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CELL SURFACE COMPLEMENT REGULATORS MODERATE EXPERIMENTAL MYASTHENIA GRAVIS PATHOLOGY

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Abstract

Introduction—Intrinsic mouse complement regulators influence the severity of passively induced experimental acquired myasthenia gravis (EAMG). To assess the potential influence of CD59b in the absence of CD59a background, we used the *mCD59ab*^{−/−} mouse model to re-evaluate mCD59 in protecting the neuromuscular junction (NMJ).

Methods—EAMG was induced with monoclonal antibody to the acetylcholine receptor (AChR) in *Daf1*^{−/−}, *CD59ab*^{−/−}, *Daf1*^{−/−} *CD59ab*^{−/−}, and wild-type C57Bl/6 mice. Animals were monitored throughout the experiment. Diaphragms were analyzed for NMJ injury.

Results—*Daf1*^{−/−} *CD59ab*^{−/−} mice required euthanasia 24 hours after disease induction because of severe weakness. Histological assessment demonstrated reduced AChR density, simplification of synaptic folds, and disrupted mitochondria. CD59ab-deficient mice demonstrated mild weakness and reduction in weight after 24 hours. In contrast, *Daf1*^{−/−} had more severe weakness at 60 hours. The NMJ of EAMG-induced *Daf1*^{−/−} and *CD59ab*^{−/−} mice demonstrated similar AChR density.

Conclusion—NMJs of CD59 and DAF mice are protected from complement-mediated injury of passive EAMG.

Keywords

complement; complement regulatory protein; myasthenia gravis; neuromuscular junction; passive EAMG

Myasthenia gravis (MG) is an autoimmune disease of neuromuscular transmission characterized by skeletal muscle weakness and fatigue upon exertion. Antibodies directed against the nicotinic acetylcholine receptor (AChR) located on the postsynaptic surface of the neuromuscular junction are the initiators of pathology in the vast majority of patients,

and the complement system is a major effector mechanism in both the human disease and animal models of experimentally acquired myasthenia gravis (EAMG).^{1,2} Membrane attack complex (MAC) deposition was identified at the neuromuscular junction (NMJ) in both EAMG and human MG over 30 years ago.³ MAC deposition at the NMJ in EAMG has been shown to be a necessary condition to produce EAMG.⁴

An array of soluble and cell surface complement regulators protect “self-cells” from the deleterious effect of complement activation. Decay accelerator factor (DAF), complement receptor 1-related gene/protein y (Crry), and CD59 are three membrane-bound proteins that restrict complement at different levels of the activation cascade. They are all concentrated at the NMJ⁵ and appear to protect it from injury produced by EAMG induced by infusion of monoclonal antibodies directed toward the AChR. Mice deficient in either DAF or CD59a (one of the two mouse CD59 genes) exhibit more severe weakness and complement deposition than wild-type (WT) mice.⁶⁻⁸ *Daf* and *CD59a* double-knockout mice demonstrate a very severe EAMG phenotype, indicating that Crry does not provide a significant shield from complement attack.^{6,8}

The advent of drugs that inhibit complement at different steps of the activation cascade has made it critical to define more precisely the involvement of complement in the pathogenesis of MG.^{9,10} DAF accelerates the decay of and thereby inactivates C3 (C4b2a and C3bBb) and C5 (C4b2a3b and C3bBb3b) convertases that assemble on self-cells.^{11,12} CD59 specifically inhibits formation of the MAC, the effector of complement-mediated cell damage, by interfering with polymerization of C9 on cell-bound C5b-8 complexes.¹³⁻¹⁵ Crry, a rodent-specific complement regulator, combines functional properties of DAF and human membrane cofactor protein (MCP), inhibiting complement at the level of the C3 convertases.¹⁶ A detailed analysis of the role of DAF and CD59 in animal models may lead to better understanding of the human disease. Evaluation of Crry is less relevant, because the protein is not present in humans and Crry deletion is lethal to the fetus.¹⁷

Previous studies from our laboratory concluded that DAF is the primary shield from complement-induced injury of the postsynaptic surface.⁶ The interpretation of previous studies on the role of complement regulators in EAMG conducted with *mCD59a*^{-/-} mice is compromised by the lack of consideration of a second isoform of *mCD59b*, designated *mCD59*.¹⁸⁻²⁰ Similar to human CD59, *mCD59a* is widely expressed among tissues. Initial studies of *CD59b* suggested that its expression is restricted to the testis, but subsequent work documented a wider expression of *CD59b* transcripts in tissues,²¹ although its expression in skeletal muscle has not been evaluated. The finding that *mCD59a* and *mCD59b* have two promoters raised the possibility of compensatory expression of the alternate gene in single *CD59a* or *CD59b* knockout mice.²⁰ Based on evidence that the expression levels of complement components and complement regulators vary and may be differentially regulated in different tissues, and on the availability of *Daf1*^{-/-} and *CD59ab*^{-/-} mice, we reassessed the relative roles of CD59 and DAF in the pathogenesis of EAMG.

METHODS

Animals

Daf1^{-/-} mice²² were provided by M.E. Medof (Case Western Reserve University), and *CD59ab*^{-/-} mice²⁰ were provided by J.A. Halperin (Harvard Medical School). Both knockout mouse strains were backcrossed 9 generations on the C57B1/6 background. *Daf1*^{-/-} *CD59ab*^{-/-} knock-outs were prepared by cross-breeding *Daf1*^{-/-} and *CD59ab*^{-/-} mice. Genotyping to assure knockout of the CD59 genes was performed as described previously.²⁰ To confirm that at least 1 copy of the Daf deletion construct was present, polymerase chain reaction (PCR) was performed with specific primers (NeoFor: 5'TGC TCC TGC CGA GAA AGT AT 3' NeoRev: 5'AAT ATC ACG GGT AGC CAA CG 3'); to assure that 2 copies of the deletion construct were present, quantitative PCR was performed with (NeoRT forward: 5' AGACAATCGGCTGCTCTGAT 3' and NeoRT reverse; 5' TCGTCC TGCAGTTCATTTCAG 3'). For all experiments, 6–8-week-old males were used. All mice were maintained in the animal facility of St. Louis University, and studies were carried out according to a protocol approved by the institutional animal care and use committee.

Real-Time Reverse Transcript PCR

Diaphragms were isolated from healthy adult WT, *Daf1*^{-/-}, *CD59ab*^{-/-}, and *Daf1*^{-/-} *CD59ab*^{-/-} mice. Total RNA was prepared in Trizol (Invitrogen, Carlsbad, California), following the manufacturer's instructions. Reverse transcription was carried out using a first-strand synthesis system (SuperScript III; Invitrogen) with oligo(dT)12-18 primers. Real-time (RT)-PCR amplification of cDNA was performed (StepOne Real-Time PCR System; Applied Systems, Carlsbad, California) with SYBR Green-based detection (Applied Biosystems). PCR primers (Table 1) were determined by Primer-BLAST (NCBI) and PrimerExpress for RT-PCR (version 3.0; Applied Biosystems). Results were based on the 2^{-Ct} formula, using the actin transcript as the internal control and WT values as the baseline.²³ All samples were performed in triplicate with 4 diaphragms from each group.

Induction of EAMG

EAMG was induced by administration of rat monoclonal anti-mouse AChR (McAb3; a gift from Vanda Lennon, Mayo Clinic²⁴), similar to our previous investigation.⁶ McAb3 is an IgG2b isotype and is known to activate complement. The antibody is directed against an extracellular epitope of the mammalian AChR, which does not include the acetylcholine binding site. At time 0, 50 μ l of purified McAb3 (4.6 mg/ml) was administered intraperitoneally. For the two independent experiments that extended to 60 hours, 5 animals were used in each group. For experiments that were terminated at 24 hours, 10 animals were used in each of the 4 groups.

Assessments

Mice were kept under close observation and sequentially evaluated daily after administration of McAb3. A generally accepted motor strength scale was used to assess for weakness: 0 = can grip and lift lid of a cage; 1 = can grip but can-not lift the lid of a cage; 2 = unable to

grip cage lid; 3 = unable to grip plus hindlimb paralysis; and 4 = severely ill with signs of imminent death.²⁵ Weights were monitored. A grip strength meter (Columbus Instruments, Columbus, Ohio) was used to assess forelimb strength. Measurements were performed with a digital force gauge (Ametek, Largo, Florida) that determines the peak force by which an animal can grasp a grid pull bar. Prior to the measurement, each mouse was exercised with 3-5 paw grips, and then 5 grips were registered by the grip strength meter. Mean grip strength (in grams) for each mouse was used for analysis.

Immunohistochemistry and Pixel Quantitation

For analysis of AChR density at the NMJs, 10- μ m cryo-sections of tibialis anterior and diaphragm from EAMG-induced mice were prepared and stained with Alexa 594-labeled bungarotoxin (BTX). To assess MAC deposition, sections were also stained with rabbit polyclonal anti-complement 5b-9 (Calbiochem). Sections were washed and incubated with Alexa 488-labeled anti-rabbit IgG (Molecular Probes).

The sections were viewed with a fluorescence microscope (BX50; Olympus, Center Valley, Pennsylvania), and digital images were captured using a Spot camera (Diagnostic Instruments, Sterling Heights, Michigan) and analyzed with ImagePro software (Media Cybernetics, Silver Springs, Maryland). Adobe Photoshop and Adobe Illustrator (Adobe Systems, Inc., Seattle, Washington) were used for preparation of figures. For analysis of pixel density, images were taken in black and white. The percentage of junctions with MAC immunoreactivity was calculated as follows: All BTX-stained areas were identified and outlined. The produced template was layered over the matching image taken for MAC immunoreactivity. MAC deposition was quantified by the pixel density in the selected area. Pixel density measurements were also used to quantitate BTX fluorescence at the neuromuscular junctions. A total of 240 NMJs were analyzed in each group based on the images from 4 animals in each group. The percent of endplates with specific pixel density was expressed in a histogram ranging by units of 10.

Electron Microscopy of NMJs

Three *Daf1^{-/-} CD59ab^{-/-}* mice were induced with McAb3 to produce EAMG. After 24 hours, tibialis anterior and diaphragm muscles were removed and fixed in 2.5% glutaraldehyde in 0.2 M sodium cacodylate buffer and postfixed in 1% osmium tetroxide, dehydrated in an ethanol series to 100%, infiltrated, and embedded (EMbed-812; Electron Microscopy Sciences, Hatfield, Pennsylvania). Semi-thin sections (0.5 μ m) were cut on an ultramicrotome (UltracutE; Reichert-Jung) using glass knives and stained with toluidine blue; silver sections were cut with a diamond knife (Diatome), placed on copper grids, and stained with 4% uranyl acetate (30 min) in 50% methanol followed by Reynolds lead citrate (15 min). Sections were examined using an electron microscope (JEM-100 CX11; JEOL USA, Inc., Peabody, Massachusetts) at 80 kV. Photographs were recorded using Kodak EM film (Eastman Kodak Co., Rochester, New York). Fifty neuromuscular junctions were assessed for nerve-muscle damage.

Statistical Analysis

Statistical differences were calculated between the 4 groups, WT, *Daf1*^{-/-}, *CD59ab*^{-/-}, and *Daf1*^{-/-} *CD59ab*^{-/-} mice. Data were analyzed and tested for statistical significance ($P < 0.05$) using one- and two-tailed Mann-Whitney *U* *t*-tests.

RESULTS

Expression Levels of 3 Mouse Complement Regulators in Diaphragm Muscle from WT and Knockout Mice

We assessed baseline transcript levels of CD59a, CD59b, Daf, and Crry in the diaphragms of the WT, *Daf1*^{-/-}, *CD59ab*^{-/-}, and *CD59ab*^{-/-} *Daf1*^{-/-} mice. Table 2 shows that change in transcript levels of the complement regulators compared with WT levels. Transcript levels of Crry and both CD59 genes in *Daf1*^{-/-} mice as well as levels of Crry and DAF in *CD59ab*^{-/-} mice were comparable to those in WT mice. Interestingly, the transcript levels of Crry were significantly increased in *Daf1*^{-/-} *CD59ab*^{-/-} as compared with WT, *Daf1*^{-/-}, and *CD59ab*^{-/-} mice ($P < 0.02$, *t*-test).

CD59ab^{-/-} *Daf1*^{-/-} Mice Develop Marked Weakness in EAMG

We induced EAMG in *Daf1*^{-/-}, *CD59ab*^{-/-}, *Daf1*^{-/-} *CD59ab*^{-/-}, and WT controls by injection of McAb3. Twenty-four hours after disease induction, *Daf1*^{-/-} *CD59ab*^{-/-} mice were profoundly weak with clinical scores of 3. These mice demonstrated inability to perform grip strength assessments, paralysis of the hindlimb, and weight loss (Fig. 1), and had to be euthanized due to extreme weakness. At this 24-hour time-point, *Daf1*^{-/-} and WT mice did not show any change in grip strength, and most had a clinical score of 0. In contrast, half of the *CD59ab*^{-/-} mice demonstrated weakness. We assessed weight of the animals in all studies at 24 hours. Ninety percent of the *Daf1*^{-/-} *CD59ab*^{-/-} mice demonstrated weight loss with an average difference of -0.66 g (± 0.54 g). The *CD59ab*^{-/-} mice demonstrated weight loss in 85% with a total average weight difference of -0.77 g (± 0.81 g). Only 50% and 15%, respectively, of the *Daf1*^{-/-} and WT mice showed minor differences (average weight fluctuation of 0.03 ± 0.25 g and 0.18 ± 0.20 g, respectively). Both *Daf1*^{-/-} *CD59ab*^{-/-} mice and *CD59ab*^{-/-} mice demonstrated statistically significant differences in weight compared with WT and *Daf1*^{-/-} ($P < 0.01$). All WT, *Daf1*^{-/-}, and *CD59ab*^{-/-} were killed concurrently with the *Daf1*^{-/-} *CD59ab*^{-/-} mice.

Extended time experiments were performed to characterize the time course of disease in the *Daf1*^{-/-}, *CD59ab*^{-/-}, and WT mice. In 2 independent experiments, *Daf1*^{-/-}, *CD59ab*^{-/-}, and WT mice demonstrated weakness in 48 hours with clinical scores of 1. After 60 hours, *Daf1*^{-/-} mice continued to decline and reached a mean clinical score of 2, whereas the WT and *CD59ab*^{-/-} had similar clinical and grip strength scores. Despite the higher clinical scores for the *Daf1*^{-/-} mice, grip strength assessment did not show differences among the groups. This may be due to the pre-dominant development of hindlimb weakness, which is not evaluated by the forelimb grip strength measure. At each time-point, weight loss was significantly greater in *CD59ab*^{-/-} mice compared with *Daf1*^{-/-} or WT mice ($P < 0.01$, according to two-tailed Mann-Whitney *U*-test) with a more dramatic difference ($6.7 \pm 3.4\%$ weight loss) in the first 24 hours after disease induction.

MAC Deposition is Distinct among *CD59ab*^{-/-}, *Daf1*^{-/-}, and *Daf1*^{-/-} *CD59ab*^{-/-} NMJs

At 24 hours, there was significantly greater MAC immunoreactivity at the neuromuscular junctions of the tibialis anterior in *CD59ab*^{-/-} mice ($P < 0.001$; Fig. 2). The *Daf1*^{-/-} *CD59a*^{-/-} mice, which developed severe weakness, had much less MAC deposition, but demonstrated MAC deposits within skeletal muscle fibers.

At 60 hours (Fig. 2), the *CD59ab*^{-/-} mice continued to show greater MAC deposition compared with *Daf1*^{-/-} (mean pixel density: 49.7 ± 11.8 in *CD59ab*^{-/-} and 46.9 ± 11.1 in *Daf1*^{-/-}, $P < 0.01$) and WT mice (43.4 ± 9.1 mean pixel density, $P < 0.001$ vs. both groups).

AChR Density Is Decreased in *Daf1*^{-/-} *CD59ab*^{-/-} Mice

Fluorescently labeled BTX was used to determine AChR density at the NMJ. At the 24-hour time-point, pixel densities of labeled NMJs from tibialis anterior of *Daf1*^{-/-} *CD59a*^{-/-} mice (138.1 ± 31.7) were markedly reduced in comparison to *Daf1*^{-/-} (155.0 ± 24.0), *CD59ab*^{-/-} (158.9 ± 27.0), or WT mice (153.3 ± 23.9 , $P < 0.001$, two-tailed Mann-Whitney *U*-test). At 60 hours, the AChR density of NMJs of WT mice was significantly greater (176.9 ± 30.5) than in either *Daf1*^{-/-} (172.2 ± 33.2) or *CD59ab*^{-/-} mice (169.7 ± 36.9 , $P < 0.05$ vs. WT). There was no difference between AChR densities of junctions from *Daf1*^{-/-} or *CD59ab*^{-/-} mice.

Electron Microscopy and Pre- and Postsynaptic Injury

Because of the severe level of weakness and the profound loss of AChR density, we performed ultrastructural analysis of diaphragm and tibialis anterior muscle from 3 24-hour EAMG-induced *Daf1*^{-/-} *CD59ab*^{-/-} mice. NMJs of both muscle types displayed simplification of synaptic folds, widening of the synaptic cleft, and electron-dense material within the cleft (likely shed postsynaptic membrane) (Fig. 3), as observed in our previous studies of complement inhibitor knockout mice⁶ and in humans with MG.²⁶ In addition, there was evidence of injury to the axon and presynaptic nerve terminal with indications of retraction of the nerve terminal and possible sloughing of presynaptic membrane. In addition, the mitochondria of the nerve terminal were dilated and demonstrated disrupted cristae. The injury to skeletal muscle was more pronounced in other regions. "Moth-eaten" fibers, which are often seen in myopathy associated with early stages of necrosis, were detected.^{27,28}

DISCUSSION

Mice with deficient intrinsic complement regulation develop more severe EAMG than WT mice. The *Daf1*^{-/-} *CD59ab*^{-/-} mice demonstrated severe weakness by 24 hours of EAMG induction with hindlimb paralysis that required euthanasia. *CD59ab*^{-/-} mice were noticeably weak at 24 hours compared with *Daf1*^{-/-} and WT mice, but by 60 hours *Daf*-deficient mice exhibited greater weakness. This indicates that DAF appears to be a more potent intrinsic complement inhibitor at the neuromuscular junction than CD59. We analyzed the NMJs at 24 hours and 60 hours and confirmed that *CD59ab*^{-/-} mice had greater MAC deposition compared with the *Daf1*^{-/-} mice, although they had similar AChR densities. Deficiency of both complement regulators led to rapid EAMG pathology. EM of the NMJ of the *Daf1*^{-/-}

CD59ab^{-/-} mice showed destroyed AChR clusters, focal muscle fiber destruction, internalization of MAC, and injury to presynaptic terminals and axons. The observation of nerve terminal injury by massive complement attack leads to the possibility that, in patients with severe weakness, such as myasthenic crisis, presynaptic compromise could contribute to the weakness in MG. Prevention of the deleterious effects of spontaneous complement activation is the primary role of complement regulatory proteins²⁹ and has been observed in hereditary neuropathies in patients presumed to have normal levels of complement inhibitors.^{16,30}

These results are in agreement with those from our original studies⁶ and from those of Morgan and colleagues,⁸ which addressed the role of complement regulators in passive EAMG. All 3 studies showed that, when complement regulators are absent, the mice have severe weakness, AChR loss, and extensive injury to the NMJs. The need to remove 2 of the complement regulators to allow this to occur suggests that these regulators may have overlapping roles.

CD59 interferes with formation of MAC complexes, the final step in the complement-mediated damage of the postsynaptic surface.¹³⁻¹⁵ In contrast, DAF acts primarily at the C3 and C5 convertases that assemble on self-cells by accelerating the decay of these enzymes.^{11,12} The development of weakness among *CD59ab*-deficient mice 24 hours after EAMG induction, but not in the DAF-deficient mice, suggests that the MAC formation on the postsynaptic surface occurs rapidly and leads to weakness. Only after prolonged antibody induced complement activation does the lack of DAF inhibition of convertases become obvious, producing greater disease severity, as determined by higher clinical scores. EAMG improves with agents that inhibit C5 activation,³¹ as compared with therapeutics that target the complement pathway earlier, such as C1q (L.L. Kusner, unpublished observations).³²

In a study of diaphragms from WT mice 48 hours after induction of EAMG, there was increased DAF1 immunoreactivity at the NMJs, whereas Crry expression increased at both the junction and sarcolemma. In marked contrast, there was no change in *CD59a* expression.⁵ Therefore, increased DAF1 expression could have a protective effect in the *CD59ab*^{-/-} mouse after EAMG induction. In contrast, no such upregulation of *CD59a* takes place to compensate for the lack of DAF1, which is consistent with our results that show greater weakness in the *Daf*-deficient strain.

These results emphasize the complexities of correlating weakness with histopathological evidence of neuromuscular junction injury. The *Daf1*^{-/-} *CD59ab*^{-/-} mice were profoundly weak and therefore could be expected to have high levels of complement deposition at the NMJ. We observed that *Daf1*^{-/-} *CD59ab*^{-/-} EAMG junctions had limited complement deposition at the NMJ but significant reduction of AChR density. Therefore, the absence of *Daf1* and *CD59ab* resulted in such severe EAMG that a significant loss of AChR occurred to the point that antibody binding could no longer deposit complement due to complement depletion. AChR densities from both the *Daf1*^{-/-} and *CD59ab*^{-/-} mice were reduced to a similar extent, although the *Daf*-deficient mice demonstrated worse clinical scores, indicating a greater impairment of neuromuscular transmission.³³

In a previous investigation we found EAMG induced in WT mice led to an increase in Crry protein expression at the NMJ and an increase in sarcolemmal Crry expression in some fibers.⁵ In evaluating *Daf1^{-/-} CD59ab^{-/-}* mice, we found that EAMG produced injury beyond the NMJ with evidence of a subpopulation of early necrotic myofibers containing large amounts of internalized MAC. However, if Crry is upregulated in certain fibers of *Daf1^{-/-} CD59ab^{-/-}* mice, they could be relatively spared. This would explain the heterogeneous pattern of muscle fiber injury in the *Daf1^{-/-} CD59ab^{-/-}* mice.

Complement inhibitors are now in their infancy with regard to application to human disease.³⁴ Figure 4 summarizes the points at which complement inhibition has been induced by either exogenous administration of a drug or targeted gene knockout in rodents in the study of EAMG. Thus far, manipulation that targets downstream portions of the cascade appears to be more effective than upstream. Administration of an anti-C1q antibody reduced the severity of EAMG but not to a dramatic extent.³² In contrast, the inhibition of C5 or C6 by antibody was found to moderate significantly the severity of EAMG.^{35,36} A construct of an Ig domain coupled to Crry is also effective in prevention of weakness caused by passive EAMG.³⁷ Further, soluble recombinant human complement receptor 1 (sCR1) treatment reduces the severity of passively induced EAMG by inhibition of complement at the C3 and C5 activation steps.²⁵ In this study we found that the severity of EAMG is worse in the first 24 hours if CD59 is absent. This would serve to enhance MAC assembly, but, as the disease develops, the absence of DAF worsens neuromuscular junction injury. Viral vector approaches have been successful in enhancing DAF expression and protecting from complement-induced injury.³⁸ We have also delivered a recombinant DAF protein to the postsynaptic surface to protect from EAMG (unpublished observation).

We used mice deficient in both CD59 isoforms and identified a more subtle distinction in the pathology of EAMG, in contrast to previous studies.^{6,8} In the absence of both CD59 isoforms, one appreciates greater weakness developing within 24 hours of EAMG induction. The similarity of the severity of weakness in DAF- and CD59-deficient strains resembles the observations of Morgan and colleagues,⁸ but at peak severity of weakness the *Daf1^{-/-}* mice are weaker than the *CD59ab^{-/-}* mice. Therefore, the results are consistent with the notion that DAF is a more potent protector of the NMJ from complement-mediated injury.

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Abbreviations

AChR	acetylcholine receptor
BTX	bungarotoxin
Crry	complement receptor 1 gene-related protein y
EAMG	experimentally acquired myasthenia gravis

MAC	membrane attack complex
MG	myasthenia gravis
NMJ	neuromuscular junction
MCP	membrane cofactor protein
PCR	polymerase chain reaction
RT-PCR	reverse transcript-polymerase chain reaction
sRC1	soluble recombinant human factor 1
WT	wild-type

REFERENCES

1. Gomez AM, van den Broeck J, Vrolix K, Janssen SP, Lemmens MA, van der Esch E, et al. Antibody effector mechanisms in myasthenia gravis—pathogenesis at the neuromuscular junction. *Autoimmunity*. 2010; 43:353–370. [PubMed: 20380584]
2. Souroujon MC, Brenner T, Fuchs S. Development of novel therapies for MG: studies in animal models. *Autoimmunity*. 2010; 43:446–460. [PubMed: 20298126]
3. Engel AG, Lambert EH, Howard FM. Immune complexes (IgG and C3) at the motor end-plate in myasthenia gravis: ultrastructural and light microscopic localization and electrophysiologic correlations. *Mayo Clin Proc*. 1977; 52:267–280. [PubMed: 870771]
4. Richman DP, Agius MA, Kirvan CA, Gomez CM, Fairclough RH, Dupont BL, et al. Antibody effector mechanisms in myasthenia gravis. The complement hypothesis. *Ann NY Acad Sci*. 1998; 841:450–465. [PubMed: 9668274]
5. Kaminski HJ, Li Z, Richmonds C, Lin F, Medof ME. Complement regulators in extraocular muscle and experimental autoimmune myasthenia gravis. *Exp Neurol*. 2004; 189:333–342. [PubMed: 15380483]
6. Kaminski HJ, Kusner LL, Richmonds C, Medof ME, Lin F. Deficiency of decay accelerating factor and CD59 leads to crisis in experimental myasthenia. *Exp Neurol*. 2006; 202:287–293. [PubMed: 16859686]
7. Lin F, Kaminski HJ, Conti-Fine BM, Wang W, Richmonds C, Medof ME. Markedly enhanced susceptibility to experimental autoimmune myasthenia gravis in the absence of decay-accelerating factor protection. *J Clin Invest*. 2002; 110:1269–1274. [PubMed: 12417565]
8. Morgan BP, Chamberlain-Banoub J, Neal JW, Song W, Mizuno M, Harris CL. The membrane attack pathway of complement drives pathology in passively induced experimental autoimmune myasthenia gravis in mice. *Clin Exp Immunol*. 2006; 146:294–302. [PubMed: 17034582]
9. Tüzün E, Huda R, Christadoss P. Complement and cytokine based therapeutic strategies in myasthenia gravis. *J Autoimmun*. 2011; 37:136–143. [PubMed: 21636248]
10. Kusner LL, Kaminski HJ, Soltys J. Effect of complement and its regulation on myasthenia gravis pathogenesis. *Expert Rev Clin Immunol*. 2008; 4:43–52. [PubMed: 20477586]
11. Hourcade DE, Mitchell LM, Medof ME. Decay acceleration of the complement alternative pathway C3 convertase. *Immunopharmacology*. 1999; 42:167–173. [PubMed: 10408377]
12. Fujita T, Inoue T, Ogawa K, Iida K, Tamura N. The mechanism of action of decay-accelerating factor (DAF). DAF inhibits the assembly of C3 convertases by dissociating C2a and Bb. *J Exp Med*. 1987; 166:1221–1228. [PubMed: 2445886]
13. Davies A, Simmons DL, Hale G, Harrison RA, Tighe H, Lachmann PJ, et al. CD59, an LY-6-like protein expressed in human lymphoid cells, regulates the action of the complement membrane attack complex on homologous cells. *J Exp Med*. 1989; 170:637–654. [PubMed: 2475570]
14. Harada R, Okada N, Fujita T, Okada H. Purification of 1F5 antigen that prevents complement attack on homologous cell membranes. *J Immunol*. 1990; 144:1823–1828. [PubMed: 2307842]

15. Holguin MH, Fredrick LR, Bernshaw NJ, Wilcox LA, Parker CJ. Isolation and characterization of a membrane protein from normal human erythrocytes that inhibits reactive lysis of the erythrocytes of paroxysmal nocturnal hemoglobinuria. *J Clin Invest.* 1989; 84:7–17. [PubMed: 2738160]
16. Kim YU, Kinoshita T, Molina H, Hourcade D, Seya T, Wagner LM, et al. Mouse complement regulatory protein Crry/p65 uses the specific mechanisms of both human decay-accelerating factor and membrane cofactor protein. *J Exp Med.* 1995; 181:151–159. [PubMed: 7528766]
17. Wu X, Spitzer D, Mao D, Peng SL, Molina H, Atkinson JP. Membrane protein Crry maintains homeostasis of the complement system. *J Immunol.* 2008; 181:2732–2740. [PubMed: 18684964]
18. Baalasubramanian S, Harris CL, Donev RM, Mizuno M, Omidvar N, Song WC, et al. CD59a is the primary regulator of membrane attack complex assembly in the mouse. *J Immunol.* 2004; 173:3684–3692. [PubMed: 15356114]
19. Qin X, Krumrei N, Grubissich L, Dobarro M, Aktas H, Perez G, et al. Deficiency of the mouse complement regulatory protein mCd59b results in spontaneous hemolytic anemia with platelet activation and progressive male infertility. *Immunity.* 2003; 18:217–227. [PubMed: 12594949]
20. Qin X, Hu W, Song W, Blair P, Wu G, Hu X, et al. Balancing role of nitric oxide in complement-mediated activation of platelets from mCd59a and mCd59b double-knockout mice. *Am J Hematol.* 2009; 84:221–227. [PubMed: 19229985]
21. Qin X, Ferris S, Hu W, Guo F, Ziegeler G, Halperin JA. Analysis of the promoters and 5'-UTR of mouse Cd59 genes, and of their functional activity in erythrocytes. *Genes Immun.* 2006; 7:287–297. [PubMed: 16541098]
22. Lin F, Fukuoka Y, Spicer A, Ohta R, Okada N, Harris CL, et al. Tissue distribution of products of the mouse decay-accelerating factor (DAF) genes. Exploitation of a Daf1 knock-out mouse and site-specific monoclonal antibodies. *Immunology.* 2001; 104:215–225. [PubMed: 11683962]
23. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods.* 2001; 25:402–408. [PubMed: 11846609]
24. Lennon VA, Lambert EH. Myasthenia gravis induced by monoclonal antibodies to acetylcholine receptors. *Nature.* 1980; 285:238–240. [PubMed: 6154894]
25. Piddlesden SJ, Jiang S, Levin JL, Vincent A, Morgan BP. Soluble complement receptor 1 (sCR1) protects against experimental auto-immune myasthenia gravis. *J Neuroimmunol.* 1996; 71:173–177. [PubMed: 8982117]
26. Engel AG. Morphologic and immunopathologic findings in myasthenia gravis and in congenital myasthenic syndromes. *J Neurol Neurosurg Psychiatry.* 1980; 43:577–589. [PubMed: 6249893]
27. Levin MI, Mozaffar T, Al-Lozi MT, Pestronk A. Paraneoplastic necrotizing myopathy: clinical and pathological features. *Neurology.* 1998; 50:764–767. [PubMed: 9521271]
28. Nagaraju K, Lundberg IE. Polymyositis and dermatomyositis: pathophysiology. *Rheum Dis Clin N Am.* 2011; 37:159–171.
29. Zipfel PF, Skerka C. Complement regulators and inhibitory proteins. *Nat Rev Immunol.* 2009; 9:729–740. [PubMed: 19730437]
30. Hafer-Macko CE, Dyck PJ, Koski CL. Complement activation in acquired and hereditary amyloid neuropathy. *J Peripher Nerv Syst.* 2000; 5:131–139. [PubMed: 11442169]
31. Soltys J, Kusner LL, Young A, Richmonds C, Hatala D, Gong B, et al. Novel complement inhibitor limits severity of experimentally myasthenia gravis. *Ann Neurol.* 2009; 65:67–75. [PubMed: 19194881]
32. Tuzun E, Li J, Saini SS, Yang H, Christadoss P. Pros and cons of treating murine myasthenia gravis with anti-C1q antibody. *J Neuro-immunol.* 2007; 182:167–176.
33. Slater CR. Structural factors influencing the efficacy of neuromuscular transmission. *Ann NY Acad Sci.* 2008; 1132:1–12. [PubMed: 18096848]
34. Emlen W, Li W, Kirschfink M. Therapeutic complement inhibition: new developments. *Semin Thromb Hemost.* 2010; 36:660–668. [PubMed: 20865643]
35. Zhou Y, Gong B, Lin F, Rother RP, Medof ME, Kaminski HJ. Anti-C5 antibody treatment ameliorates weakness in experimentally acquired myasthenia gravis. *J Immunol.* 2007; 179:8562–8567. [PubMed: 18056404]

36. Biesecker G, Gomez CM. Inhibition of acute passive transfer experimental autoimmune myasthenia gravis with Fab antibody to complement C6. *J Immunol.* 1989; 142:2654–2659. [PubMed: 2703710]
37. Hepburn NJ, Chamberlain-Banoub JL, Williams AS, Morgan BP, Harris CL. Prevention of experimental autoimmune myasthenia gravis by rat Crry-Ig: a model agent for long-term complement inhibition in vivo. *Mol Immunol.* 2008; 45:395–405. [PubMed: 17651804]
38. Kaname Y, Tani H, Kataoka C, Shiokawa M, Taguwa S, Abe T, et al. Acquisition of complement resistance through incorporation of CD55/decay-accelerating factor into viral particles bearing baculovirus GP64. *J Virol.* 2010; 84:3210–3219. [PubMed: 20071581]
39. Tuzun E, Li J, Saini SS, Yang H, Christadoss P. Pros and cons of treating murine myasthenia gravis with anti-C1q antibody. *J Neuro-immunol.* 2007; 182:167–176.

FIGURE 1.

Physical assessment of EAMG mice. Mice were immunized to induce EAMG by the injection of McAb3. The weight, grip strength, and clinical scores were determined throughout the experimentation period. Values for WT, *Daf1*^{-/-}, and *CD59ab*^{-/-} ($n = 5$ /group) are shown as 1 of 2 experiments lasting 60 hours. The values for the *Daf1*^{-/-} *CD59ab*^{-/-} mice ($n = 10$) are from 1 experiment, which was terminated at 24 hours because of the severity of weakness (the *Daf1*^{-/-} *CD59ab*^{-/-} mice were too weak to undergo grip strength evaluation). The mean weight (A), grip strength (B), and clinical score (C) of animals in each group during the course of EAMG (* $P < 0.01$ and ** $P < 0.001$, by Mann-Whitney U -test).

FIGURE 2.

Membrane attack complex assessment after induction with McAb3. (A) At 24 hours after EAMG, fluorescent-labeled BTX was used to localize the neuromuscular junction and labeling performed with an antibody directed against MAC in the tibialis anterior muscle of WT, *Daf1*^{-/-}, *CD59ab*^{-/-}, and *Daf1*^{-/-} *CD59ab*^{-/-} mice (original magnification $\times 40$). MACs were analyzed at (B) 24 hours and (C) 60 hours after induction with McAb3. Pixel density of fluorescence of MAC deposition at NMJs from tibialis anterior muscles was determined. A distribution plot was constructed based on the percentage of NMJs expressing a specific pixel density. A total of 240 NMJs were analyzed in each group based on the images from 4 animals in each group. The percent of endplates with specific pixel density was expressed in a histogram ranging by units of 10 (see text). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

FIGURE 3.

Electron microscopy of skeletal muscle from EAMG-induced *Daf1*^{-/-} *CD59ab*^{-/-} mice.

(A)–(F) Neuromuscular junctions from EAMG-induced *Daf1*^{-/-} *CD59ab*^{-/-} mice with arrows identifying areas of mitochondrial swelling in the postsynaptic region, arrow heads identifying areas of nerve retraction, and asterisks identifying presynaptic regions with disrupted cristae (×25,000). (D)–(F) Disruption of tibialis anterior myofibers (×8000).

FIGURE 4.

Schematic illustration of inhibition of the complement pathway in EAMG. Complement regulators and the therapeutic methods of EAMG modulation are shown.^{25,31,35,36,39}

Table 1

Primer sets for complement regulator expression assessment.

Gene	Accession	Forward primer	Reverse primer
<i>Actin</i>	NM_007393	5'-GAGTACGATGAGTCCGGCCCCCT-3'	5'-ACGCAGCTCAGTAACAGTCCGC-3'
<i>Daf</i>	NM_010016	5'-ACTGCGGCTCAAAACAGCTCCAGC-3'	5'-GGTGATGGCCGAGTCCTAGGCG-3'
<i>CD59a</i>	NM_007652	5'-GCTGCTTCTGGCTGTGTTCTG-3'	5'-GGTTGGAAACAGTGGTAGCATGT-3'
<i>CD59b</i>	NM_181858	5'-ACTGCCCTGTTGCCTTGGATCAG-3'	5'-AGACAGCCAGAAGCAGCAGGAGT-3'
<i>Crry</i>	NM_013499	5'-TACCTACCGCTGCAACACTGA-3'	5'-GGCTCACCCACCAGGTAAA-3'

Primers were selected and screened by Primer-BLAST and PrimerExpress software for real-time PCR 3.0 and produced by Integrated DNA Technologies (Coralville, IA).

Table 2

Relative gene expression of complement regulatory factors.

	<i>Crry</i>	<i>CD59a</i>	<i>CD59b</i>	<i>Daf</i>
<i>Daf</i> ^{-/-}	1.19 ± 0.28	1.38 ± 0.08	0.90 ± 0.11	–
<i>CD59ab</i> ^{-/-}	1.19 ± 0.27	–	–	1.36 ± 0.44
<i>Daf1</i> ^{-/-} <i>CD59ab</i> ^{-/-}	2.20 ± 0.45*	–	–	–

Values are based on relative expression compared with WT mice.

* $P < 0.02$ (*t*-test)