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Administration of bone marrow stromal cells ameliorates experimental autoimmune myasthenia gravis by altering the balance of Th1/Th2/Th17/Treg cell subsets through the secretion of TGF-β

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ABSTRACT

Bone marrow stromal cells (BMSCs) are strong candidates for cell therapy against human autoimmune diseases. Intravenous administration of syngenic BMSCs to EAMG-model rats effectively ameliorated the disease, partially through a TGF-β-dependent mechanism. The proliferative ability of T or B cells from EAMG rats was inhibited by BMSCs at proper cocultured ratios. And the imbalance of Th1, Th2, Th17 and Treg cell subsets accompanied with the development of EAMG was corrected by the administration of BMSCs. These results provide further insights into the pathogenesis of MG, EAMG, and other immune-mediated diseases, and support a potential role for BMSCs in their treatment.

1. Introduction

In the past few years, bone marrow stromal cells (BMSCs, also called bone mesenchymal stem cells) have come into the limelight due to their multi-lineage differentiated potential, including osteoblasts, adipocytes, chondrocytes (Pittenger et al., 1999), skeletal myocytes, and neurons (Woodbury et al., 2000) under the appropriate conditions, and also because of their characteristic immunomodulatory ability exerted on many T cell, B cell, NK, and dendritic cell (Glennie et al., 2005; Krampera et al., 2003; Corcione et al., 2006; Aggarwal and Pittenger, 2005; Jiang et al., 2005). BMSCs present at low frequency ($1/10^4$–$1/10^5$) (Pittenger et al., 1999) in the adult bone marrow of many species, and they are now thought to be promising candidate for use in strategies directed toward tissue engineering, repair of damaged tissues, gene therapy, and therapy of immune-mediated disorders (Pittenger et al., 1999; Woodbury et al., 2000; Le Blanc and Ringden, 2005; Dezawa et al., 2005).

Human MSCs (hMSCs) administered *in vivo*, assisted hematopoietic engraftment (Le Blanc and Ringden, 2005), while *in vitro* hampered graft-versus-host disease, probably due to their inhibitive activity on immune system (Aggarwal and Pittenger, 2005; Le Blanc and Ringden, 2005; Krampera et al., 2006). Systemic administration of BMSCs in mice suffering from experimental autoimmune encephalomyelitis (EAE), a disease model of multiple sclerosis that is mediated by self-reactive T cells, results in highly effective disease amelioration associated with a profound suppression of effector T cells and the induction of peripheral tolerance (Zappia et al., 2005). BMSCs may also inhibit the maturation and function of dendritic cells (Jiang et al., 2005; Zappia et al., 2005), suggesting that activated T cells are not the only targets of BMSCs. It has been further demonstrated that hMSCs can significantly affect the proliferation, differentiation, and chemotactic behavior of normal mature B cells (Corcione et al., 2006). Though the mechanisms mediating such effects are still not fully understood, it is likely that both cell-to-cell contact and the transmission of soluble factors are involved in supporting lymphocyte inhibition. One candidate for the latter mechanisms is transforming growth factor beta (TGF-β), which arrests the cell cycle in the G1 phase, thereby inhibiting cell proliferation and triggering apoptosis (Hocevar and Howe, 1998; Zhang et al., 2002). TGF-β is a strong immunosuppressive factor that is expressed by stimulated BMSCs; it can be inhibited *in vitro* by the addition of anti-TGF-β antibody (Krampera et al., 2006; Beyth et al., 2005).

Experimental autoimmune myasthenia gravis (EAMG) is a B-cell-mediated, T-cell-dependent autoimmune disease of the neuromuscular junction, in which the nicotinic acetylcholine receptor (AChR) serves as the autoantigen: it thereby represents the paradigmatic model for myasthenia gravis (MG) (Lennon et al., 1975; De Baets et al., 2003; Baggi et al., 2004). There has not yet been a successful therapeutic approach to this model that has been based on targeting the AChR-specific T or B cells. In view of the immunoregulatory function of BMSCs, we designed this study to determine whether they could ameliorate the pathological features of EAMG *in vivo* and *in vitro*.

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2. Materials and methods

2.1. Animals

Female Lewis rats weighing 160–180 g were purchased from Vital River Laboratory Animal Co. Ltd. (Beijing, PR China) and maintained at Harbin Medical University in sterile microisolator cages under specific-pathogen-free conditions at 21±2 °C and 45±5% humidity. All animal handling and experimental procedures were performed in accordance with the guidelines of the Care and Use of Laboratory Animals published by the China National Institute of Health.

2.2. Antigens

A synthetic peptide corresponding to region α97–116 of the rat AChR α subunit, R-AChR peptide 97–116 (DGDFAIVKFTKVLLDYTGHI) was synthesized by AC Scientific, Inc. (Xian, China) as described previously (Baggi et al., 2004). The myelin basic protein (MBP) 68–86 peptide (YGSLPQKSQRSQDENPV) was synthesized at Sangon Ltd. (Shanghai, China).

2.3. Immunization protocols

Female Lewis rats (160–180 g) were randomly divided into three groups with 8 rats in each. The EAMG and BMSC-administered groups were immunized subcutaneously at the base of tail with the R-AChR 97–116 peptide (50 μg/rat), emulsified in complete Freund's adjuvant (CFA, Sigma-Aldrich, U.S.A.) supplemented with 1 mg of *Mycobacterium tuberculosis* strain H37Ra/rat (Difco, Detroit, MI) in a total volume of 200 μl (day 0). They were boosted on day 30 with the same peptide in incomplete Freund's adjuvant (IFA, Sigma-Aldrich, U.S.A.) (Baggi et al., 2004). The CFA control group was injected with an emulsion in which Dulbecco's phosphate-buffered saline (D-PBS, PH=7.4) was substituted for the R-AChR peptide, but which was otherwise identical.

2.4. Clinical evaluation

After the first immunization, each animal was weighed on alternate days until sacrificed (6–8 weeks). Disease severity was scored by measuring muscular weakness in a blinded fashion. Clinical scoring was based on the presence of tremor, hunched posture, muscle weakness, and fatigability. Fatigability was assessed after exercise (repetitive paw grips on the cage grid) for 30 s. Disease severity was expressed as follows (Lennon et al., 1975; Baggi et al., 2004): Grade 0: normal muscle strength; Grade 1: mildly decreased activity, weak grip, fatigable; Grade 2: weakness, hunched posture at rest, decreased body weight, tremor; Grade 3: severe generalized weakness, marked decrease in body weight, moribund; Grade 4: dead. Rats with intermediate signs were assigned grades of 1.5, 2.5 or 3.5, as appropriate. Results are expressed as the mean score for each group at each time point.

2.5. Preparation of BMSCs

Bone marrow stromal cells (BMSCs) were generated from bone marrow aspirates of normal female Lewis rats (80–100 g). Briefly, whole marrow from the femurs and tibias was flushed in MesenCult Basal Medium, supplemented with Mesenchymal Stem Cell Stimulatory Supplements (StemCell Technologies Inc., U.S.A.), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco, UK). Cultures were incubated at a final concentration of 3×10^7 nucleated cells per ml at 37 °C in a 5% CO_2 humidified incubator (SANYO, Japan) for 72 h, at which time nonadherent cells were removed and fresh media was added to the adherent cells every 3 to 4 days. When 80% confluent, adherent cells were trypsinized (0.05% Trypsin at 37 °C for 5 min, Calbiochem, San Diego, CA), and expanded until a homogenous population was obtained after 2 to 3 weeks of culture. Before further expansion and experimental use, BMSCs were tested for their ability to differentiate into adipocytes and osteoblasts as previously described. Oil red O and von Kossa dyes (Sigma, Aldrich) were used to identify adipocytes and osteoblasts, respectively (Pittenger et al., 1999).

2.6. Transplantation of BMSCs

On the day of the second immunization, rats in the BMSC-administered group were injected with a total of 1×10^7 normal donor BMSCs (BMSCs were trypsinized, collected, suspended in 0.5 ml PBS, then injected into the tail vein). Rats of the CFA and EAMG groups injected with the same volume of PBS. All rats were weighed and monitored for clinical scores every other day until sacrificed.

2.7. Lymphocyte proliferation assay

Popliteal, inguinal, axillary, and paraaortic lymph nodes were harvested aseptically from all rats 56 days after the first immunization, and processed into single cell suspensions. Triplicate aliquots (200 μl) of mononuclear cell (MNC) suspensions, each containing 4×10^5 cells, were placed into 96-well round-bottom microtiter plates (Nunc, Copenhagen, Denmark) in EAMG culture medium, which included 85% RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% L-glutamine (Sigma, Santa Clara, CA, U.S.A.), 1% sodium pyruvate, 1% non-essential amino acids, 2×10^{-5} M 2-mercaptoethanol (2-ME, Amresco, Solon, OH, U.S.A.) and 1% penicillin-streptomycin (Gibco, Paisley, U.K.). 10 μl aliquots of R-AChR peptide, MBP, or Concanavalin A (Con A) were added in triplicate to the appropriate wells at a final concentration of 10 μg/ml (AChR or MBP) or 5 μg/ml (Con A). Wells used as negative controls received PBS only. Cells were incubated at 37 °C in 5% CO_2 for 72 h; a 10 μl aliquot containing 1 μCi ^{3}H-thymidine (specific activity, 60 Ci/mmol; China Institute of Atomic Energy, Beijing) was added prior to the final 18 h of culture.

In another set of experiments, R-AChR-specific T and B cells were isolated from the EAMG group using magnetic beads, as instructed by the manufacturer (R&D Systems, Inc., U.S.A.). Negatively selected cells consisted, on average, of greater than 95% B cells and T cells, as assessed by flow cytometric analysis (FACS). A total of 10^4 purified R-AChR specific T or B lymphocytes were stimulated with R-AChR peptide (10 μg/ml) in the presence or absence of BMSCs, which were irradiated (30 Gy) with Co^{60}, at the final ratios of T:BMSCs=10:1, and B:BMSCs=1:1 in a total volume of 200 μl EAMG culture medium per well in triplicate. TGF-β neutralizing antibody (10 μg/ml) was added to abolish the inhibitive activities of TGF-β secreted by BMSCs. The incubation was continued and ^{3}H-thymidine was added 18 h prior to the end of the 72-hour culture.

Cells described above were harvested onto glass-fiber filters for the assay of radioactivity by a liquid β-scintillation counter (Perkin-Elmer, Wellesley, MA). All experiments were performed a minimum of 3 separate times. The results were expressed as mean counts per minute (cpm)±SD.

2.8. Cytokine ELISA

Quantitative analysis of interferon (IFN)-gamma, IL-4, IL-6, IL-17 and TGF-β levels was performed by enzyme-linked immunosorbent assay (ELISA) using commercially available kits (Shanghai Senxiong Biotech Industry Co. Ltd., China). Measurements were made on serum samples from the three groups of rats, as well as on the fresh supernatants derived from 3-day culture of purified T cells, alone or in the presence of BMSCs (at a ratio of BMSCs:T=1:10, with or without anti-TGF-β antibody, 10 μg/ml). Results were expressed as the mean cytokine concentration (picograms per milliliter)±SD.

2.9. Enumeration of anti-AChR IgG antibody secreting cells

R-AChR-specific B cells were isolated from the EAMG group using magnetic beads, and co-cultured in 96-well round-bottom plates in the

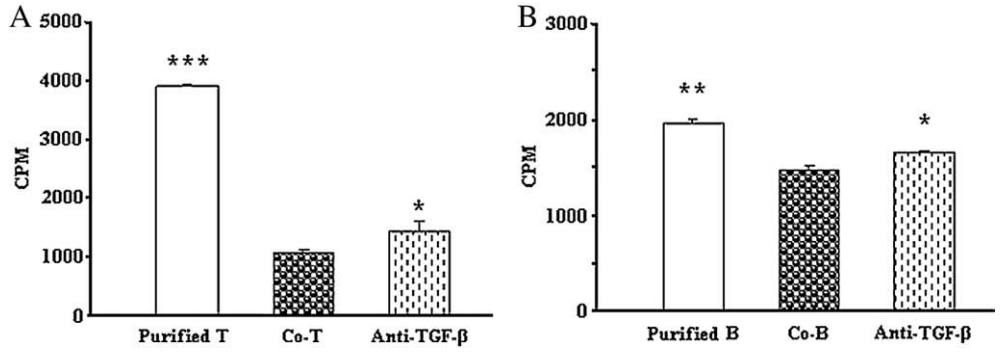


Fig. 1. T and B lymphocyte proliferation is inhibited by normal BMSCs. Purified suspensions of T cells (A) and B cells (B) from EAMG rats were co-cultured for 72 h with (middle bars) or without (left bars) normal irradiated syngenic BMSCs at a ratio of 10:1 or 1:1, respectively, in the presence of R-AChR97–116 peptide. Bars on the right represent conditions identical to those represented by the middle bars but with the addition of the anti-TGF-β antibody. Cell proliferation was assessed by ^{3}H-thymidine incorporation. Values are expressed as mean counts per minute (CPM) ± SD of triplicates of 4 independent experiments. (*P<0.05, **P<0.01, ***P<0.001).

presence or absence of BMSCs suspensions, which were irradiated (30 Gy) with Co^{60} at the final ratios of B:BMSCs = 1:1 in a total volume of 200 μl EAMG culture medium supplemented with R-AChR peptide (10 μg/ml) per well in triplicate for 72 h. Besides, BMSCs/B cells co-cultured group with the addition of anti-TGF-β (10 μg/ml) to abolish the inhibitive activities of TGF-β secreted by BMSCs was designed. Immunoglobulin production at the single-cell level was investigated by enzyme-linked immunospot assay (ELISPOT) as previously described with some modifications (Corcione et al., 2006). Briefly, nitrocellulose bottomed microtiter plates (Millipore, Bedford, MA) were coated overnight at 4 °C with 100 μl aliquots per well of R-AChR 97–116 peptide (10 μg/ml in PBS). After washing thrice with PBS, aliquots of 100 μl incubated cells were added in triplicate wells for 24 h at 37 °C in 5% CO_2 and humidified atmosphere. Then, the wells were emptied, washed and incubated with rabbit anti-rat IgG (1/400; Sigma Chemical Co.) overnight at 4 °C. After that biotinylated swine anti-rabbit IgG (1/500; Dakopatts, Copenhagen, Denmark) was added for 2 h at 20 °C, followed by and avidin-biotin peroxidase complex (ABC; 1/200; Dakopatts) for 1 h at 20 °C. After peroxidase staining the red-brown immunospots, which indicated anti-AChR IgG production by individual cell, were counted in a blinded fashion by using a dissection microscope, values obtained were standardized to numbers of spots per 10^5 MNC.

2.10. Flow cytometry

For the assay of intracellular cytokine synthesis and expression of extracellular molecules, FACS analysis was performed as described previously (Lohr et al., 2006), with some modifications.

Briefly, for *in vivo* studies designed to distinguish the Th subsets among different groups, purified single-cell suspensions from 3 different groups were incubated for 5 h with of Brefeldin A (1:1000 dilution, eBioscience Inc., San Diego, CA, USA), an inhibitor of intracellular protein transport. T cells were first incubated extracellularly with fluorescein isothiocyanate (FITC)-conjugated anti-rat-CD4 (eBioscience, San Diego, USA) for 30 min at 4 °C. After fixation and permeabilization, intracytoplasmic staining was carried out using one of the following fluorescently labeled Abs: PE-conjugated anti-rat-IFN-γ (BD Biosciences), anti-rat-IL-4 (BD Biosciences), anti-Foxp3 antibody (BD Biosciences) and rabbit-anti-rat-IL-17, which was followed by Cy3-conjugated anti-rabbit-IgG (Sigma-Aldrich) as the second antibody.

For *in vitro* tests, purified T cells from EAMG rats were cultured in different groups: purified T culture group, T+BMSCs co-culture group and T+BMSCs+anti-TGF-β antibody group, at the indicated ratio for 72 h, and then stained T cells with CD4-IFN-γ, CD4-IL-4, CD4-IL-17, and CD4-Foxp3 as described above.

Samples were analyzed within 24 h with BD FACScan (BD Biosciences) using Cell Quest software (BD Biosciences). Isotype-matched, PE- and FITC-conjugated mAbs of irrelevant specificity were tested as negative controls.

2.11. Statistical analysis

Differences between groups were analyzed by a two-tailed Student's t-test for paired and unpaired data, respectively. P<0.05 was considered statistically significant.

3. Results

3.1. Isolation of homogeneous BMSCs

The BMSCs used in our experiment were able to differentiate into adipocytes and osteocytes when cultured in the appropriate media (data not shown) (Pittenger et al., 1999).

3.2. BMSCs inhibit the proliferation of T and B cells

The ability of BMSCs to suppress normal T and B cell function has been previously demonstrated (Woodbury et al., 2000; Glennie et al., 2005; Krampera et al., 2003; Corcione et al., 2006; Hocevar and Howe, 1998; Le Blanc et al., 2003). However, there is no consensus as to whether BMSCs are able to inhibit AChR-specific T and B cells. As a first step, therefore, we used R-AChR97–116 peptide to challenge T or B cells from EAMG rats in the presence and absence of irradiated BMSCs.

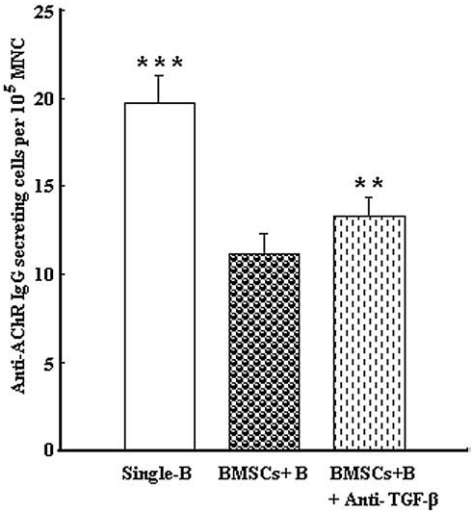


Fig. 2. Effects of BMSCs on immunoglobulin production. The anti-AChR IgG antibody secreting by R-AChR-specific B cells, incubated with R-AChR peptide (10 μg/ml) in the presence or the absence of BMSCs and the anti-TGF-β antibody (10 μg/ml), was detected by ELISPOT. Compared with the single-cultured B cell, BMSCs inhibited the immunoglobulin secretion by B cells, and anti-TGF-β antibody partially reversed the inhibition of BMSCs on B cells by their secretion of TGF-β. Results are expressed as mean values ± SD of three independent experiments (**P<0.01, ***P<0.001).

BMSCs inhibited the specific response of both T cells and B cells obtained from EMAG rats to the R-AChR 97–116 peptide. This effect was detectable at a T: BMSC ratio of 10:1 (Fig. 1A), and a B: BMSC ratio of 1:1 (Fig. 1B), as measured by ^{3}H-thymidine incorporation (Corcione et al., 2006; Le Blanc et al., 2003). These coculture ratios were used for all subsequent experiments in vitro.

A number of studies have suggested that the inhibitory effects of BMSCs are mediated by soluble factors, particularly TGF-β (Krampera et al., 2003, 2006; Rasmusson, 2006). To determine whether this was true under our assay conditions, we cultured purified T or B cells with BMSCs at the indicated ratios (Corcione et al., 2006; Le Blanc et al., 2003), in the presence or absence of TGF-β neutralizing antibody. We found that TGF-β neutralizing antibody indeed abrogated the inhibitory effect of BMSCs on both T cells (Fig. 1A) and B cells (Fig. 1B) to some extent, however not thoroughly. This indicated that the mechanism by which BMSCs inhibit the proliferative effect of AChR-specific T and B cells partially depends on the secretion of TGF-β.

3.3. Effects of BMSCs on immunoglobulin production

The anti-AChR IgG antibody secreting B cells were detected by B-ELISPOT. Compared with the single-cultured B cells, the immunoglobulin secretion was inhibited by BMSCs addition, and the anti-TGF-β antibody could effectively reverse the inhibition of BMSCs on B cells to

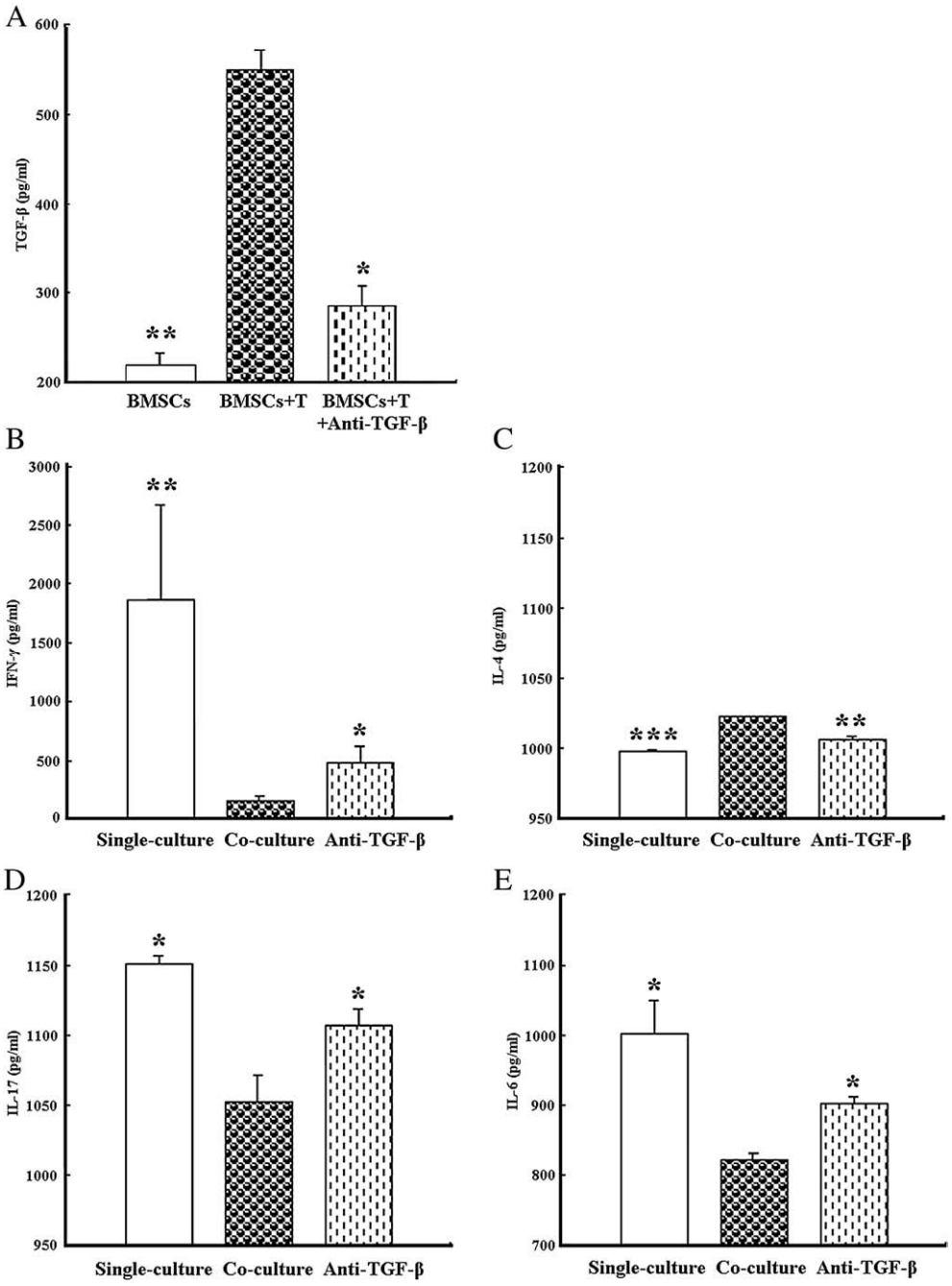


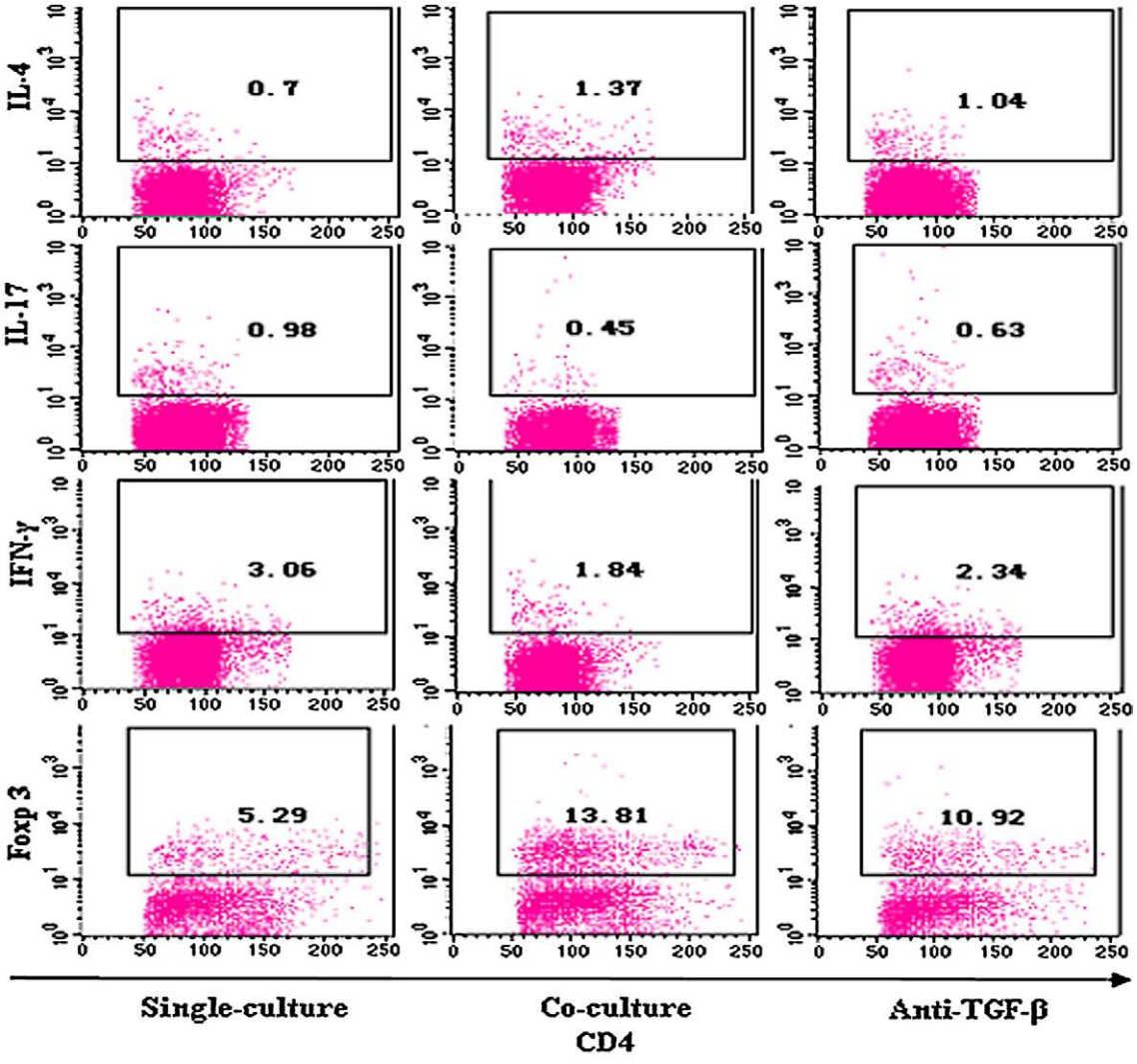
Fig. 3. Cytokine secretion. AChR-specific T lymphocytes were obtained from EAMG rats 56 d after the first immunization, BMSCs originated from normal Lewis rats. The concentration of TGF-β in the supernatants of BMSCs, BMSCs:T=1:10, BMSCs+T+anti-TGF-β was evaluated, results indicated that the TGF-β secretion was elevated when co-cultured BMSCs with T lymphocytes, compared with BMSCs single-cultured group, and the addition of anti-TGF-β antibody would effectively downregulate the TGF-β concentration, but not thoroughly (A). T lymphocytes were incubated for 72 h in the presence or absence of BMSCs (with or without anti-TGF-β antibody, 10 μg/ml). Bars show mean cytokine concentrations (pg/ml±SD) measured in the supernatant obtained from 3 different experiments: (B) IFN-γ; (C) IL-4; (D) IL-17; (E) IL-6 (*P<0.05, **P<0.01, ***P<0.001).

some extent, even not thoroughly, indicating that the inhibitive effect of BMSCs on B cell immunoglobulin production was partially by the secretion of TGF-β (significant statistical differences were found in Fig. 2).

3.4. Cytokine secretion by T lymphocytes in the presence of BMSCs

First of all, we measured the TGF-β concentration in the culture of BMSCs alone or in co-culture with T lymphocytes in the presence or absence of anti-TGF-β antibody (10 μg/ml). Results proved that the secretion of TGF-β, which was elevated by mixing BMSCs and T cells together, could be effectively reduced by the addition of anti-TGF-β antibody (Fig. 3A).

Then, a single-cell suspension of T lymphocytes obtained from EAMG rats was incubated for 72 h with R-AChR peptide (10 μg/ml), alone or in the presence of BMSCs (with or without anti-TGF-β antibody, 10 μg/ml). Supernatants were collected for the detection of IFN-γ (Th1), IL-4 (Th2),



	Groups		
	Single-culture	Co-culture	Anti-TGF-β
CD4-IL-4	0.70 ± 0.040 ***	1.37 ± 0.092	1.04 ± 0.078 *
CD4-IL-17	0.98 ± 0.014 **	0.45 ± 0.035	0.63 ± 0.029 *
CD4-IFN-γ	3.06 ± 0.014 **	1.84 ± 0.127	2.34 ± 0.184 *
CD4-Foxp3	5.29 ± 0.429 ***	13.80 ± 0.960	10.92 ± 0.509 *

Fig. 4. The effect of BMSCs on the distribution of Th cells. (Top) T-cell-enriched populations were cultured for 72 h: alone (left panel); in coculture with normal irradiated syngenic BMSCs (medium panel); or in coculture with the addition of the anti-TGF-β antibody (right panel). Expression of IFN-γ, IL-4, IL-17, and Foxp3 on CD4+ T cells was detected by FACS as indicated. Data were obtained from 4 different experiments, and results (bottom) are expressed as the median percentage double-positive cells ± SD. (*$P<0.05$, **$P<0.01$, ***$P<0.001$).

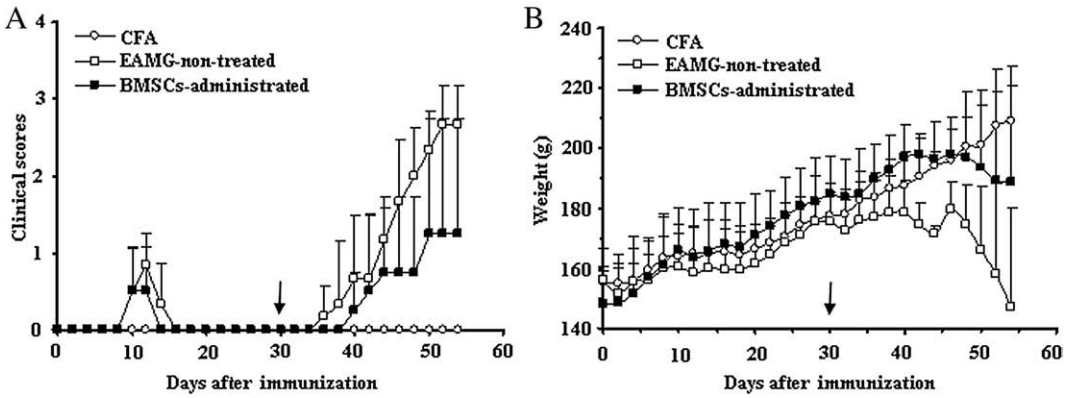


Fig. 5. Clinical score and body weight in BMSC-treated and control EAMG model rats. Clinical manifestations (A) and body weight (B) were measured in normal rats (○), EAMG rats (□), and EAMG rats treated with BMSCs (■). The arrows indicate day of the booster injection with R-AChR97–116 peptide and of simultaneous administration of BMSCs. Note the difference in the timing and severity of EAMG symptoms when rats were treated with BMSCs. Error bars refer to statistical comparison between (□) and (■). Data were obtained from 4 different experiments ($P_{\text{clinical score}}<0.01$, $P_{\text{body weight}}<0.001$).

IL-17 (Th17) and IL-6 (secreted by Th2 and Th17) secretion. We found that the concentration of the following cytokines was significantly decreased in the presence of BMSCs compared to the single culture group: IFN-γ (Fig. 3B); IL-17 (Fig. 3D); and IL-6 (Fig. 3E). IFN-γ was the most strongly attenuated. In contrast, the IL-4 concentration was elevated (Fig. 3C). Meanwhile, compared with the co-culture groups, the addition of anti-TGF-β antibody could partially abolish the effect of BMSCs on the cytokines secretion. Taken together, these results suggest coculture with BMSCs diminishes the Th1 and Th17 subsets and increases the Th2 (IL-4) subset, sectionally by their secretion of TGF-β.

3.5. Altered distribution of T cell subsets by BMSCs

To further investigate the influence of BMSCs on subsets of AChR-specific T cells, we purified T cells and cultured them with or without BMSCs, in the presence of anti-TGF-β antibody for 72 h at the indicated ratio. We then measured the expression of IFN-γ, IL-4, IL-17, and Foxp3 on CD4+ T cells by Facs (Fig. 4). Coculture with BMSCs was associated with a reduction in the proportion of CD4+ T cells secreting IFN-γ and IL-17, and an increase in the proportion of cells secreting IL-4 and Foxp3. This indicated that coculture with BMSCs corresponded to a decrease in the Th1 (IFN-γ) and Th17 (IL-17) subsets and an increase in the Th2 (IL-4) and Treg (Foxp3) subsets, a finding that was in accordance with the results of the ELISA assay of IFN-γ, IL-4, and IL-17 in vitro. The addition of anti-TGF-β antibody partially reversed the BMSC-mediated alteration in the distribution of Th1/Th2/Th17/Treg cells: the relative proportion of CD4+-IL-4- and CD4+-Foxp3-expressing cells were diminished, and those of the CD4+-IL-17-, CD4+-IFN-γ-expressing cells were increased, compared to the coculture group receiving no anti-TGF-β antibody (Fig. 4).

3.6. Administration of BMSCs ameliorates EAMG

In the view of the striking inhibition of T-cell and B-cell activation by BMSCs, as well as their influence on the distribution of Th subsets, we wondered whether this might allow BMSCs, to have an influence in vivo on a B-cell-mediated autoimmune disease such as EAMG. Symptoms in EAMG model rats began to appear after the second immunization (Fig. 5A), indicating a coincident activation AChR-specific T and B cells. We reasoned that BMSCs given at this time point would have a therapeutic, but not a protective, influence. To test this hypothesis, we injected BMSCs intravenously via the tail vein on the day of the second immunization. In the BMSC-treated group of EAMG model rats, the disease severity (clinical scores) (by Mann–Whitney *U* test, Fig. 5A) were markedly reduced and the body weight (by Mann–Whitney *U* test, Fig. 5A) were obviously increased compared to untreated model rats. The BMSC-treated rats showed no increased frequency of concomitant infectious disease or cancer through a maximum observation period of 4 months, indicating that BMSC treatment had not evoked immunodeficiency. BMSC treatment led to a reduced proliferation of lymphocytes in response to R-AChR 97–116 peptide, in comparison with untreated EAMG model rats (Fig. 6).

We used FACS to compare the cytokine expression of lymphocytes from EAMG rats and control rats immunized with CFA alone. We found that the EAMG rats had higher CD4+-IFN-γ (Th1) and CD4+-IL-17 (Th17) expressing cells, and lower CD4+-IL-4 (Th2) and CD4+-Foxp3 (Treg) expressing cells (Fig. 7). Treatment of EAMG animals with BMSCs led to a decrease in CD4+-IFN-γ and CD4+-IL-17 (Th17) secreting cells, and an increase in CD4+-IL-4 (Th2) and CD4+-Foxp3 (Treg) (Fig. 7). We measured an increase in serum IFN-γ (Fig. 8A), IL-4 (Fig. 8B), and IL-17 (Fig. 8C) in the BMSC-treated group as compared to the untreated EAMS group. However, the production of IL-6 (Fig. 8D) was reduced in the treated group. This apparently contradictory result suggests that lymphocytes are not the only cells that secret these cytokines and indicates a need to further study of the mechanisms of IL-17 and IFN-γ in EAMG.

4. Discussion

In this study, for the first time, we demonstrated that the administration of BMSCs could influence the pathogenic features of

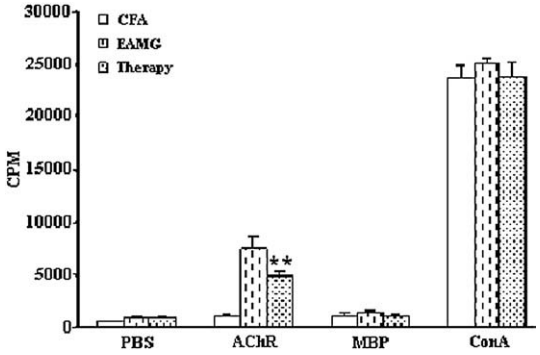
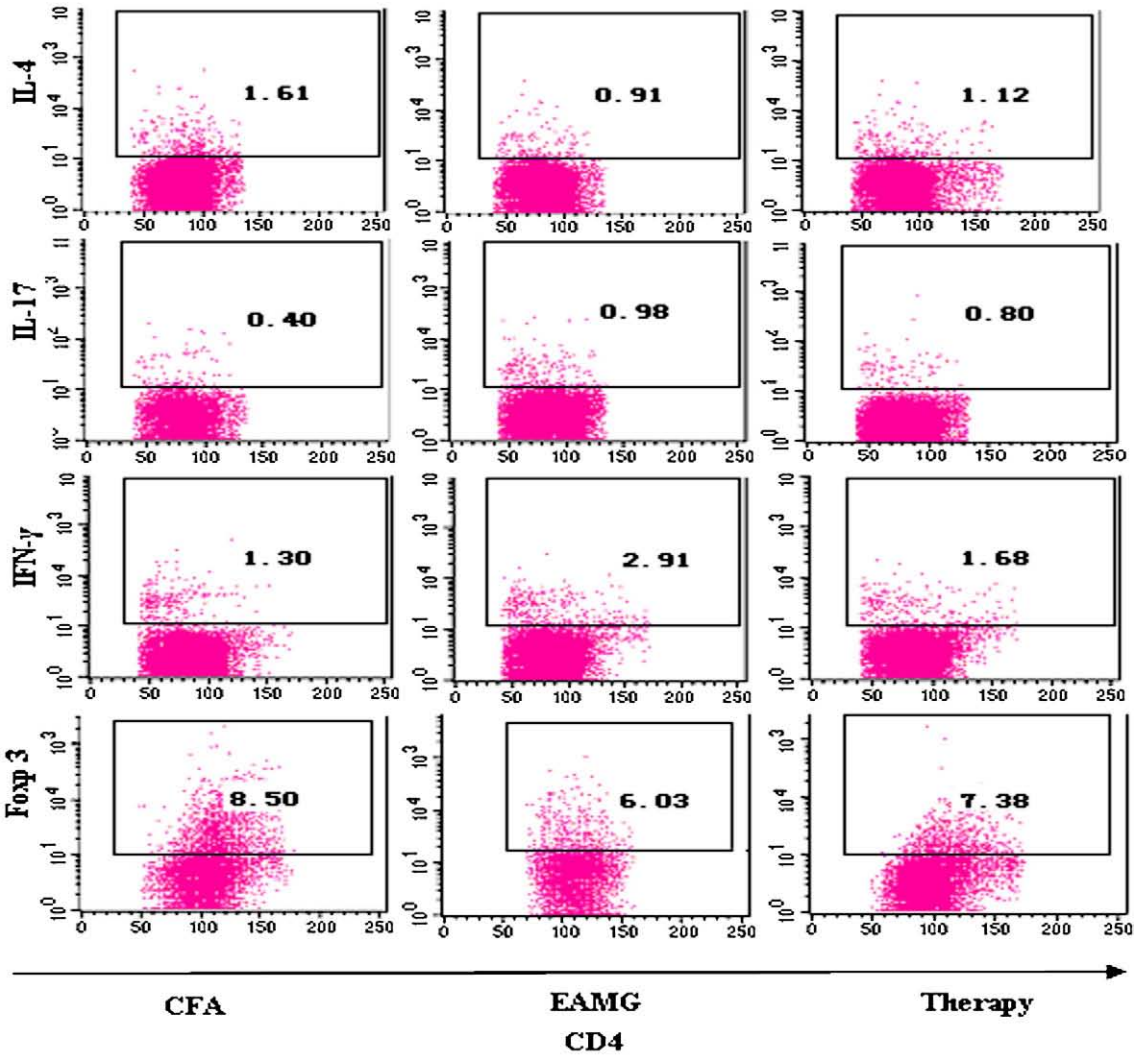


Fig. 6. Proliferative response of lymphocytes to peptides R-AChR97–116 in 3 different treated groups. Proliferative response of rat LNCs to: PBS (negative control); the immunogen R-AChR 97–116; MBP (uncorrelated peptide); and ConA (positive control). For each condition, cells from three groups of rats were tested: control-immunized with CFA (left); R-AChR 97–116-immunized (middle); and R-AChR 97–116-immunized and treated in vivo with BMSCs. Note the difference in the proliferative response to the immunizing peptide between EAMG rats and BMSC-treated rats. (As compared to the EAMG group obtained from 4 different experiments: $^{**}P<0.01$, $^{***}P<0.001$).



	Groups		
	CFA	EAMG	BMSCs-treated
CD4-IL-4	1.16 ± 0.200 *	0.91 ± 0.028	1.12 ± 0.071 *
CD4-IL-17	0.40 ± 0.073 * * *	0.98 ± 0.014	0.80 ± 0.042 *
CD4-IFN-γ	1.30 ± 0.154 * * *	2.91 ± 0.085	1.68 ± 0.395 *
CD4-Foxp3	8.50 ± 0.491 * *	6.03 ± 0.200	7.38 ± 0.318 *

Fig. 7. Altered distribution of Th cells in EAMG and BMSC-treated rats. (Top) FACS analysis of expression of IFN-γ, IL-4, IL-17, and Foxp3 on CD4+ T cells derived from: normal rats (left), EAMG group (middle), and EAMG rats treated with BMSCs (right). Data were obtained from 4 different experiments, and results (bottom) are expressed as median percentage of double-positive cells ± SD. (*P<0.05, **P<0.01, ***P<0.001).

EAMG, a B-cell-mediated model of MG (Lennon et al., 1975; De Baets et al., 2003). BMSCs treatment not only led to a relief in the clinical severity of the disease model, but also partially restored the disordered balance of Th1/Th2/Th17/Treg cell subsets, and inhibited the proliferative activity of T and B cells from EAMG in vitro, an effect we have shown to be mediated by the secretion of TGF-β.

Since their immunomodulatory capabilities have recently been identified, BMSCs are believed to hold promise in the therapy of autoimmune diseases and other immune disorders (Corcione et al., 2006; Aggarwal and Pittenger, 2005; Jiang et al., 2005; Le Blanc and Ringden, 2005; Zappia et al., 2005; Wang et al., 2008). This ability has been shown to be mediated primarily by secreted factors such as TGF-β, HGF, IDO, PGE2, IL-10, although cell-to-cell contact may also be required (Rasmusson, 2006; Meisel et al., 2004). It has recently been determined that the transforming growth factor β, TGF-β, which is secreted by BMSCs, is an essential regulator of cell proliferation, survival and apoptosis, depending on the cellular context that has been mostly investigated (Krampera et al., 2003; Hocevar and Howe, 1998; Zhang

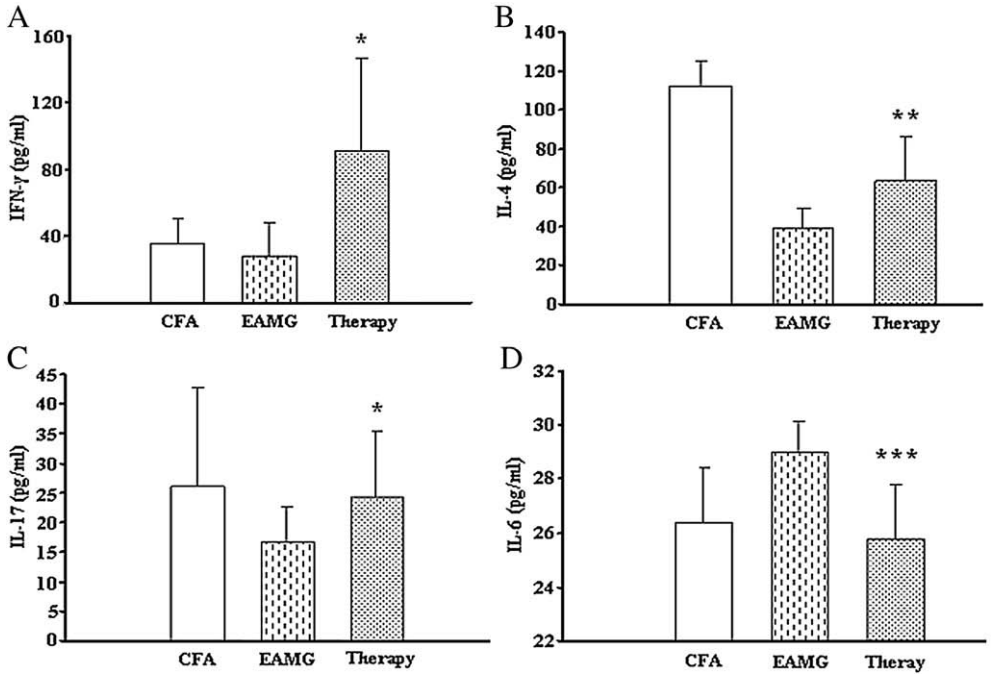


Fig. 8. Cytokine levels in the sera of EAMG and BMSC-treated rats. Serum concentrations of IFN-γ (A), IL-4 (B), IL-17 (C) and IL-6 (D), from control (left bars), EAMG (middle bars), and BMSC-treated EAMG rats (right bars). Data are expressed as the mean ± SD of 3 independent experiments. (As compared with EAMG group: $*P<0.05$, $**P<0.01$, $***P<0.001$).

et al., 2002). In this study, we confirmed, in proliferation assays, that the BMSC-mediated suppression of the proliferation of AChR-specific T and B cells was partially dependent on the secretion of TGF-β in the co-culture system (Fig. 1); meanwhile, TGF-β also reduced the immunoglobulin secretion of B cells (Fig. 2).

Although MG, as well as its animal model, EAMG, is a typical autoantibody-mediated disease, T helper cells specific for AChR, and the cytokines they secrete, may play a pathogenic role by permitting and facilitating the synthesis of high-affinity anti-AChR antibodies (Milani et al., 2006). Traditionally, Th cells were divided into the Th1 and Th2 functional subsets, based on whether they express IFN-γ (Th1) or IL-4 (Th2) (Saoudi et al., 1999), however, as Th 17 cells, the IL-17-producing Th subset, have been identified, the traditional classification of Th cells has been supplemented (Oboki et al., 2008; Bi et al., 2007). The identification of Th 17 cells have provided new insight into our understanding of the development of autoimmune diseases as well as immune responses, and thereby led to revision of the classic Th1/Th2 paradigm (Liu et al., 2005; Kramer and Gaffen, 2007).

IFN-γ appears to promote EAMG: it has been shown to be necessary for the induction of EAMG in mice by *Torpedo californica* AChR (TAChR); the localized expression of IFN-γ transgenic in the neuromuscular junction induces an MG-like syndrome (Gu et al., 1995); and IFN-γ receptor knockout mice, but not IL-4-deficient mice, are resistant to EAMG (Zhang et al., 1999; Balasa et al., 1998). In contrast, alteration of the balance in favor of Th2 cells has been shown to protect against EAMG (Milani et al., 2006, 2003). IL-4 appears to be involved in the differentiation of AChR-specific regulatory CD4+ T cells, which can prevent the development of EAMG and its progression to a self-maintaining, chronic autoimmune disease (Milani et al., 2006, 2003). Tolerance procedures in mice have been found to be effective in preventing EAMG through the induction of Th1 unresponsiveness, which was associated with an upregulation of Th2 cytokine synthesis (Saoudi et al., 1999). While the FACS and ELISA results obtained in this study (Figs. 3 and 4) are in agreement with this model, there have been reports which do not agree with the respective roles of Th1 and Th2 cells it proposes. Some studies have found that IL-4 was not required either for the generation of a pathogenic anti-AChR humoral immune response or for progression of clinical EAMG in IL-4(−/−) gene knockout mice, and found no direct or circumstantial evidence indicating a role of IL-4 in the modulatory or immunosuppressive circuits in MG (Balasa et al., 1998).

Experiments with gene-deficient mice indicate that IL-17 and Th17 cells are crucial in the pathogenesis of classical Th1-mediated autoimmune disorders, and Th2-mediated allergic disorders, as well as EAMG, a B cell mediated autoimmune disease (Oboki et al., 2008; Wang et al., 2007; Komiyama et al., 2006). The differentiation of Th17 cells have been shown to depend on another CD4+ subset, the regulatory T cells (Treg). CD4+ Foxp3+ regulatory T cells suppress effector T cells in various pathological conditions, including inflammation, autoimmunity, cancer, and organ transplantation (Askenasy et al., 2008). They are especially important in maintaining self-tolerance and inhibiting the development of autoimmunity (Wang et al., 2007; Sakaguchi, 2005). The expression of Foxp3 and the potency of Treg cells might inhibit the proliferation of autoreactive T cells in EAMG, thereby providing a potentially superior strategy for the treatment of EAMG (Liu et al., 2005). IL-6, another cytokine secreted by Th2 and Th17 cells, has also been shown to be an essential factor in EAMG. IL-6-deficient mice are resistant to EAMG and IL-6 in vivo have been shown to regulate production of AChR-specific IL-2, IFN-γ, and IL-10, which facilitate the development and maintenance of the autoimmune response to AChR (Deng et al., 2002). In addition, IL-6 promotes the generation and development of IL-17-producing CD4+ T cells and suppresses the development of Treg cells development (Nishihara et al., 2007).

Abundant data thus indicate that Th1, Th2, Th17, and Treg cells are involved in the pathogenesis of EAMG, via a complex web of interactions among the cells and their cytokines. This would suggest that the development of the disease, or its return toward the normal state following successful treatment, would be associated with a shift in the relative abundance of the four subsets. Our results (Figs. 7 and 8) are in accordance with this prediction. Furthermore, we found that that the administration of BMSCs both ameliorates the symptoms of EAMG (Fig. 5), and, both in vivo and in vitro, increases the proportions of IFN-γ (Th1) and IL-17 (Th17) expressing cells, and decreases those of the IL-4 (Th2) and Foxp3 (Treg) expressing cells (Figs. 4 and 7).

In some cases, the results of measurement of secreted cytokines carried out in this study were in accordance with the proposed model

and with a therapeutic role for BMSCs: IFN-γ, IL-17 and IL-6 were decreased following BMSCs treatment (Fig. 3B, D, E), while the potential protective factor IL-4 was increased (Fig. 3C). However, the IFN-γ and IL-17 concentrations in the sera of different groups appeared inconsistent with the model (Fig. 8A, C). One explanation may be that lymphocytes are not the only cells secreting these cytokines, with other lymphoid organs becoming involved as the severity of EAMG increases to have more broad systemic effects. To eliminate these complications we focused on in vitro analyses, in which we co-cultured AChR-specific T cells with BMSCs in the presence or absence of anti-TGF-β antibody. We detected an alteration in the cytokine secretion and Th subsets by BMSCs that was similar to our earlier results, and we were additionally able to show that the addition of the anti-TGF-β antibody could reverse this effect. Differentiation of both Treg and Th17 cell requires TGF-beta, but depends on opposing activities: at low concentrations, TGF-beta synergizes with interleukin IL-6 and IL-21 to promote IL-23 receptor expression, favoring Th17 cell differentiation, while high concentrations of TGF-beta repress IL-23 receptor expression and favor Foxp3+ Treg cells (Zhou et al., 2008), and this theory just consistent with our data.

Taken together, our data indicate that an alteration in the balance of Th1, Th2, Th17, and Treg contributes to the development of EAMG, and that the intravenous administration of BMSCs can ameliorate the severity of EAMG and, in a process dependent on the secretion of TGF-β, presenting to inhibit the proliferation of AChR-specific T and B cells, reduce the ability of immunoglobulin secretion of B cells, normalize the distribution of the four Th subsets and their corresponding cytokines. The immunoregulatory function of BMSCs thus appears to represents a promising strategy for cell therapy of myasthenia gravis and other autoimmune diseases, which is central to human health and disease, and provides novel insights into new therapeutic interventions.

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