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Inhibitory IgG receptor FcγRIIB fails to inhibit experimental autoimmune myasthenia gravis pathogenesis

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Abstract

Deficiency of the inhibitory FcγRIIB renders mice susceptible to autoimmune disorders characterized with cellular infiltration of target tissue. To analyze the role of FcγRIIB in an antibody-mediated autoimmune disease, experimental autoimmune myasthenia gravis (EAMG), FcγRIIB knockout (KO) and wild-type mice were immunized with acetylcholine receptor (AChR). In contrast with previous reports, FcγRIIB KO mice were mildly resistant to EAMG despite preserved anti-AChR antibody production and neuromuscular junction complement deposition capacity. EAMG resistance was associated with reduced lymph node cell IL-6 and IL-10 production and increased $CD4^{+}CD25^{+}$ cell ratios in lymph nodes. Our data suggest that FcγRIIB promotes antibody-mediated autoimmunity.
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Keywords: Myasthenia gravis; FcγRIIB; IL-6; IL-10; Autoimmunity

1. Introduction

Myasthenia gravis (MG) and its animal model, experimental autoimmune myasthenia gravis (EAMG) are both antibody and complement-mediated autoimmune diseases (Christadoss and Dauphinee, 1986; Conti-Fine et al., 2006; Vincent and Drachman, 2000). There is substantial evidence showing that activation of the complement cascade via the classical complement pathway by anti-acetylcholine receptor (AChR) antibodies triggers the damage of the AChR at the postsynaptic neuromuscular junction (NMJ), ultimately leading to AChR loss and subsequent muscle weakness and fatigue (Tuzun et al., 2003; Romi et al., 2005; Tsujihata et al., 1989). This response is brought by a complex network of immune system components, including cytokines and complement factors, initiating and modulating the activities of T, B and dendritic cells (Christadoss and Dauphinee, 1986; Conti-Fine et al., 2006; Vincent and Drachman, 2000). The emerging evidence suggests that the circulating immune complex (CIC) is also a key component of this network. CIC levels are significantly elevated in both EAMG mice and MG patients and are correlated with the severity of muscle weakness (Casali et al., 1976; Mathai et al., 2000; Tuzun et al., 2006a, 2004). We have recently demonstrated that Fcγ receptor (FcγR) III knockout (KO) mice are resistant to EAMG induction (Tuzun et al., 2006b), suggesting that CIC affects EAMG induction by not only activating the classical complement pathway but also by influencing the activities of immune cells via FcγRs.

Due to the crucial role of FcγRIII in promoting inflammation, FcγRIII KO mice are resistant to various autoimmune disorders (Tarzi et al., 2003; Kagari et al., 2003; Kaplan et al., 2002; Hall et al., 2001; Nabbe et al., 2003). In contrast, FcγRIIB KO

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mice are generally highly susceptible to autoimmune diseases (Muhlfeld et al., 2003; Nakamura et al., 2003, 2000) suggesting that FcγRIIB antagonizes the inflammatory response against self-tissue and its deficiency dampens the inhibition of the immune system, which eventually enhances the immune attack against the target tissue and increases disease severity. However, most of the previous studies with FcγRIIB KO mice have been performed on autoimmune diseases primarily mediated by cell mediated immunity and characterized with cellular infiltration of the target tissue. To characterize the role played by FcγRIIB in a classical antibody-mediated autoimmune disease, we induced EAMG in FcγRIIB KO mice by AChR-immunization. In this manuscript, we show that FcγRIIB KO mice develop less severe myasthenic symptoms as compared to wild-type (WT) mice following immunization with AChR, suggesting that FcγRIIb is acting in favor of antibody-mediated autoimmunity.

2. Materials and methods

2.1. AChR and mice

AChR was purified from the electric organ of *Torpedo californica* by α-neurotoxin affinity column (Wu et al., 1997). Seven- to 8-week-old FcγRIIB KO mice in the B6 background and WT B6 mice were purchased from Taconic Farm (Germantown, NY). FcγRIIB KO mice have been produced by back-crossing to B6 mice for 12 generations (N12). All animals were housed in the viral antibody-free barrier facility at the University of Texas Medical Branch and maintained according to the Institutional Animal Care and Use Committee Guidelines.

2.2. Induction and clinical evaluation of EAMG

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. On day 30 and 60, all mice were boosted with 20 μg AChR in CFA s.c. at four sites on the back. Mice were screened for clinical EAMG on a daily basis. Screening was performed separately by two authors, JL and ET [blind observer]. For clinical examination, mice were left 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 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. For objective measurement of muscle strength, mice were first exercised with 40 paw grips on cage top grid. Following exercise, mice were made to grasp a grid attached to a dynamometer (Chatillon Digital Force Gauge, DFIS 2, Columbus Instruments, Columbus, OH). The maximal force applied to the dynamometer while pulling the mouse by its tail until it lost its grip on the grid was recorded. Clinical EAMG was also confirmed by i.p. administration of 50 μl neostigmine bromide, along with atropine sulfate in PBS, and observing improvement in muscle strength.

2.3. RIA to measure muscle AChR content

The total concentration of AChR per mouse carcass was determined according to a previously published method and expressed as picomole (pM) of [^{125}I]-labeled α-bungarotoxin (BTX) binding sites (Wu et al., 1997). Aliquots (0.1 ml) of [^{125}I] BTX-labeled (5×10^{-9} M), Triton X-100 solubilized mouse muscle extracts, with and without benzoquinonium (10^{-3} M), were mixed with 10 μl of mouse anti-AChR serum. The resulting complex was precipitated by goat anti-mouse serum and then centrifuged. Radioactivity of the pellet was counted in a Packard gamma counter (Packard Instrument Co., Meriden, CT), and cpm values of samples with benzoquinonium were subtracted from cpm values of samples without benzoquinonium. The concentration of AChR was expressed as pM of [^{125}I] α-BTX-binding sites per gram of mouse carcass.

2.4. ELISA for anti-muscle AChR antibodies and isotypes

IgM, IgG, IgG1, IgG2b and IgG2c antibody isotypes to mouse muscle AChR were evaluated by ELISA, using a previously described method (Tuzun et al., 2003). Affinity-purified mouse AChR (0.5 μg/ml) was coated onto a 96-well microtiter plate in 0.1 M carbonate bicarbonate buffer overnight at 4 °C. Diluted serum samples of 100 μl (1:500) were added and incubated at 37 °C for 90 min. Horseradish peroxidase (HRP)-conjugated anti-mouse IgM, IgG, IgG1, IgG2b and IgG2c (Caltag Laboratories, Burlingame, CA) (1:1000) were added and then incubated at 37 °C for 90 min. Subsequently, the peroxidase indicator substrate 2,2′-azino-bis-(3-ethylbenzothiazoline 6-sulfonate) substrate (ABTS) solution in 0.1 M citric buffer (pH 4.35) was added in the presence of H_2O_2, and mixture was allowed to develop color at room temperature in the dark. Plates were read at a wavelength of 405 nm. Normal mouse serum (collected from mice before immunization) was used for the background determination.

2.5. Immunohistochemical staining for splenic germinal centers

Four-micron-thick sections of 10% formalin-fixed and paraffin-embedded spleens were prepared. Sections were deparaffinized and rehydrated. Endogenous peroxidase activity was quenched with 3% H_2O_2 in methanol. Sections were blocked for nonspecific binding with normal goat serum diluted 1/20 in DAKO antibody diluent (Dako, Carpinteria, CA) for 15 min. Antigen retrieval was done with DAKO Target Retrieval Solution in steam for 20 min. Sections were then cooled down on the bench top for 20 min, rinsed two to three times with distilled water, and transferred to TBS. Slides were then incubated for 30 min with biotinylated-peanut agglutinin (PNA) (Vector Laboratories, Burlingame, CA) diluted 1/250 in DAKO antibody diluents and washed, followed by a second incubation with streptavidin–HRP. Bound conjugates were visualized with DAKO Liquid

diaminobenzidine substrate–chromogen for 5 min when a brown color for PNA-positive cells was obtained. For all staining steps, a DAKO Autostainer was used. Slides were counterstained for 2 min with Mayer's modified hematoxylin diluted 1/5 in distilled water.

2.6. Measurement of serum C3 and C3-CIC levels

Ninety-six-well microtiter plates (Dynatech Laboratories, Alexandria, VA) were covered with goat antibody to mouse C3 (ICN Biomedicals/Cappel, Aurora, OH) overnight at 4 °C. The plates were then blocked with 2% BSA in PBS at room temperature for 30 min. Diluted (1/10 in PBS-0.05% Tween 20) serum samples were added and incubated at 37 °C for 90 min. After four washes, HRP-conjugated goat anti-mouse C3 complement (ICN Biomedicals/Cappel) diluted 1/500 in PBS/0.05% Tween 20, was added and incubated at 37 °C for 90 min for C3. ABTS substrate solution in 0.1 M citric buffer (pH 4.3) in the presence of H_2O_2 was added, and color was allowed to develop at room temperature in the dark. Plates were read at a wavelength of 405 nm using a Dynatech ELISA reader, and the results were expressed as OD values. Serum C3-CIC levels were determined with the same principle and with the same capture antibodies, with the difference that HRP-conjugated goat anti-mouse IgG (Caltag Laboratories) was used as a secondary antibody.

2.7. Detection of IgG, C3 and membrane attack complex (MAC) deposits at the NMJ

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 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, Temecula, CA), goat anti-mouse complement C3 (ICN-Cappel, Aurora, OH) or rabbit anti-human C5b-9 (MAC) (Calbiochem, San Diego, CA) (diluted 1/1000) to colocalize IgG or complement deposits in NMJ. Anti-IgG and anti-C3 antibodies were FITC-conjugated. For detection of MAC deposits, the muscle tissues were further incubated with Oregon green-conjugated goat anti-rabbit IgG (Molecular Probes). The sections were washed and viewed in a fluorescence microscope (Olympus IX-70 with a DP-11 digital camera). The NMJ deposit percentages were calculated by dividing the number of deposits with the number of BTX-binding sites for each examined muscle section (5 sections per mouse) and multiplying by 100. Then, the average values for each group and deposit were compared for statistical significance.

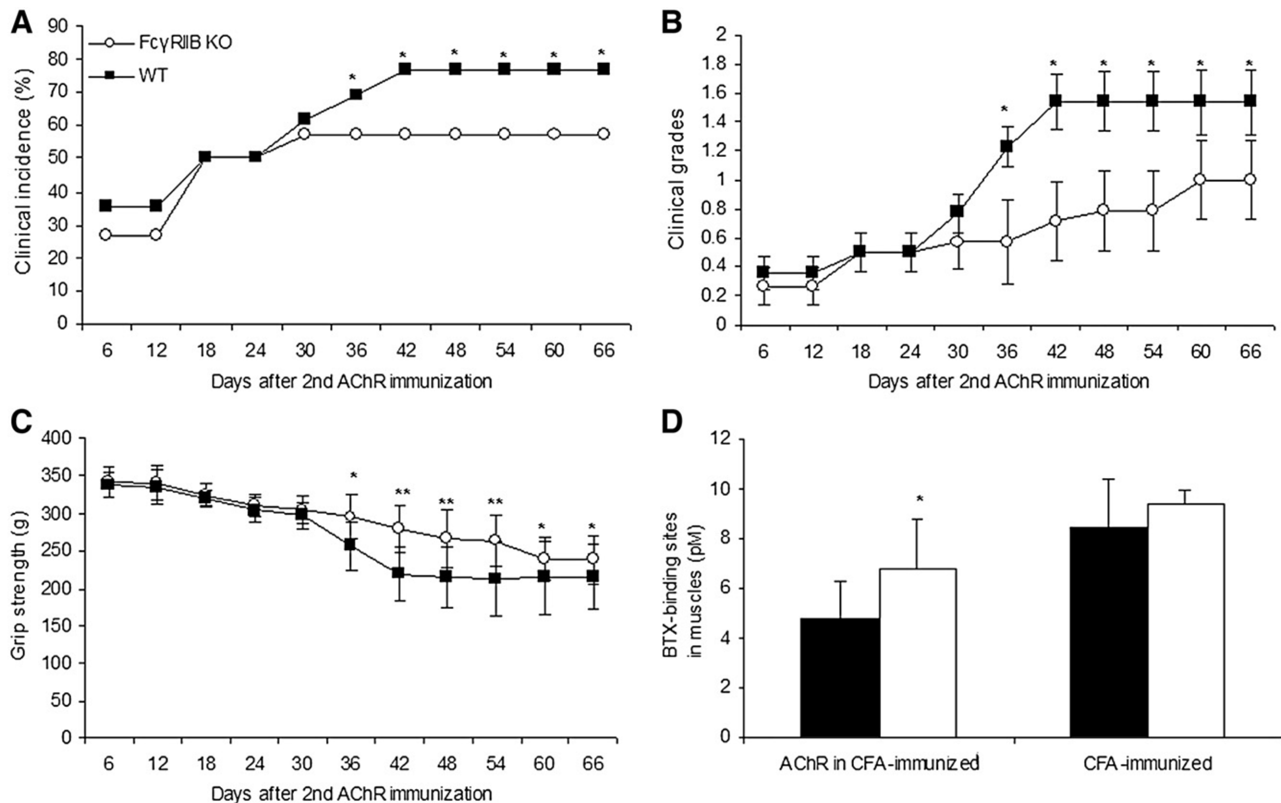

Fig. 1. Kinetics of the accumulated EAMG clinical incidence (A), severity (B), grip strength (C) and muscle AChR content (D) of AChR-immunized WT and FcγRIIB KO mice. FcγRIIB KO mice had significantly lower clinical incidence and severity and higher average grip strength as compared to WT mice from day 36 to 66 (A–C). At termination, WT mice had significantly reduced levels of functional muscle AChR compared to FcγRIIB KO mice (D). *, indicates $p<0.05$, **, $p<0.01$, the bars indicate standard errors. One representation of two independent experiments.

2.8. Lymphocyte proliferation assay

Inguinal, popliteal, and axillary LNC were collected at termination of the experiment. The cells (2×10^5 cells/well) were seeded in triplicate into 96-well, round-bottomed microtiter plates in 0.2 ml of RPMI 1640 medium with or without *Torpedo* AChR (2.5 μg/ml), supplemented with 10% fetal calf serum, penicillin G (100 U/ml) streptomycin (100 μg/ml), L-glutamine (2 mM), 2-mercaptoethanol (3×10^{-5} M), and HEPES buffer (25 mM). The cells were cultured for 5 days at 37 °C in humidified 5% CO_2-enriched air, and pulse-labeled with [3H]TdR (1 μCi per well) for 16 h before harvesting. The ^{3}H incorporation was determined in a Beckman beta scintillation counter (Beckman Coulter Inc., Fullerton, CA). The results are expressed as cpm.

2.9. Cytokine ELISA

At termination, draining LNCs (inguinal, popliteal, and axillary) from individual mice were cultured in 48-well, round-bottomed plates in the presence of *Torpedo* AChR (2.5 μg/ml) for 72 h and the supernatants were collected for IL-2, IL-6, IL-10

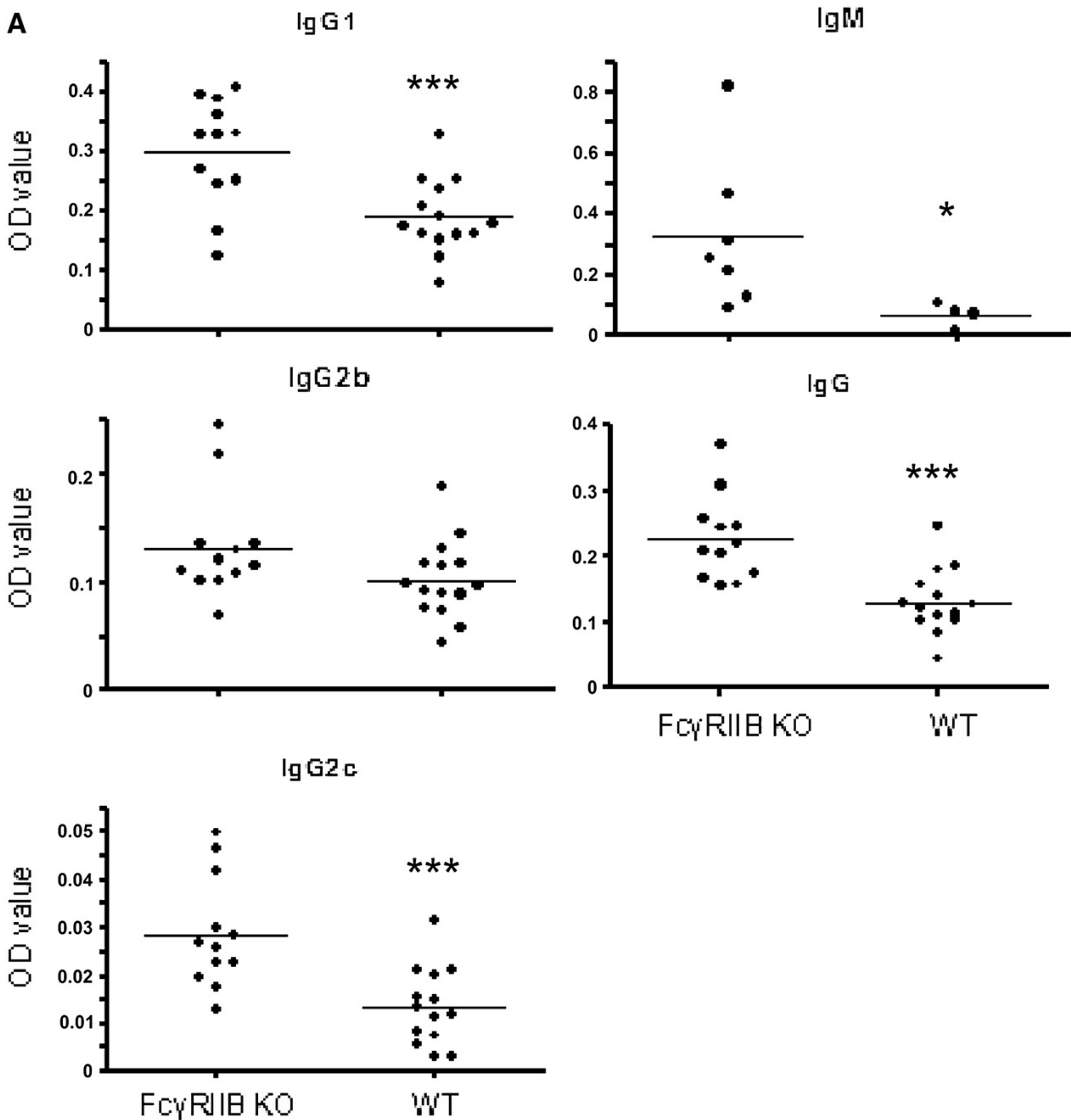

Fig. 2. Predominant elevation of serum anti-AChR IgM and IgG1 antibodies in AChR-immunized FcγRIIB KO mice on days 15 (A) and 45 (B) following second AChR-immunization. The anti-AChR IgG2b levels in sera of FcγRIIB KO and WT mice were comparable at both time points. Anti-mouse AChR isotype titers were determined using ELISA on mouse affinity-purified AChR-coated plates. *, indicates $p<0.05$, ***, $p<0.001$, horizontal lines indicate the mean values for each group. One representation of two independent experiments.

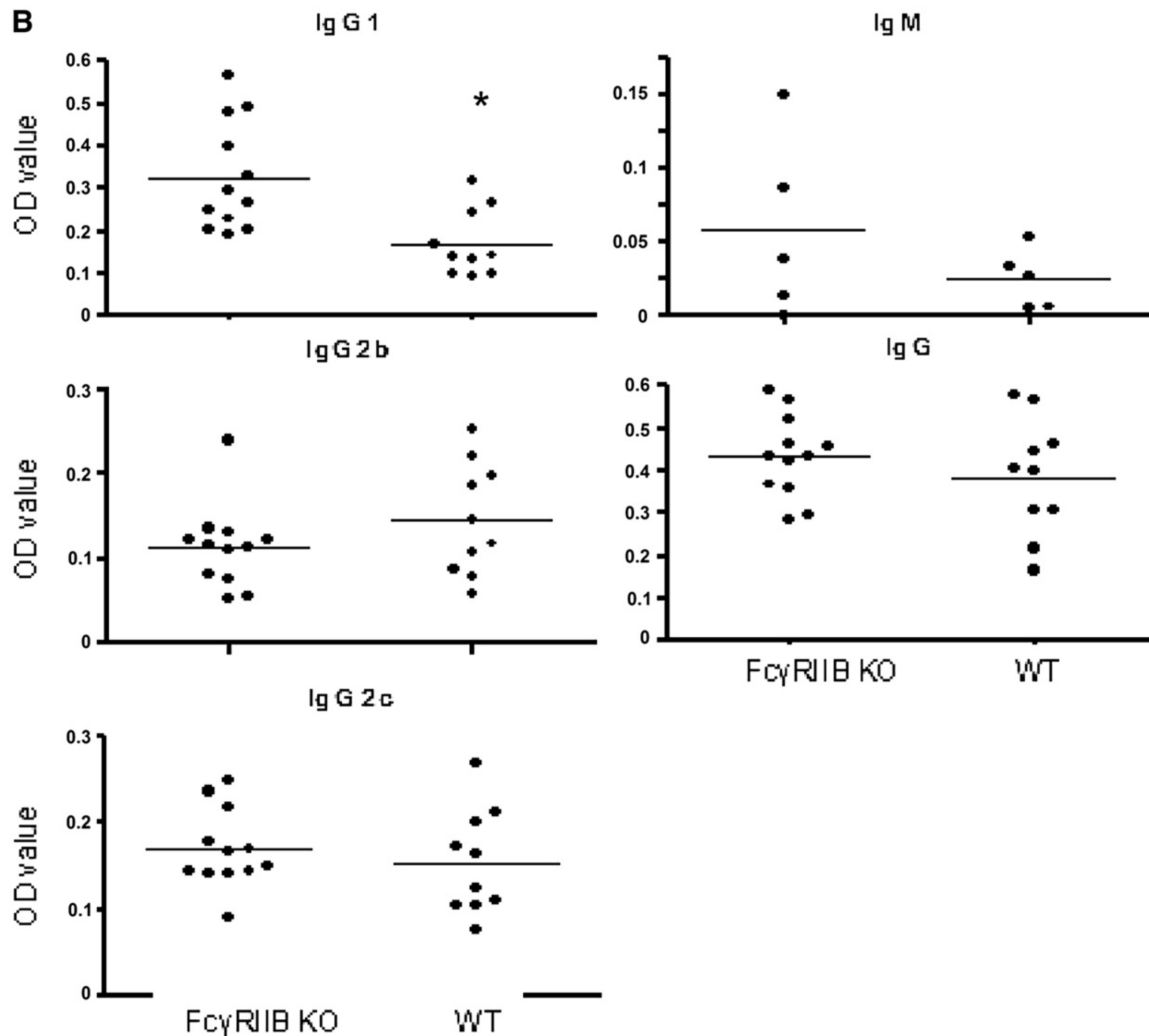

Fig. 2 (*continued*).

and IFN-γ measurements by ELISA, as described before (Tuzun et al., 2003).

2.10. Flow cytometry for CD4, CD19, CD11c and CD25

Single-cell suspensions of LNC were incubated for 30 min with the following anti-mouse antibodies: FITC-conjugated anti-CD4 (with or without PE-conjugated anti-CD25), PE-conjugated anti-CD19 or FITC-conjugated anti-CD11c (all from BD Biosciences, San Jose, CA). PE- or FITC-conjugated isotypes were used for controls. Cells were washed twice and then fixed with 2% paraformaldehyde and analyzed by FACStation flow cytometry (BD Biosciences, San Jose, CA).

2.11. Statistical analysis

To determine the significance of the observed results, three statistical tests were used. Clinical EAMG incidence was compared using the Fisher's exact test, clinical grades were compared using Mann–Whitney *U* test, and all other parameters were compared using Student's *t* test.

3. Results

3.1. AChR-immunized FcγRIIB KO mice are relatively resistant to EAMG

FcγRIIB KO ($n=4$) and WT ($n=13$) B6 mice were immunized on days 0, 30 and 60 with AChR in CFA. Eight of 14 (57%) FcγRIIB KO mice and 10 of 13 (77%) WT mice developed EAMG. The incidence of EAMG in FcγRIIB KO mice was significantly reduced than that of WT mice (at termination $p<0.05$ by Fisher's exact test) (Fig. 1A). EAMG severity was also reduced in FcγRIIB KO mice compared to WT mice. Severe grade 3 muscle weakness was only observed in WT mice and not in FcγRIIB KO mice. The difference among groups in clinical severity attained statistical significance starting on day 36 after 2nd AChR-immunization ($p<0.05$ by Mann–Whitney *U*) (Fig. 1B). Moreover, in the same time frame, average grip strength values of FcγRIIB KO mice were significantly higher than those of WT mice (Fig. 1C). Our data suggest that the deficiency of FcγRIIB gene fails to augment disease severity, and rather suppresses EAMG severity.

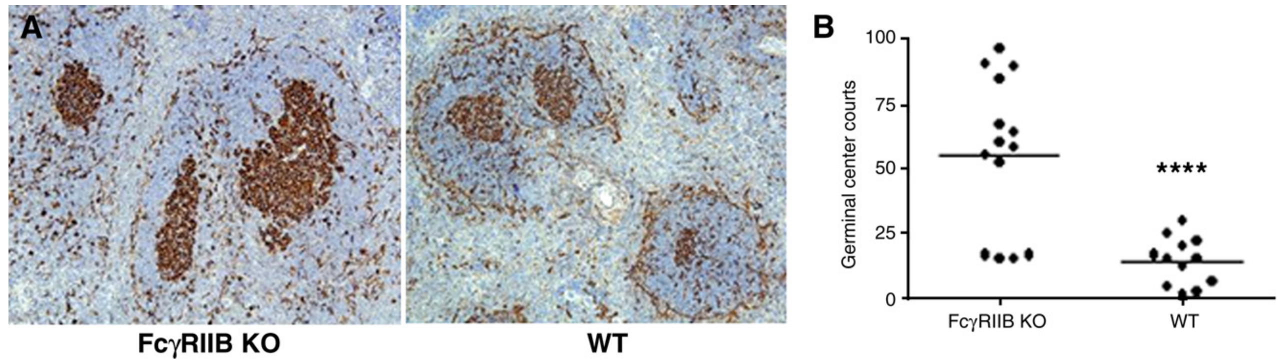

Fig. 3. Splenic germinal center size (A) and number (B) are enhanced in FcγRIIB KO mice. Spleens from FcγRIIB KO and WT mice were isolated at termination and stained with biotynilated-PNA. One representation of 14 PNA-stained spleens from FcγRIIB KO and 13 spleens from control WT mice. Original magnification, ×250. Horizontal lines indicate the mean values for each group, **** indicates $p<0.0001$.

The primary pathology in MG and EAMG is a significant reduction of muscle AChR due to antibody- and complement-mediated attack to the NMJ. The number of muscle BTX-binding sites, which reflects the amount of functionally available muscle AChR, was measured in both groups at termination. Mice ($n=6$) from both strains only immunized with CFA were used as controls. In line with the clinical observations, the muscle tissue of AChR-immunized FcγRIIB KO mice had significantly higher ($p<0.05$ by Student's t test) BTX-binding sites than that of WT mice (Fig. 1D), indicating that FcγRIIB KO mice had higher functional muscle AChR content. There was no difference between the muscle AChR contents of non-immunized FcγRIIB KO and WT mice.

3.2. FcγRIIB gene deficiency augments anti-AChR IgM and IgG1 but not IgG2b production

Serum anti-AChR IgM, IgG, IgG1, IgG2b and IgG2c isotype levels were detected by ELISA. On day 15 following the second AChR-immunization, the levels of all anti-AChR antibody isotypes except anti-AChR IgG2b, were significantly higher in sera of FcγRIIB KO mice as compared to WT mice (Fig. 2A). On day 45, following the third AChR-immunization, FcγRIIB KO mice still had higher serum anti-AChR IgG1 antibody isotype levels than WT mice (Fig. 2B). The data indicate that FcγRIIB gene deficiency augmented the IgM and non-complement fixing IgG1, but not complement fixing IgG2b anti-AChR antibodies.

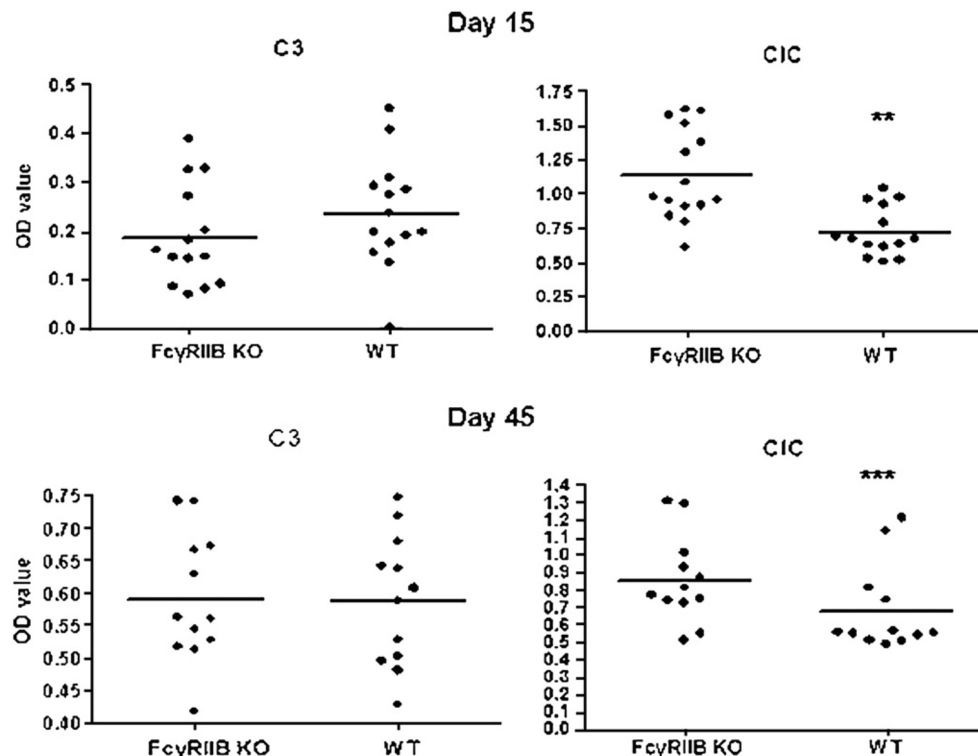

Fig. 4. Serum C3 and C3-conjugated CIC levels of FcγRIIB KO and WT mice, 15 and 45 days following 2nd AChR-immunization. **, indicates $p<0.01$, ***, $p<0.001$, horizontal lines indicate the mean values for each group. One representation of two independent experiments.

3.3. FcγRIIB KO mice have increased number and size of germinal centers

Spleens from FcγRIIB KO and WT mice were isolated at termination and germinal centers were detected by immunohistochemistry using PNA as a marker. The numbers and sizes of PNA+ splenic germinal center follicles of FcγRIIB KO mice were significantly greater than those of WT mice (Fig. 3), in consistency with the higher serum levels of anti-AChR antibodies in KO mice.

3.4. FcγRIIB KO mice have enhanced CIC levels and reduced NMJ complement and IgG deposits

AChR-immunized FcγRIIb KO mice had elevated serum C3-CIC levels as compared to WT mice on days 15 and 45 following 2nd AChR-immunization. However, serum C3 levels of FcγRIIB KO and WT mice were identical (Fig. 4). To clarify the mechanism by which FcγRIIB gene deficiency confers protection against EAMG induction, we double stained frozen muscle sections of mice with BTX (binds to NMJ) and antibodies directed against IgG, C3, or MAC (C5b-C9). Similar to WT mice, AChR-immunized FcγRIIB KO mice displayed all three types of deposits at their NMJs (Fig. 5A), suggesting that FcγRIIB KO mice are resistant to EAMG induction despite the accumulation of IgG and complement deposits at NMJs. Additionally, FcγRIIB KO mice displayed comparable NMJ C3, IgG and MAC deposits to WT mice (Fig. 5B). Therefore, the reduced pathogenicity but not the quantity of antibodies appear to be causing EAMG resistance in FcγRIIB KO mice.

3.5. EAMG resistance of FcγRIIB KO mice is associated with reduced LNC IL-6 and IL-10 production

At termination, LNCs from FcγRIIB KO and WT mice were stimulated *in vitro* with or without AChR and measured for lymphocyte proliferation and cytokine, IFN-γ, IL-2, IL-6 and IL-10 secretion. LNCs of FcγRIIB KO and WT mice showed comparable proliferative responses to AChR (*data not shown*). On the other hand, LNCs of FcγRIIB KO mice exhibited significantly enhanced IFN-γ and IL-2 (Fig. 6A,B) and suppressed

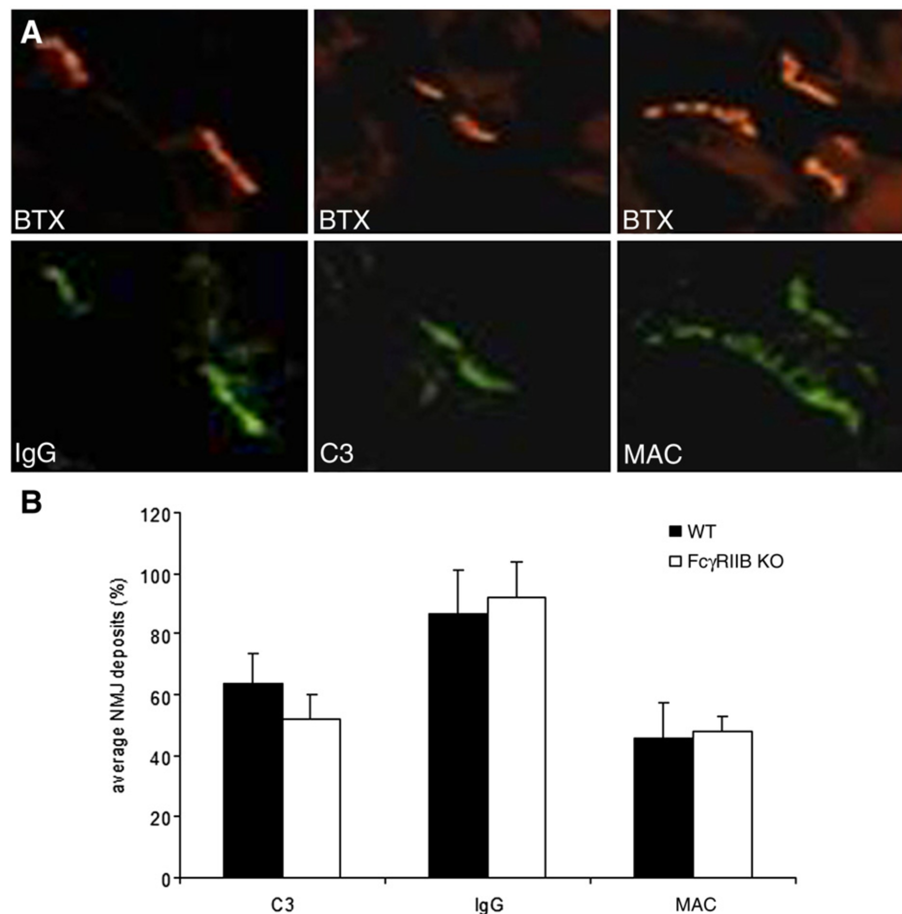

Fig. 5. IgG, C3 and MAC deposits at the NMJs of AChR-immunized FcγRIIB KO mice (A). Frozen muscle sections were stained for IgG, C3 and MAC (bottom panels, all green fluorescence) and the NMJs were co-localized by BTX (top panels, red fluorescence) (magnification for all, ×200). The immunofluorescence data represent one of 5 sections for each mouse. FcγRIIB KO mice had similar amounts of C3, IgG and MAC deposits at their NMJs as WT mice (B). The bars indicate standard errors. One representation of two independent experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

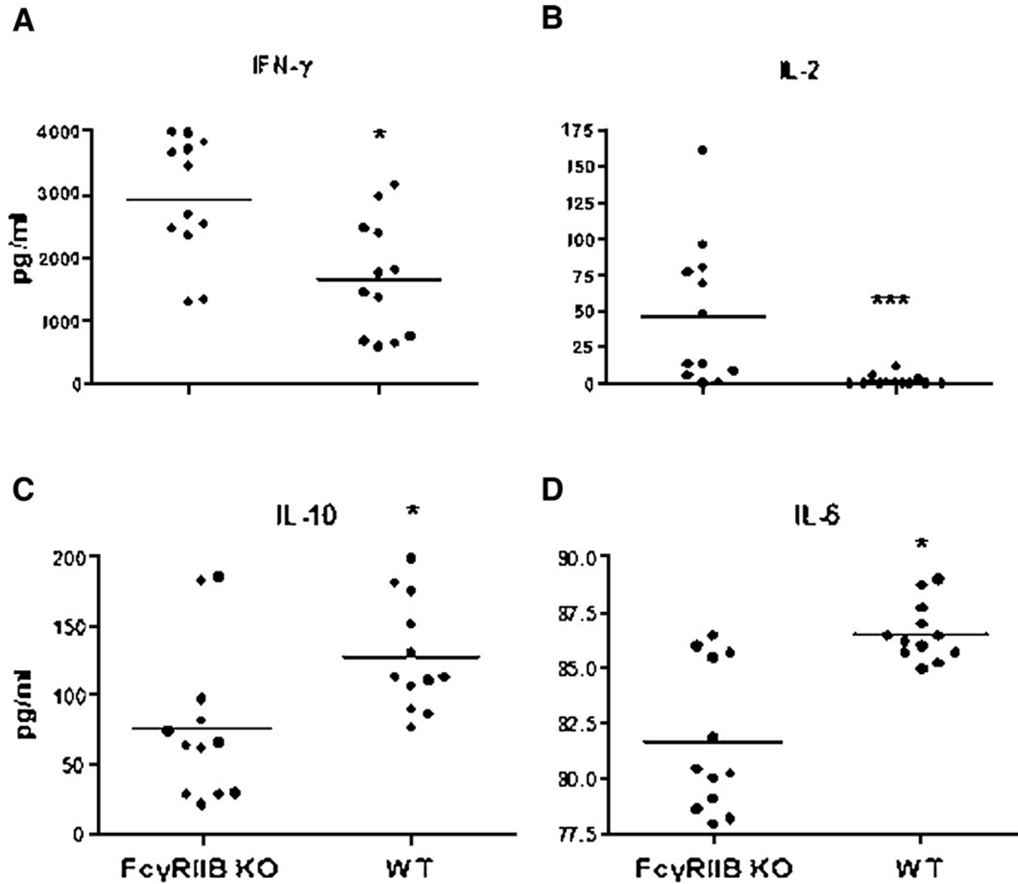

Fig. 6. Cytokine responses of LNC collected from AChR-immunized FcγRIIB KO and WT mice at termination. FcγRIIB KO mice revealed increased IFN-γ (A) and IL-2 (B) and decreased IL-10 (C) and IL-6 (D) responses to AChR stimulation. Results are given as cytokine production in supernatants in pg/ml for each mouse. *, indicates $p<0.05$, ***, $p<0.001$, horizontal lines indicate the average values for each group. One representation of two independent experiments.

IL-6 and IL-10 (Fig. 6C,D) production as compared to those of WT mice.

3.6. $CD4^+CD25^+$ T cells are elevated in FcγRIIB KO mice

There were no significant differences between the percentages of lymph node $CD4^+$, $CD19^+$ and $CD11c^+$ cell populations of AChR-immunized FcγRIIB KO and WT mice, suggesting that T cell, B cell and dendritic cell populations are not impaired in FcγRIIB gene deficiency. Alternatively, FcγRIIb KO mice had significantly more $CD4^+CD25^+$ LNCs than WT mice (Fig. 7).

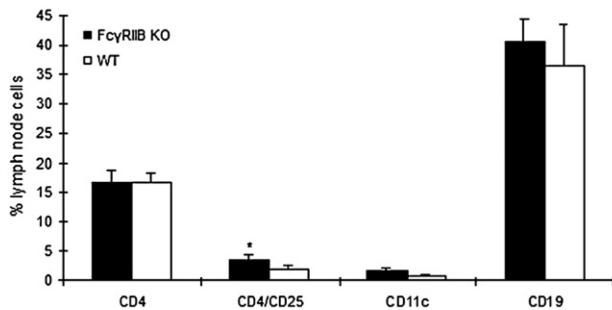

Fig. 7. The lymph node T, B and dendritic cell ratios of AChR-immunized FcγRIIB KO and WT mice estimated by FACS analysis. The lymph nodes of FcγRIIB KO mice contained significantly increased $CD4^+$ $CD25^+$ cells as compared to WT mice. Results are expressed as the percentage of positive cells for each marker. Data represent one of two similar experiments. * indicates $p<0.05$, bars indicate standard errors.

4. Discussion

IgG-FcγR interactions are known to be critical for the humoral and cellular immune responses and are implicated in promotion of inflammation in autoimmune diseases (Dijstelbloem et al., 2001; Brauweiler and Cambier, 2003). Four different classes of FcγRs have been identified in mice; FcγRI (CD64), FcγRII (CD32), FcγRIII (CD16) and FcγRIV. While FcγRI displays high affinity for the Fc region of IgG and restricted isotype specificity, FcγRII and FcγRIII have low affinity and broader isotype binding pattern. FcγRIV has an intermediate affinity and restricted subclass specificity (Nimmerjahn et al., 2005).

Among these receptors, FcγRIIB and III have been shown to be involved in various immune functions such as macrophage phagocytosis, neutrophil activation, immune complex clearance and antigen presentation to T cells. FcγRIIB is expressed on all myeloid cells, except T cells and NK cells and also it is the only

antibody binding Fc receptor on B cells. It is crucial for the expansion of autoactive B cells, maturation of B cells to plasma cells and maturation of dendritic cells (Dijstelbloem et al., 2001; Brauweiler and Cambier; 2003; Nimmerjahn et al., 2005). Due to the significant importance of FcγRIII functions in inflammatory response, FcγRIII KO mice are naturally resistant to a number of autoimmune disorders including autoimmune nephritis (Tarzi et al., 2003) and autoimmune arthritis (Kagari et al., 2003; Kaplan et al., 2002; Nabbe et al., 2003). FcγRIII gene deficiency also renders mice resistant to EAMG and this resistance was associated with decreased C3, IgG and C5b-9 deposits at NMJ, reduced serum C1q, C3 and CIC levels and LNC IL-6 production (Tuzun et al., 2006b).

In contrast, FcγRIIB KO mice have been constantly shown to have an increased susceptibility to autoimmune disorders such as membranoproliferative glomerulonephritis (Muhlfeld et al., 2003), collagen-induced arthritis (Nakamura et al., 2003) and Goodpasture's syndrome (Nakamura et al., 2000). However, our results have shown for the first time that AChR-immunized FcγRIIB KO mice are significantly resistant to antibody-mediated EAMG. This contradiction might be due to the fact that previously studied disease models are characterized with cellular infiltrates or non-complement fixing IgG1 antibody. On the other hand, EAMG mice have little or no inflammatory cells in their muscle tissue and muscle weakness develops solely by antibody and complement-mediated NMJ destruction. Therefore, our results also imply that FcγRIIB is involved in immunological function(s) essential for induction of autoimmunity in an antibody-mediated disorder.

Immune complexes have long been shown to induce IL-6 and IL-10 production by an FcγRIIB-dependent mechanism (Tejde et al., 2004; Ronnelid et al., 2003) and IL-6 and IL-10 KO mice are significantly resistant to EAMG induction (Deng et al., 2002; Poussin et al., 2000). Therefore, reduced LNC release of these two cytokines would be expected to be associated with EAMG resistance of FcγRIIB KO mice. However, deficiency of these cytokines is associated with reduced humoral immunity and anti-AChR antibody production and FcγRIIB KO mice have preserved serum antibody and C3 levels, suggesting that reduced IL-6 and IL-10 production is not a major factor in EAMG resistance. The suppressive role of $CD4^{+}CD25^{+}$ cells in autoimmune disorders including MG has been well established (Sun et al., 2004; Aruna et al., 2005; Liu et al., 2005). As shown for the first time in our experiments, FcγRIIB gene deficiency acts in favor of generation of lymph node $CD4^{+}CD25^{+}$ T cells, which might also have contributed to EAMG resistance of FcγRIIB KO mice.

Just like FcγRIII KO mice (Tuzun et al., 2006b), FcγRIIB KO mice have increased germinal center numbers, serum anti-AChR antibody and CIC levels. All these data suggest that FcγRIIB and FcγRIII both have an inhibitory effect on anti-AChR antibody production. In contrast with FcγRIII, which increases C3 production (Tuzun et al., 2006b), FcγRIIB does not influence serum C3 levels. Similar results have been obtained in collagen-induced arthritis, systemic lupus erythematosus and Goodpasture's syndrome models (Nakamura et al., 2000, 2003; Clynes et al., 2005). Additionally, AChR-immunized FcγRIIB KO mice display increased serum anti-AChR IgM levels. These results suggest that both FcγRIIB and III play a regulatory role on antibody production and isotype switching probably by their direct effects on germinal center cells. High serum anti-AChR antibody and CIC levels and preserved complement production and NMJ C3 and IgG deposit accumulation abilities might seem to be contradicting reduced EAMG incidence and severity observed in FcγRIIB KO mice. However, in both MG and EAMG, serum anti-AChR antibody levels are not correlated with the severity of disease suggesting that the pathogenicity but not the quantity of these antibodies in serum determine the degree of muscle weakness. For instance, anti-AChR antibodies of FcγRIIB KO mice might not be suppressing AChR functions or activating the complement cascade as efficiently as those of WT mice. This assumption is best supported with the finding that muscle BTX-binding sites of AChR-immunized FcγRIIB KO mice are better preserved than those of WT mice. Notably, just like FcγRIIB KO mice, anti-AChR antibody production and lymphocyte proliferation capacities of EAMG resistant IL-10 KO and FcγRIII KO mouse strains were comparable to or greater than those of WT mice (Tuzun et al., 2006b; Poussin et al., 2000). These mice also displayed reduced LNC IL-6 and/or IL-10 production following AChR-immunization. Whether reduced LNC IL-6 and IL-10 production and/or increased $CD4^{+}CD25^{+}$ T cell populations might contribute to this phenomenon remains to be determined.

Higher anti-AChR IgG1 levels observed in FcγRIIB KO mice might even have a somewhat protective role in EAMG. The complement-mediated NMJ destruction initiated by anti-AChR IgGs is the most crucial component of EAMG pathogenesis. The non-complement activating IgG1 and complement-activating IgG2 isotypes compete on the target NMJ AChR. Therefore, higher anti-AChR IgG1 levels might be protecting FcγRIIB KO mice from NMJ destruction by preventing IgG2b binding and thereby decreasing the complement-mediated muscle cell membrane lysis. In line with this assumption, EAMG resistant IL-6 KO mice (Deng et al., 2002), DBA/1 mice protected from experimental autoimmune encephalomyelitis by myelin oligodendrocyte glycoprotein vaccination (Wallberg et al., 2003) and *Torpedo* AChR/Alum immunized B6 mice with mild EAMG severity (Milani et al., 2006) all have significantly higher IgG1 isotypes in their sera than control mice.

Human FcγRII gene shows allelic heterogeneity and some FcγRII genotypes are associated with MG susceptibility or resistance (van der Pol et al., 2003), suggesting that FcγRII is also involved in human MG pathogenesis. In conclusion, our results are providing the first direct genetic evidence on the role of FcγRIIB on EAMG pathogenesis and showing for the first time that certain FcγRIIB-mediated immune functions act in favor of specific humoral immunity.

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