IL-12 is involved in the induction of experimental autoimmune myasthenia gravis, an antibody-mediated disease

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IL-12 has been shown to be involved in the pathogenesis of Th1-mediated autoimmune diseases, but its role in antibody-mediated autoimmune pathologies is still unclear. We investigated the effects of exogenous and endogenous IL-12 in experimental autoimmune myasthenia gravis (EAMG). EAMG is an animal model for myasthenia gravis, a T cell-dependent, autoantibody-mediated disorder of neuromuscular transmission caused by antibodies to the muscle nicotinic acetylcholine receptor (AChR). Administration of IL-12 with *Torpedo* AChR (ToAChR) to C57BL/6 (B6) mice resulted in increased ToAChR-specific IFN-γ production and increased anti-ToAChR IgG2a serum antibodies compared with B6 mice primed with ToAChR alone. These changes were associated with earlier and greater neurophysiological evidence of EAMG in the IL-12-treated mice, and reduced numbers of AChR. By contrast, when IL-12-deficient mice were immunized with ToAChR, ToAChR-specific Th1 cells and anti-ToAChR IgG2a serum antibodies were reduced compared to ToAChR-primed normal B6 mice, and the IL-12-deficient mice showed almost no neurophysiological evidence of EAMG and less reduction in AChR. These results indicate an important role of IL-12 in the induction of an antibody-mediated autoimmune disease, suggest that Th1-dependent complement-fixing IgG2a anti-AChR antibodies are involved in the pathogenesis of EAMG, and help to account for the lack of correlation between anti-AChR levels and clinical disease seen in many earlier studies.

Key words: Experimental autoimmune myasthenia gravis / IL-12 / Th1 / IgG2a / Muscle nicotinic acetylcholine receptor

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1 Introduction

Myasthenia gravis (MG) is a T cell-dependent, autoantibody-mediated autoimmune disorder characterized by weakness and fatigability of voluntary muscles [1]. These symptoms follow an autoimmune attack directed against the nicotinic acetylcholine receptor (AChR) on the post-synaptic end plate leading to reduced AChR numbers. Antibodies and T cells specific for AChR epitopes can be detected in the blood from MG patients [2–5], while IgG and complement components have been localized at the end-plate junction [6]. The loss of functional AChR that occurs in MG patients is therefore thought to result from a T helper cell-induced antibody response to AChR, leading to complement-mediated lysis of AChR-rich membranes [6]. In addition, increased degradation of AChR and pharmacological block of AChR function contribute to the pathogenesis of disease [2, 7].

Differentiated mouse $CD4^+$ T cells produce a restricted set of cytokines, allowing their subdivision into two discrete populations: Th1, characterized by the secretion of IL-2 and IFN-γ, and Th2, which selectively produce IL-4, IL-5 and IL-10 [8]. Th2 cells play a predominant role in

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Abbreviations: AChR: Muscle nicotinic acetylcholine receptor **EAMG:** Experimental autoimmune myasthenia gravis **EMG:** Electromyography **PDR:** Positive decremental response **RST:** Repetitive stimulation test

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immediate-type hypersensitivity, as IL-4 is the critical stimulus inducing a switch to IgE antibody production [9]. Conversely, Th1 cells activate macrophages, mediate delayed-type hypersensitivity and promote IgG2a synthesis [10]. Th1 cells are considered to be involved in the induction of experimental autoimmune diseases such as EAE, insulin-dependent diabetes mellitus and collagen-induced arthritis [11] as well as in human organ-specific autoimmune diseases [12], whereas Th2 cells may have a protective role [11]. The development of Th1 and Th2 cells is primarily influenced by the cytokine milieu during the initial phase of the immune response, in which IL-12 and IL-4, respectively, play a decisive role [9, 13].

IL-12 is a heterodimer composed of two covalently linked glycosylated chains, p35 and p40, encoded by distinct genes [14, 15]. This cytokine, produced predominantly by activated monocytes and dendritic cells but also by other cell types such as neutrophils [16], enhances proliferation and cytolytic activity of NK and T cells, and stimulates their IFN-γ production [17]. Most importantly, IL-12 induces the development of Th1 cells *in vitro* [18, 19] and *in vivo* [20]. In addition, IL-12 is a potent cofactor stimulating growth, IFN-γ synthesis, and cell adhesion of already differentiated Th1 cells [21]. The key role of IL-12 in the induction of Th1 cell-mediated autoimmune diseases is clearly documented in several experimental models [22]. Evidence for the critical role of endogenous IL-12 in the pathogenesis of autoimmune diseases is provided by the reduced incidence and severity of collagen-induced arthritis in IL-12-deficient mice [23].

A similar distinction between Th1 and Th2 cells also applies in humans [24], but their role in MG has not been clarified. The involvement of both AChR-reactive Th1 and Th2 cells has been documented [25, 26] but it is not yet clear which are essential in driving the production of pathogenic anti-AChR antibodies. Experimental autoimmune myasthenia gravis (EAMG) is an animal model of MG, induced by immunization with purified AChR, usually from the *Torpedo* electric organ (ToAChR) [27]. In this study, we analyzed the role of Th1 cells in EAMG by evaluating the Th cell phenotype, IgG1 and IgG2a antibodies, and neurophysiological and biochemical evidence of EAMG in IL-12-treated, IL-12-deficient, and normal C57BL/6 (B6) mice primed with ToAChR. The results indicate that IL-12 plays an important role in the pathogenesis of EAMG and suggest that anti-AChR IgG2a antibodies induced by IL-12-driven Th1 cells specific for ToAChR are critically involved.

2 Results

2.1 The anti-ToAChR Th1 response induced by priming with ToAChR is enhanced in mice co-injected with IL-12 and is reduced in IL-12-deficient mice

Lymph node cells (LNC) from B6 mice immunized with ToAChR 15 days previously, responded *in vitro* to ToAChR (Fig. 1A). The ToAChR-induced T cell proliferation was enhanced in LNC from mice immunized with ToAChR plus 0.5 or 5 μg/mouse IL-12, as compared to LNC from mice immunized with ToAChR alone. LNC from mice injected with 5 μg/mouse IL-12 only did not show any proliferative response to ToAChR. IFN-γ was determined in supernatants from the same cultures. LNC from ToAChR-primed mice secreted IFN-γ dose-dependently in response to ToAChR (Fig. 1B). The IFN-γ production was 50-fold higher in mice immunized with ToAChR and 5 μg IL-12 (range: 0.4–52.6 ng/ml) and 15-fold higher in mice immunized with ToAChR and 0.5 μg IL-12 (range: 0–15.3 ng/ml) as compared to mice immunized ToAChR only (range: 0–0.9 ng/ml).

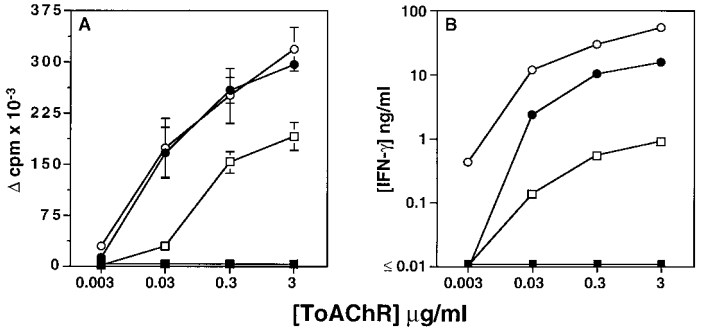

Figure 1. Proliferative responses and IFN-γ production by LNC from ToAChR-primed B6 mice restimulated *in vitro* with ToAChR. B6 mice immunized 15 days before culture with ToAChR plus 0.5 (●) or 5 (○) μg /mouse IL-12 show higher proliferative responses as well as IFN-γ production in response to ToAChR stimulation compared to mice immunized with ToAChR alone (□). Mice injected with 5 μg IL-12 alone (■) did not show any proliferative response against ToAChR or any IFN-γ production.

Three months after immunization, $CD4^+$ T cells from ToAChR-primed mice were restimulated *in vitro* with antigen and analyzed by cytofluorimetry for intracytoplasmic production of IFN-γ and IL-4 following stimulation with PMA/ionomycin. Only 1.2 % of ToAChR-restimulated LNC from mice primed with ToAChR and IL-12 were IL-4-producing $CD4^+$ cells compared to 4 % in LNC from B6 mice receiving ToAChR alone. The IFN-γ-producing cells were not different between the two groups. To test the role of endogenous IL-12 in the response to ToAChR, we performed a similar analysis on ToAChR-restimulated cells from B6 IL-12-deficient mice. T cells from IL-12 p40-deficient mice showed higher (6 %) ToAChR-stimulated IL-4- and lower (4 %) IFN-γ-producing $CD4^+$ cells compared to values of 4 % and 11 %, respectively, in cells from normal B6 mice (Fig. 2, upper panels). Differences in cytokine secretion were also demonstrated in the 72-h culture supernatants of cells from these mice (Fig. 2, lower panel). ToAChR-stimulated cells from mice injected with ToAChR and IL-12 secreted fivefold less IL-4 compared to mice immunized with ToAChR alone, whereas IL-4 secretion was slightly higher in IL-12-deficient mice. IFN-γ secretion was threefold higher in mice given IL-12 than in mice immunized with AChR alone, and nearly undetectable in LNC from IL-12p40-deficient mice, consistent with previous results in IL-12p40-deficient mice [28]. The quantitative discrepancies between cytokine secretion and intracytoplasmic cytokine production (Fig. 2) are probably related to the stimulation by antigen only (lower panel) or by antigen followed by PMA/ionomycin (upper panel), respectively. Indeed, a direct comparison between cytokine levels secreted, after 3 days of culture, in response to antigen only or to antigen followed by a 4-h incubation with PMA/ionomycin has demonstrated only a slightly higher cytokine production in the latter group, but no qualitative differences (not shown).

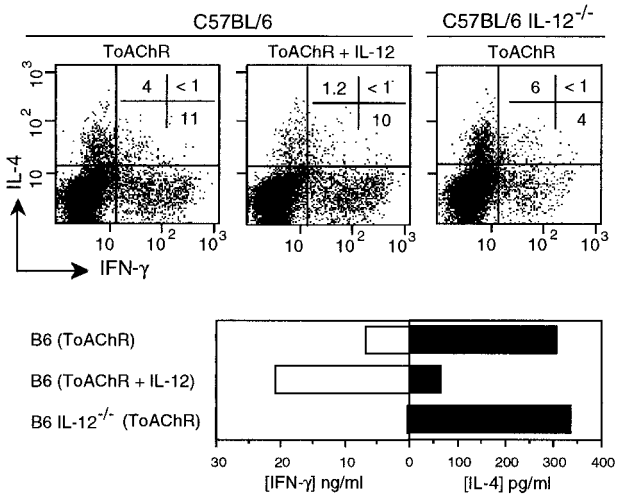

Figure 2. IL-4 and IFN-γ-producing cells. LNC from B6 mice primed with ToAChR (upper left panel) or ToAChR and IL-12 (upper central panel), and LNC from IL-12-deficient B6 mice primed with ToAChR (upper right panel) were cultured at 6×10^5 cells/well with 3 μg/ml ToAChR. After 3 days of culture, LNC were washed, rested for 24 h and restimulated with PMA/ionomycin for 4 h with brefeldin A added for the last 2 h. Cells were then fixed and stained for intracellular IFN-γ and IL-4, as well as for surface CD4, as described in Sect. 4.4. Percentages of IFN-γ-producing cells detected by FCM analysis are shown in the abscissa and of IL-4 producing cells in the ordinate. Secretion of IFN-γ and IL-4 by ToAChR-stimulated cells (lower panel) was assessed in 72-h culture supernatants by two-site ELISA.

2.2 Electromyographic response to repetitive nerve stimulation

None of the mice were overtly weak, probably because of the high safety factor for neuromuscular transmission in this species [27]. To determine whether there were subclinical changes in the efficiency of neuromuscular transmission, repetitive nerve stimulation was performed, before and after giving d-tubocurarine to reduce the safety factor (Fig. 3). None of the ToAChR-immunized mice, or mice injected with IL-12 alone, showed a positive decremental response (PDR) 15 days after immunization (not shown). After the first boost, however, statistically significant PDR were seen, both before and after d-tubocurarine (Fig. 4A), in 2/7 and 9/21 mice co-injected with IL-12 but were less frequent or absent in wild-type or IL-12-deficient B6 mice injected with ToAChR alone (Table 1). In all mice exhibiting PDR the electromyography (EMG) abnormalities were reversed within 15–30 min following edrophonium chloride administration (not shown). After the second boost (Fig. 4B), a statistically significant PDR was observed in 5/13 IL-12-injected but only 1/6 IL-12-deficient mice. After d-tubocurarine administration, a substantial number of both wild-type and IL-12-treated mice showed PDR (Table 1). These results indicate that administration of exogenous IL-12 accelerates the onset of EAMG in wild-type mice, whereas the disease does not develop in the majority of mice lacking endogenous IL-12.

2.3 Reduced muscle AChR content correlates with reduced EMG response

The amount of AChR in unimmunized B6 mice was 795 ± 203 (mean ± SD, $n = 6$) fmol/ml of extract. In mice given ToAChR alone, the AChR content was reduced to 405.1 ± 115.7 fmol/ml (49 %, $n = 9$), not significantly dif-

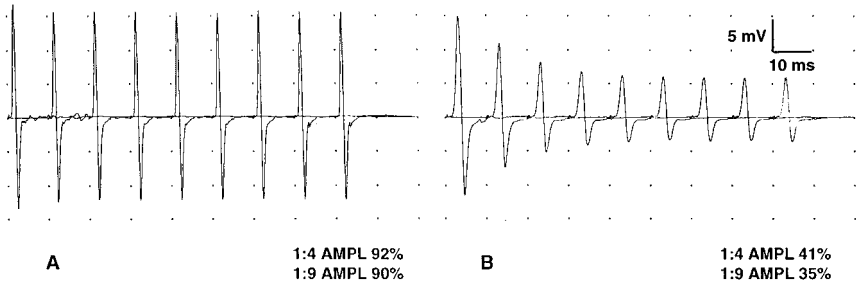

Figure 3. Electrophysiological assessment of EAMG. (A) shows a representative RST in a B6 mouse in which no PDR was recorded in basal conditions. The trace in (B), recorded in the same mouse 5 min after i.p. administration of 0.15 μg/g d-tubocurarine, shows a PDR of more than 10 % in the fourth and in the ninth evoked response amplitude (AMPL) compared to the first response, indicating a disturbance in neuromuscular transmission. The percent values refer to the amplitude ratio between either the fourth or the ninth and the first compound motor action potential during RST. The values in the ordinate are in mV and the values in the abscissa ms, as indicated in the scale.

ferent from that in mice co-injected with IL-12 in which it was 366.0 ± 116.3 fmol/ml (39 %, n = 9). However, the AChR content in IL-12-deficient mice, 478.9 ± 113.8 fmol/ml (54 %, n = 9), was significantly higher than that in mice co-injected with IL-12 ($p < 0.05$, Student's t test for unpaired data). Overall, the reduced EMG response correlated with the reduction in muscle AChR (p = 0.01, Fig. 5), suggesting that the neurophysiological signs reflect the immune-mediated pathological damage at the neuromuscular junction.

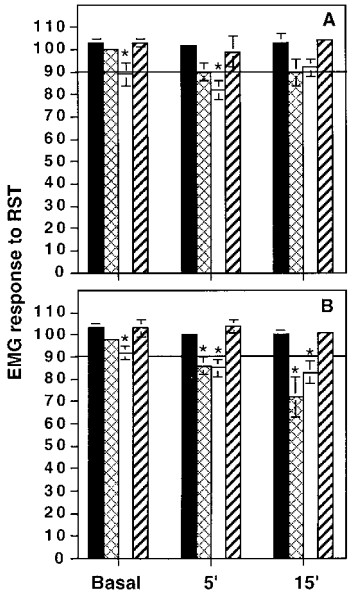

Figure 4. Electrophysiological assessment of EAMG. The EMG response to RST was recorded after the first (A) and the second (B) boost in B6 mice injected with CFA only (■), To AChR (▩), ToAChR plus IL-12 (□), or in IL-12-deficient B6 mice injected with ToAChR (▨). The EMG response was recorded after the 9th stimulus at 5 Hz in basal conditions and 5 min or 15 min after i.p. administration of 0.15 μg/g d-tubocurarine. The results represent the mean decrement and SEM of 6–21 mice/group. * $p < 0.05$ *vs.* B6 mice injected with CFA only or IL-12-deficient B6 mice injected with ToAChR. Line drawn at 10 % decrement.

2.4 IL-12 increases IgG2a antibodies to AChR

Th1 cells favor the production of antibodies bearing IgG2a isotype, whereas Th2 cells promote IgG1 antibody responses. We analyzed the anti-ToAChR serum antibody isotypes every 2 weeks for 3 months in the three groups of mice. Compared to results in mice immunized with ToAChR alone, IgG2a ToAChR-specific antibodies were detected earlier and rose higher in mice co-injected

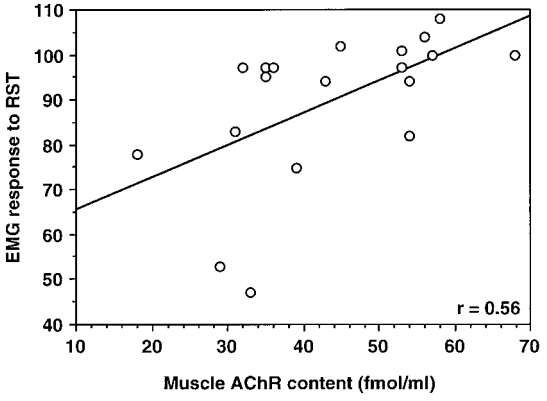

Figure 5. Relationship between EMG response to repetitive stimulation and muscle AChR content. ToAChR-primed mice were analyzed, after the second boost, as described in Fig. 3. There was a significant positive correlation between EMG response and muscle AChR content (p = 0.01).

Table 1. Decremental muscular response recorded by repetitive nerve stimulation test

Boosts received	Type of recording	B6 ToAChR	B6 IL-12$^{-/-}$ ToAChR	B6 ToAChR + IL-12
One	Basal	0/6* (0)	0/9 (0)	2/7 (29)
	After d-tubocurarine**	4/13 (31)	1/8 (12)	10/21 (48)
Two	Basal	0/8 (0)	1/6 (17)	5/13 (38)
	After d-tubocurarine	6/8 (75)	0/5 (0)	7/12 (58)

* Values (%) represent the number of positive *vs.* total number of mice analyzed.
** Administration of d-tubocurarine was performed as described in Sect. 4.5.

with IL-12 (Fig. 6A), whereas IgG1 anti-ToAChR antibodies were slightly reduced (Fig. 6B). Conversely, IL-12 p40-deficient mice showed reduced production of anti-ToAChR IgG2a and highly increased IgG1 antibodies as compared with ToAChR-primed normal B6 mice. No anti-ToAChR serum antibodies were found in mice injected with IL-12 only (data not shown). The relationship between the IgG isotypes of anti-ToAChR antibodies in individual mice is shown in Fig. 6C.

As in previous studies [27], antibodies reacting with extracted mouse AChR were highly variable, with titers ranging from 0.1 to 2.7 nM (data not shown). However, there was a significant correlation between the serum levels and the proportion of muscle AChR that had IgG bound to it ($r = 0.88$; $p < 0.001$, $n = 21$, Fig. 7), with higher mean serum levels and IgG bound in IL-12-deficient mice than in the other two groups (data not shown). To investigate why this bound antibody was not more pathogenic, we examined the IgG subclasses by indirect immunofluorescence. IgG2a was demonstrated at muscle end plates in 3/4 (75 %) B6 mice immunized with ToAChR and in 4/6 (67 %) mice immunized with ToAChR and IL-12, but only in 1/7 (14 %) B6 IL-12-deficient mice. The difference between the latter two groups was statistically significant ($p < 0.05$; χ^2 test). Conversely, IgG1 was found at neuromuscular junctions in all B6 IL-12-deficient mice, but only in 50–75 % of the other two groups.

3 Discussion

This study provides evidence for the involvement of Th1 cells and IL-12 in the pathogenesis of EAMG, an animal model for the autoantibody-mediated human autoimmune disease MG. These findings were unexpected because EAMG is an antibody-mediated autoimmune disease, and IL-12 might have been predicted to amelio-

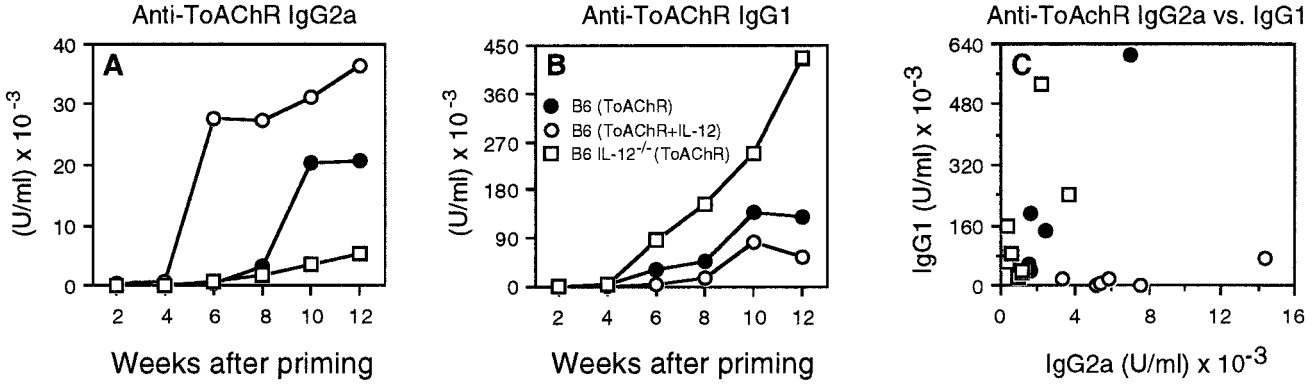

Figure 6. Anti-ToAChR IgG1 and IgG2a serum antibody isotypes. Anti-ToAChR IgG2a (A) and IgG1 (B) antibodies were measured in B6 mice primed with ToAChR (●) or ToAChR plus IL-12 (○), and in IL-12-deficient B6 mice primed with ToAChR (□). Mice were bled every 2 weeks after priming and ToAChR-specific antibodies were quantified by ELISA in pooled sera from each group. Results are expressed as serum isotype concentration in U/ml. (C) shows anti-ToAChR IgG2a and IgG1 serum antibody levels in individual mice.

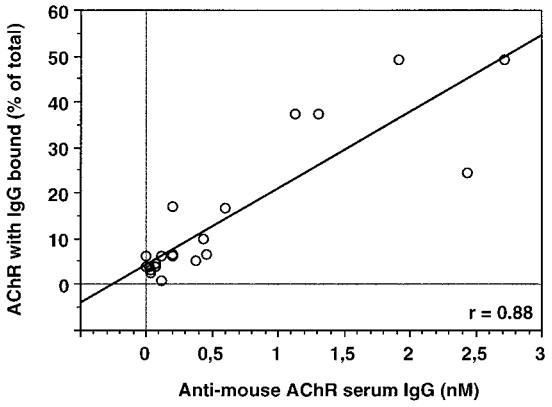

Figure 7. Correlation between anti-mouse AChR serum IgG and the proportion of muscle AChR that had IgG bound to it in ToAChR-primed mice. ToAChR-primed mice were analyzed after the second boost as described in Sect. 4.6.

rate rather than accelerate EAMG. However, when we immunized C57BL/6 mice with purified AChR, we found that co-administration of IL-12 produced greater AChR loss and greater evidence of neuromuscular defect than immunization with purified AChR alone. Both groups of mice showed Th1 responses to ToAChR with low IL-4 secretion, raised serum IgG2a antibodies to ToAChR, and deposits of IgG2a at the neuromuscular junctions. By contrast, IL-12-deficient mice, which exhibited little neurophysiological evidence of EAMG, had increased numbers of IL-4-producing cells, with reduced numbers of IFN-γ-producing cells and almost undetectable IFN-γ secretion *in vitro*. Moreover, the serum anti-AChR antibodies in IL-12-deficient mice were predominantly IgG1, and almost only IgG1 was deposited at the neuromuscular junctions. Thus, these results suggest that EAMG in normal C57BL/6 mice is associated with Th1-dependent IgG2a antibodies, that the disease can be enhanced by exogenous IL-12, and that immunization with AChR in IL-12-deficient mice is associated principally with less pathogenic IgG1 antibodies.

IL-12 has been shown to be involved in the pathogenesis of several autoimmune diseases that are principally T cell mediated, notably EAE [29], insulin-dependent diabetes mellitus [30], collagen-induced arthritis [31], autoimmune colitis [32] and uveoretinitis [33]. In these conditions Th1 cells are considered as effector cells capable of inducing disease via secretion of pro-inflammatory cytokines. However, so far, IL-12 has never been implicated in any antibody-mediated autoimmune disease. The development of lupus nephritis in New Zealand B/W F_1 mice could be prevented by anti-IL-4 but not by anti-IL-12 antibodies [34]. Even the IL-12-induced acceleration of spontaneous lupus-like autoimmune disease in MRL/lpr mice has been considered to be due to increased production of nitric oxide rather than autoantibodies [35].

It is possible that Th1 cells can directly attack the neuromuscular junction in IL-12-treated mice. IFN-γ-deficient mice were resistant to EAMG and this was associated with greatly reduced levels of both IgG1 and IgG2a antibodies specific for mouse AChR [36]. Conversely, transgenic expression of IFN-γ at the neuromuscular junction provoked an autoimmune humoral response resembling MG, characterized by infiltration of mononuclear cells and by autoantibody deposition at motor end plates, but these mice did not have detectable antibodies to mouse AChR [37]. In sera from our B6 mice injected with ToAChR and IL-12, however, there were raised levels of antibodies to ToAChR and to mouse AChR, and muscle AChR had IgG bound to them consistent with a pathogenic role for antibodies. A possible explanation for the pathogenicity of IL-12-induced Th1 cells in EAMG is therefore that they induce increased production of complement-fixing anti-ToAChR IgG2a antibodies. IL-12 has been shown to up-regulate by 2–3 orders of magnitude the *in vivo* synthesis of complement-fixing IgG subclasses [38]. Complement-fixing anti-ToAChR antibodies could thus play an essential role in reducing the efficiency of neuromuscular transmission to an extent that results in d-tubocurarine-induced decrement. In mice, in contrast to humans, the safety factor for neuromuscular transmission is high and obvious weakness is only seen when the efficacy of transmission is severely impaired [27]. Therefore, in mice given IL-12 the extra deficit induced by complement-mediated damage to the neuromuscular junction must have been sufficient to uncover the defect in transmission. By contrast, in IL-12-deficient mice, the serum anti-ToAChR antibodies were predominantly of IgG1 isotype, mainly IgG1 was found at the neuromuscular junctions and AChR were less reduced. Thus, the deficit in neuromuscular transmission correlates with the Th1-dependent IgG2a anti-ToAChR antibodies rather than the Th2-dependent IgG1 antibodies.

These results are important because they may explain the lack of correlation between antibody levels and clinical severity that has been reported in many previous studies of EAMG [27]. Few studies have looked at the relative roles of different subclasses of anti-AChR antibodies, particularly with respect to their potential to induce complement-dependent damage. In mice with anti-AChR secreting hybridomas, antibody bound to AChR and AChR loss was achieved with mAb of IgG1, 2a and 2b subclasses [39], but the effects were not titrated. With passive transfer of rat mAb to rats, it appeared that the epitope specificity was more important than the subclass [40], but antibodies of the IgG2b subclass, the rat analogue of mouse IgG2a, were not

tested. On the other hand, rat IgG2b anti-AChR mAb enhanced presentation of AChR to specific T cells, whereas other subclasses had little effect [41]. EAMG can also be induced in mice by injection of purified mouse AChR without adjuvant [42]. It will be interesting to see whether this form of the disease is also associated with Th1-dependent IgG2a antibodies.

Are these results relevant to the human disease? Although AChR-specific $CD4^+$ cells from MG patients have been shown to secrete both Th1 (IFN-γ) and Th2-type (IL-4) cytokines indicating that both subsets, as well as Th0 cells, may be involved in disease pathogenesis [25, 26, 43], anti-AChR antibodies show a predominance of the Th1-dependent complement-fixing IgG1 isotype [44], the human counterpart of mouse IgG2a [10]. In addition, the concentration of IgG1 antibodies increases significantly with MG severity [45] and complement-dependent mechanisms are clearly important [6]. Nevertheless, the pathogenicity of individual human antibodies has not yet been examined.

In IL-12-deficient mice the defective Th1 cell development is in itself sufficient, in most cases, to prevent EAMG. $CD4^+$ as well as $CD8^+$ T cells were found to secrete IFN-γ and IL-4 and both cytokines have been implicated in the development of EAMG [46]. This is consistent with results of AChR-induced immunointervention in EAMG. Tolerance induction by oral or nasal administration of AChR has been shown to reduce AChR-reactive IFN-γ and IL-4 while enhancing TGF-β mRNA-expressing cells and this was associated with decreased muscular weakness [47]. However, our results do not support a pathogenic role for IL-4-producing cells in EAMG. In fact, IL-12-deficient B6 mice primed with ToAChR, which develop almost no EAMG, show an increased percentage of ToAChR-stimulated IL-4-producing cells as compared to controls. Nevertheless, EAMG clearly develops in a few IL-12-deficient mice, in analogy with results obtained in the collagen-induced arthritis model [23]. It would be interesting to see whether other cytokines, such as IGIF/IL-18 [48], may contribute to the disease process.

In conclusion, the present results indicate that EAMG involves an IL-12-dependent autoimmune response mediated by AChR-specific Th1 cells which promote the synthesis of pathogenic, complement-fixing, IFN-γ-driven anti-AChR antibody isotypes. Targeting IL-12 may therefore be beneficial not only in T cell-mediated [49], but also in antibody-mediated autoimmune diseases.

4 Materials and methods

4.1 Mice and immunizations

Six to eight week-old B6 mice of either sex were obtained from Charles River (Calco, Italy). IL-12 p40-deficient B6 mice (B6 $IL12^{-/-}$) [28] were kindly provided by J. Magram (Hoffmann-La Roche Inc., Nutley, NJ). Mice were immunized s.c. in the flanks and at the tail base with 0.2 ml of an emulsion containing 50 μg ToAChR, obtained using a modification of the method described by Klimkowsky [50] and kindly provided by B. Fairclough (Davis, CA), in IFA (Difco, Detroit, MI) supplemented with 4 mg/ml of *Mycobacterium tuberculosis* (strain H37Ra; Difco). Mice were boosted with ToAChR in IFA 1 and 2 months after the first immunization. IL-12 was incorporated in the emulsion of the first immunization as indicated. Recombinant mouse IL-12 was produced in serum-free medium by transfected CHO cells and purified by sequential chromatography as described [51]. The IL-12 batch used was >95 % pure, as assessed by SDS-PAGE analysis, and contained <5 U/mg endotoxin, as determined by the *Limulus* amebocyte assay. IL-12 was kindly provided by M.K. Gately (Hoffmann-La Roche Inc., Nutley, NJ).

4.2 T cell proliferation

For T cell proliferation assays, draining lymph nodes were removed and 4×10^5 cells/well were cultured in 96-well culture plates (Costar, Cambridge, MA) in synthetic HL-1 medium (Ventrex Laboratories, Portland, ME) supplemented with 2 mM L-glutamine and 50 μg/ml gentamicin (Sigma, St. Louis, MO) with the indicated ToAChR concentrations. Cultures were incubated for 3 days in a humidified atmosphere of 5 % CO_2 in air and pulsed 8 h before harvesting with 1 μCi [^{3}H]dThd (40 Ci/nmol, The Radiochemical Centre, Amersham, GB). Incorporation of [^{3}H]dThd was measured by liquid scintillation spectrometry.

4.3 Cytokine determination

IFN-γ and IL-4 were quantified by two-site sandwich ELISA. For IFN-γ, polyvinyl microtiter plates (Falcon 3012) were coated with AN-18.17.24 mAb [52] in carbonate buffer. Samples (5 μl/well) diluted in test solution (PBS containing 5 % FCS and 1 g/l phenol) were incubated together with 50 μl peroxidase-conjugated XMG1.2 mAb [53]. After overnight incubation at room temperature, bound peroxidase was detected by 3,3',5,5'-tetramethylbenzidine (Fluka Chemical Co., Ronkonkoma, NY), and adsorbance read at 450 nm with an automated microplate ELISA reader (MR5000, Dynatech Laboratories Inc., Chantilly, VA). For IL-4 determination two-site ELISA was performed with paired mAb from PharMingen. For capture the mAb were BVD4-1D11 or 11B11. Samples were titrated in test solution and incubated overnight at 4 °C. To detect bound IL-4, plates were incubated

with biotinylated BVD6-24G2 mAb in test solution. After washing, the bound biotinylated antibodies were revealed by an additional 30-min incubation with alkaline phosphatase-conjugated streptavidin (Jackson Immunoresearch Laboratories, Avondale, PA) diluted 1/5000. The plates were washed again and incubated with the developing substrate *p*-nitrophenylphosphate disodium (Sigma) in diethanolamine buffer pH 9.6. The reaction was stopped by adding 50 μl/well 3N NaOH and absorbance was read at 405 nm. Cytokines were quantified from two to three titration points using standard curves generated by purified recombinant mouse cytokines and results expressed as cytokine concentration in ng/ml. Detection limits were 15 pg/ml for IFN-γ, and 3 pg/ml for IL-4..

4.4 ToAChR-induced intracellular synthesis of IFN-γ or IL-4

Immune LNC (6×10^5 cells/well) were cultured in 96-well culture plates in synthetic HL-1 medium with 30 μg/ml ToAChR. After 72 h of culture cells were harvested, washed and recultured for additional 72 h in RPMI 1640 medium supplemented with 2 mM L-glutamine, 50 μg/ml gentamicine, 50 μM 2-ME (Fluka Biochemica) and 10 % FCS (Sigma). After culture, living cells separated on Ficoll gradients were restimulated with PMA (1 μg/ml) and ionomycin (50 ng/ml) for 4 h at 37 °C, with 10 μg/ml brefeldin A (Novartis Ltd., Basel, Switzerland) added for the last 2 h to prevent egress of newly synthesized proteins from the endoplasmic reticulum. Cells were resuspended in PBS containing 10 μg/ml brefeldin A before adding an equal volume of 4 % formaldehyde. After fixing for 20 min at room temperature, cells were stained for IFN-γ and IL-4 using the method described by Openshaw et al. [54]. Reagents for intracytoplasmic staining contained 1 % FCS, 0.5 % saponin (Sigma) and 0.1 % sodium azide, and all incubations were performed at room temperature. Cells were washed, preincubated for 10 min with PBS/FCS/saponin, and then incubated with FITC-conjugated rat anti-mouse IFN-γ (XMG1.2, PharMingen) and PE-conjugated rat anti-mouse IL-4 (11B11, PharMingen) or with FITC- and PE-labeled rat IgG1, kappa isotype controls (R3-34, PharMingen). After 30 min, cells were washed twice with PBS/FCS/saponin and then with PBS containing 5 % FCS without saponin to allow membrane closure. Cell membranes were then stained with CyChrome-labeled anti-CD4 (L3T4, PharMingen) for 15 min at room temperature. Analysis was performed with a FACScan® flow cytometer (Becton Dickinson and Co. Mountain View, CA) equipped with CellQuest software and 50 000 events were acquired.

4.5 Electrophysiological assessment of EAMG

Mice were anesthetized by i.p. injection of chloral hydrate (100 mg/g weight), placed under a heating lamp and immobilized with tape. A ground electrode was placed on the back of the mouse. EMG was performed by using a Sapphire EMG machine (Vickers Medelec, Woking, GB). The sciatic nerve was stimulated with a needle-electrode inserted into sciatic notch area. A monopolar recording wire electrode was used to record compound muscle action potential (cMAP) from the gastrocnemius muscle with an anode wire electrode inserted s.c. into the tendon of the above muscle. Repetitive stimulation test (RST) was performed by delivering one set of two 3 Hz and one set of two 5 Hz supramaximal stimulations with a 1-min interval between each stimulation. Evoked cMAP were recorded simultaneously in the same period. A PDR was defined as decrement equal or greater than 10 % in the fourth and in the ninth evoked response amplitude, compared to the first response. In the case of a negative response, 0.15 μg/g d-tubocurarine was administered i.p. and an identical test stimulation was performed 5 min and 15 min later to assess whether suboptimal doses of this ACh antagonist produced a decremental response. In the case of a significant (> 10 %) PDR, 0.15 μg/g edrophonium chloride, an anticholinesterase inhibitor that prevents the breakdown of ACh, was administered i.p. and reversal of decrement monitored for the following 30 min.

4.6 Quantitation of AChR in mouse muscle tissue

Leg muscles were extracted and AChR measured as described previously [27]. One leg muscle from each mouse was weighed and homogenized in 100 mM phosphate buffer pH 7.2. The volume was made up to 50 ml and the homogenate centrifuged at 13 000 rpm for 30 min. The pellet was extracted in 2 % Triton X-100 (approximately 1 ml for every gram of tissue) in PBS for 2 h or overnight, and centrifuged as above. The volume of the extract was measured and aliquots labeled with 5 nM ^{125}I-α-bungarotoxin (BuTx) (Amersham, specific activity 220 Ci/mmol). AChR was precipitated by addition of the G10 anti-AChR mAb [55] followed by sheep antiserum to mouse IgG. The precipitate was washed and counted on a gamma counter. Results were expressed as fmol of ^{125}I-α-BuTx binding sites precipitated/ml of muscle extract. Historical controls were included (A. Vincent, unpublished data, 1996). In parallel assays, the amount of AChR that had IgG bound to it was measured by immunoprecipitation in the presence of carrier normal mouse serum only (*i.e.* without G10 mAb), as previously described [27].

4.7 Determination of anti-ToAChR Ig isotypes

Anti-ToAChR IgG1 and IgG2a isotypes were determined in serum samples by ELISA. Ninety-six-well plates were coated with membrane-bound ToAChR (0.1 μg/ml) diluted in PBS. After blocking, serum samples diluted in PBS containing 5 % BSA and 0.5 % Tween-20 were titrated and incubated for 2 h at room temperature. After incubation the plates were extensively washed and biotin-conjugated anti-

IgG1 or -IgG2a goat anti-mouse isotype-specific antibodies (Southern Biotechnology Ass., Birmingham, AL) were added. Plates were then washed again extensively and the bound anti-isotypic antibodies were revealed by alkaline phosphatase-conjugated streptavidin. The reaction was developed using *p*-nitrophenylphosphate disodium and adsorbance read at 405 nm with an automated microplate ELISA reader. Standard curves were generated using pooled anti-ToAChR sera and results expressed as arbitrary units per ml (U/ml), 1 U corresponding to 50 % maximum absorbance. Serum antibody cross-reacting with mouse AChR was tested by immunoprecipitation of ^{125}I-α-BuTx-labeled mouse muscle extracts as previously described [42].

4.8 Immunofluorescence

Extensor muscles from the posterior legs of mice were dissected, oriented for cross-section, embedded in O.C.T. compound tissue-TEK (Miles Laboratories Inc., Elkahart, IN) and immediately frozen in liquid nitrogen-cooled isopentane. Cryostat sections (10 μm thick) were first blocked with a solution containing PBS and BSA 2 % and then double-stained with fluorescent antibodies overnight at 4 °C. FITC-conjugated α-BuTx (Amersham) was used to localize the end-plate AChR while AChR-bound mouse IgG1 or IgG2a were stained with PE-conjugated goat anti-mouse IgG1 or IgG2a antibodies (DAKO, Milan, Italy).

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