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Full Length Research Paper

Tα146-162-iMDC in intervention of experimental autoimmune myasthenia graves in terms of B cell activation

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To explore the mechanism of Tα146-162-iMDC in intervention of experimental autoimmune myasthenia gravis (EAMG) in terms of B cell activation through use of dendritic cells pulsed with Talpha146-162 in EAMG. Thirty-four healthy male C57BL/6J mice aged 6 to 8 weeks were randomized into model group (Group A), intervention group (Group B) and control group (Group C). Dendritic cells were cultured and loaded with Tα146-162 for intervention in Group B. From initial immunization to 90th day when the experimentation was terminated, the severity of EAMG was evaluated and the morbidity was calculated. Cbl-b mRNA was detected, and Syk and Lyn protein expression and phosphorylation were assessed. Anti-AChR IgM, total IgM, IgG1, IgG2b, and IgG2c were detected on 15d, 45d and 75d after initial immunization. The morbidity of EAMG in Group A was higher than in Group B (P<0.05), while no EAMG occurred in Group C. Cbl-b mRNA in the spleen and lymph nodes was statistically significantly higher in Group A than in Group C (P<0.01), while higher in Group B than in Group A (P<0.05), but lower than in Group C (P<0.05). Syk protein expression and phosphorylation in Group A was higher than in Group C (P<0.01), while lower in Group B than in Group A (P<0.05) and higher than in Group C (P<0.05). Lyn protein expression and phosphorylation in Group A was lower than in Group C (P<0.01), while higher in Group B than in Group A (P<0.05), and lower than in Group C (P<0.050). Anti-AChR antibody in Groups A and B was apparently higher than Group C (P<0.01) after immunization. There was no significant difference in anti-AChR IgM between Groups A and B at different time points (P>0.05). Anti-AChR IgG and IgG1 decreased in Group B compared to Group A at different time points (P<0.05, Day 15; P<0.05, Days 45 and 75, P<0.01). After secondary immunization, anti-AChR IgG2b decreased in Group B compared to Group A at different time points (P<0.05, Day 45; P<0.01, Day 75). No statistically significant difference was noted in Anti-AChR IgG2c between Groups A and B at different time points (P>0.05). Cbl-b suppresses secretion of subtypes of anti-AchR antibodies and B cell activation through negative regulation of B cell antigen receptor (BCR) signal pathway. Induction of B cell tolerance is a possible mechanism of intervention of Tα146-162-iMDC in EAMG.

Key words: Experimental autoimmune myasthenia graves (EAMG), Cbl-b, anti-AChR antibody, B cell activation.

INTRODUCTION

Experimental autoimmune myasthenia gravis (EAMG) is a classical animal model for myasthenia graves (MG) (Yang et al., 2000). B cell antigen receptor is a surface marker of B cells that is essential for activation of B cells (Vilen et al., 2002). Ubiquitin E3 ligase Casitas B lineage lymphoma b (Cbl-b), a member of the RING-type ubiquitin ligase family can promote ubiquitination of protein tyrosine kinase (PTKs) that activates receptors including the B and T cell receptors for antigen and the widely distributed receptors for the Fc portion of immunoglobulins, negatively regulating B cell antigen

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receptor (BCR) signal pathway and inducing B cell immune tolerance (Ruschmann et al., 2010). B cell activation and secretory anti-AChR antibody are essential for onset of MG/EAMG. Serum antibodies in a single patient are closely correlated with severity of the disease; the type of antibodies also determines prognosis of the patient. As an important cell type in development of EAMG, B cells decide the development and the recovery of the disease. Thus AchR targeted specific B cell treatment may be a potential choice in the future.

In the current study, EAMG was intervened with immature bone marrow dendritic cells (iMDCs) loaded with Tα146-162. The expression of Cb1-b mRNA in the spleen and lymph nodes, dynamic changes of Syk and Lyn protein expression and phosphorylation mediated by BCR and different types of anti-AChR subtypes in serum were detected to investigate possible mechanism of Tα146-162-iMDC in intervention of EAMG in terms of B cell activation to provide theoretical and experimental basis for antibody-specific treatment.

MATERIALS AND METHODS

Animals and reagents

Thirty-four specific pathogen free (SPF) inbred line healthy male C57BL/6J mice aged 6 to 8 weeks (weight: 20 to 24 g) were used in the study. Fifteen SPF healthy male C57Bl/6J mice were purchased from SLAC Laboratory Animals (weight: 18 to 22 g). T-AchR was distilled and purified from California numbfish. Tolerant peptide Tα146-162 was Leu-Gly-Ile-Trp-Thr-Tyr-Asp-Gly-Thr-Lys-Val-Ser-Ile-Ser-Pro-Glu-Ser with purity (HPLC)≥95% (52968, GLS).

Methods

Thirty-four C57BL/6J mice were randomized into three groups: model group (Group A, n = 12), intervention group (Group B, n = 12) and control group (Group C, n = 10). T-AChR emulsion was injected substaneously through shoulder and foot pad on Days 0, 30 and 60 to induce EAMG in Group A. In Group B, the animals were injected with T-AChR emulsion as in Group A. They were intervened with iMDCs loaded with Tα146-162 (50 μg/ml) at Days 3, 33 and 63. In Group C, complete Freund's adjuvant (CFA) and phosphate buffered saline (PBS) were injected subcutaneously on Days 0, 30 and 60; 1 ml PBS was injected subcutaneously on Days 3, 33 and 63. The severity of EAMG was evaluated according to Berman et al. (1980). Dendritic cells (DCs) were harvested from fifteen C57BL/6J mice aged 4 to 6 weeks that were independent from grouping.

Culturing DCs

According to the methodology detailed by Wang et al. (2009a), bone marrow was distilled from C57BL/6J mice and DCs were cultured. Fluorescent antibody was added to label CD11c, CD40, CD86 and MHC-II. Fluorescent-labeled Ig was used as control. DCs were detected through FACS caliber (BD, Franklin Lakes, New Jersey, USA). The phenotype of DCs was assessed using CellQuest software. 50 μg/ml Tα146-162 was added to be loaded in DCs.

Expression of Cbl-b mRNA by RT-PCR

Primers for Cb1-b were sense: 5'GGGTCTCAGAGGCAATG3'; antisence: 5'TTCCTACCAGC TCCAACA3'. Primers for β-actin were sense: 5'CTGTCCCT GTATGCCTCTG 3'; antisence: 5'GGATGTCAACGTCAC -ACTTC 3'. Products for them were 585 and 451 bp, respectively. The spleen and lymph nodes were harvested at Day 90. Total RNA was distilled using Trizol, amplified by PCR and reversely transcripted. Reaction consisted of 30 cycles of predenaturalization at 95°C for 3 min, denaturalization at 94°C for 1 min, annealing at 53°C for 40 s and extension at 72°C for 45 s. A final extension was done at 72°C for 7 min. PCR products underwent gel electrophoresis. The optical density was analyzed using Tanon GIS-2020 Analysis System (Tanon Co., Shanghai, China).

Syk and Lyn protein expressions and phosphorylation by Western blot

Proteins were extracted from lymph nodes and the spleen at Day 90. Protein concentration was measured through BCA method. After denaturalization of proteins, they underwent 10% SDS-PAGE electrophoresis and transferred to nitrocellulose filter for 1.5 to 2 h using wet transfer at 150 mA. 3% BSA + 5% skimmed milk was added for blocking at 37°C for 1 h. Rabbit anti-mouse Syk antibody, anti-Lyn antibody and HRP-p-Tyr antibody (1:500) were added for shaking at 4°C overnight. After rinsing with PBS, horse radish peroxidase labeled goat anti-rabbit secondary antibody (1:2000) was added and placed at room temperature for 1 to 1.5 h. After rinsing with TBS, ECL reagent was added for film processing and development. GIS gel image analyzer V 3.74 was used to analyze films, with β-actin (1:2000) as the internal reference.

Measurement of serum anti-AChR antibody

At Day 0 (prior to initial immunization), Days 15, 45 and 75 after initial immunization, blood was sampled from caudal vein in Groups A, B and C. Anti-AChR antibody (HRP-labeled goat anti-mouse IgG, IgG1, IgG2b, IgG2c and IgM) was measured using ELISA.

Statistical analysis

Measurement data were expressed as $\overline{X} \pm s$. All statistical analyses were done using SPSS 13.0. Comparison among multiple groups was performed using ANOVA. Inter-group comparison was done using SNK method. Semi-quantitative scores were compared using Mann-Whitney U test. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Growth and morphology of DCs

After DCs were cultured for 24 h, round monocytes growing in cluster adhering to the wall were observed under the inverted phase contrast microscope. After they were cultured for 3 d, many small and round cells adhered loosely to the plate in cluster; they grew increasingly larger. 6 d later, they grew irregularly adhering to the wall, with thorns on the surface. Flow cytometry found $CD11_C^+$, $CD40^{low}$, $CD86^{low}$ and $MHC\text{-}II^{low}$,

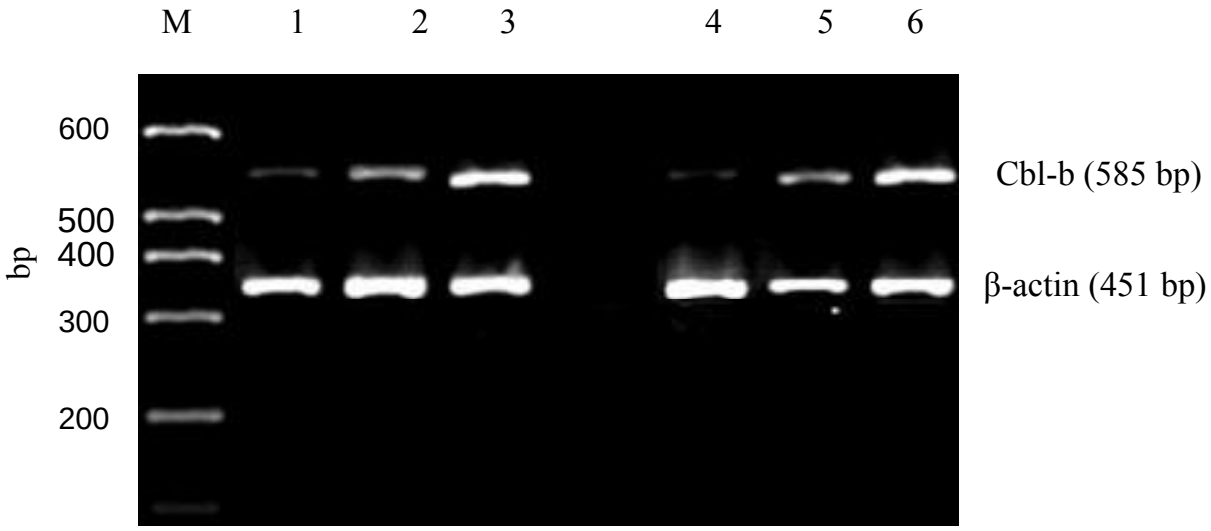

Figure 1. Expression of Cbl-b mRNA in the spleen and lymph nodes in three groups. M: Marker; 1: Spleen in the model group; 2: Spleen in the intervention group; 3: Spleen in the control group; 4: Lymph nodes in the model group; 5: Lymph nodes in the intervention group and 6: Lymph nodes in the control group.

demonstrating they were iMDC.

Behaviors of mice

The model mice presented with different degrees of amyasthenia. They had few activities, consumed less food, and became lean with crooked posture and even systemic failure. Symptoms were partially relieved after use of neostigmine.

Incidence of EAMG and clinical scoring

No EAMG developed in Groups A and B. At Day 39, 16.7% mice were sick in Group A, and 8.3% were sick at Day 57, in a postponed manner compared to Group A. When experimentation was terminated, 75% mice were sick in Group A, and 25% in Group B, showing a statistically significant difference between two groups ($p<0.05$). Before they were sacrificed, one died because of serious illness in Group A, but none died in Group B. There was a statistically significant difference in the clinical score between two groups since Day 66 ($p<0.05$). The two groups achieved a score of 1.67±1.15 versus 0.33±0.65 ($p<0.01$).

Cbl-b mRNA expression in the spleen and lymph nodes in three groups

Cbl-b mRNA in the spleen and lymph nodes was higher in Group A than in Group C ($p<0.01$), while it was higher in Group B than in Group A ($p<0.05$), but lower than in Group C ($p<0.05$) (Figure 1).

Syk protein expression and phosphorylation in the spleen and lymph nodes in three groups

Syk protein expression in the spleen and lymph nodes in Group A was higher than in Group C ($p<0.01$), while it was lower in Group B than in Group A ($p<0.05$), and higher than in Group C ($p<0.050$). Syk protein phosphorylation in the spleen and lymph nodes in Groups A and B was higher than in Group C. Syk protein phosphorylation in the spleen and lymph nodes in Group B was lower than in Group A, but still higher than in Group C (Figure 2).

Lyn protein expression and phosphorylation in the spleen and lymph nodes in three groups

Lyn protein expression and phosphorylation in Group A was lower than in Group C ($p<0.01$), while it was higher in Group B than in Group A ($p<0.05$), and lower than in Group C ($p<0.050$). Syk protein phosphorylation in the spleen and lymph nodes in Groups A and B was higher than in Group C. Lyn protein phosphorylation in the spleen and lymph nodes in Group B was higher than in Group A, but still lower than in Group C (Figure 3).

Change of anti-AChR antibody in three groups

There was no significant difference in anti-AChR IgM between Groups A and B at different time points ($p>0.05$). Anti-AChR antibody in Groups A and B was apparently higher than Group C ($p<0.01$) at Days 15, 45 and 75 after immunization (Figure 4). Anti-AChR IgG in Groups A and B was higher than in Group C at different time points

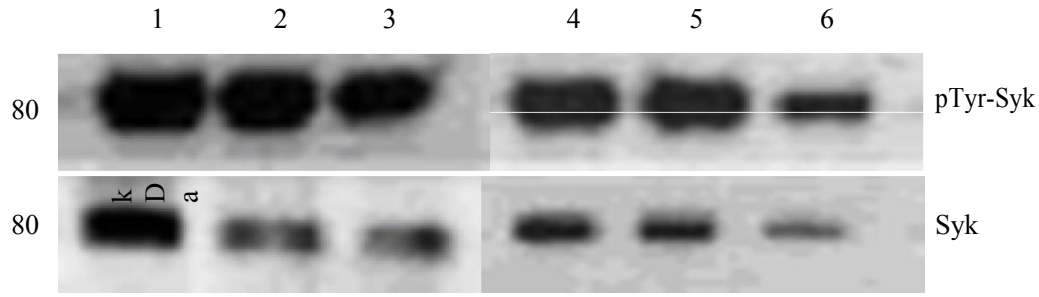

Figure 2. Syk protein and phosphorylation in the spleen and lymph nodes in three groups. M: Marker; 1: Spleen in the model group; 2: Spleen in the intervention group; 3: Spleen in the control group; 4: Lymph nodes in the model group; 5: Lymph nodes in the intervention group and 6: Lymph nodes in the control group.

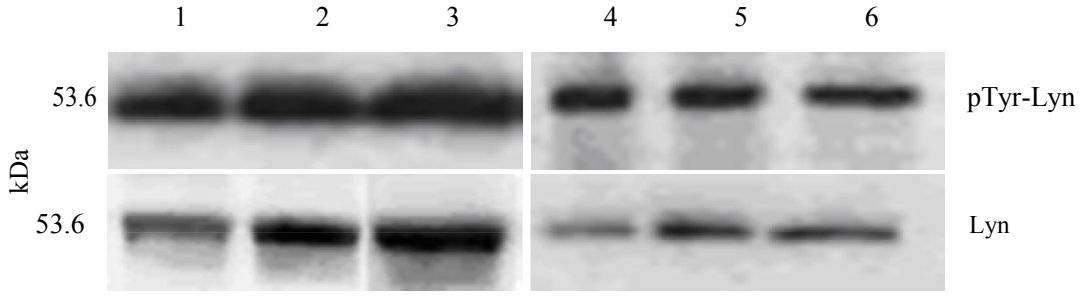

Figure 3. Lyn protein and phosphorylation in the spleen and lymph nodes in three groups. M: Marker; 1: Spleen in the model group; 2: Spleen in the intervention group; 3: Spleen in the control group; 4: Lymph nodes in the model group; 5: Lymph nodes in the intervention group and 6: Lymph nodes in the control group.

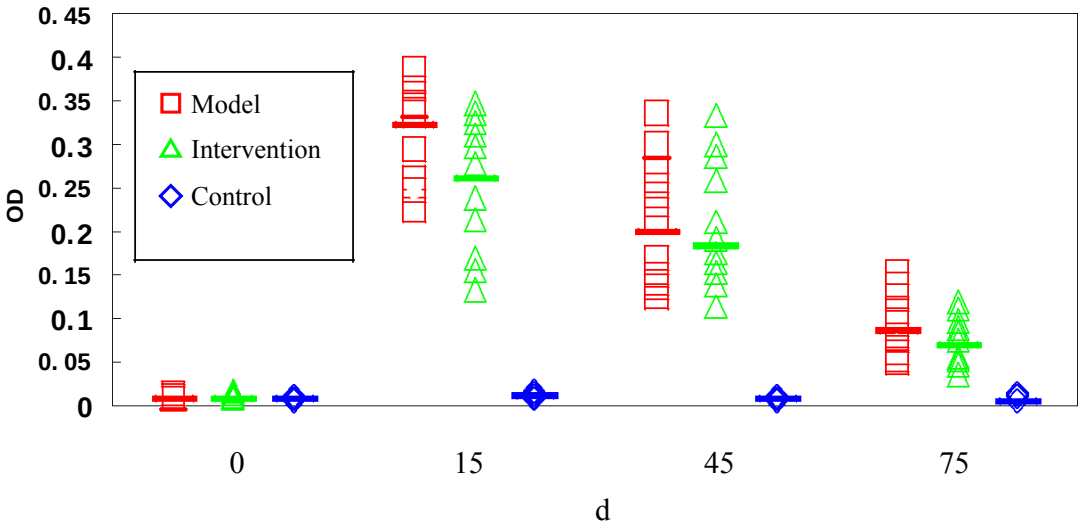

Figure 4. IgM in different groups at different time points.

(p<0.05). Anti-AChR IgG decreased in Group B compared to Group A at different time points (p<0.05, Day 15; p<0.05, Days 45 and 75, p<0.01) (Figure 5). Anti-AChR IgG1 in Groups A and B was higher than in Group C at different time points (p<0.05). Anti-AChR IgG1 decreased in Group B compared to Group A at different

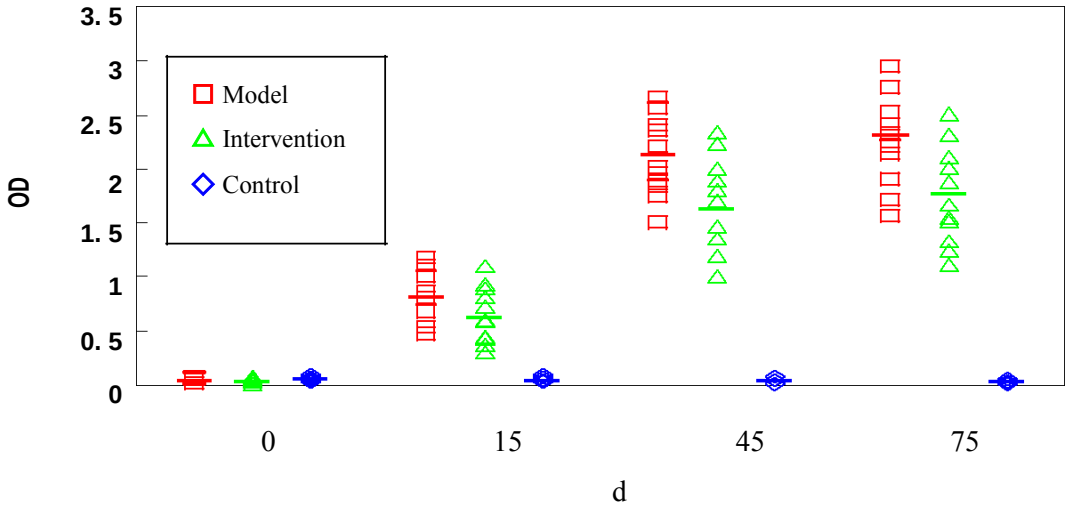

Figure 5. IgG in different groups at different time points.

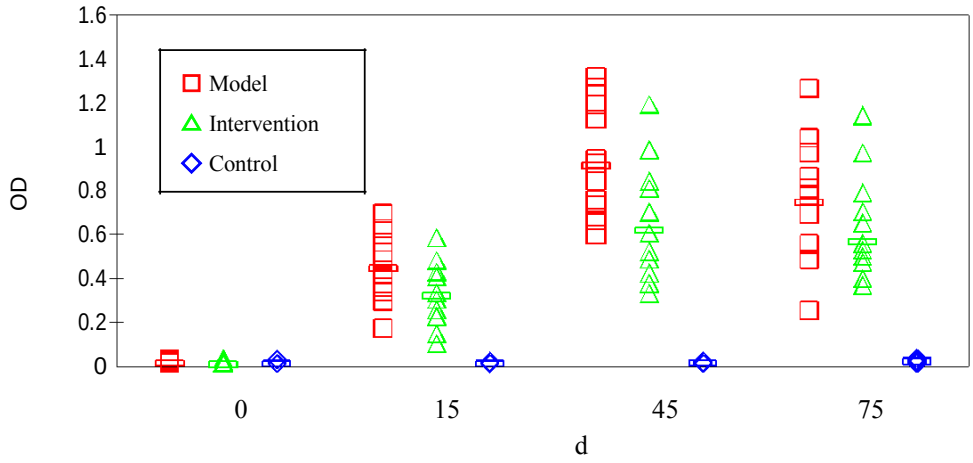

Figure 6. IgG1 in different groups at different time points.

time points ($p<0.05$, Day 15; $p<0.05$, Days 45 and 75, $p<0.01$) (Figure 6). Anti-AChR IgG2b in Groups A and B was higher than in Group C at different time points ($p<0.01$). After secondary immunization, anti-AChR IgG2b decreased in Group B compared to Group A at different time points ($p<0.05$, Day 45; $p<0.01$, Day 75) (Figure 7). No statistically significant difference was noted in anti-AChR IgG2c between Groups A and B at different time points ($p>0.05$), while in both groups, anti-AChR IgG2c was higher than in Group C ($p<0.01$) (Figure 8).

DISCUSSION

EAMG treated with Tα146-162-iMDC

MG is an autoimmune neuromuscular disease in neuromuscular junction leading to fluctuating muscle weakness and fatigability. The EAMG animal model is often established for research on MG. We induced the model in C57BL/6J mice through injection of Torpedo Californica AChR (T-AChR) and CFA emulsion. When T-AChR was used to immunize C57BL/6J mice, the 146-162 peptide (Tα146-162) in the subunit of T-AChR α is important for immune response (Atassi and Oshima, 1997; Wu et al., 1997). Immature DCs are shown to induce peripheral tolerance (Steinbrink et al., 2002). In animal models of experimental autoimmune encephalomyelitis, non-obesity diabetes and transplantation rejection, iMDCs loaded with small dose of antigen/immune peptide can inhibit immune reactions (Jiang et al., 2003; Horwitz et al., 2004). High dose of Tα146-162 successfully induces immune tolerance through the mucous membrane in C57BL/6J mice

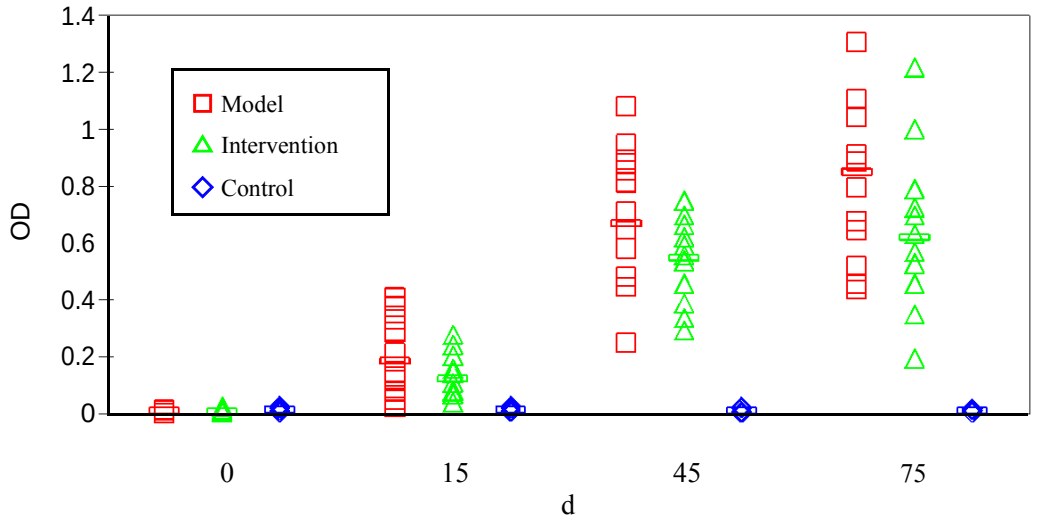

Figure 7. IgG2b in different groups at different time points.

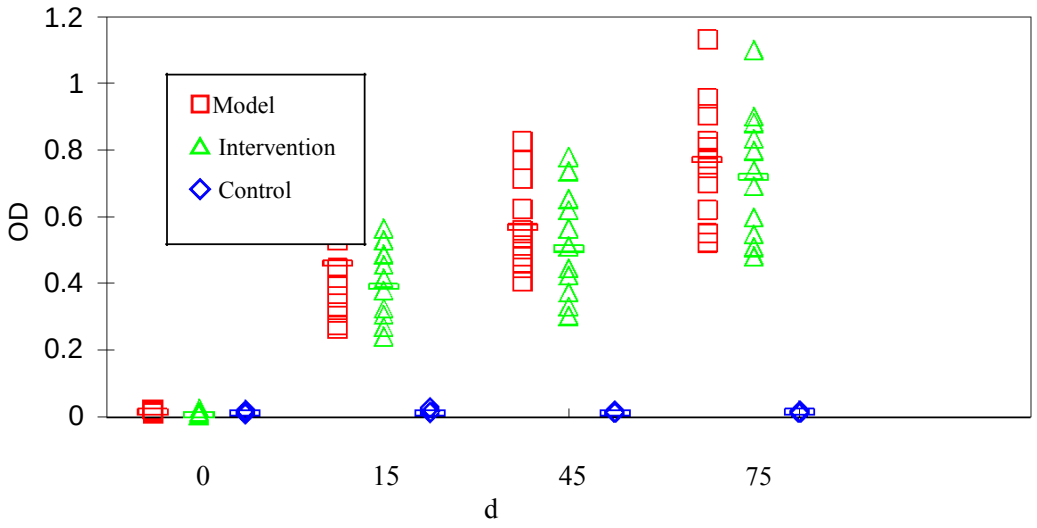

Figure 8. IgG2c in different groups at different time points.

immunized with T-AChR, and thus delays the development of EAMG through inhibiting Th1 (type 1 T-helper lymphocyte) cells and IgG2b (Wu et al., 1997). DCs are a key group of antigen presenting cells that are not only initiators of immune response but also inducers of immune tolerance. Their biological function is correlated with maturity of cells; especially iDCs induce peripheral tolerance.

Previously iDCs are thought to regulate polarizatioin of T cells and promote T cell tolerance such as apoptosis of activated T cells. Tr cells have an impact on activation of B cells and lead to secretion of anti-AChR antibody by B cells (Suzuki et al., 2007). In the study, mice in the intervention group were sick later than those in the model group with a decreased incidence and a lower clinical score, showing iMDCs loaded with Tα146-162 successfully inhibited EAMG, which is consistent with previous studies (Wang et al., 2009b).

Effect of Tα146-162-iMDC on Cbl-b expression

Cb1-b is widely expressed by immune cells that suppresses activation of lymphocytes and prevents autoimmune diseases. Regulation of B cell signal pathway may be crucial for induction and/or maintenance of peripheral immune tolerance and autoimmune response. In the current research, Cbl-b mRNA in the model group was significantly lower than in the control group, while it was higher in the intervention group than

in the model group, indicating that decreased Cbl-b expression weakens negative regulation of B cell activation, thus leading to strengthened B cell activation and EAMG in mice. Tα146-162-iMDC itself can inhibit B cell activation through strengthening the expression of Cbl-b. It has been demonstrated that Tα146-162-iMDC can induce Tr cells that initiate negative regulation of BCR through Cb1-b and thus inhibit B cell activation (Mellor et al., 2004). It is hypothesized that Tα146-162-iMDC induces high Cb1-b expression through inducing Tr cells and inhibits BCR initiation to deactivate B cells thus causing antigen-specific immune tolerance.

BCR signal transduction in EAMG

BCR consists of membrane-bound immunoglobulin and Igα/β heterodimer. As a surface marker for B cells, it activates PTKs after crosslinking reaction that is a major biochemical reaction in antigen receptor mediated B cell activation and important for subsequent cell reaction (Craxton et al., 1999). In the BCR signal pathway, PTKs, such as Syk and Lyn are important: decreased Syk influences BCR signal and B cell activity (Flores-Borja et al., 2005), while Lyn is closely correlated with proliferation of B cells. Syk, a non-Src PTK, is important for IgM mediated signal pathway. It is not only a target protein for downregulating Src family kinase, but also an upstream factor for Lyn in immature B cells as lack of Syk in B cells results in loss of reaction to BCR stimuli (Kroczek et al., 2010; Shao et al., 2004). This study found that Lyn protein expression and phosphorylation decreased in the model group compared to the control group. Following intervention by Tα146-162-iMDC, Lyn expression was higher than in the model group, showing Tα146-162-iMDC may increase Lyn expression and phosphorylation, or accompanied with influence from Cb1-b. In the model group, Syk protein expression and phosphorylation increased apparently compared to the control group, suggesting the negative regulation of BCR by Cb1-b and Lyn weakens, while positive regulation by Syk is strengthened. Tα146-162-iMDC may increase Cbl-b and Lyn as well as Lyn phosphorylation to inhibit positive regulation of BCR from Syk.

Inhibition of BCR signal pathway by Cb1-b

Cb1-b inhibits BCR signal early and decouple BCR and multiple downstream signal. Following BCR crosslinking, Cb1-b promotes ubiquitination of phosphorylated Syk, weakens and thus downregulates BCR signal (Kitaura et al., 2007). Following BCR crosslinking, Igα in the Igα/Igβ is phosphorylated, leading to recruitment of Syk by Igα SH2 and phosphorylation (Yasuda et al., 2000). This may also be why Syk protein expression and phosphorylation increased in the model group. Syk denaturalization also influences connection between Cbl-b and Igα, thus regulating multiple downstream signal pathways. Thus, through enhanced negative regulation of BCR signal by Cb1-b to suppress autoimmune B cell activation and induce B cell tolerance is a possible mechanism of intervention in EAMG by Tα146-162-iMDC.

Change of T-AchR antibody

High dose of Tα146-162 can inhibit function of Th1 cells and production of IgG2b and postpone EAMG (Wu et al., 1997). iDCs can induce peripheral tolerance and T cell tolerance including T cell polarization, the latter having an impact on B cell activation and causing secretion of anti-AChR antibody by B cells (Suzuki et al., 2007). The direct cause of MG/EAMG is anti-AChR antibody secreted by B cells. Research finds that anti-AChR antibody binds to Tα146-162 to propel degradation of AChR and block its binding to Ach, and especially subtypes of anti-AChR IgG have a major role in disease development (Yang et al., 2005). In this study, there was no statistically significant difference in anti-AChR IgM and IgG2c between the model group and the intervention group at different time points. The concentration of anti-AChR IgG and IgG1 in the intervention group decreased statistically significantly after initial immunization, while the anti-AChR IgG2b also reduced following secondary immunization. Anti-AChR-Ab is secreted by active B cells. With antigen stimulation, IgM changes to IgG, IgA, and IgE secreted by B cells, recognized as class switch (Yang et al., 2005). Following AChR immunization, compared to the model group, anti-AChR IgM in the intervention group decreased significantly, while IgG and IgG2b did not change much, indicating mice with Tα146-162-iMDCs is able to respond initially to AChR (IgM), but lead to no change of anti-AChR IgM to IgG, especially IgG1 and IgG2b. In the current study, serum IgG2b increased apparently in EAMG mice. IgG2b is complement binding antibody and its complement inhibitor with Tα146-162-iMDCs can have strong inhibition of EAMG (Tüzün et al., 2003; Deng et al., 2002).

IgG1 does not bind to the complement, so its decrease alleviates clinical symptoms through other approaches, such as blocking binding of AChR and Abs in neuromuscular junction to prevent influx of AChR in cells, or inhibiting binding between anti-AChR antibody and AChR functional area to prevent blocking of neuromuscular transmission (Yang et al., 2005). Anti-AChR IgG, IgG1and IgG2b decreased in the intervention group compared to the model group, indicating that Tα146-162-iMDCs can intervene IgG1 and IgG2b subtypes to influence onset and development of EAMG and Tα146-162-iMDCs as well as Tr cells induced by them strengthen negative regulation of BCR by Cb1-b and inhibit activation of autoimmune B cells that are able to secrete IgG1 and IgG2b subtypes, leading to decrease

of IgG1 and IgG2b. This may also be a possible mechanism of intervention in EAMG by Tα146-162-iMDC.

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