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Costimulation blockade by combining CTLA4Ig with anti-CD40L mAb markedly inhibits the inflammatory response of experimental autoimmune myocarditis

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Abstract

The aim of this study was to investigate the effect of costimulation blockade with cytotoxic T-lymphocyte-associated-antigen 4-immunoglobulin (CTLA4Ig) and anti-CD40L monoclonal antibody (anti-CD40L mAb) on an experimental autoimmune myocarditis (EAM) mouse model. Characteristics of myocardial tissue were observed by hematoxylin and eosin (H&E) staining. The messenger RNA (mRNA) levels of CTLA4, CD40L, IFN-γ, and IL-4 were detected by real-time fluorescence quantitative polymerase chain reaction (RT-qPCR). Serum concentrations of IFN-γ and IL-4 were determined by ELISA. After immune intervention, the inflammatory score, mRNA levels of CTLA4 and CD40L, and IFN-γ level were decreased. Furthermore, these parameters in the combinational intervention group (blockade by CTLA4Ig and anti-CD40L mAb) were significantly decreased, compared to the single intervention group (blockade by CTLA4Ig or anti-CD40L mAb). However, after costimulation, blockade serum IL-4 levels were increased. Therefore, costimulation blockade by combination CTLA4Ig and anti-CD40L mAb could more effectively inhibit the inflammatory response of EAM than single use of CTLA4Ig or anti-CD40L mAb.

Keywords

anti-CD40L mAb, autoimmune myocarditis, costimulation blockade, CTLA4Ig

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Introduction

Experimental autoimmune myocarditis (EAM) is a T cell-mediated disease.[1] Myocarditis usually leads to fatal events including progressive heart failure and sudden death.[2] The pathogenesis of myocarditis is still not clear, but growing evidence has suggested that excessive activation of T cells is an important initiator and mediator for EAM.[3] Therefore, inhibiting activation of T cells may effectively reduce the inflammatory response of cardiomyocytes.[4]

Cytotoxic T-lymphocyte-associated-antigen 4 (CTLA4, CD152) is expressed on the surface of activated T cell membrane and has a 10–20-fold higher binding affinity to CD80 (B7-1) and CD86 (B7-2) than CD28. Due to its engagement of negative costimulatory signal (co-inhibitory signal), CTLA4Ig has been the most widely used to regulate the immune response through blockade of the CD28–B7 pathway.[5] Also, the CD40 ligand (CD40L or CD154) binds to CD40, which also plays an important role in costimulation.[6] Studies have shown that costimulation blockade with CTLA4Ig and anti-CD40L

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monoclonal antibody (anti-CD40L mAb) efficiently prevented acute rejection and extended survival of grafts.[6] However, in myocarditis, the effect of blockade of CD28–B7 or CD40–CD40L pathways, especially using combination CTLA4Ig and anti-CD40L mAb, is still not clear.

In this study, in order to investigate the effect of costimulation blockade by combination CTLA4Ig with anti-CD40L mAb on inflammatory response of EAM, the model of EAM was used and the inflammatory response was assessed.

Material and methods

Animals

Male 7-week-old specific pathogen free (SPF) BALB/c mice (weight range, 18–22 g) were purchased from Silaike Laboratory Animal Co., Ltd (Shanghai, PR China) and maintained in Laboratory Animal Center, Fujian Medical University, PR China. Throughout the studies, the protocols for these experiments were approved by the special committee on Animal Welfare of Fujian Medical University.

Induction of EAM and costimulation blockade

In order to induce EAM, mice were immunized twice as previously described,[2] with some modifications. Briefly, the left groin and armpit on day 0 and right groin and armpit on day 7 were injected subcutaneously multiple times with 0.1 mg per mouse of the porcine cardiac myosin (Sigma Chemicals, St Louis, MO, USA) emulsified 1:1 with Freund's complete adjuvant (Sigma Chemicals). BALB/c mice were randomly divided into six groups including ten mice each. In the EAM group, mice were injected with porcine cardiac myosin and PBS (0.2 mL per mouse); in the single intervention groups, including EAM + CTLA4Ig and EAM + anti-CD40L mAb groups, mice were injected with porcine cardiac myosin and CTLA4Ig (10.0 mg/kg of body weight; BD Pharmingen, USA) in the EAM + CTLA4Ig group and mice were treated with porcine cardiac myosin and anti-CD40L mAb (10.0 mg/kg of body weight; BD Pharmingen, USA) in the EAM + anti-CD40L mAb group; in the combined intervention group (EAM + CTLA4Ig + anti-CD40L mAb group), mice were injected with porcine cardiac myosin, CTLA4Ig (5.0 mg/kg of body weight), and anti-CD40L mAb (5.0 mg/kg of body weight); in the EAM + IgG group, mice were injected with porcine cardiac myosin and IgG (10.0 mg/kg of body weight; BD Pharmingen, USA); in the normal group, BALB/c mice were injected with the complete Freund's adjuvant. The mice tail vein injections of PBS, CTLA4Ig, anti-CD40L mAb, and IgG were on days 0, 1, and 2 in the whole experiment. All animals were killed on day 21, and their hearts were removed freshly and aseptically for detection. Before killing, the blood of these mice was collected via retro-orbital bleeding and the serum was prepared for measurement.

Histopathological examination

After the mice were sacrificed, the hearts were fixed with perfusion of 3.8% formaldehyde, embedded in paraffin, sectioned into 4-μm slices, and stained with hematoxylin and eosin (H&E) for histological examination. The severity impairment of myocarditis was graded in a double-blind manner by two independent investigators, according to a semi-quantitative scale based on the presence of inflammatory cell infiltration and accompanying cardiac myocyte necrosis.[7] The detailed scoring system was as follows: grade 0, no; grade 1, <10% of the heart section is involved; grade 2, 10–30%; grade 3, 30–50%; grade 4, 50–90% and grade 5, >90%.

Measurement of CTLA4, CD40L, IFN-γ, and IL-4 messenger RNA (mRNA) levels

For extraction of total RNA, the hearts of mice were snapfrozen in liquid nitrogen immediately after excision and stored at −80°C until used. The preserved heart was homogenized and total RNA was isolated with Trizol (Invitrogen, Carlsbad, CA, USA). The first strand of complementary DNA (cDNA) was synthesized from total RNA using a PrimeScript 1st strand cDNA synthesis kit (TaKaRa Bio, Inc., Otsu, Shiga, Japan) according to a standard protocol. The mRNA levels of CTLA4, CD40L, IFN-γ, and IL-4 were measured by quantitative real-time polymerase chain reaction (RT-qPCR). The reaction mixture consisted of cDNA from 100 ng of total RNA, 10 μM of each primer indicated in Table 1, and 10 μL 2 × SYBR (TaKaRa Bio, Inc., Otsu, Shiga, Japan) for a final volume of 20 μL. β-actin was amplified as an internal control. The

Table 1. Primer sequences for RT-qPCR.

	Primer sequence (5'–3')	Fragment sizes (bp)
CTLA4	Forward: TAAGCGAAGCCAACAGTAATGCAG Reverse: GCTGCTAGCCAACACCACTGAA	134
CD40L	Forward: TAAGCGAAGCCAACAGTAATGCAG Reverse: T GACTCGAAGGCTCCCGATTAG	178
IFN-γ	Forward: CGGCACAGTCATTGAAAGCCTA Reverse: GTTGCTGATGGCCTGATTGTC	199
IL-4	Forward: TCTCGAATGTACCAGGAGCCATATC Reverse: AGCACCTTGGAAGCCCTACAGA	183
β-actin	Forward: CATCCGTAAAGACCTCTATGCCAAC Reverse: ATGGAGCCACCGATCCACA	171

fold change ($2^{-\Delta\Delta CT}$) was calculated to evaluate the mRNA levels of CTLA4, CD40L, IFN-γ, and IL-4.

Measurement of serum IFN-γ and IL-4 levels

The serum levels of IFN-γ and IL-4 were evaluated with ELISA kits (Mouse IFN-γ and IL-4 ELISA Kits; R & D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Statistical analysis

All data were analyzed with SPSS version 16.0 (SPSS Inc., Chicago, IL, USA). Variables were presented as mean ± SD and analyzed by one-way analysis of variance (ANOVA) test and student's t-test among groups. For all tests, a two-sided *P* value <0.05 was considered to be statistically significant.

Results

Costimulation blockade ameliorates myocardial injury

Histological analysis was performed to evaluate inflammatory infiltration in the heart (Figure 1a). The hearts in the EAM and EAM + IgG groups showed the features of acute myocarditis characterized by inflammatory infiltration of the myocardium, cardiac myocyte necrosis, and fibrosis. However, compared to the EAM and EAM + IgG groups, myocardial inflammatory cell infiltration in myocardial tissues was much milder in the single intervention group, which showed limitations of inflammatory cell infiltration, especially in the combined intervention group, which showed low mononuclear cell infiltration.

The inflammatory scores of myocardial tissue are shown in Figure 1b. The inflammatory scores of the EAM and EAM + IgG groups were 3.3 ± 0.73 and 3.4 ± 0.77, respectively. There was no difference between these two groups (P = 0.7694). After immune intervention, the inflammatory scores of the EAM + CTLA4Ig, EAM + anti-CD40L mAb, and EAM + CTLA4Ig + anti-CD40L mAb groups (0.85 ± 0.23, 1.11 ± 0.41, and 0.2 ± 0.20, respectively) were significantly decreased compared to EAM groups (P <0.01). Moreover, the inflammatory score of the combined intervention group was lower than the single intervention group (P <0.01), while there was no difference between the EAM + CTLA4Ig group and EAM + anti-CD40L mAb group (P = 0.1028).

Costimulation blockade decreased the mRNA levels of CTLA4 and CD40L in myocardial tissues

In order to verify that the CD28–B7 and CD40–CD40L pathways are involved in the development of EAM, we examined the expression of CTLA4 and CD40L on hearts with EAM. The mRNA levels of CTLA4 and CD40L are shown in Figure 2. The expression of these two molecules was significantly enhanced in the hearts of EAM and EAM + IgG groups compared with normal group (P <0.01). After immune intervention, they were significantly decreased compared to the EAM group (P <0.01). Moreover, the mRNA levels of CTLA4 and CD40L of the combined intervention group were lower than single intervention group (P <0.05), while there were no differences between the EAM + CTLA4Ig group and the EAM + anti-CD40L mAb group (P >0.05).

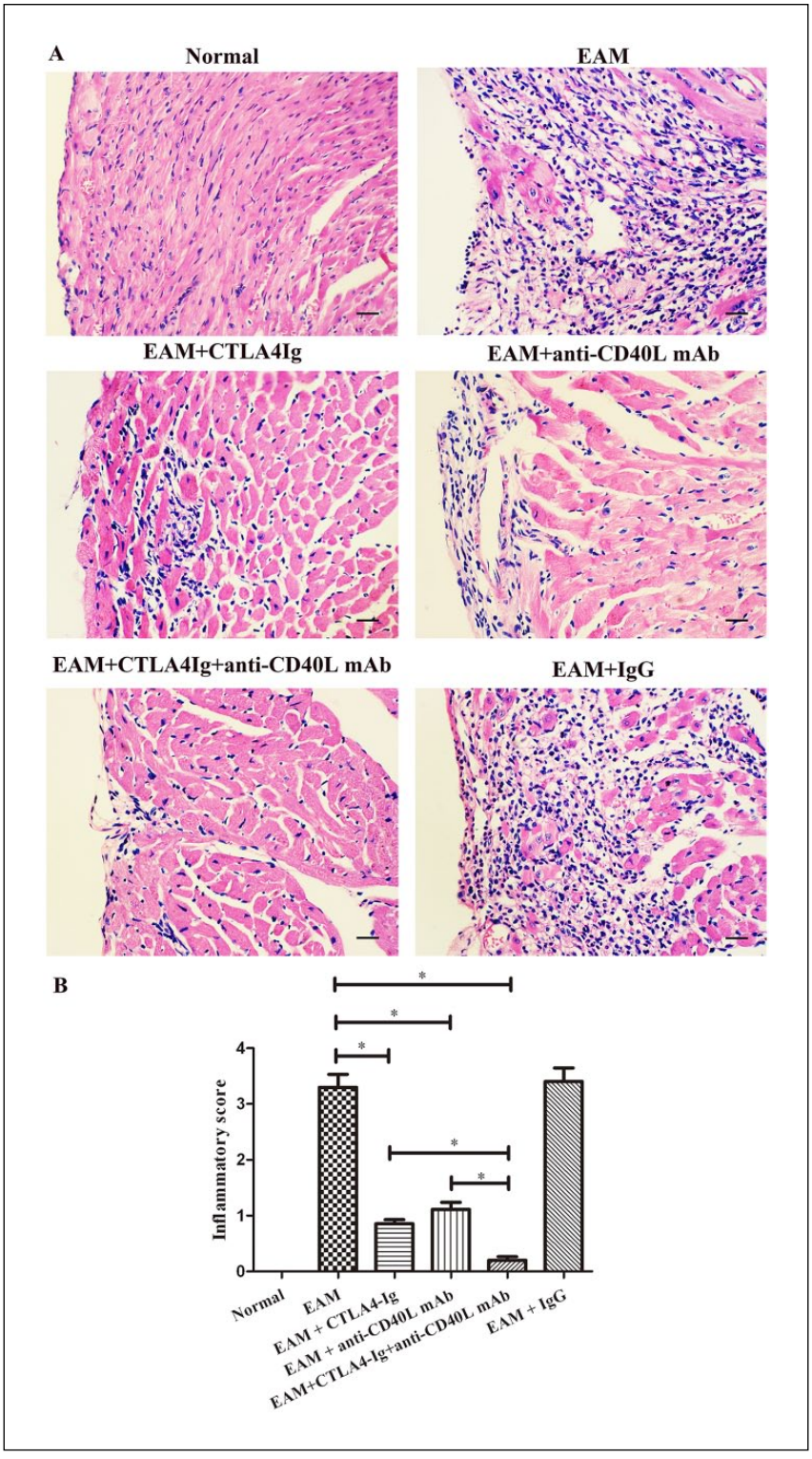

Figure 1. Costimulation blockade ameliorates myocardial injury. (a) The myocardial tissues were stained with H&E. One representative image was shown for each group. H&E staining showed that myocardial cells were arranged in neat rows and there were no necroses nor inflammatory cell infiltration in the myocardial tissue of the normal group. The EAM and EAM + IgG groups showed features of acute myocarditis, which were disordered arrangement and extensive infiltration of inflammatory cells in myocardial tissues, myocardial interstitium, and interstitium under the epicardium. Infiltration of inflammatory cells was decreased after immune intervention, especially in combined intervention group. Scale bar = 50 μm. (b) Inflammatory scores of myocardial tissue. The severity impairment of myocarditis was graded according to a semi-quantitative scale based on the presence of inflammatory cell infiltration and accompanying cardiac myocyte necrosis. Values are presented as mean ± SD (*P <0.01).

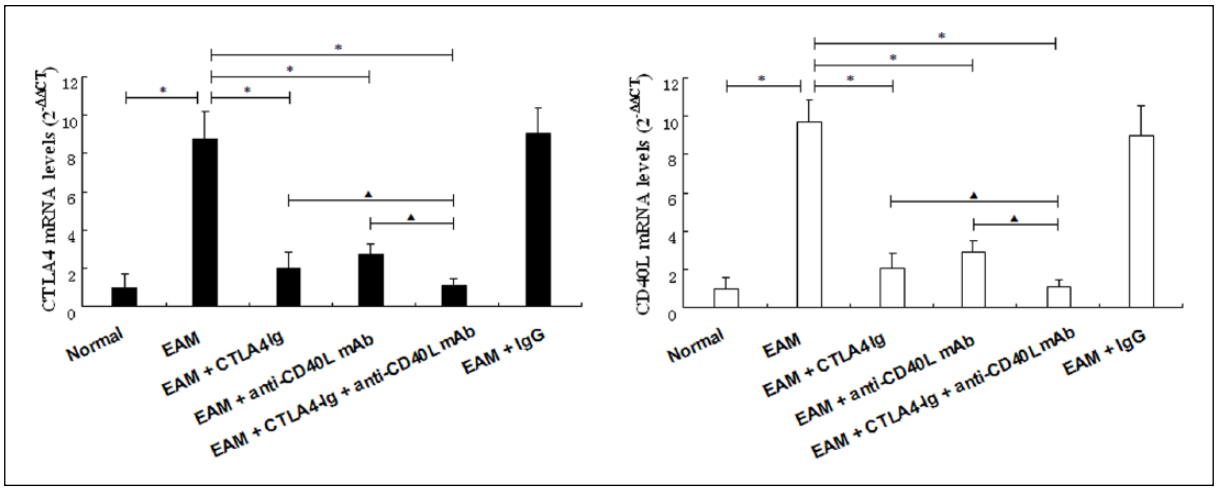

Figure 2. Costimulation blockade decreased the mRNA levels of CTLA4 and CD40L in myocardial tissues. The mRNA levels of CTLA4 and CD40L were measured by RT-qPCR. β-actin was amplified as an internal control. The fold change (2-ΔΔCT) was calculated to evaluate the mRNA levels. Values are presented as mean ± SD (*P <0.01, ▲P <0.05).

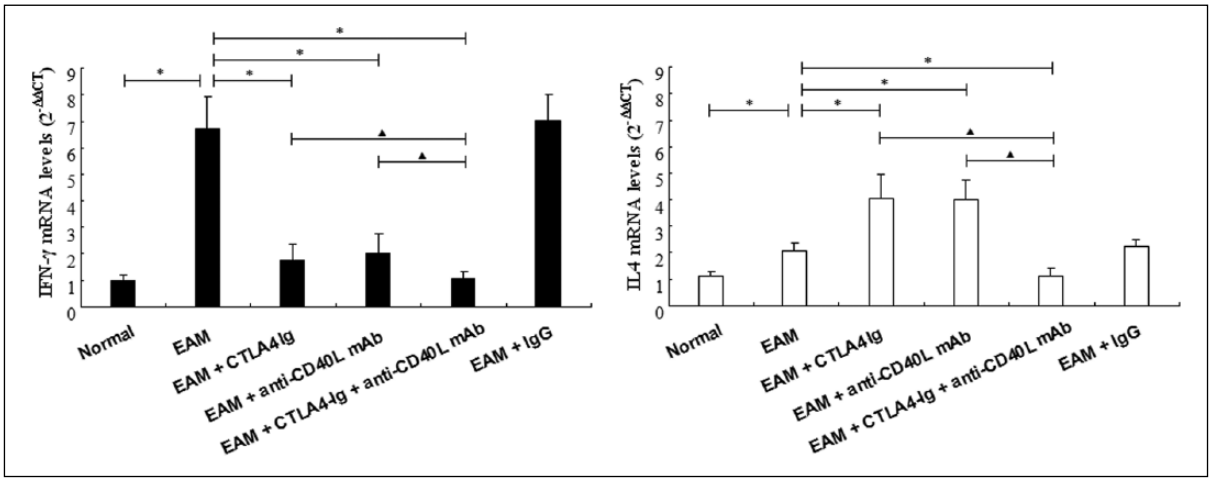

Figure 3. Costimulation blockade modulates the mRNA expression of IFN-γ and IL-4 in myocardial tissues. The mRNA levels of IFN-γ and IL-4 were measured by RT-qPCR. β-actin was amplified as an internal control. The fold change (2-ΔΔCT) was calculated to evaluate the mRNA levels. Values are presented as mean ± SD (*P <0.01, ▲P <0.05).

Costimulation blockade modulates the mRNA expression of IFN-γ and IL-4 in myocardial tissues

The mRNA levels of IFN-γ and IL-4 are shown in Figure 3. The mRNA levels of IFN-γ and IL-4 in the EAM or EAM + IgG group were significantly higher than normal control (P <0.01), especially the Th1 cytokines IFN-γ. The mRNA expression of Th1 cytokines IFN-γ was decreased (P <0.01), while Th2 cytokines IL-4 was increased (P <0.01) after single intervention. However, the mRNA levels of IFN-γ and IL-4 were decreased (P <0.05) after combined intervention.

Costimulation blockade significantly decreased serum levels of IFN-γ and upregulated serum levels of IL-4

The serum IFN-γ and IL-4 levels are shown in Figure 4. The serum IFN-γ levels were significantly higher in the EAM or EAM + IgG group than in the normal group (P <0.01). However, there were no significant differences for serum IL-4 levels among the EAM, EAM + IgG, and normal control groups. After immune intervention, the concentrations of serum IFN-γ were significantly decreased compared to the EAM group (P <0.01). Moreover, it was lower in the combined intervention group than

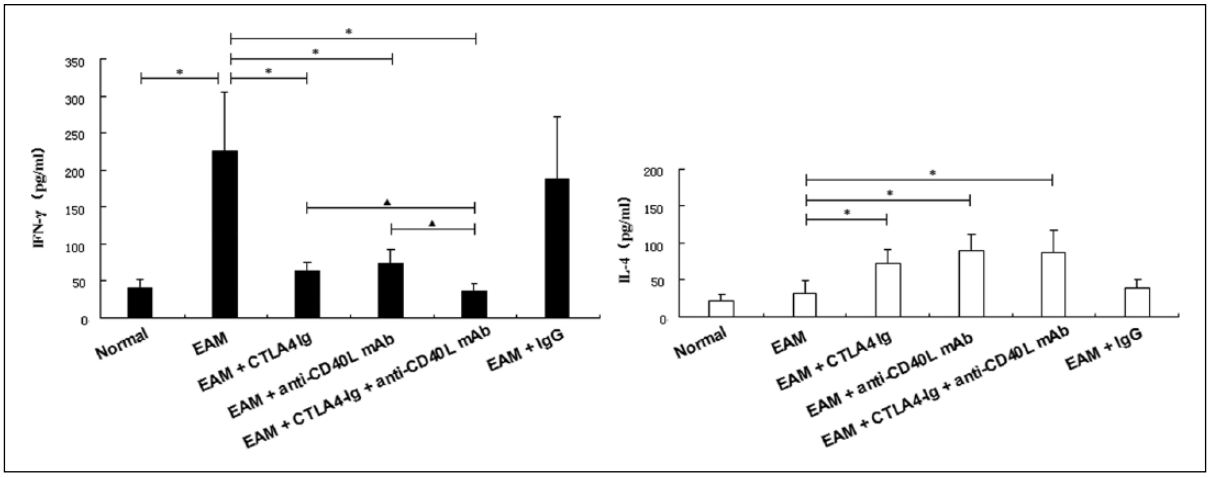

Figure 4. Costimulation blockade modulated the serum levels of IFN-γ and IL-4. Sera were tested for IFN-γ and IL-4 concentration by ELISA. Values are presented as mean ± SD (*P <0.01, ▲P <0.05).

the single intervention group (P <0.05). Contrary to serum IFN-γ, the results showed that the concentrations of serum IL-4 were significantly increased after immune intervention compared to EAM (P <0.01), while there were no differences among the EAM + CTLA4Ig, EAM + anti-CD40L mAb and EAM + CTLA4Ig + anti-CD40L mAb groups.

Discussion

This study illustrated the effect of inhibition inflammatory response on mice with EAM through costimulation blockade by CTLA4Ig and anti-CD40L mAb. The study showed that the combination of CTLA4Ig with anti-CD40L mAb could effectively reduce inflammatory response on mice with EAM.

CTLA4Ig and anti-CD40L mAb effectively suppress the development of EAM. In agreement with Matsui et al.,[8] this study showed that inflammatory infiltration and scores in mouse myocardial tissue were significantly decreased after immune intervention compared to the EAM or IgG control groups. Moreover, the inflammatory scores of the combination intervention group were lower than the single intervention group, which reflects that combination CTLA4Ig with anti-CD40L mAb is really a viable option for controlling inflammatory response.

Cytokines play an important role in the development and severity of autoimmune myocarditis.[9] Th1 cytokines, such as IFN-γ, have predominant functions during the progressive phase of acute myocarditis,[10–12] while Th2 cytokines such as IL-4 have an inhibitory effect in EAM.[3] In line with Massilamany et al.,[13] this study showed that IFN-γ was decreased while IL-4 was increased after single intervention. Our results indicate that early immune intervention may shift the Th1/Th2 balance toward Th2; our results are also consistent with Kishimoto et al.[14] Our study also found that not only was the mRNA level of IFN-γ decreased after combined intervention, but also that of IL-4. The possible reason was that the proliferation of T cells in myocardial tissues were suppressed after blocking the two costimulatory signal pathways, and the T cell anergy was increased, which led to the a reduction in the secretion of both Th1 and Th2 cytokines.

In conclusion, costimulation blockade by combination CTLA4Ig with anti-CD40L mAb can regulate the balance of Th1/Th2 and effectively inhibit the development of EAM in the early stages of EAM. Although the conclusion was obtained from an animal model, and further clinical evaluation is needed, it provides evidence for the treatment of EAM using a new application of costimulation blockade by combining CTLA4Ig with anti-CD40L mAb.

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Declaration of conflicting interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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References

1. Valaperti A, Marty RR, Kania G et al. (2008) CD11b+ monocytes abrogate Th17 CD4+ T cell-mediated experimental autoimmune myocarditis. *Journal of Immunology* 180: 2686–2695.
2. Schmerler P, Jeuthe S, Oh-I D et al. (2014) Mortality and morbidity in different immunization protocols for experimental autoimmune myocarditis in rats. *Acta Physiologica* 210: 889–898.
3. Han L, Guo S, Wang Y et al. (2014) Experimental drugs for treatment of autoimmune myocarditis. *Chinese Medical Journal* 127: 2850–2859.
4. Hoetzenecker K, Zimmermann M, Hoetzenecker W et al. (2015) Mononuclear cell secretome protects from experimental autoimmune myocarditis. *European Heart Journal* 36: 676–685.
5. Weaver TA, Charafeddine AH and Kirk AD. (2008) Costimulation blockade: Towards clinical application. *Frontiers in Bioscience* 13: 2120–2139.
6. Pilat N, Sayegh MH and Wekerle T. (2011) Costimulatory pathways in transplantation. Seminars in Immunology 23: 293–303.
7. Cihakova D, Sharma RB, Fairweather D et al. (2004) Animal models for autoimmune myocarditis and autoimmune thyroiditis. *Methods in Molecular Medicine* 102: 175–193.
8. Matsui Y, Inobe M, Okamoto H et al. (2002) Blockade of T cell costimulatory signals using adenovirus vectors prevents both the induction and the progression of experimental autoimmune myocarditis. *Journal of Molecular and Cellular Cardiology* 34: 279–295.
9. Penta KL, Fairweather D, Shirley DL et al. (2015) Low-dose mercury heightens early innate response to coxsackievirus infection in female mice. *Inflammation Research* 64: 31–40.
10. Su Z, Sun C, Zhou C et al. (2011) HMGB1 blockade attenuates experimental autoimmune myocarditis and suppresses Th17-cell expansion. *European Journal of Immunology* 41: 3586–3595.
11. Watanabe K, Sukumaran V, Veeraveedu PT et al. (2011) Regulation of inflammation and myocardial fibrosis in experimental autoimmune myocarditis. *Inflammation & Allergy Drug Targets* 10: 218–225.
12. Liu X, Liu M, Yuan W et al. (2013) Anti-viral effects of curcumin on influenza A virus-induced myocarditis via inhibiting Wnt/β-catenin signaling. *Central European Journal of Immunology* 38: 328–335.
13. Massilamany C, Gangaplara A, Steffen D et al. (2011) Identification of novel mimicry epitopes for cardiac myosin heavy chain-alpha that induce autoimmune myocarditis in A/J mice. *Cellular Immunology* 271: 438–449.
14. Kishimoto C, Nimata M, Okabe TA et al. (2013) Immunoglobulin treatment ameliorates myocardial injury in experimental autoimmune myocarditis associated with suppression of reactive oxygen species. *International Journal of Cardiology* 167: 140–145.