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Statin-modified dendritic cells regulate humoral immunity in experimental autoimmune myasthenia gravis

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ABSTRACT

We previously demonstrated that atorvastatin induced immature dendritic cells (DCs) derived from spleen in vitro. Administration of these tolerogenic DCs led to amelioration of experimental autoimmune myasthenia gravis (EAMG). The protective effect was mainly mediated by inhibited cellular immune response, including up-regulated regulatory T cells and shifted Th1/Th17 to Th2 cytokines. The present study employed atorvastatin-modified bone marrow-derived DCs (AT-BMDCs) to explore the effect of tolerogenic DCs on humoral immune response of EAMG and further elucidate the underlying mechanisms. Our data showed that AT-BMDCs reduced the quantity and the relative affinity of pathogenic antibodies, suppressed germinal center response, decreased follicular helper T cells and IL-21, and increased regulatory B cells. These results suggest that AT-BMDCs ameliorate EAMG by regulating humoral immune response, thus providing new insights into therapeutic approaches of myasthenia gravis and other autoimmune diseases.

1. Introduction

Myasthenia gravis (MG) is a classical organ specific autoimmune disease, which is antibody-mediated, T cell-dependent and complement-involved. It impairs the postsynaptic nicotinic acetylcholine receptor (AchR) at the neuromuscular junction (NMJ), leading to neuromuscular transmission failure, thereby inducing muscle weakness and fatigability (Berrih-Aknin et al., 2014). Experimental autoimmune myasthenia gravis (EAMG), induced by immunization of AchR purified from *Torpedo californica* (Lennon et al., 1975) or synthetic peptide corresponding to region 97–116 of the rat AChRα subunit (R97–116 peptide) (Baggi et al., 2004), serves as a well-established animal model used to explore the immunopathological mechanisms and therapeutic strategies of human MG.

Current immunosuppressive agents for MG treatment, such as corticosteroids and cytotoxic reagents, are more pan-immunosuppressive than specific cell targeted, thus causing numerous side effects. Pathogenic autoantibodies (mostly antibodies against AchR) are detected in a majority of MG patients and damage the neuromuscular transmission via many mechanisms (Meriggioli and Sanders, 2009), indicating that antibody-secreting B cells are essential for the development of MG. Accumulating evidence demonstrates that B cell targeted therapy via elimination of B cells with drugs such as rituximab (anti-CD20 mAb) can relieve symptoms of MG patients (Iorio et al., 2014), which supports the idea that autoreactive B cells may be a prominent candidate for targeted therapies in MG. Moreover, a distinct T cell subset, named follicular helper T (Tfh) cells and characterized by expression of transcription factor B-cell lymphoma 6 (Bcl6), surface molecules including CXC chemokine receptor 5 (CXCR5), inducible T-cell costimulator (ICOS) and programmed death protein-1 (PD-1), and cytokine IL-21, is indicated to supply direct assistance for antibody production of B cells (Crotty, 2011). The contributing role of Tfh cells has been confirmed in many antibody-mediated autoimmune diseases, such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) and autoimmune thyroid diseases (Ma et al., 2012; Simpson et al., 2010; Zhu et al., 2012). Recent studies have also reported an aberrant expansion of Tfh population in MG patients (Luo et al., 2013; Zhang et al., 2014a). Taken together, these findings suggest the pathogenic role of humoral immune response mediated by B cells and Tfh cells in MG.

Recently, the discovery of regulatory B cells (Breg) has challenged the conventional roles of B cells as antigen-presenting cells and antibody-secreting cells (Mauri and Ehrenstein, 2008). Adoptive transfer of Breg attenuates severity of some autoimmune diseases in animal models, such as experimental autoimmune encephalomyelitis (EAE) (Matsushita et al., 2010), type 1 diabetes (T1DM) (Hussain and

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Delovitch, 2007) and collagen-induced arthritis (CIA) (Yang et al., 2012). Sun et al. have reported that IL-10 producing Breg (B10 cells) decrease in MG patients and those who respond well to rituximab treatment exhibit rapid repopulation of B10 cells (Sun et al., 2014). However, data are still scarce about the exact role of Breg in MG.

Previously, our study reported that atorvastatin induced immature dendritic cells (DCs) derived from spleen in vitro and administration of these tolerogenic DCs ameliorated EAMG by inhibiting proliferation of lymphocytes, up-regulating regulatory T cells (Treg), shifting Th1/Th17 to Th2 response and decreasing levels of pathogenic antibodies (Li et al., 2013). The previous study focused on the effect of tolerogenic DCs on cellular immunity, however, the exact mechanisms by which tolerogenic DC treatment triggered reduction of antibodies deserve further investigations. Furthermore, given that bone marrow generates more abundant and immature DCs with higher trafficking property than spleen (Emmanouilidis et al., 2006; Fields et al., 1998), and most studies employing DCs prefer bone marrow-derived dendritic cells (BMDCs) to spleen-derived dendritic cells (SpDCs), we used BMDCs in this study instead of SpDCs in the previous study. Collectively, the aim of the present study was to explore the effect of atorvastatin-modified BMDCs (AT-BMDCs) on humoral immune response in EAMG and we attempted to explore the underlying mechanisms.

In this study, we found that atorvastatin successfully induced immature BMDCs in vitro, of which CD80, CD86 and MHC II molecules were inhibited. AT-BMDC treatment led to amelioration of ongoing EAMG. AT-BMDCs suppressed the quantity and the relative affinity of anti-R97-116 IgG as well as germinal center (GC) response. In addition, Tfh cells and IL-21 were decreased by AT-BMDC treatment. Interestingly, the amount of $CD19^+$ B cells was slightly influenced, while Breg were increased by AT-BMDCs. Our data suggest that AT-BMDCs could ameliorate EAMG by regulating humoral immune response and might provide a potential approach and new evidence for MG therapies.

2. Results

2.1. Atorvastatin induces immature BMDCs in vitro

To determine whether atorvastatin could induce immature BMDCs, an experiment in vitro was conducted. BMDCs were cultured with or without atorvastatin and then analyzed by flow cytometry (FACS). In the presence of atorvastatin, CD80 (89.77 ± 1.98%), CD86 (82 ± 5.31%) and MHC II (90.23 ± 1.27%) expressed on AT-BMDCs were inhibited compared with those on 0-BMDCs cultured in the absence of atorvastatin (CD80, 50.93 ± 3.01%, CD86, 51 ± 4.02%, MHC II, 63.20 ± 9.04%; $t = 18.693$, $p < 0.001$; $t = 8.042$, $p < 0.01$ and $t = 5.132$, $p < 0.05$, respectively) (Fig. 1A).

2.2. AT-BMDC treatment suppresses the development of ongoing EAMG

To explore the effect of AT-BMDCs on EAMG, AT-BMDCs, 0-BMDCs and the same volume of serum-free medium were transferred into EAMG rats on days 5 and 16 post immunization (p.i.). Rats began to exhibit clinical signs on about day 13 p.i., and all immunized rats exhibited EAMG manifestations at different degrees on day 17 p.i. At the end of the experiment, the average clinical score was 1.0 ± 0.35 in the AT-BMDC group, while 1.6 ± 0.42 in the 0-BMDC group and 1.8 ± 0.27 in the EAMG group ($p < 0.05$ vs AT-BMDC group). Rats in the AT-BMDC group exhibited lower scores compared with the EAMG group and differences were statistically significant on day 13, 24, 34, 36, 38 and 42 p.i. ($p < 0.05$ for all comparisons). When compared with the 0-BMDC group, the AT-BMDC group showed lower clinical scores, however, the difference was not significant. Moreover, there was no significant difference between the 0-BMDC group and the EAMG group (Fig. 2).

2.3. AT-BMDC treatment inhibits anti-R97-116 IgG and germinal center response in EAMG rats

To confirm the effect of AT-BMDCs on humoral immune response in EAMG rats, we initially measured the quantity and relative affinity of anti-R97-116 IgG. As shown in Fig. 3A, rats in the AT-BMDC group produced less anti-R97-116 IgG compared with rats in the EAMG and 0-BMDC groups ($F = 8.455$; $p < 0.05$ and $p < 0.01$, respectively). Moreover, the relatively affinity was determined by ELISA using thiocyanate elution and showed similar results ($F = 9.602$; $p < 0.01$ vs EAMG group and $p < 0.01$ vs 0-BMDC group) (Fig. 3B). There was no difference between the EAMG group and the 0-BMDC group. In brief, both quantity and affinity of anti-R97-116 IgG were inhibited by AT-BMDC treatment.

Germinal centers (GCs) are discrete structures within B-cell follicles of secondary lymphoid organs, where many important issues in humoral immune response occur. B cells in GCs possess high ability of binding with peanut agglutinin (PNA), consequently, PNA is commonly used to identify GCs (Carter and Myers, 2008). As shown in Fig. 3C, there were many clusters of PNA^+ GC-B cells in spleen sections of rats from the EAMG group and the 0-BMDC group. In contrast, PNA^+ GC-B cells were significantly decreased in the AT-BMDC group, which implied that the formation of GCs was abolished by AT-BMDC treatment.

2.4. AT-BMDC treatment decreases Tfh cells and IL-21^+ cells in EAMG rats

As Tfh cells play an essential role in humoral immune response, we assessed Tfh cells to ascertain whether they were influenced by AT-BMDC treatment. Tfh cells were analyzed by FACS and characterized by expression of CD4, CXCR5 and ICOS. The percentage of Tfh cells among $CD4^+$ cells in the AT-BMDC group was lower than that in the EAMG group and the 0-BMDC group ($F = 8.237$; $p = 0.065$ and $p < 0.01$, respectively) (Fig. 4A). In addition, we detected the percentages of IL-21^+ cells among lymph node mononuclear cells (MNC). As shown in Fig. 4B, there were less IL-21^+ cells in the AT-BMDC group compared with the EAMG group and the 0-BMDC group ($F = 7.924$; $p < 0.01$ for both comparisons).

2.5. AT-BMDC treatment increases Breg in EAMG rats

To investigate the effect of AT-BMDCs on Breg, we further tested Breg defined as $CD19^+$ $Foxp3^+$ (B-Foxp3) and $CD19^+$ IL-10^+ (B10) cells in EAMG rats. The results showed that AT-BMDCs decreased $CD19^+$ B cells compared with the 0-BMDC group ($H = 6.87$; $p < 0.05$). However, when compared with the EAMG group, there was a slight decrease without significant difference (Fig. 5A). Interestingly, the percentages of B-Foxp3 cells were increased in the AT-BMDC group and the 0-BMDC group ($H = 8.749$; $p < 0.05$ vs EAMG group for both comparisons) (Fig. 5B). B10 cells were also slightly up-regulated in the AT-BMDC group compared with the EAMG group and the 0-BMDC group, however, there was no significant difference (Fig. 5C).

3. Discussion

DCs play a key role in inducing immunity as the most efficient antigen-presenting cells, however, the fact that DCs also induce tolerance has been well established and accepted, which at least partially depends on their maturation state (Manicassamy and Pulendran, 2011). Generally, mature DCs tend to induce immunity, whereas immature DCs induce tolerance. Previously, we found that atorvastatin could induce immature SpDCs in vitro and AT-SpDCs successfully ameliorated EAMG by up-regulated Treg cells and shifted Th1/Th17 to Th2 cytokines (Li et al., 2013). Another study showed similar effects of AT-SpDCs on experimental autoimmune neuritis (EAN) by inhibiting cellular immune response (Xu et al., 2014). The aim of this study was to identify the effect of atorvastatin on BMDCs and the mechanisms by which tolerogenic DCs regulate humoral immunity in EAMG. Results showed