuppresses an ongoing disease (see below). This study has also elucidated the role of spacial conformation in immunogenicity, which forms the basis of many antigen-specific immune-therapies.

We have also considered quite early the idea of regulating EAMG by anti-idiotypic antibodies. The first AChR-specific anti-idiotypic antibodies were induced by us by immunization with syngeneic spleen cells educated *in-vitro* with AChR [28]. These studies demonstrated a broad cross-reactivity between idiotypes of anti-AChR antibodies from different mouse strains, as well as with anti-AChR antibodies from other species.

At later stages of our research we have elicited anti-idiotypic antibodies against polyclonal and monoclonal anti-AChR antibodies, characterized their *in vitro* and in vivo specificities and examined their therapeutic activity on EAMG. These studies will be described in the following chapter.

3. Therapies attempted

Among the many studies on EAMG that we have carried out during the last two decades, we chose to concentrate in this review, mainly on those studies in which some therapeutic approaches have been proposed or employed. The common treatment for MG and for many other autoimmune diseases involves the use of symptomatic treatments and general immunosuppression. Currently, the first-line symptomatic treatment for MG is the use of anticholinesterase drugs, which inhibit the acetylcholine esterase at the neuromuscular junction (NMJ) and increase the concentration of available acetylcholine (ACh), thus enhancing neuromuscular transmission. In terms of immunomodulatory treatments, thymectomy is used in some cases for early onset and thymoma MG, whereas immunosuppressive agents such as glucocorticoids and Imuran are used in many cases of MG. Plasmapheresis and intravenous immunoglobulin (IVIG) are equally effective and well tolerated in the treatment of moderate and severe MG relapses [29]. All these available MG treatments share their unspecific mechanism of action and occasionally may present severe side effects. Some of the major adverse effects linked to immunosuppression are osteoporosis, hypertension, gastrointestinal discomfort and psychiatric changes.

As mentioned, the availability of an experimental autoimmune disease model for myasthenia gravis (EAMG) in which the autoantigen, AChR, is identified and characterized, provides an excellent experimental system for testing and developing new treatment modalities for MG. During the last decades many of our studies aimed at developing novel therapeutic modalities for MG that would specifically or preferentially eliminate the autoimmune response without affecting the function of the entire immune system, with hopefully minimal side effects. Such treatments could be applied either alone or in conjunction with lower doses of immunosuppressive drugs. Herein, we summarize some of our efforts to develop new treatment modalities for EAMG.

3.1. Antigen-specific treatments

Our first attempt to treat EAMG by an antigen-specific immunotherapy was performed in 1978 using intra-dermal injections of a denatured Torpedo AChR preparation [27]. This chemically modified AChR derivative (reduced and carboxymethylated AChR (RCM-AChR)) did not induce any myasthenic symptoms when injected into rabbits. Nevertheless, it prevented the induction of EAMG and immunosuppressed an ongoing disease. The structural similarity to, or cross-reactivity of the applied therapeutic agent with the pathogenic auto-antigen, forms the basis for many of the antigen-specific therapies for autoimmune diseases.

Exposure of mucosal surfaces to an antigen is known to lead to systemic tolerance to the same antigen [30]. We have shown that mucosal (nasal or oral) administration of native Torpedo AChR before immunization with AChR, modulated EAMG and suppressed humoral and cellular responses to AChR [31,32]. Feeding with Torpedo AChR during the acute phase of EAMG resulted in

inhibition of the clinical manifestation of EAMG, associated with a paradoxical enhancement of the AChR-antibody responses, in line with previous findings [33]. The priming effect on autoantibody levels induced by feeding with the xenogeneic and highly immunogenic Torpedo AChR and its limited availability hampered this application for therapeutic purposes in humans, suggesting that an easily available syngeneic molecule with less immunogenic potential may be required for safe and effective immunotherapy of myasthenia in humans.

The cloning of the mammalian AChR in the early 1980s led to the mapping of regions within the AChR molecule, which play a key role in the cellular and humoral autoimmune response in myasthenia. It was found that the α-subunit of the AChR molecule is the main target of the autoimmune response and that a large portion of the antibodies to AChR is directed to a specific sequence within this domain (residues 67–76), which has been accordingly termed main immunogenic region (MIR) [22]. These findings enabled the use of fragments and peptides from selected regions of AChR, rather than the whole AChR molecule, for specific suppression of EAMG.

We have employed a recombinant fragment from the extracellular portion of the human AChR α-subunit (residues 1–210), known to be the target for the majority of the anti-AChR antibodies in MG, to induce mucosal tolerance in rat EAMG. We first showed that such recombinant fragments could protect AChR in TE671 cells, from accelerated degradation induced by anti-MIR monoclonal or rat anti-AChR polyclonal antibodies and could also modulate the passive transfer of EAMG induced by monoclonal antibodies in Lewis rats [34]. We have then demonstrated that nasal or oral administration of these AChR-derived recombinant fragments prevents EAMG in rats when administered before the induction of disease, and immunosuppresses an ongoing disease when treatment is initiated at the acute or chronic EAMG [31,35]. Suppression of EAMG was accompanied by a marked decrease in AChR-specific T-cell proliferative responses and IL-2 production, as well as a decreased anti-self AChR antibody titer. The underlying mechanism for the mucosal tolerance induced by the AChR fragments is active suppression and not clonal anergy [31,32,35] (Table 1). Suppression of EAMG was mediated by a shift from a Th1 to a Th2/Th3 response and down-regulation of co-stimulatory factors. We have further demonstrated that the spatial conformation of the mucosally administered tolerogen is important in determining its efficacy in suppressing EAMG [32].

The next question we addressed was the importance of the chemical nature of the fed antigen for the induction of systemic tolerance. It should be noted that mucosal administration of an antigen can also induce systemic immunity by activation of Ag-specific Th2 cells, resulting in stimulation of Ag-specific B cells and synthesis of pathogenic anti-self Abs [36,37]. This may be especially dangerous when treatment is initiated in ongoing autoimmunity; in which activated autoreactive B and T cells already exist. Indeed, we have demonstrated that oral administration of an AChR fragment with a conformation similar to that of the native protein, during ongoing EAMG, induces active immunity and exacerbates EAMG pathogenesis, while feeding with a less native fragment induces tolerance [32]. This suggests that the spatial conformation of an orally administered tolerogen should be given careful attention when considering oral treatment for the induction of systemic tolerance.

Since application of a xenogeneic recombinant fragment may have limitations when considered as a possible approach for the treatment of human myasthenia, we have tested the suppressive potential of feeding rats by a syngeneic fragment corresponding to the extracellular region of the rat AChR *a*-subunit (R*a*1-205). This fragment was as effective as the formerly described human xenogeneic fragment in suppressing ongoing EAMG and the underlying mechanism of immunomodulation by Rα1-205 was similar but not identical to that of the xenogeneic human fragment. It induced a shift from Th1 to Th2 regulation but there was no elevation of the Th3-type cytokine TGF-β which was increased in Hα1-205-treated rats [38] (Table 1).

Some microbial peptides mimicking T-cell epitopes of the AChR a-subunit were also shown to have suppressive effects on EAMG [39]. Pretreatment of rats by a peptide derived from *H. influenzae*, selected from protein databases on the basis of its partial homology (50%) to an identified T-cell epitope of the human AChR α-subunit (residues 170–182), attenuated the induction and progression of EAMG. This may suggest that a non-pathogenic microbial mimicry peptide could serve as an immuno-modulator of the autoimmune attack on host antigens and could thereby affect the progression of antibody-mediated autoimmune diseases such as MG.

3.2. Anti-idiotypes

Antibodies and autoreactive T cells are found at low levels in normal individuals and are thought to be kept at bay by T regulatory (Treg) cells and a network of idiotypic and anti-idiotype-bearing antigen receptors on lymphocytes as well as idiotypic anti-idiotypic antibodies. Disruption of this network by genetic, environmental and other unknown factors is thought to result in autoimmune diseases. An obvious, ideal and specific therapy for such disorders would be to harness this regulatory network to re-establish immunologic homeostasis [40]. In practice, however, this is not an easy task as most autoimmune diseases involve polyclonal responses to self-antigen as well as epitope spreading [41,42]. We have shown that "vaccination" of mice with a certain idiotype prior to induction of EAMG by Torpedo AChR led to suppression of this particular idiotype and to a reduced overall anti-AChR titer in the treated mice [43]. However, since active immunization would not be considered for the treatment of MG patients, we have analyzed the effect of passively transferred rabbit anti-idiotypes [44].

We demonstrated that anti-idiotypes raised against polyclonal anti-AChR antibodies isolated from a rabbit with EAMG were successful in preventing the initial development of EAMG and possibly in modifying existing disease [20]. We have further employed monoclonal anti-AChR antibodies and their respective anti-idiotypes for evaluation of their role in the maintenance and regulation of EAMG [43–45]. Anti-idiotypes were raised in mice against several well-characterized anti-AChR monoclonal antibodies (mAbs). In binding experiments, the anti-idiotypic antibodies inhibited the binding of AChR only to the immunizing idiotype. However, a less restricted specificity was found in in vivo experiments. Challenging mice producing anti-idiotypes with AChR has demonstrated that pre-immunization with either polyclonal or monoclonal anti-AChR antibodies resulted in a reduction of the

Table 1
Effect of antigen-specific mucosal tolerance and blockade of cytokines and co-stimulatory factors.

Treatment	EAMG suppression	T-cell response	Antibody titer	Cytokines			CTLA-4
				Th1	Th2	Th3	
Xenogeneic antigen-specific mucosal tolerance	+++	↓	↓↓	↓	↑	↓	↓
Syngeneic antigen-specific mucosal tolerance	+++	↓	–	↓	↑	–	↓
Anti-CD40L	++	–	↓	↓	–	–	↑
Anti-IL-18	++	↓	±	↓	–	↑	↑

overall anti-Torpedo AChR and anti-muscle AChR titers, which was greater than would be expected from the representation of each of the respective idiotypes in the polyclonal anti-AChR serum. This may imply that in addition to the immunizing idiotype, other anti-AChR idiotypes are also suppressed.

To test the therapeutic effect of passively transferred anti-idiotypes, an EAMG-like disease has been first induced in chicken hatchings by transfer of mAb 5.5, which is directed to the AChR binding site [23]. Administration of rabbit anti-idiotypic antibodies against mAb 5.5 could reverse the neuromuscular block induced in the chicken hatchings by this mAb. Thus, specific anti-idiotypes raised against mAb 5.5 were demonstrated to prevent the induction of EAMG by a subsequent injection of mAb 5.5. Also, administration of anti-idiotypes against mAb 5.5 to chickens in which EAMG has been induced by mAb 5.5 led to a recovery from myasthenic symptoms. These results suggest that passive transfer of the appropriate anti-idiotypes may be potential in the regulation of myasthenia.

3.3. IVIG and its active sub-fractions

IVIG administration has been beneficially used in a variety of autoimmune diseases including MG [46–48], although its mode of action is still not clear. In order to delineate its mechanism of action and attempt to identify the active component in IVIG, we have first shown that IVIG has a suppressive effect on the clinical symptoms of ongoing EAMG that is associated with decreased AChR-specific cellular and humoral immune reactivity. Immunological analyses suggested that IVIG modulates EAMG by suppressing Th1 and B-cell proliferation but probably not by generation of Treg [49,50].

We have employed the EAMG model and have isolated a specific active fraction from pooled normal human immunoglobulins and have analyzed its immunosuppressive activity. We showed that chromatography of pooled human IVIG on immobilized immunoglobulins, isolated from either EAMG rats (Fig. 1) or from MG patients, results in a complete depletion of the suppressive activity of the IVIG preparation [51,52]. The suppressive activity could be partially recovered upon reconstitution of the activity-depleted IVIG with the eluted minute IVIG fractions that had been adsorbed onto the EAMG- or MG-specific columns (Fig. 1). These studies demonstrated that a disease-specific anti-immunoglobulin fraction present in IVIG preparations is essential for the suppressive effect of IVIG.

3.4. Immunomodulation by co-stimulatory molecules, cytokines and chemokines

Although antigen-specific treatment would be ideal for targeting the autoimmune response without affecting the entire immune system, in an already established disease, the antigen-specific approach might need to be supported by direct modulation of key immunological factors.

Cumulative studies on T- and/or B cell-mediated autoimmunity suggest that cytokine network and co-stimulatory signaling are important in disease pathogenesis. We showed that successful suppression of EAMG is accompanied by a shift in the cytokine profile and by down-regulation of co-stimulatory factors and that regulation of these molecules can be considered as a strategy for modulation of the autoimmune response in myasthenia. Therefore, we have tested the potential of this approach by treating myasthenic rats with antibodies either to pro-inflammatory cytokines [53], co-stimulatory factors [54] or chemokines [55]. We found that such treatments resulted in suppression of disease but acted via different mechanisms that could complement each other.

3.4.1. CD40L

CD40L is expressed on activated $CD4^+$ T cells, whereas CD40, the receptor for CD40L, is expressed on various APCs such as B cells, dendritic cells, and macrophages [56]. CD40L is involved in contact-dependent T cell help and is a predominant B cell co-stimulatory molecule expressed on activated T cells [57]. The role of CD40-CD40L in EAMG was studied in CD40L knockout mice ($CD40L^{-/-}$) [58], in which mice were completely resistant to EAMG induction and had diminished Th1 and Th2 responses as well as severely impaired T cell-dependent AChR-reactive B cell response. We have shown that treatment of rats with anti-CD40L antibodies starting at either the acute or chronic stage of EAMG leads to suppression of clinical symptoms of EAMG. The underlying mechanism of this

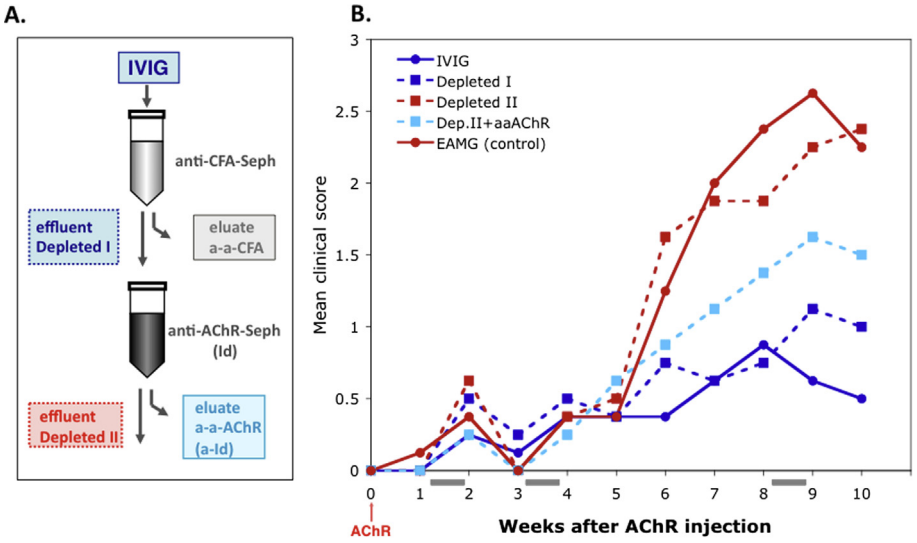

Fig. 1. A. Schematic presentation of IVIG fractionation on rat IgG columns. (A similar procedure has been used for fractionation of IVIG on human MG immunoglobulin [52]). B. Fractionation and reconstitution of the suppressive activity of IVIG on EAMG following chromatography on rat IgG columns. Mean clinical score of AChR-immunized rats ($n = 8$ for each group) following treatment with IVIG. Representative of five similar independent experiments. Bars at the bottom represent each a five-day treatment.
Figure taken from Fuchs et al., *J Neuroimmunol* [52].

suppression seems to be mediated by down-regulation of B7-2 and up-regulation of CTLA-4 levels. Anti-CD40L antibodies affected the humoral response, decreased Th1 but had no effect on Th2 and on T-cell proliferation [54] (Table 1).

3.4.2. IL-18

IL-18 is a pleiotropic pro-inflammatory cytokine that plays a key role in IFN-γ production and IL-12-driven Th1 phenotype development. IL-18 was reported to be involved in the pathogenesis of several diseases. In EAMG, IL-18 knockout mice were shown to be resistant to induction of disease [59]. We addressed the question whether antibodies to IL-18, given at different stages of EAMG, have an effect on disease progression, and attempted to study the underlying mechanism of this treatment. We have shown that anti-IL-18 treatment suppresses EAMG progression when treatment was initiated before the induction of EAMG or at either the acute or chronic stage of EAMG [53]. The suppression of EAMG by anti-IL-18 treatment seems to be mediated by increased levels of the immunosuppressive TGF-β as well as decreased AChR-reactive Th1 type cellular responses. The levels of Th2-type cytokines (IL-4 and IL-10) did not change by anti-IL-18 Ab treatment. The suppression was accompanied by down-regulation of CD40L and up-regulation of CTLA-4, a key negative immunomodulator (Table 1).

3.4.3. IP-10/CXCR3

The importance of chemokines and chemokine receptors in the pathogenesis of autoimmunity has been initially suggested in a number of animal models and was later supported by genetic evidence and clinical studies in humans. Chemokines constitute a superfamily of chemoattractant cytokines that mediate leukocyte recruitment into tissues in homeostasis and inflammation [60]. IFN-γ inducible protein 10 (IP-10) is a primary response gene that belongs to the CXC chemokine superfamily. It is a highly inducible chemoattractant for activated T cells, but has pleiotropic activities such as stimulation of monocytes and natural killer cells, bone marrow progenitor maturation, modulation of adhesion molecule expression and inhibition of angiogenesis [61]. We have found using microarray analyses (Fig. 2) that IP-10 is overexpressed in LNC of EAMG rats. Quantitative real time RT-PCR confirmed these findings and revealed up-regulated mRNA levels also of Mig and their common receptor, CXCR3 as well as of TNF-α and IL-1β that act synergistically with IFN-γ to induce IP-10 [55]. The relevance of IP-10/CXCR3 signaling in myasthenia was validated by similar observations in MG patients. A significant increase in IP-10 and CXCR3 mRNA levels in both thymus and muscle were observed in myasthenic patients compared to age-matched controls. CXCR3 expression in PBMC of MG patients was markedly increased in CD4+ but not in CD8+ T cells or CD19+ B cells. In order to assess the potential of IP-10/CXCR3 signaling to serve as a drug target in MG, we tested the effect of interference with IP-10/CXCR3 signaling in rat EAMG [62]. Two different approaches were used: (1) blocking IP-10 by IP-10-specific antibodies and (2) inhibiting the CXCR3 chemokine receptor by a CXCR3 antagonist. Treatment by either of these reagents led to suppression of ongoing EAMG. Treatment by IP-10-specific antibodies led to decreased mRNA expression of IP-10 and CXCL9 and increased expression levels of CXCR3 and the IP-10 inducer, IFN-γ, but had no significant effect on AChR-specific responses. Treatment by the CXCR3 antagonist led to a reduction in humoral and cellular AChR-specific responses but had no significant effects on the expression levels of CXCR3 and its ligands [55].

3.4.4. IL-6

IL-6 is a pleiotropic inflammatory cytokine produced by T cells, monocytes, macrophages and synovial fibroblasts and

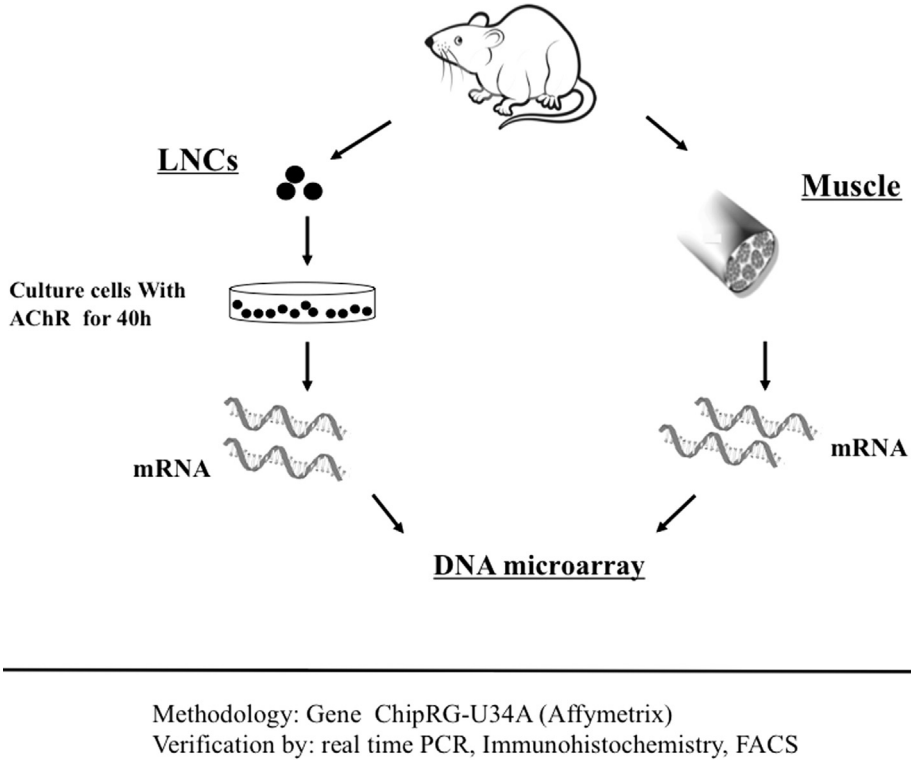

Fig. 2. A schematic diagram illustrating sample collection and preparation for DNA microarray analysis. Total RNA from popliteal LNC and muscle samples were collected from healthy control rats or from EAMG rats when they reached a clinical score of 2. Two RNA samples were used for each group, and each sample consisted of a pool from three individual rats. The preparation of cRNA, hybridization, washing, and labeling were performed according to the manufacturer's instruction. After scanning with the HP GeneArray scanner, the fluorescence intensity of each probe was quantified using MicroArray Suite 4.0 (Affymetrix). Genes showing a fold difference of greater than 2 between samples from EAMG and healthy rats were selected as leads for further evaluation by RT-PCR and for monitoring of their protein products by FACS and immunohistochemistry.

mediates various functions through its specific receptor (IL-6R). It acts as a regulator of B and T cell functions. The pro-inflammatory cytokine IL-6 is a potent factor in switching immune responses in vivo from the induction of Treg to pathogenic Th17 cells, two lymphocyte subsets with opposing activities in autoimmune diseases. We studied the Treg and Th17 cell compartments in EAMG and healthy control rats in order to assess whether the equilibrium between Treg and Th17 cells is perturbed in the disease [63]. We found that Th17 cell-related genes are up-regulated and Treg-related genes are down-regulated in EAMG. The shift in favor of Th17 cells in EAMG could be reversed by antibodies to IL-6. Administration of anti-IL-6 antibodies to myasthenic rats suppressed EAMG when treatment started at the acute or at the chronic phase of disease (Fig. 3). Suppression of EAMG by anti-IL-6 antibodies was accompanied by a decrease in the overall rat anti-AChR antibody titer and by a reduced number of B cells as compared with control treatment. Administration of anti-IL-6 antibodies led to down-regulation of several Th17-related genes including IL-17, IL-17R, IL-23R and IL-21 but did not affect the number of Treg cells in the lymph nodes. These data identify IL-6 as an important target for modulation of autoimmune responses [63].

3.5. *Phosphodiesterase inhibitors: therapeutic and steroid-sparing agents*

The identification of new genes that are associated with the induction and/or progression of EAMG may provide new drug targets for the treatment of myasthenia. The application of high-density oligonucleotide microarray technology to the research of autoimmune diseases enabled us to address questions ranging from fundamental disease mechanisms to improved treatment modalities (Fig. 2). Using the DNA microarray and RT-PCR analyses comparing myasthenic and healthy rats, we found higher levels of certain phosphodiesterases (PDE) types in LNC and muscles of EAMG rats [64].

PDEs are enzymes degrading the second messenger cAMP, which mediates and regulates essential intracellular processes There are 11 different PDE subtypes, but immune cells predominantly express PDE4, PDE3, and, to a lesser extent, PDE7 [65]. Since an increase in cAMP has been shown to inhibit inflammatory and immunological processes, PDEs have been proposed as targets for therapeutic intervention in pathologies such as allergies and autoimmune diseases [66]. Quantitative real-time PCR analysis indicated that EAMG is characterized by an increase in PDE subtypes 1, 3, 4, and 7 in LNC, and of PDE subtypes 2–4, and 7 in muscles [64]. A similar up-regulation of PDE was also observed in human MG [67]. The changes in PDE expression in EAMG and MG provided the rationale for our attempts to suppress and immunomodulate EAMG by pentoxifylline (PTX), a general PDE inhibitor [64]. This suppression was associated with down-regulation of humoral and cellular AChR-specific responses as well as down-regulation of PDE4, TNF-α, IL-18, IL-12, and IL-10 in LNC and of PDEs 1, 4, 7, and TNF-α in muscles. The expression of Foxp3, a transcription factor essential for $CD4^{+}CD25^{+}$ Treg function, was increased in splenocytes although the number of these cells remained unchanged. PTX also reduced the expression of the endopeptidase cathepsin-1, a marker of muscle damage, in EAMG muscles [64]. Since PTX is already being used in patients with other disorders with no serious side effects, these results justify well-designed clinical trials to test the efficacy of treating MG patients by PTX.

In a subsequent study, we evaluated the therapeutic potential of a combination of suboptimal doses of methylprednisolone (Solumedrol) and PTX in rat EAMG. This combined treatment resulted in a pronounced suppressive effect on EAMG and was by far more effective than each of these drugs administered separately at suboptimal doses [68]. The suppressive effect on EAMG was accompanied by decreased humoral and cellular responses to AChR as well as down-regulated mRNA expression levels of Th1 cytokines and IL-10 in LNC and of PDE-4 and cathepsin-1 in the muscle. These studies demonstrate the effectiveness of PTX not only as a potential

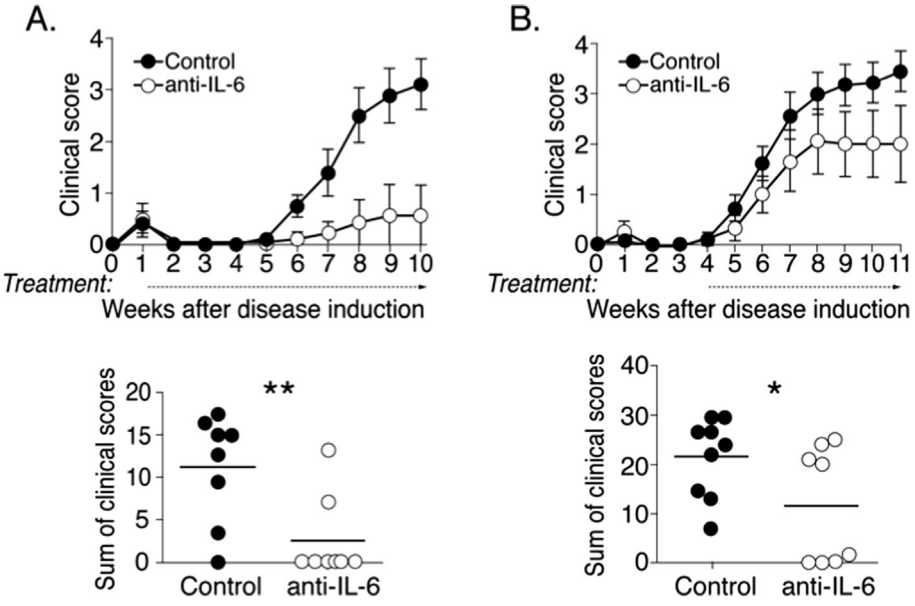

Fig. 3. Suppression of EAMG by anti-IL-6 treatment. Mean clinical scores (upper panel) and sums of cumulative weekly clinical score values for individual rats (lower panel) of rats treated by anti-IL-6 or normal goat IgG starting at the acute (A) or at the chronic phase (B) of EAMG. $N = 8$ for each group. Data represent one out of two or three independent experiments. $P < 0.0001$ in A and $P < 0.001$ in B. Analyzed by the two-way ANOVA test.
Figure taken from Aricha et al., *J Autoimmun* [63].

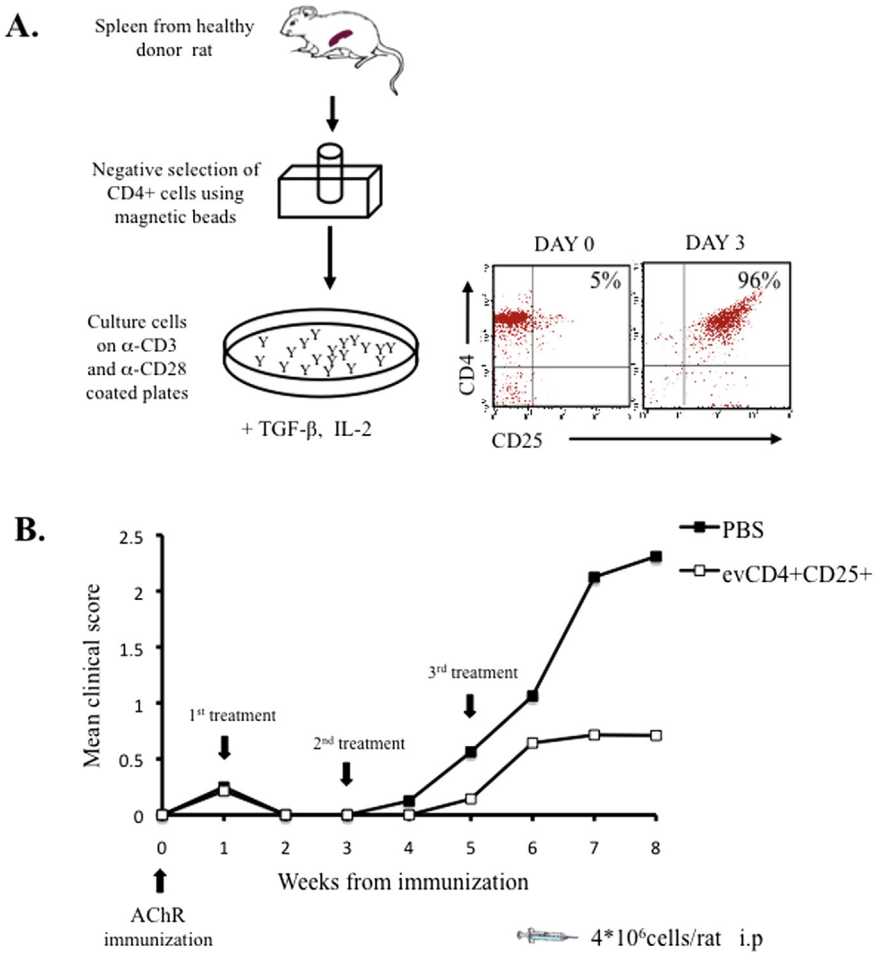

Fig. 4. A. Schematic illustration of ex-vivo generation of $CD4^+CD25^+$ Treg cells. B. Suppression of EAMG by *ex-vivo* generated Treg cells from healthy donors. Rats were administered i.p. with *ex vivo* generated $CD4^+CD25^+$ cells (evCD4$^+$CD25$^+$; 4×10^6/rat), or with PBS, starting 4 days after EAMG induction and every two weeks thereafter. Mean clinical scores of the different groups ($n = 8$ for each group). Representative of three independent experiments.
Figure taken from Aricha et al., *J Immunol* [80].

drug for MG but also as a steroid-sparing agent in the management of myasthenia [64,68].

3.6. Regulatory T cells

A subpopulation of suppressive $CD4^+$ T cells, termed regulatory T cells (Treg), has been recognized to play a central and prominent role in the generation and maintenance of peripheral tolerance. Most endogenous $CD4^+$ Treg constitutively expresses the CD25 molecule (IL-2 receptor alpha-chain).

Abnormalities in Treg, either in number or in function, have been reported in a series of human autoimmune diseases and in their corresponding experimental models. There is accumulating evidence that abnormalities within the Treg compartment are involved also in the pathogenesis of MG. Several groups have evaluated peripheral blood lymphocytes (PBL) and thymuses from patients to determine Treg number and function. Most of these studies have reported on normal to decreased number of $CD4^+CD25^{high}$ cells in PBL of MG patients compared to healthy controls [69–71]. A functional impairment of thymic Treg cells was found in the thymus of MG patients and may thus be involved in the onset of the autoimmune process [72]. Moreover, following successful immunosuppression or thymectomy, MG patients have elevated number of $CD4^+CD25^{high}$ cells compared to healthy controls [69,71].

In the mouse and rat EAMG, it was demonstrated by us and by others that Treg cells are involve in the suppressive action of various effective therapies [64,73–78]. Moreover, we found that the frequency of $CD4^+CD25^+Foxp3^+$ Treg cells within the spleen and PBL was decreased in EAMG rats and that Treg cells from myasthenic rats were less effective than Treg cells from controls in suppressing the proliferation of $CD4^+$T effector cells in response to ConA and of B cells in response to LPS [79]. These observations suggest that modulation of the $CD4^+CD25^+$ cell compartment could play a key role in the treatment of myasthenia.

As the number of naturally occurring Treg cells is low we developed an approach for large-scale *ex vivo* generation of functional Treg cells (Fig. 4A) [80]. Treg cells were generated *ex vivo* from $CD4^+$ splenocytes by stimulation with anti-CD3 and anti-CD28 antibodies in the presence of TGF-β and IL-2. The obtained cells expressed high levels of CD25, CTLA-4, and Foxp3, and were capable of suppressing *in vitro* proliferation of T cells from myasthenic rats in response to AChR. Administration of such *ex-vivo* generated Treg cells to myasthenic rats inhibited the progression of EAMG (Fig. 4B) and led to down-regulation of humoral AChR-specific responses and to decreased IL-18 and IL-10 expression. The number of $CD4^+CD25^+$ cells in the spleen of treated rats

remained unchanged, but the subpopulation of $CD4^+CD25^+$ cells expressing Foxp3 was significantly elevated [80]. These findings imply that Treg cells play a critical role in the control of myasthenia and could thus be considered as potential agents for the treatment of MG patients.

As ex-vivo generated Treg cells from sick animals do not have the same in vivo suppressive capacities as those from healthy donors, we have extended our research one step further toward autologous cellular treatment of patients. The objective of a recent ongoing study in our lab is to develop a protocol for generating Treg cells from sick objects that would be able to suppress effectively the disease in vivo. In this project, bone marrow (BM) cells were cultured in the presence of GM-CSF and gave rise to a population of CD11c+ (BMDCs), which expanded upon co-culture with $CD4^+$ T cell to a highly expressing (70–90%) $Foxp3^+$ Treg cells. *In vitro* assay showed a dose-dependent manner in the suppression of T effector cells proliferation and was similar in extent, whether Treg cells were obtained from either healthy or sick donors. In addition, both Treg cells inhibited similarly the secretion of IFN-γ from activated splenocytes. Preliminary experiments show that *i.v.* administration of ex-vivo generated Treg cells to EAMG rats, modulate the disease following a single treatment. Similar disease inhibition was achieved when Treg cells were taken from either healthy or sick donors. The disease suppression is accompanied by reduced levels of total AChR-specific antibodies in the serum and elevated numbers of Treg cells in the spleen. We hope that this experimental modality can be considered as a personalized treatment approach for human myasthenia.

4. Concluding remarks

In this review in a volume in honor of Michael Sela and Ruth Arnon we report on studies from our lab on EAMG. This paper is part of a special issue devoted to Michael Sela and Ruth Arnon and is part of a yearly effort by the Journal of Autoimmunity to recognize truly distinguished figures in immunology, scientists that have contributed to basic research with its implications to patient care; previous honorees have included Ian Mackay, Noel Rose, Pierre Youinou and Abul Abbas [81–83].

The subject of the acetylcholine receptor (AChR) and its associated autoimmune disease, myasthenia gravis (MG), has been a fascinating one. We feel lucky to have been introduced to it at its early stages. In this review we have chosen to concentrate mainly on a particular part of our research in which several treatment approaches for EAMG have been proposed or employed. We feel that this is in line with the interest of Michael Sela and Ruth Arnon, who more than anybody else, have excelled in translating their research work into practice. I have been privileged to be associated with both Michael and Ruthie throughout my career. Michael has been and remained my mentor who introduced me to the exciting world of scientific research and taught me how to apply our education in chemistry for our research in immunochemistry and immunology. Both Michael and Ruthie have been my close colleagues and friends during all my years at the Weizmann Institute and serve as role models.

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