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*J Immunol* 2003; 171:3847-3854; doi: 10.4049/jimmunol.171.7.3847

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Genetic Evidence for Involvement of Classical Complement Pathway in Induction of Experimental Autoimmune Myasthenia Gravis

Erdem Tüziin,* Benjamin G. Scott,* Elzbieta Goluszko,* Stephen Higgs, † and Premkumar Christadoss2*  

Abs to acetylcholine receptor (AChR) and complement are the major constituents of pathogenic events causing neuromuscular junction destruction in both myasthenia gravis (MG) and experimental autoimmune myasthenia (EAMG). To analyze the differential roles of the classical vs alternative complement pathways in EAMG induction, we immunized C3−/−, C4−/−, C3+/−, and C4+/− mice and their control littermates (C3+/+ and C4+/+ mice) with AChR in CFA. C3−/− and C4−/− mice were resistant to disease, whereas mice heterozygous for C3 or C4 displayed intermediate susceptibility. Although C3−/− and C4−/− mice had anti-AChR Abs in their sera, anti-AChR IgG production by C3−/− mice was significantly suppressed. Both C3−/− and C4−/− mice had reduced levels of B cells and increased expression of apoptosis inducers (Fas ligand, CD69) and apoptotic cells in lymph nodes. Immunofluorescence studies showed that the neuromuscular junction of C3−/− and C4−/− mice lacked C3 or membrane attack complex deposits, despite having IgG deposits, thus providing in vivo evidence for the incapacity of anti-AChR IgGs to induce full-blown EAMG without the aid of complements. The data provide the first direct genetic evidence for the classical complement pathway in the induction of EAMG induced by AChR immunization. Accordingly, severe MG and other Ab- and complement-mediated diseases could be effectively treated by inhibiting C4, thus leaving the alternative complement pathway intact. The Journal of Immunology, 2003, 171: 3847–3854.

Myasthenia gravis (MG) and its animal model experimental autoimmune myasthenia (EAMG) are Ab-mediated autoimmune neuromuscular diseases. In both MG and EAMG, Abs are produced by B cells activated by acetylcholine receptor (AChR)-specific T cells (1–3). In the current view of MG and EAMG, anti-AChR Abs are suggested to destroy AChR located at the neuromuscular junction (NMJ) by the aid of complement. This proposition is based upon the observation that the deposits of IgG, C3, and membrane attack complex (MAC) are colocalized at the limb or intercostal muscle NMJ of both patients with MG and mice with EAMG (4–6). Moreover, it has been demonstrated that C5-deficient mice are highly resistant to EAMG induction (7); inhibition of C3 by cobra venom factor inhibits disease induction (8); and mice lacking the complement inhibitor decay-accelerating factor are more susceptible to EAMG induction (9). Collectively, these reports show that at least in the murine model of MG, activation of the terminal lytic complement complex (C5 to C9) is required for muscle AChR destruction, which is the primary pathology of the disease.

In EAMG, it is unclear whether anti-AChR Abs are activating the complement cascade (namely C3) by the classical pathway or other pathways. We hypothesized that the classical pathway plays a key role in EAMG development because this pathway is known to be directly activated by immune complexes. Anti-AChR Abs bind to their target Ags in the NMJ, thus forming immune complexes, which might potentially activate the classical pathway.

To demonstrate direct genetic evidence for the critical role of the classical pathway in EAMG pathogenesis, C4−/−, C4+/−, and C4+/+ littermate mice in the C57BL/6J background were immunized with AChR in CFA. AChR-immunized C3−/−, C3+/−, and C3+/+ littermate mice in the C57BL/6J background were used as a positive control, because C3 is the target molecule in the complement cascade activated by all pathways. Our findings provide the first direct genetic evidence for a key role for the involvement of C4 and therefore the classical pathway in the development of EAMG induced by AChR immunization.

Materials and Methods

AChR and mice

AChR was purified from the electric organ of Torpedo californica by an α-neurotoxin affinity column (10, 11). Torpedo AChR α-chain peptide (α146–162) was synthesized in the Protein Core Laboratory at the University of Texas Medical Branch (Galveston, TX). Seven- to 8-wk-old C3−/−, C4−/− mice (backcrossed to C57BL/6J mice for six generations) and C3+/−, C4+/− littermates in the C57BL/6J background were immunized with AChR in CFA. AChR-immunized C3−/−, C3+/−, and C3+/+ littermate mice in the C57BL/6J background were used as a positive control, because C3 is the target molecule in the complement cascade activated by all pathways. Our findings provide the first direct genetic evidence for a key role for the involvement of C4 and therefore the classical pathway in the development of EAMG induced by AChR immunization.

Abbreviations used in this paper: MG, myasthenia gravis; C3, C4, and C5, complement components; AChR, acetylcholine receptor; FasL, Fas ligand; LNC, lymph node cell; MAC, membrane attack complex; NMJ, neuromuscular junction.
the University of Texas Medical Branch and maintained according to the Institutional Animal Care and Use Committee Guidelines.

**Induction and clinical evaluation of EAMG**

For in vivo studies, all mice were anesthetized and immunized with 20 μg AChR emulsified in CFA (Difco, Detroit, MI) s.c. at four sites (two hind footpads and shoulders) on day 0. All of the mice were boosted with 20 μg AChR in CFA s.c. at four sites on the back on days 30 and 60 (second and third immunizations). Mice were screened for clinical EAMG on a daily basis. For clinical examination, mice were kept for 3 min on a flat platform and were observed for signs of EAMG. Clinical muscle weakness was graded as follows: grade 0, mouse with normal posture, muscle strength, and mobility; grade 1, normal at rest, with muscle weakness characteristically shown by a hunchback posture, restricted mobility, and difficulty to raise the head after exercise, consisting of 20–30 paw grips on cage top grid; grade 2, mouse showed grade 1 symptoms without exercise during observation period on flat platform; grade 3, dehydrated and moribund with grade 2 weakness; and grade 4, dead.

**RIA for anti-muscle AChR Ab**

Serum samples were collected after the second (day 44) and third (day 74) immunizations with AChR in CFA. The Ab response to mouse muscle AChR was measured by RIA, according to the overnight version of a previously described method (10, 11). The anti-AChR Ab levels were expressed in nmol of α-bungarotoxin (BTx) binding sites bound per liter of serum for individual mice.

**ELISA for IgG isotypes**

Affinity-purified mouse AChR (0.5 μg/ml) purified from mouse carcasses in our laboratory was coated onto a 96-well microtiter plate (Dynatech Immulon 2; Dynatech Laboratories, Chantilly, VA) with 0.1 M carbonate bicarbonate buffer (pH 9.6) overnight at 4°C. The plates were blocked with 2% BSA in PBS at room temperature for 30 min. Serum samples diluted 1/1000 in PBS/0.05% Tween 20 were added and incubated at 37°C for 90 min. After four washes, HRP-conjugated goat anti-mouse IgG isotypes (IgG, IgG1, IgG2b; Caltag, San Francisco, CA) diluted 1/1000 in PBS/0.05% Tween were added and incubated at 37°C for 90 min. Subsequently, ABTS (indicator) solution in 0.1 M citric buffer, pH 4.3, in the presence of H₂O₂ was added, and color was allowed to develop at room temperature in the dark. Absorbance values were measured at a wavelength of 405 nm, using a Dynatech ELISA reader, and the results were expressed as OD values.

**ELISA for serum total IgG levels**

This assay was performed in the same manner as mentioned above, except that the plates were coated with goat anti-mouse IgG (Chemicon International, Temecula, CA) with 0.1 M carbonate bicarbonate buffer (pH 9.6) overnight at 4°C. After incubation with serum samples, HRP-conjugated goat anti-mouse IgG Ab (Caltag) directed to a different determinant than the primary coating Ab, was added in 1/1000 dilution in PBS/0.05% Tween.

**Detection of IgG, C3, and MAC deposits at the NMJ by immunofluorescence microscopy**

Sections (10 μm thick) were obtained from forelimb muscle samples of mice, frozen in liquid nitrogen, and stored at −80°C. Slides were allowed to air dry and then were fixed in cold acetone. After washing with PBS, the sections were incubated with tetramethylrhodamine-conjugated α-BTX (Molecular Probes, Eugene, OR) (1/500 dilution) for 1 h at room temperature to label the NMJ. Sections were then incubated for 1 h at room temperature in the presence of goat anti-mouse IgG (Chemicon International), goat anti-mouse complement C3 (ICN-Cappel, Aurora, OH), or rabbit anti-human C5b-9 (MAC) (Calbiochem, San Diego, CA) (Abs diluted 1/1000) to colocalize IgG or complement deposits in NMJ. Anti-IgG and anti-C3 Abs were FITC conjugated. For detection of MAC deposits, the muscle tissues were further incubated with Oregon green-conjugated goat anti-rabbit IgG (Molecular Probes). Because human and mouse complement components involved in MAC are known to be highly homologous, we used anti-human MAC Ab to detect MAC deposits in NMJ (12). The sections were washed and viewed in a fluorescence microscope (Olympus IX-70 with a DP-11 digital camera).

**Cytokine ELISA**

Inguinal, popliteal, and axillary lymph node cells (LNC) were collected at termination of the experiments, and the cells were exposed in vitro in 48-well plates to AChR (2.5 μg/ml) and immunodominant peptide α146–162 peptide (40 μg/ml) and incubated for 3 days. Culture supernatants were collected for determination of IL-2, IL-6, IL-10, and IFN-γ levels by ELISA. ELISA plates (Dynatech Laboratories) were coated with 0.1 μg/well of anti-IL-2, anti-IL-6, anti-IL-10, or anti-IFN-γ Abs (BD Pharmingen, San Diego, CA) overnight at 4°C. Plates were blocked with 10% FBS in PBS for 1 h at room temperature. Supernatant samples were then added in duplicate and incubated overnight at 4°C. A secondary biotinylated specific anti-cytokine Ab (BD Pharmingen) was then added (0.1 μg/well), directed to determinants different from that recognized by anti-cytokine Ab used for coating. Development of coloration was conducted using avidin-peroxidase, followed by the substrate consisting of 0.3 mg/ml ABTS (Boehringer-Mannheim, Indianapolis, IN). Development of color occurred at room temperature in the dark. Serially diluted recombinant cytokines were used to establish a standard curve. Plates were read at OD₅₅₀, and results were expressed as cytokine concentration (picograms per milliliter).

**FACS for CD4, CD8, CD19, Fas, Fas ligand (Fasl), and CD69**

Single-cell suspensions of LNC were incubated for 30 min with one of the following anti-mouse Abs: PE-conjugated anti-CD4, anti-CD19, anti-Fasl, anti-CD69, FITC-conjugated anti-CD8, anti-Fas (all from BD Pharmingen). PE- or FITC-conjugated isotypes were used for controls. Cells were washed twice and then were fixed with 2% paraformaldehyde and analyzed by FACScan flow cytometry (BD Biosciences, San Jose, CA).

**Annexin V staining to detect apoptotic cells**

Single-cell suspensions of LNC (10⁶) from naïve C3⁻/⁻ and C4⁻/⁻ mice and control littermates were triple stained with PE-conjugated anti-CD19 Ab, FITC-conjugated annexin V, and 7-amino actinomycin D (7-AAD) (BD Pharmingen). One million cells were washed twice with cold PBS (0.15 M NaCl, 5×10⁻⁵ M CaCl₂ containing 0.05% sodium azide and 0.5% FBS (FACS buffer)). Cells were stained with 1 μg PE-conjugated anti-CD19 in 100 μl FACS buffer for 30 min on ice. The cells were washed once with FACS buffer and stained in the dark with 5 μg annexin V-FITC in 100 μl binding buffer at room temperature for 15 min. Without any further washing, 20 μl 7-AAD was added 20 min before FACS analysis. Backgrounds were established by staining with standard isotype control matched by species, fluorochrome, and isotype. Then we calculated the percentage of apoptotic cells of the indicated live cell population. Analyses were performed on FACSScan (BD Biosciences).

**Statistical analysis**

To determine the significance of the observed results, three statistical tests were used. Incidences of clinical EAMG were compared using the Fisher’s exact test; clinical scores were compared using Mann-Whitney U test; serum Abs levels (ELISA), cytokine levels (ELISA), and percentages of cells in the lymph node (FACS) were compared using Student’s t test.

**Results**

AChR-immunized C3⁻/⁻ and C4⁻/⁻ mice were highly resistant to EAMG, whereas C3⁻/⁻ and C4⁻/⁻ mice exhibited intermediate susceptibility.

To induce EAMG, in the first experiment, C3⁻/⁻, C3⁺/⁻, C3⁺/+ mice; in the second experiment, C3⁻/⁻, C3⁺/⁻, C3⁺/+ mice; and in the third experiment, C4⁻/⁻, C4⁺/⁻, C4⁺/+ mice and C3⁻/⁻ mice were immunized with AChR in CFA. C3⁻/⁻ or C4⁻/⁻ mice were highly resistant to EAMG (p values for clinical incidences as compared with control littermates were 0.0001 and 0.0005, respectively, and p values for clinical scores as compared with control littermates were less than 0.0001 for C3 and C4 gene knockout mice), whereas the disease incidences for C3⁻/⁻, C3⁺/⁻, C3⁺/+ mice and C4⁻/⁻ mice were 63, 86, 56, and 88%, respectively (Fig. 1). Additionally, C3⁺/⁻ and C4⁺/+ mice had lower disease incidences and clinical scores, which shows that even partial deficiency of C3 or C4 protects mice to some extent from EAMG induction. However, these differences were not statistically significant. The data demonstrate the first direct genetic evidence for the involvement of C3, C4, and thus the classical complement pathway in the development of clinical EAMG following immunization with AChR.
C3 gene disruption reduces both the AChR-specific total IgG and IgG2b responses, whereas C4 gene deficiency only reduces the anti-AChR IgG2b response.

Sera from individual mice were collected at day 74 after the first immunization with AChR. The anti-AChR IgG Ab response was measured first by RIA. Then anti-AChR IgG, IgG isotypes (IgG1, IgG2b), and total serum IgG were detected by ELISA. Interestingly, AChR-immunized C4+/– mice possessed similar amounts of total anti-AChR IgG levels (RIA) in their serum samples as their control C4+/+ littermates, whereas C3+/– mice displayed suppressed anti-AChR IgG production (p = 0.0049) (Fig. 2A). Values of anti-AChR IgG obtained from RIA and ELISA (data not shown) were highly comparable. In contrast, IgG2b production against AChR was inhibited significantly in both C3+/– (p = 0.047) and C4+/– mice (p = 0.029) (Fig. 2C). These results reveal that in C4+/– mice, EAMG resistance may not be solely associated with the classical complement pathway, but also with diminished levels of complement-binding IgG2b Abs. Just like anti-AChR IgG levels, total serum IgG and anti-AChR IgG1 levels were similar to that of control littermates in C4+/– mice, whereas C3+/– mice showed decreased IgG production as compared with their control littermates (p = 0.0003) (Fig. 2, B and D), which suggests that C4+/– mice do not have a significantly impaired IgG production.

**Absence of C3 and MAC deposits on the NMJ of AChR-immunized C3+/– and C4+/– mice**

To further clarify the importance of C3 or C4 deficiency in EAMG resistance despite the presence of serum anti-AChR IgGs, we doubly stained frozen muscle sections of all strains of mice with α-BTxs (binds to NMJ) and Abs directed against either IgG, C3, or MAC. All mice displayed IgG deposits on the NMJ and all C3+/+ and C4+/+ littermates had C3 and MAC deposits, whereas C3 and MAC deposits could not be detected on the NMJ of C3+/– and C4+/– mice (Fig. 3). The absence of C3 and MAC deposits, despite the presence of IgG on the NMJ of C3+/– and C4+/– mice, implicates C3 and MAC as playing a critical role in NMJ destruction in EAMG. Additionally, IgG bound to the NMJ without complement is not sufficient to induce optimal AChR destruction and clinical EAMG. Because C3+/– and C4+/– mice did not have any C3 or MAC deposits in the NMJ, we did not attempt to quantify C3 and MAC deposits.

**LNC of C3+/– mice, but not of C4+/– mice, reveal a suppressed cytokine production in response to AChR challenge**

We also investigated whether resistance to EAMG is also related with reduced AChR-specific IL-6, IL-10, or IFN-γ (all implicated in EAMG) production. C3+/– mice produced reduced cytokine responses (IL-6, IL-10, and IFN-γ) to Ag stimulation following immunization with AChR, whereas the cytokine production by C4+/– mice was identical with that of control littermates (Fig. 4). In contrast, IL-2 production was unaffected in all strains. These results give further proof to the fact that the disease resistance of C3+/– mice is associated with reduced anti-AChR Ab and cytokine response, besides deficiency of C3. However, the resistance of C4+/– mice is dominantly related to inactivation of the classical complement pathway and suppressed anti-AChR IgG2b production.
Both C3−/− and C4−/− mice have more T cells and reduced B cells in their lymph nodes after AChR immunization

T and B cell ratios of the lymph nodes of all strains of mice were estimated by detecting the expression of CD4, CD8, and CD19 molecules using flow cytometry analysis. For this purpose, lymph nodes obtained from nonimmunized and AChR-immunized mice were used. As illustrated in Fig. 5A, both nonimmunized C3−/− and C4−/− mice had decreased levels of B cells. In AChR-immunized mice, both C3−/− and C4−/− mice had decreased amounts of B cells and at the same time their T cell levels increased significantly (Fig. 5B).

LNC of C3−/− and C4−/− mice contain higher amounts of apoptosis-inducer molecules and apoptotic B cells

Because the role and significance of C3 and C4 in apoptosis are well known, we investigated the status of the apoptotic molecules in all strains of nonimmunized mice. For this purpose, the LNC expressions of Fas, FasL, and CD69 (which is not only an early activation marker for T cells, but also an apoptosis-inducer molecule) were analyzed by flow cytometry. Both C3−/− and C4−/− mice had slightly but not significantly increased amounts of Fas+ cells, whereas the amounts of FasL and CD69 molecules had been prominently raised. The increases were more prominent in C3−/− mice (Fig. 6A). Increased levels of FasL and CD69 molecules might be due to an increased demand for ongoing apoptotic events, which has been augmented by increased amounts of negative selection of B cells as a result of C3 or C4 deficiency. Consistent with this interpretation, FACS analysis showed increased amounts of apoptotic (annexin V-positive) cells, apoptotic (CD19-positive) B cells (Fig. 6B), and apoptotic (CD3-positive) T cells (data not shown) in both complement knockout mice. Again, the level of apoptotic B cells in C3−/− mice was more prominent than C4−/− mice. Thus, the increased level of apoptotic B cells correlates with significant suppression of anti-AChR Abs in AChR-immunized C3−/− mice. The percentages of necrotic LNC (as assessed by 7-AAD staining) had also been increased in C4−/− mice (data not shown), suggesting that there is also an increase in LNC necrosis in these mice.

Discussion

It has long been established that IgG, C3, and MAC are bound to the NMJ of MG patients (4, 5). These Ab and complement deposits are well known to contribute to NMJ damage and to the reduction of AChR. However, the complement components that cause MG have never been studied in detail, and the dominance of complement pathways (classical vs alternative) in EAMG induced by AChR immunization has not been duly investigated.

There is scant evidence about the role of complements in MG. Some authors have reported that some haplotypes carrying certain C4 alleles are associated with MG (13). Decreased serum C4 levels have been observed in a group of patients with MG and rodents with active disease (14–16). This decrease may potentially be related to the excessive consumption of C4, and C4 might thus be playing a specific role in pathogenesis of MG at least in a subgroup of patients.

There are two experiments focused on the development of clinical disease and AChR loss in soluble complement receptor 1-treated mice (17) and C4-deficient guinea pigs (18) with EAMG,
which has been induced passively by administration of mAbs to AChR. In both studies, the severity of the muscle weakness and AChR loss has been significantly diminished. However, soluble complement receptor 1 is not a specific C4 inhibitor, and it also blocks C3 and C5 (17). Moreover, in both studies, the disease has been induced by passive transfer, which does not accurately mimic the immunopathogenesis of human MG. In passive transfer studies, a very high concentration of anti-AChR Abs is transferred to the recipient, saturating the NMJ and thus inducing EAMG. However, in human MG, serum Ab levels may not be high enough to block all receptors; there is no correlation between Ab levels and clinical severity, and Abs cause disease by different mechanisms, as discussed below. In EAMG induced by AChR immunization, mice have limited amount of serum anti-AChR IgG, which possibly induces disease in a way similar to human MG. Thus, passive transfer studies may not be showing the specific pathological consequences taking place in NMJ following the blockade of complement activation. Our results provide the first direct genetic evidence of the significance of the classical pathway in the pathogenesis of EAMG. Because C4 is also involved in the lectin pathway, our results do not dissect the role of this pathway. Binding of MBL to pathogens triggers the activation of the lectin pathway, leading to cleavage of C4 and C2, which induces the formation of the C3 convertase, activation of C3, and thus the rest of the complement cascade. Because this pathway is known to be activated predominantly by bacteria (19), we plausibly suggest that this pathway is not involved in EAMG induction. Although our results suggest that alternative pathway cannot induce EAMG, this pathway might still be important in human MG, because human beings are not living in viral Ab-free environments as our mice. Moreover, alternative pathway can be activated by pathogens, and some pathogens have been suggested to induce certain autoimmune

FIGURE 3. Resistance to EAMG in C3⁻/⁻ and C4⁻/⁻ mice was associated with the absence of C3 and MAC at their NMJs. Muscle sections obtained from mice of different strains (indicated at the top) immunized with AChR were stained for mouse IgG, C3, and MAC (right column, all green fluorescence). The NMJs were localized by α-BTx (left column, red fluorescence) (magnification for all, ×100). Although both knockout and control littermate muscle sections reveal IgG binding, muscle sections of C3⁻/⁻ and C4⁻/⁻ mice were lacking C3 and MAC, which are crucial in disease induction (pictures from nonstained areas (right) were taken from the same location with BTx binding sites (left) under green fluorescent light). For these experiments, muscle samples of seven mice were used from each strain, and at least four sections from each individual mouse were studied. The immunofluorescence data represent one of 7 × 4 sections for each strain.

FIGURE 4. Cytokine responses of LNC collected from AChR-immunized mice at termination were significantly reduced in C3⁻/⁻ and C4⁻/⁻ mice. The cells were stimulated in vitro by peptide α146-162 or whole AChR molecule. Results are given as cytokine production in supernatants in pg/ml for each mouse. Results obtained for individual mice of each strain were compared using Student’s t test. Asterisks on the top of the bars indicate p values for the comparisons between knockout mice and their control littermates. *, Indicates p < 0.05; **, indicates p < 0.01; and bars indicate SEs. One representation of two independent experiments.
diseases. Additionally, our mice may not necessarily be pathogen free, and the alternative pathway might have caused EAMG in the single C4−/− mouse with clinical disease. Therefore, alternative pathway might be playing a more subtle role in EAMG induction, and we still recommend the investigation of this pathway’s role in EAMG.

The deficiency of classical complement pathway factors has been most strongly related with systemic lupus erythematosus (20, 21) and other immune complex-mediated diseases (22). C4 deficiency could thus be expected to increase EAMG incidence rather than decreasing it. The fact that C4−/− mice are resistant to EAMG could indicate that C4 deficiency may not be associated with the development of all autoimmune diseases, but only with classical immune complex-mediated autoimmune diseases, such as systemic lupus erythematosus.

Anti-AChR Abs are found in ~90% of MG patients (23), and are suggested to play an important role in functional loss by blocking the receptor (24), by increasing receptor endocytosis (25), or by activating the complement-mediated inflammatory destruction of the NMJ (26). It is uncertain whether Abs could cause clinical symptoms without the aid of the complement system. We suggest that our model would be a valuable way of testing this hypothesis. EAMG-resistant C3−/− and C4−/− mice did not possess C3 or MAC deposits at the NMJ, but still had IgGs (Fig. 3), which strongly suggests that at least in the vivo animal model of disease, IgG cannot induce disease in most cases without the help of complement. Moreover, C4−/− mice, but not C3−/− mice, had normal levels of anti-AChR IgG and total IgG (Fig. 2, A and D). Because C4−/− mice are highly, but not entirely, resistant to disease, Abs might still be important in disease induction by themselves alone at least in a certain fraction of immunized mice. It is also plausible that the classical complement pathway is critical for severe forms of MG, and in the absence of complement, anti-AChR IgG alone could be sufficient to induce mild forms of MG, including ocular MG.

C3- and C4-deficient mice have been demonstrated to have diminished Ab responses to T cell-dependent Ag stimulation and a failure in Ab isotype switching (characterized by decreased serum IgG levels), all of which can be recovered by increased concentrations of the Ag used for immunization (27). Our data clearly indicate that AChR immunization does not affect total IgG and IgG1 isotype production against AChR in C4−/− mice. It is also noteworthy that Ab production of C3−/− mice is significantly affected by the same amount and dosage of Ag used possibly as a result of their disabled cytokine production skills (especially for IL-6).

In our experiments, C3−/− mice were unable to produce normal amounts of cytokines, including IL-6, IL-10, and IFN-γ, all of which are important for EAMG induction (28, 29). This indicates that C3 may act not only as a central figure in the complement cascade, but also as an important immune mediator by up-regulating IFN-γ and IL-6. It is already known that IL-6 and C3 activate one another. In the current study, we have shown that C3 deficiency caused a reduction in IL-6 levels (possibly via its receptor CR1/2), just as IL-6 deficiency caused a reduction in C3 levels (28).

Interestingly, B cell counts of C3- and C4-deficient mice were reduced, whereas T cell counts of these mice increased even more prominently than those of control littermates, after AChR immunization. We suggest that this T cell increase was to compensate...
reduced B cell levels. There is growing evidence that complements are important in stimulation of B cells by Ags in the lymphoid tissues and inadequately stimulated B cells undergo apoptosis in their absence (30). This may explain why reduced B cell counts were observed in C3- and C4-deficient mice, and it may also elucidate the reason for the increase in the expression of molecules involved in apoptosis. Increased amounts of apoptotic B cells might also indicate that resistance of C3\(^{-/-}\) mice to EAMG induction is associated with increased elimination of autoreactive B cells from the lymph nodes. Because this elimination is relatively low in C4\(^{-/-}\) mice, they might be capable of producing sufficient amount of anti-AChR Abs. The fact that both C3\(^{-/-}\) and C4\(^{-/-}\) mice have increased frequency of apoptotic T cells (data not shown) is challenging this assumption. However, the ratio of apoptotic lymph node CD3\(^+\) T cells for knockout vs control littermate is less than 1.2 times for both knockout mouse groups, whereas ratio for apoptotic CD19\(^+\) B cells in knockout and control mice is >2.5 times. Besides, C4\(^{-/-}\) mice also have increased amounts of necrotic cells (data not shown), suggesting that B cell loss is not only associated with apoptosis.

It may seem paradoxical that C3\(^{-/-}\) mice have reduced cytokine production despite increased lymph node T cell counts. However, cytokine levels reflect the in vitro activity of AChR-specific T and B cells, whereas cell counts by FACS do not reflect the AChR-specific T and B cell population. It is possible that T cells not specific for AChR have expanded in the LNC population of C3\(^{-/-}\) mice. This expanded T cell population does not contribute to disease.

C4 is found within the H-2S region (class III). Although the C4 or C3 genes are specifically deleted in these strains in the B6 background (after sex backcross generations to C57BL/6), neighboring genes could have been affected, and one could argue that the disease protection in C4 or C5 gene knockout mice could be due to an H-2 effect. Within the H-2 region of B6, only I-A\(^b\)-gene controls susceptibility to EAMG (31, 32), and B6 mice do not express I-E molecules, because of \(\alpha_E\) gene deletion. MHC class I genes do not influence EAMG susceptibility, because \(B_2\)-microglobulin-deficient mice lacking MHC class I molecules demonstrated susceptibility to EAMG, like the wild-type B6 mice (33).

In our experiments, we observed that LNC of C4\(^{-/-}\) and C3\(^{-/-}\) mice have I-A\(^b\) expression like their control littermates (data not shown), as evaluated by FACS analysis. AChR immune lymphocytes of C4\(^{-/-}\) mice responded to AChR and its dominant peptide, and produced cytokines (Fig. 4) and AChR-specific IgG, just like the wild-type mice. Thus, C4\(^{-/-}\) mice class II MHC molecules (I-A\(^b\)) functioned properly. Therefore, resistance to EAMG in C4\(^{-/-}\) mice is primarily due to deficiency of the classical complement pathway and partial reduction of IgG2b anti-AChR Abs, because C4\(^{-/-}\) mice had intact AChR-specific cellular and IgG responses, which are influenced by the I-A\(^b\) allele of C4\(^{-/-}\) mice.

Our results indicate that the inhibition of C4 may be an effective way of treating not only severe MG, but also other Ab- and complement-mediated diseases (e.g., pemphigus, Goodpasture’s syndrome, hemolytic anemia, thrombocytopenic purpura) without interfering with the entire complement system. C3 can be activated via the alternative pathway, which is activated by viruses and bacteria. Thus, even with a deficiency in C4, the adaptive host defense against pathogens may be expected to remain unaffected and the complement system may be expected to continue to clear viruses, apoptotic cells, and tumor cells. However, the classical complement pathway is also known to be involved in both host defense against infection and disposal of immune complexes (34, 35), which suggests that its inhibition may result in increased susceptibility to lethal infection (35) and autoimmune disease. Interestingly, C4-deficient mice (but not C3-deficient mice) can effectively mediate opsonization, phagocytosis, and protective immunity to streptococci in the presence of passively transferred specific Abs (35), implicating that in the deficiency of the classical complement pathway, the immune system may be sufficient for an effective immune response to pathogens (35). Treatment trials for EAMG by using specific classical pathway inhibitors are, therefore, warranted to ascertain the place of C4 inhibition in MG treatment. Severe MG is treated by plasmapheresis or IVIg, which are expensive and invasive methods and provide only temporary benefit. C4 inhibition may be less expensive way to treat severe MG, and long-term remissions could be attained.

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