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DAF/CD55 and Protectin/CD59 modulate adaptive immunity and disease outcome in experimental autoimmune myasthenia gravis

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ABSTRACT

The role of regulators of complement activity (RCA) involving CD55 and CD59 in the pathogenesis of experimental autoimmune myasthenia gravis (EAMG) remains unclear. CD55 and CD59 restrict complement activation by inhibiting C3/C5 convertases' activities and membrane attack complex formation, respectively. Actively immunized EAMG mice deficient in either CD55 or CD59 showed significant differences in adaptive immune responses and worsened disease outcome associated with increased levels of serum cytokines, modified production of acetylcholine receptor antibodies, and more complement deposition at the neuromuscular junction. We conclude that modulation of complement activity by RCA represents an alternative in controlling of autoimmune processes in EAMG.

1. Introduction

Myasthenia gravis (MG) is a disorder of the neuromuscular transmission mediated by antibodies against the acetylcholine receptor (AChR) (Gomez et al., 2010). It is the best known neurological autoimmune disease where the AChR receptor breakdown is executed by a direct block of the binding sites, enhanced endocytosis and degradation or complement mediated lysis of the neuromuscular junction (NMJ) (Conti-Fine et al., 2006).

Several lines of evidence indicate that formation of AChR specific antibodies and subsequent complement mediated lysis of the NMJ is the major determinant of disease severity (Chamberlain-Banoub et al., 2006; Morgan et al., 2006). The pathogenic effect of complement activation on MG and EAMG is validated by various studies (Romi et al., 2005; Christadoss et al., 2008; Willcox et al., 2008). As a result MG patients show at the NMJ deposits of IgG, C3 complement component and membrane attack complex (MAC) (Sahashi et al., 1980; Barohn and Brey, 1993; Nakano and Engel, 1993). A functionally simplified NMJ structure in human MG is a more likely consequence of complement-mediated injury (Engel, 2004).

However, there is a limited knowledge that particular regulators of complement activity (RCA) such as decay accelerating factor DAF/CD55 and Protectin/CD59 affect disease progression or modulate the adaptive immune responses. DAF/CD55 and Protectin/CD59 interrupt complement activation at the two distinct locations. DAF/CD55 inhibits complement activity (Medof et al., 1984) by interfering with the C3 and C5 convertases' activities (Miwa and Song, 2001). Protectin/CD59 inhibits membrane attack (MAC) formation by preventing binding of C9 to the C5b-8 complex (Farkas et al., 2002). DAF and Protectin are expressed ubiquitously on almost every host cell (Terstappen et al., 1992; Navenot et al., 1997) and protect host tissues from complement mediated damage. Since both DAF and Protectin are expressed on muscle fibers (Navenot et al., 1997; Louboutin et al., 2003) and tissues exposed to complement (Hinchliffe et al., 1998; Harris et al., 2000) their role in EAMG pathogenesis is greatly anticipated (Lin et al., 2002).

EAMG represents a reliable model of human disease and is well suited for investigating the mechanisms underlying disease pathology (Kusner et al., 2008). The majority of previous studies studied the function of RCA in a passive EAMG model where the development of an adaptive immune response could not be examined (Kaminski et al., 2004; Kaminski et al., 2006; Morgan et al., 2006). In our study, we utilize an active EAMG model which closely mimics the onset and outcome of human MG. Additionally, an active model of EAMG in mice deficient in DAF/CD55 and Protectin/CD59$_{a/b}$ allows us to assess how RCA modulate the adaptive immune response and contribute to EAMG pathology.

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Deficiency in DAF/CD55 and Protectin/CD59$_{a/b}$ affected the clinical outcome of EAMG where a more severe disease phenotype, particularly in DAF/CD55$^{-/-}$ EAMG, was observed. EAMG mice deficient in specific RCA exhibited differences in adaptive immune responses and had stronger deposition of C3, C3b/iC3b/C3c, C5b-9 (MAC) complement components at the site of pathology — the NMJ.

2. Material and methods

2.1. Mice

Mice deficient in decay accelerating factor (DAF/CD55$^{-/-}$) (Lin et al., 2001) and Protectin (Protectin/CD59$_{a/b}^{-/-}$) (Qin et al., 2009) were bred in house. Wild-type (C57BL/6) breeders were purchased from the Jackson Laboratory (Bar Harbor, ME). Mice were housed and maintained in a pathogen-free environment at the Saint Louis University Department of Comparative Medicine. All experiments were performed according to the protocols approved by SLU IACUC.

2.2. Induction and clinical evaluation of experimental autoimmune myasthenia gravis (EAMG)

The acetylcholine receptor was purified from the electric organs of *Torpedo californica* (*tAChR*) by affinity chromatography (Wu et al., 2001). Eight to ten week old male mice were used for experiments. EAMG was induced by 4 subcutaneous injections of 20 μg *tAChR* emulsified in complete Freund's adjuvant (CFA) (Difco, Voigt Global Distributions, KS) in a total volume of 200 μl. Mice were immunized along the back subcutaneously, at the base of the tail and boosted twice with 20 μg of *tAChR* in incomplete Freund's adjuvant 4 and 8 weeks after primary immunization. Control (CTRL) mock immunized mice received an equal volume of PBS in CFA or IFA.

Mice were screened for the clinical disease outcome on weekly basis. Following the active immunizations, EAMG and CTRL mice were weighed and assessed for muscle weakness. A grip strength meter (Columbus Instruments, Columbus, OH) was used to assess forelimb muscle strength. Measurements were performed with a grip strength meter (Columbus Instruments, Columbus, OH) and DFE digital force gauge (Ametek, Largo, FL) was used to detect the peak force when animals grasp a grid pull bar. Prior to the measurement, each mouse was exercised with 10–20 paw grips and then the final 5 grips were recorded and analyzed.

2.3. ELISA

Conventional ELISA was used for the detection of anti-AChR specific complement fixing antibodies in blood serum. Serum levels of anti-AChR antibodies were examined at the end of the experiment (Day 63). A 96 well Nunc plates (Fisher Scientific, Pittsburgh, PA) were coated overnight at 4 °C with 10 μg/ml of purified AChR. After three washes with PBS Tween, plates were blocked for 2 h at room temperature (RT) with 200 μl PBS Tween 20 (Sigma, Saint Louis, MO). Mouse serum samples in triplicates at dilution 1:500 were added (100 μl/well) and incubated at RT for 90 min. After washes with PBS-Tween, the plates were incubated for another 90 min with HRP conjugated goat anti-mouse Abs (IgG, IgG$_1$, IgG$_{2b}$; 1:2000; Alpha Diagnostics, San Antonio, TX). The color reaction was developed with SureBlue TMB substrate and stopped with TMB stop solution (KPL Inc., Gaithersburg, MA). Stopped reactions were read on a Tecan Infinity M200 reader (Tecan Group Ltd., Durham NC). Absorbances were measured at 450 nm and the results were expressed in O.D. values.

2.4. ELISPOT

Plates coated with primary capture antibodies specific for IFN-γ and IL-4 from BD™ Elispot Kits (BD Biosciences, San Jose, CA) were used for detection of cytokine secreting cells. Single cell suspensions of splenocytes (5×10^5/well) in 100 μl of complete RPMI-1640 medium were added in triplicates and incubated for 24 h with recall antigen of the *tAChR* (at the concentrations 10; 1.0; 0.1 and 0.01 μg/well). For determination of negative or positive spot production, cells were also incubated either with or without mitogen (Con A, 1 μg/ml). After three washes with PBS-Tween buffer, cells were stained for 2 h with secondary biotin labeled IFN-γ and IL-4 antibodies. Followed by another three washes, streptavidin-HRP was added and plates were incubated for 60 min. ELISPOT plates were developed with a BD™ Elispot AEC substrate sets (BD Biosciences, San Jose, CA). Spots were counted on an Immunospot Image Analyzer using Beta 4.0 version software (ELISPOT Image Analyzer, Cellular Technology Ltd., Cleveland, OH).

2.5. Cytometric Bead Array (CBA)

The BD™ CBA Mouse Th1/Th2/Th17 Cytokine Kit (BD Biosciences, San Jose) was used to measure IL-2, IL-4, IL-6, IFN-γ, TNF, IL-17 and IL-10 protein levels. The procedure was carried out according to the manufacturer's protocol (CBA™, BD Biosciences, San Jose, CA). Serum samples from individual mice were collected at Day 63 p.i. Total 25 μl of serum was mixed with 25 μl of assay diluent. Then, 50 μl of cytokines capture beads and 50 μl of PE-labeled detection antibody were added to each diluted serum and incubated for 2 h at RT. Cytokine standard solutions from the BD™ CBA Kits were diluted from concentrations of 0 to 5000 pg/ml. After incubation in the dark at RT for 2 h, all cytokine standards and samples were washed twice with wash buffer. Finally, bead pellets with captured cytokines were resuspended in 300 μl of wash buffer and read on a BD FACSArray Analyzer (BD Biosciences, San Jose, CA). Acquired data were further analyzed with FCAP Array™ software (Soft Flow Inc. Minneapolis, MN).

2.6. Immunofluorescence staining for IgG and C3, C3b/iC3b/C3c, C5b-9 (MAC) complement components

Mouse diaphragms were embedded in OCT Compound Tissue-Tek (Fisher Scientific, Pittsburgh, PA) and frozen in liquid N_2-cooled 2-methybutane. Tissue samples were stored at −80 °C until further use. For immunofluorescence analysis of C3, C3 fragments (C3b/iC3b/C3c) and C5b-9 (MAC) deposition at the NMJ, 10 μm cryosections of mouse diaphragms were mounted on Superfrost^{Plus} slides. Slides were allowed to air dry and tissues were fixed in cold acetone for 5 min. After three washes with PBS, sections were blocked with 3% BSA in PBS for at least 1 h. Tissues were further stained with FITC conjugated anti-mouse IgG (Sigma, Saint Louis, MO) and C3 (MP Biomedicals, Solon, OH) antibodies. For recognition of C3b/iC3b/C3c rat anti-mouse monoclonal antibody [clone 3/26 recognizes C3b/iC3b/C3c but C3c is not retained in tissue Ramaglia et al., 2012; Hycult Biotech, Plymouth Meeting, PA] and rabbit anti-mouse C5b-9 (EMD Biosciences, San Diego, CA) polyclonal antibodies were used. These primary antibodies were diluted at 1:200 and 1:300. For C3b/iC3b/C3c and C5b-9 (MAC) staining, sections were labeled with Alexa^{488} conjugated goat anti-rat and goat anti-rabbit secondary antibodies (1:500; Invitrogen, Carlsbad, CA), respectively. Finally, Alexa Fluor^{596} labeled bungarotoxin (1:1000; BTX, Invitrogen, Carlsbad, CA) was used to visualize NMJ. After three washes, sections were coverslip with Fluorogel II with DAPI mounting medium (Electron Microscopy Sciences, Hatfield, PA) and viewed by an Olympus fluorescence microscope (60× magnification; Olympus Inc., PA, USA). Captured microphotographs were analyzed with Image Pro software (Media Cybernetics, Silver Springs, MD). Results were expressed as percentage of C3, C3b/iC3b/iC3c and C5b-9 positive deposits presented at the BTX labeled NMJs.