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Ablation of IL-17 expression moderates experimental autoimmune myasthenia gravis disease severity☆

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ABSTRACT

An array of cytokines influences the pathogenesis of early onset myasthenia gravis (MG) and its animal model, experimental autoimmune myasthenia gravis (EAMG). Patients with MG, in particular those with more severe weakness, have elevations of the pro-inflammatory cytokine IL-17 in the blood. We assessed the role of IL-17A in autoimmunity by inducing EAMG in mice with knockout of IL-17 and found a reduction of EAMG severity, but not a complete ablation of disease. The IL-17^{ko} mice had no evidence of weakness, low levels of acetylcholine receptor antibodies, and retention of acetylcholine receptor at the neuromuscular junction. Splenic germinal center size was reduced in EAMG IL-17^{ko} mice along with elevations of Foxp3 and BCL-6 gene expression, suggesting a shift away from pro-inflammatory signals. The results emphasize the importance of IL-17 in EAMG development and that IL-17 independent pathways drive the autoimmune reaction.

1. Introduction

Myasthenia gravis (MG) is a prototypical, antibody mediated autoimmune disorder of the neuromuscular junction (NMJ) with several subtypes based on age, autoantibody status, and thymus pathology [1,2]. Thymic hyperplasia with germinal center (GC) formation is the pathological hallmark of patients with early onset of disease and antibodies directed against the acetylcholine receptor (AChR). Experimental autoimmune MG (EAMG) mimics early-onset MG of humans with development of weakness, AChR antibodies after immunization of purified AChR, and evidence of IgG deposition at the neuromuscular junction [3]. EAMG mice do not develop thymic hyperplasia; however, splenic pathology demonstrates enhanced GC formation [4].

A complex network of signals influence disease pathogenesis in MG patients and EAMG animals with pro-inflammatory cytokine IL-17 emerging as a significant contributor in supporting autoimmunity. We identified elevations in circulating IL-17A among patients with MG compared to normal controls [5] consistent with a smaller investigation [6], and further, IL-17A elevations were associated with generalized weakness. Increased serum levels of IL-17A were more common in the subgroup of patients with thymic hyperplasia. Thymic Treg cells from MG patients have an increase in expression of the IL-17 family potentially due to a fluctuation between the T reg and T eff cells [7], and the stimulation of CCR6(+) memory T cells lead to IL-17 production in contrast to controls [8]. We utilize an IL17 knockout mouse (IL17^{ko}) to further assess the role of IL-17A in EAMG.

2. Materials and methods

2.1. Animal ethics statement

Eight-week-old female C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) and IL-17^{ko} mice (Y. Iwakora, Tokyo University, Tokyo, Japan) with baseline weights ranging between 15 and 19 g were used. Mice were maintained in isolator cages with free access to food and water. All animals were housed in The George Washington University Animal Research Facility in accordance with IACUC, AALAS, and AAALAT standards regulating housing conditions, cage cleaning procedure, air purity, humidity, temperature, feed quality, and light dark cycles. A veterinarian was available to monitor the animals during the course of the study. Animal use was approved by The George Washington University Institutional Animal Care and Use Committee (Permit No. A247), and all experimental outcomes are reported using the quality assurance guidelines set by NINDS [7].

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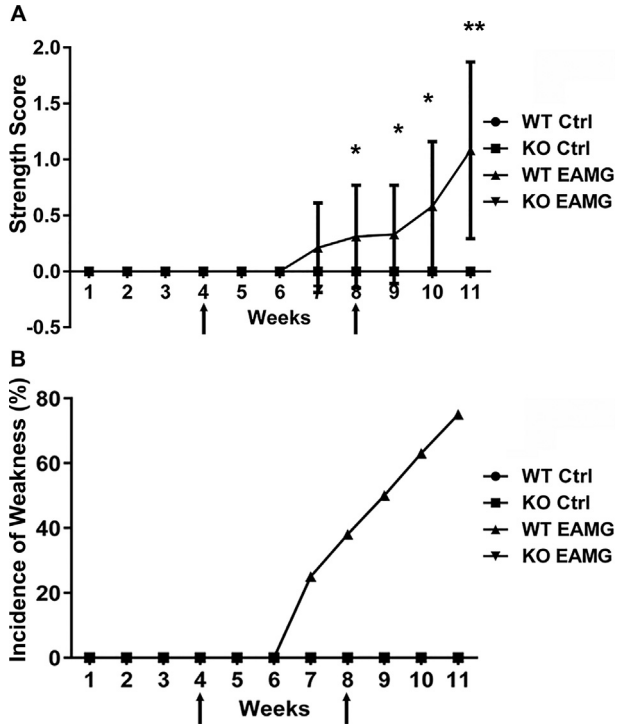

Fig. 1. Weakness scores. Weakness score (A) and percentage of animals showing weakness (B) of 31 animals in four groups (WT Ctrl n = 7, WT EAMG n = 8, KO Ctrl n = 8, KO EAMG n = 8) shown over an 11-week period for mice immunized with tAChR/CFA/PBS or CFA/PBS. Second and third immunizations with tAChR/IFA/PBS or IFA/PBS occurred at days 28 and 56, as indicated with arrows. Error bars represent mean and standard deviation. One-tailed, unpaired t-tests were performed on strength scores to indicate statistical significance (*p < 0.05, **p < 0.01).

2.2. Induction and evaluation of EAMG

Based on an a priori power analysis, four groups of eight mice were established WT EAMG, WT Control, KO EAMG, and KO Control. Mice of each strain were randomly assigned to control and experimental groups. AChR, isolated from the electric organs of *Torpedo californica* through affinity chromatography [8], was used to induce EAMG. A total injection volume of 200 µl consisted of 50 µg *Torpedo* AChR (tAChR) in complete Freunds' adjuvant (CFA; Sigma-Aldrich, St. Louis, MO) and on the first day of the experiment administered subcutaneously in four equal doses at the shoulder and at the base of the tail. Subsequent immunizations at weeks 4 and 8 were performed similarly using 20 µg tAChR in incomplete Freunds' adjuvant (IFA; Sigma) 100 µl. Control groups received equal injection volumes of 1:1 mix of phosphate buffered saline (PBS; Sigma) and CFA on the same days. The mice were assessed in a blinded fashion biweekly for weeks 1–6 and 8 and triweekly for weeks 7 and 9–11. The evaluations consisted of weight measurement and determination of a generally accepted weakness scale (0 = no weakness after exercise, 1 = normal at rest, but weak after exercise, 2 = weakness at rest, 3 = severe weakness with paralysis, 4 = found dead or euthanized) [9]. The mice underwent euthanasia on day 72 using CO_2 asphyxiation followed by cervical dislocation. Blood was collected by cardiac puncture and stored 4 °C. The spleen was removed, washed with PBS, placed in liquid N_2-cooled 2-methybutane OCT, and stored at −80 °C until use (Thermo Fisher Scientific, Waltham, MA). Tibialis anterior was removed and prepared in a similar fashion to the spleen for immunohistochemical staining.

2.3. Mouse AChR isolation

C2C12 cells (D. Beeson, University of Oxford, Oxford, UK) were grown in DMEM (ATCC, Manassas, VA) with 10% Fetal Bovine Serum (FBS; Gibco, Fisher), 1% Penicillin-Streptomycin (Pen-Strep; Gibco, Fisher), and 1% L-glutamine (Gibco, Fisher). For differentiation into myotubes, the medium was changed to 2% FBS in DMEM. After ten days, cells were isolated and the cell suspension centrifuged at 3000*g* for 10 min at 4 °C. The supernatant was discarded, and the pellet was suspended in 50 mM NaCl (Fisher), 5 mM EDTA (Sigma), and 1x protease inhibitor (Sigma) in PBS (100 mg pellet to 200 µl buffer). The suspension was homogenized using a polytron (Kinematica, Lucerne, Switzerland). The homogenized cells were then diluted to 1 ml of buffer

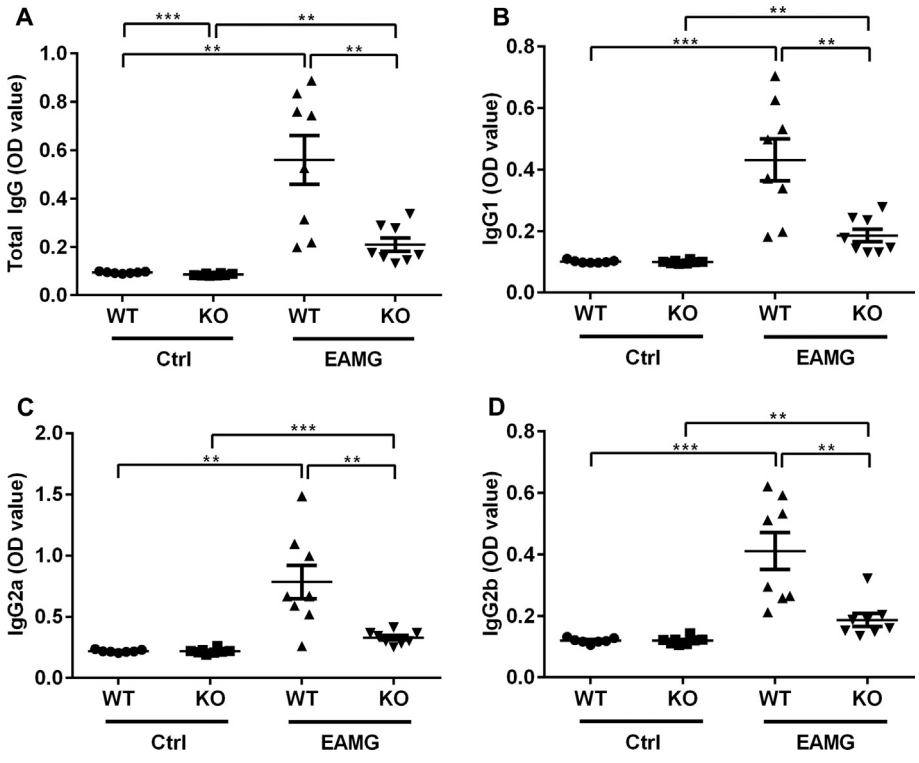

Fig. 2. Anti-tAChR Immunoglobulins. anti-tAChR antibody serum concentrations were measured using ELISA. Total IgG (A), IgG1 (B), IgG2a (C), and IgG2b (D) were evaluated from 31 animals in four groups (WT Ctrl n = 7, WT EAMG n = 8, KO Ctrl n = 8, KO EAMG n = 8). One-tailed, unpaired t-tests were performed to determine statistical significance (*p < 0.05, **p < 0.01, ***p < 0.001), and error bars depict mean and SEM.