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Evaluation of AD-MSC (adipose-derived mesenchymal stem cells) as a vehicle for IFN-β delivery in experimental autoimmune encephalomyelitis

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ABSTRACT

Interferon-β (IFN-β) is commonly used as a disease modifying drug for the treatment of relapse-remitting multiple sclerosis (RR-MS). However, the underlying mechanism by which IFN-β mediate this immunosuppressive effect is still unknown. In this study, we analyzed the effects of genetically modified adipose-derived mesenchymal stem cells (AD-MSCs) expressing murine interferon beta (MSCs-VP/IFN-β) on the animal model of MS, experimental autoimmune encephalomyelitis (EAE). Lymph node mononuclear cells and serum were examined by using RT-PCR and ELISA methods to measure the production of IL-10 and IL-17 gene and protein expression, respectively. Our results indicated that in the MSCs-VP/IFN-β treated group induction of Tregs and IL-10 and reduction of IL-17 were significant. Taken together, we showed that using AD-MSCs expressing IFN-β as an anti-inflammatory agent, offer evidence supporting that the stem cell therapies in EAE conceivably will improve the valuable effects of IFN-β in this autoimmune disease.

1. Introduction

Multiple sclerosis (MS) and its animal model, experimental autoimmune encephalomyelitis (EAE), is an autoimmune disease that results in progressive demyelinating disorder of the central nervous system (CNS) [1]. The most common treatment approach for multiple sclerosis mainly modulates the immune system. Immunomodulatory properties of mesenchymal stem cells (MSCs) are one of the most fundamental features of these cells, as they can suppress many functions of immune cells. Furthermore, their innate capabilities to migrate to the site of chronic inflammation provide new opportunities for cell based therapy for treatment of autoimmune disease [2–3]. Adipose-derived mesenchymal stem cells (AD-MSCs) are adult non-hematopoietic stem cells (HSC) that could differentiate into multiple lineages, including chondrocytes, adipocytes and osteoblasts.

Due to their particular immunomodulatory properties, MSCs has been emerged as a promising tool for clinical applications such as tissue engineering and cell and gene therapy in several autoimmune diseases such as MS [4–5]. Several experiments showed that mouse bone marrow derived MSCs have a potential to ameliorate chronic and relapsing form of EAE [6–7]. Recent data indicated that MSCs can mediate anti-inflammatory properties by the production of anti-inflammatory cytokines such as TGF-β. Moreover, besides to their wide availability, AD-MSCs could serve as a safe autologous source for stem cell transplantation [8–10]. It is now generally accepted that both Th1 and Th17 cells, play a critical role in MS and EAE pathogenesis [11–12]. Several lines of evidence indicated that bone marrow derived-MSCs could ameliorate EAE by preventing Th1 and Th17 cell population [13–14]. IFN-β is the most commonly prescribed FDA-approved treatment for relapsing-remitting form of MS (RR-MS) [15]. However, the results of the several

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clinical trials showed that the side effects of IFN-β therapy such as common flu-like syndrome, liver function abnormalities, leukopenia, and depression restricted the using of IFN-β in about 60% of patients [16]. Moreover, at least 10–50% of patients do not respond to treatments by IFN-β [17]. Several experiments have reported that treatments with any forms of the recombinant IFN-β results in the development of neutralizing antibodies, which consequently leads to the destruction of IFN-β [18–19]. Evidence from other experiments has revealed that IFN-β has an anti-inflammatory impact on Th1 and Th17 cells [20–21]. Notably, impaired Treg cell production and function is one of the most common features of MS pathogenesis [22]. Thus, we explored the therapeutic application of genetically modified adipose-derived MSCs containing lentiviral particle expressing IFN-β (AD-MSCs-VP/IFN-β) in EAE animals. Considering that the impairments of Tregs in MS patients, we investigated whether AD-MSCs-VP/IFN-β contributes to Tregs induction in EAE mice.

2. Materials and methods

2.1. Isolation, culture and differentiations of murine MSCs

Previously described methods were used for MSCs isolation [23] briefly, murine AD-MSCs were obtained from inguinal fat of 6–8 weeks old C57BL/6 female mice. Tissue digestion was done at 37 °C with 1 mg/ml collagenase type I (Gibco). And suspension centrifuged at 1200 *g* for 20 min. AD-MSCs were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin (Invitrogen), 100 g/ml streptomycin (Invitrogen), and 2 mM L-glutamine (Invitrogen). After 48 h medium was replaced and non-adherent cells were removed. And fresh media were added every three days. After 3 weeks, when the cells were reached at 70–80% confluences, AD-MSCs detached by incubation in 0.25% trypsin/EDTA for 2 min at 37 °C.

All the experiments are performed on cells at passages 3 to 4. For multilineage differentiation; we assessed AD-MSCs for their ability to differentiate into osteocytes and adipocytes under the especial conditioned media. Adipogenic differentiation was induced in the pre-plated cells by adding dexamethasone 10 M and insulin (6 ng/ml) and, in the case of osteogenic differentiation ascorbic acid (50 μg/ml), sodium β-glycerophosphate (10 mM) and dexamethasone 10 M (Sigma Aldrich). After 3 weeks, plates were washed with 1× PBS, fixed with 4% paraformaldehyde, and stained with Oil Red O and Alizarin Red for adipogenic and osteogenic differentiation, respectively.

2.2. Flow cytometric analysis

Murine AD-MSCs was characterized by the positive expression of CD105, CD73, CD44 and CD90 (Thy1), and they were checked for negative expression of hematopoietic stem cell marker, e.g. CD45 and CD11b as a myeloid cell marker. For the immunophenotyping analysis, AD-MSCs washed with PBS and incubated with the specific mAbs for 30 min at 4 °C. For each test, 10,000 events were analyzed. (All antibodies were purchased from eBioscience Co.) For Tregs, according to the manufacturer's instructions: after isolation of the spleen and draining lymph nodes of study groups, mono-nuclear cells were harvested and stained with eBioscience mouse regulatory T cell staining kit #2 containing anti-mouse CD4-FITC, anti-mouse CD25-PE, anti-mouse Foxp3-APC (FJK-16s; eBioscience) and anti-mouse CD127-PE-CY7(eBioscience). All examinations performed by using flow cytometry (FACSCanto, Becton Dickinson) and analyzed by Flowjo software (Treestar Inc., USA).

2.3. Lentiviral vector construction, packaging and purification

HIV-1-based, VSV-G pseudo typed lentiviral vector carrying a mouse IFN-β gene cDNA were constructed as previously described [24]. In short, mouse IFN-β cDNA (clone ID; 8734144) was purchased from thermo scientific and sub-cloned. The transfer vector, pCDH-513B-1 (System Bioscience, Mountain View, CA, United States) containing cPPT-CMV-GFP-eF1-pur, according to manufacturer's instructions was used for reflection of our interest. IFN-β containing vector was digested with ECORI to release the 650-bp fragment of interest gene and this piece sub-cloned into the multiple cloning site (MCS) of PCDH transfer vector (both vectors have digested with ECORI). The correctness of obtained IFN-β cDNA was confirmed by sequencing and comparing it with the GenBank accession number; BC119395. Then the IFN-β cDNA was inserted between the ECORI site in the PCDH vector, and the constructed plasmid was transformed into *Escherichia coli* competent cells (DH5a) and cultured for 16 h in Amp-resistant agar plate and the Amp-resistant clones were selected by colony-PCR method using IFN-β and eF1 (elongation factor) forward and reverse primers, respectively. The selected colony was used for extraction and sequencing. After these junctures, the confirmed vector was produced by transient transfection of a three-plasmid system into HEK293 cell line. For viral particle production, three plasmids including: ps-PAX2.2 for packaging (Addgene plasmid 12260), pMD2.G for envelope (Addgene plasmid 12259) and transfer vector (PCDH-IFN-β) were transfected into HEK293 cell line by the calcium phosphate precipitation method and culture medium was replaced 16 h post-transfection. Supernatants were collected 24 h, 48 h and 72 h, filtered with 0.2 filters, pooled and concentrated by PEG-6000 and vector titration was determined by flow cytometric method based on the expressing green fluorescent protein (GFP) as transducing units per ml (TU/ml). All steps were previously described in depth [25].

2.4. MSC transduction and MTT assay

Cells were seeded in 6-well plates at a density of 6×10^4 cells in 2 ml complete DMEM medium containing 6 μg/ml polybrene (Sigma) and virus particles were added to the cells with different multiplicities of infection (MOI, we used MOI = 100 in our experiment for transduction of AD-MSCs). After centrifugation at 2000 rpm, plates incubated at 37 °C for 12 h and the cultures were replaced with fresh media. Infected MSCs were evaluated by fluorescent microscope and selected by incubation in medium containing 2 μg/ml of puromycin (Sigma) and selected cells were cultured and expanded until they reach to acceptable confluences [26]. After transduction stage, adipose-derived mesenchymal stem cells containing viral particle expressing IFN-β (AD-MSC-VP/IFNβ) was assessed for viability by using MTT assay. Transduced AD-MSCs was seeded in 96-well plates at density of 8×10^3 cells per well and incubated at 37 °C for 24 h. MTT dye was added, and incubation was performed for 4 h and the reaction was stopped with the addition of DMSO, and the plate was read at 570 nm by a micro-plate reader (Bio-Rad).

2.5. EAE induction and treatment protocols

For EAE induction, we used C57Bl/6 female mice 6–8 weeks old as described [27]. Briefly a total volume of 200 ml emulsion containing a 1:1 ratio of MOG33–55 (final concentration; 250 μg, Sigma)/CFA (complete Freund's adjuvant, Invitrogen) was administered into two different flanking sites to each mouse. Pertussis toxin (200 ng/ml) (Invitrogen; P2980) was injected intra-peritoneal (i.p.) at 0 and 48 h post-immunization. All animals were checked for weight loss every day and graded according to a standard clinical index: 0 = no clinical signs; 0.5 = partially limp tail; 1 = paralyzed tail; 2 = hind limb paresis; 2.5 = one hind limb paralyzed; 3 = both hind limbs paralyzed; 3.5 = hind limbs paralyzed and weakness in forelimbs; 4 = forelimbs paralyzed and 5 = moribund or dead. Mice were randomly divided into four groups and were injected through the tail vein (IV) on day 16 and 18 after immunization (5×10^5/each time): EAE untreated ($n = 8$), MSCs ($n = 8$, 1×10^6 cells/each mouse), MSCs-VP ($n = 8$,

1×10^6 cells/each mouse) MSCs-VP/IFNβ ($n = 8$, 1×10^6 cells/each mouse). Animals were sacrificed on days 23 (as a peak of disease severity; $n = 5$) [28] and 55 (chronic phase; $n = 3$) after post immunization. To cytokine assay, before sacrificing them blood samples were drawn from the heart of the mice. All mice were anesthetized with an injection of 250–300 ml of a ketamine/xylazine mixture by i.p. injection.

2.6. Western-blot

Transduced AD-MSCs was used for Western blotting experiments as described [29]. Cells were scraped from culture plate and washed with PBS and lysed by prepared lysis buffer containing 20 mM Tris–HCl, 150 mM NaCl, 1% Triton X-100, and protease inhibitor was added. After centrifugation for 20 min at 12,000 *g*, supernatants were collected. Protein extraction carried out according to Pierce BCA Protein Assay Kit (Thermo Scientific). 40 μg protein was loaded, and electrophoresis on 12% SDS-PAGE was accomplished and transferred onto a polyvinylidene difluoride membrane (PVDF, Invitrogen). The membrane was blocked for 2 h with 5% BSA using Tris-buffered saline containing 0.1% Tween 20 and incubated with a monoclonal rat anti-mouse IFN-β antibody (Abcam) for overnight at 4 °C. The membrane was washed and followed by incubation for 2 h with an anti-rat secondary antibody (Abcam), and protein band was illuminated by using chemiluminescent kit (Amersham, Bioscience). For normalization, in the same membrane, β-actin was used as a loading control.

2.7. Proliferation assays and cytokine ELISA

Isolated spleen and draining lymph node cells (10×10^6 cells/ml for each mouse) were harvested from mice of every group and stained with 5 μM (final concentration) of CFSE (Invitrogen) for 15 min at 37 °C. Then the reaction was quenched by the addition of 5 volumes of ice-cold culture media to the cells and followed by 5-minute incubation on ice. After 3 times washing with PBS/0.1% BSA, the cells were re-suspended in fresh RPMI1640 medium and cultured for 1 to 3 days at 37 °C and 5% CO_2 in the presence or absence of 50 ng/ml antigenic peptide (MOG35–55 peptide). For culture, 3×10^5 cells plated in 96-well Microtiter plates and after 72 h, total lymphocytes are harvested and the CFSE profile was measured by flow cytometric analysis. For cytokine ELISA assay, harvested cells (1×10^6 per well) co-cultured with the MOG peptide (50 ng/ml) in complete RPMI1640 medium in 48-well plates. After 72 h, supernatants were collected and stored in −70 °C until used for measurement of IL-17 and IL-10 using ELISA kits (eBioscience). According to the manufacturer's instructions, capture antibodies (anti-mouse IL-10 and anti-mouse IL-17A, eBioscience) were coated by incubation overnight at 4 °C. After aspiration and washing, samples and provided standards were added, and incubation was done for 2 h at room temperature. Then biotinylated detecting antibodies added and detection was accomplished by using Avidin-HRP (eBioscience) and substrate (1× TMB) (Sigma). Absorbance was read at 450 nm using a microplate reader (Bio-Rad). The samples for ELISA were examined in triplicate. For measurement of systemic IFN-β level, serum samples were collected at days 23 and 55 after immunization and used for ELISA measurements. There were no significant differences found between four groups ($P > 0.05$; data were not shown).

2.8. Real-time PCR

The lymphocytes of the spleen or draining lymph nodes was used for total RNA extraction (TRIzol, Invitrogen) and extracted RNA was changed into the cDNA by using cDNA synthesis kit (Fermentas Life Science). The protocol an primer sequences were described in depth in our earlier published article [30]. Generally, the cDNA was examined by real-time PCR using SYBR Green Master Mix (Takara Bio) and β2m used as an internal control.

Cycling was performed on a Qiagen thermocycler (Rotor-Gene Q, Qiagen) using a two or three-step PCR protocol (depends on related Tm for each primer) under the following condition: an initial holding at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and at 60 °C for 1 min.

2.9. Histology

The brains were removed from mice on days 23 and 55 after post-immunization and fixed with 10% formalin for histological analysis. For further processes and scoring leukocyte infiltration, we used the described method by Calida DM et al. [31]. Brain tissues were

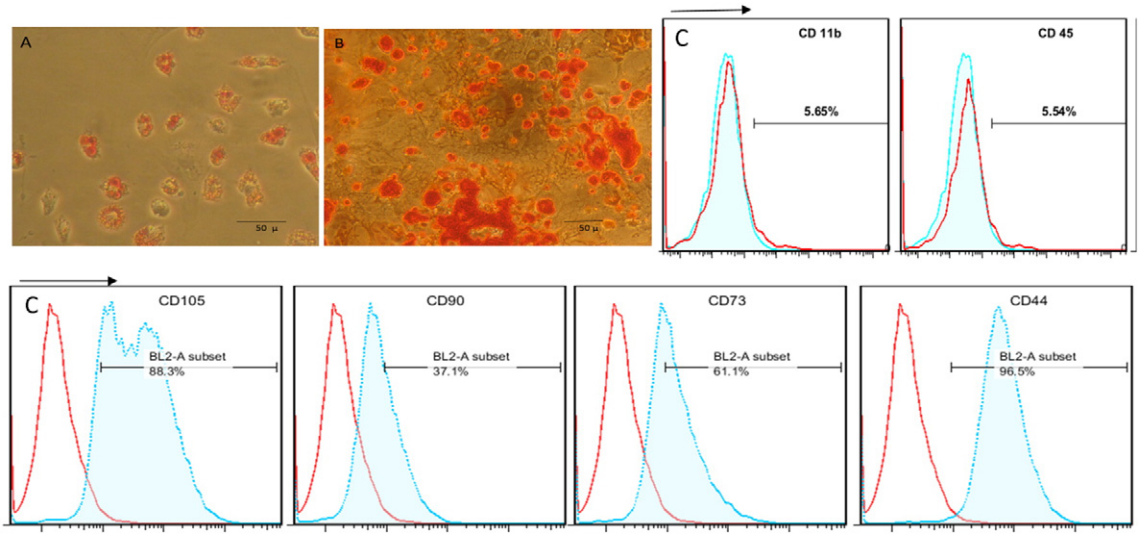

Fig. 1. Multilineage differentiation and immunophenotyping of AD-MSCs. AD-MSCs obtained from C57BL/6J mouse were cultured in the conditioned medium and their multilineage differentiation capacity was confirmed. For adipogenic differentiation, the 2nd passage AD-MSCs stained with oil red (A). And osteogenic differentiation confirmed with Alizarin Red (B). Scale bars, 50 μm. For immunophenotyping profile of AD-MSCs, The 2nd passage cells were harvested and stained with known positive markers for MSCs; CD44, CD90, CD73 and CD105 and stained for negative markers; CD11b and CD45 (C). More details are described in Materials and methods.

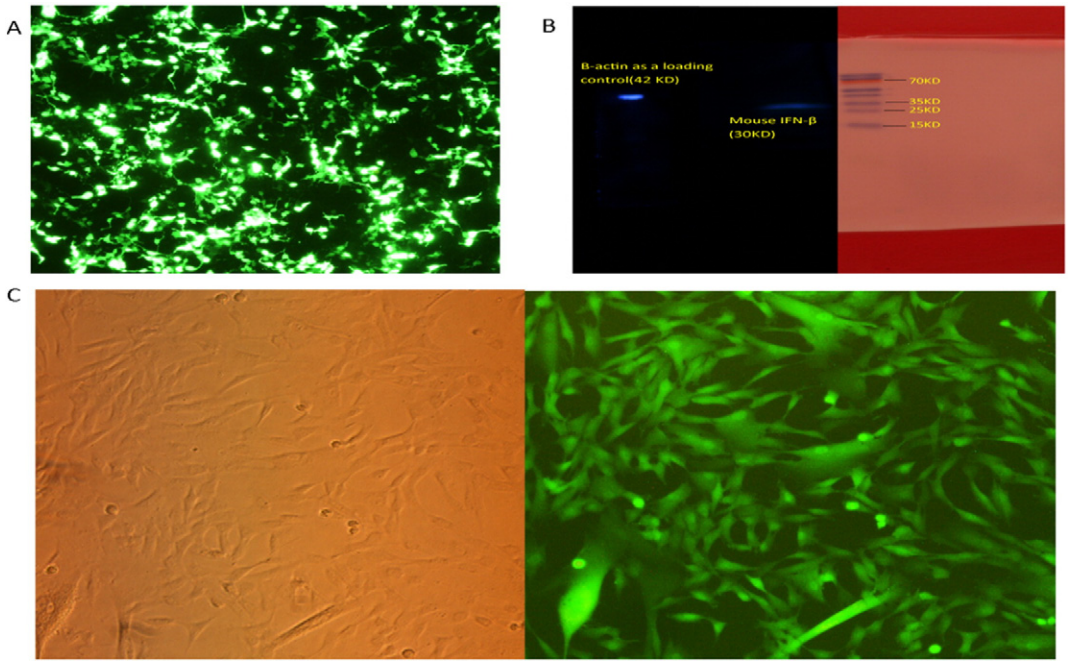

Fig. 2. Transfection, western blot and transduction of AD-MSCs. Transfection efficiency of HEK-293 cell line, was determined by the expression of GFP using a fluorescent microscope (A). The fate of AD-MSCs ("C" left) after transduction ("C" right) was assessed by the expression of GFP protein in fluorescent microscopic view and the in vitro expression analysis of IFN-β protein assessed by western blotting (B). Protein marker (right lane) and a 30 kDa IFN-β in the middle lane is confirmed the expression of this protein.

stained with hematoxylin and eosin (H&E). Infiltration in every section was classified as follows: −; none, +; faint, ++; moderate, +++; intense, ++++; very intense. At least 3 to 5 slides were analyzed for each mouse.

3. Statistical analysis

Differences between groups were determined by one-way ANOVA. Student *t*-test was used in the case of comparing two groups and data

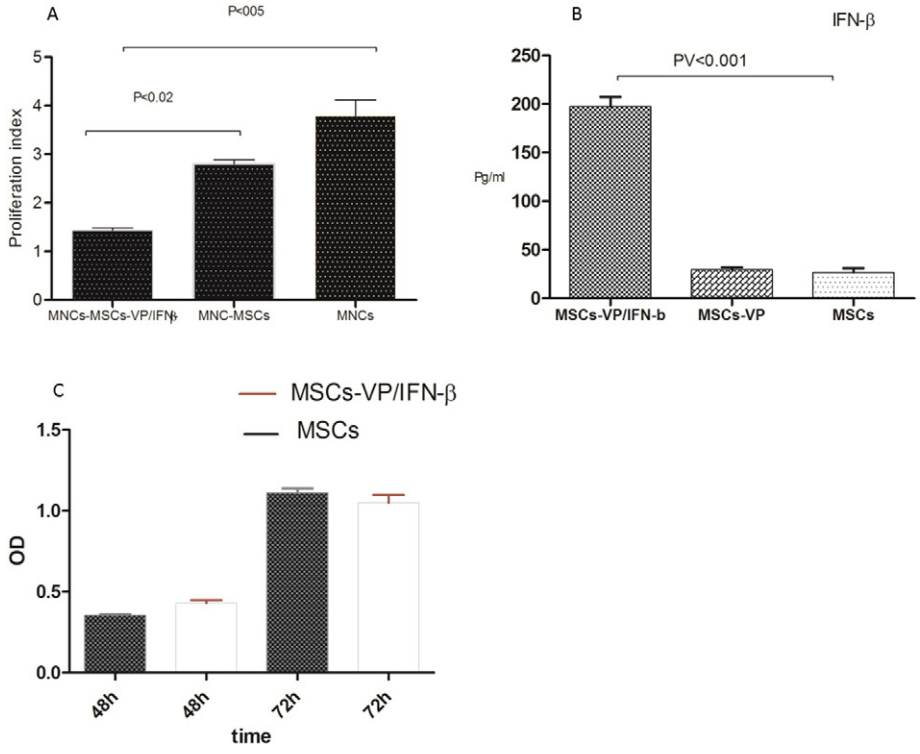

Fig. 3. Functional assay and MTT, and IFN-β production. IFN-β could act as an anti-proliferative agent, thus spleen mononuclear cells (MNCs) of EAE mice were isolated and co-cultured with MSCs and MSCs-VP/IFN-β in the presence of 20 μg/ml of MOG peptide for 72 h. Cell proliferation (A) was measured by the CFSE method as described in more details in the Materials and methods section. Statistically significant differences between MSCs and MSCs-VP/IFN-β groups were observed. For the measurement of the possible toxicity effect of IFN-β on MSCs. The MTT assay (C) was assessed at 48 h and 72 h. We did not find significant differences between MNC-MSCs and MNCs-MSCs-VP/IFN-β. And this is confirmed that the presence of IFN-β, has no harmful effects on MSCs. For the measurement of IFN-β expression by transduced MSCs, an ELISA was performed with supernatants after 72 h culture with different groups (B). All tests were performed in triplicate.

were analyzed by Prism software (GraphPad, San Diego, CA). Data were expressed as mean ± SD and when the Pv < 0.05, results reflected statistical significance.

4. Results

4.1. Characterization of murine adipose-derived MSCs

AD-MSCs were obtained from C57BL/6J mouse inguinal adipose tissue according to an established protocol with slight modifications [32]. AD-MSCs exhibited continuous proliferation and flow cytometric analysis of these cells showed the presence of CD73, CD90, CD44 and CD105, but they were negative for CD11b and CD45 (Fig. 1C). For more characterization, the capacity of multilineage differentiation of these cells into adipocytes and osteoblasts, were examined under the appropriate conditioned medium (Fig. 1A & B).

4.2. Lentiviral vector-mediated expression of mouse IFN-β in mouse AD-MSCs

Murine IFN-β gene was sub-cloned in a lentiviral-based vector, and this construct used for transfection of HEK-293 cell line (Fig. 2A). Supernatant, containing viral particles, from transduced cells were harvested and used to transduction of AD-MSCs. After three days, cultured, MSC-VP/IFN-β was assessed by western blot analysis. As depicted in Fig. 2B, a 30 kDa molecular weight band of IFN-β protein confirmed the presence of the transgenic protein. For exclusion of toxic effects of the transduced gene on AD-MSCs, the IFN-β transduced and intact MSCs were analyzed by MTT assay (Fig. 3C). The anti-proliferative properties of IFN-β were examined by co-culturing MSC-VP/IFN-β and MSCs with spleen derived mononuclear cells (MNCs) using CFSE proliferation assay and the results showed significant differences between MSCs and MSCs-VP/IFNβ treated groups (Fig. 3A).

4.3. Therapeutic administration of MSC-VP/IFN-β reduced the severity of disease in EAE

To investigate the effects of IFN-β along with an immunomodulatory agent for EAE treatment, IFN-β gene transduced MSCs (MSCs-VP/IFN-β) was administered to the EAE mice. In addition, we used two other groups that just received MSCs alone or viral backbone transduced MSCs (MSCs-VP) for comparison. The amount of IFN-β expression in supernatant media was measured by ELISA in each group (Fig. 3B). The clinical signs and scores between groups that received MSCs and untreated EAE are significant (Fig. 4D).

To evaluate mononuclear cells (MNCs) infiltration, the brain tissue of all groups was stained with H&E method. Our results indicated that the cell infiltration in MSCs-VP/IFNβ treated group were significantly lower than the other groups (Fig. 4B & C); both MSCs and MSCs-VP/IFN-β have $P < 0.01$ compared to untreated mice (A). However, we did not find significant differences between groups that treated with MSC and MSCs–VP/IFN-β. Clinical and pathological observations are depicted in the Table 1.

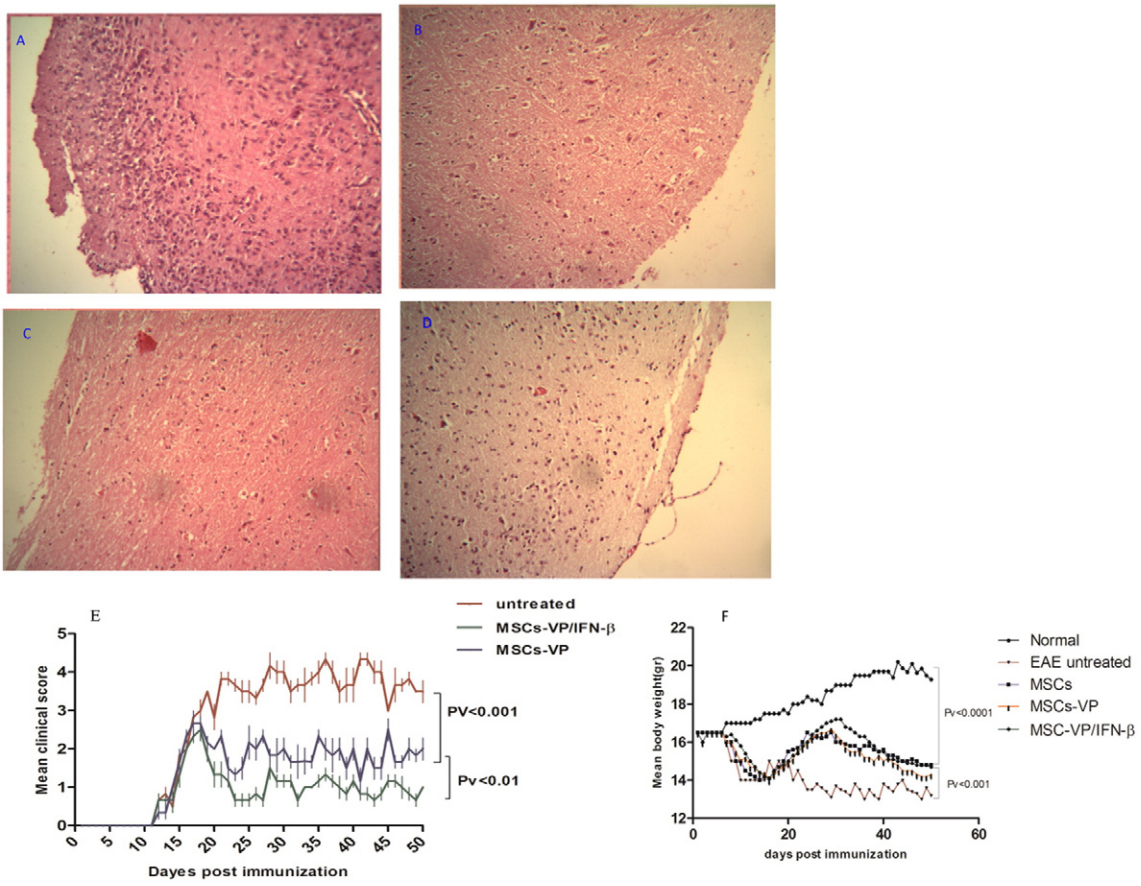

Fig. 4. Therapeutic administration of MSCs-VP/IFN-β ameliorates EAE severity. Brain tissue of all groups was harvested, and 6 μm sections were stained with H&E. Untreated mice showed severe cellular infiltration (A) and in MSCs-VP (B), MSCs-VP/IFN-β (C) and MSCs (D) treated groups ($n = 5$/each group). Statistical results showed that MSCs-VP/IFN-β treatment significantly reduced the cell infiltration compared to EAE or MSCs-VP and MSCs treatment. Cellular infiltrations are down-regulated when compared with the untreated group ($P < 0.01$). Magnification ×100. Mice were immunized with 300 μg of MOG35–55 peptides in CFA. Clinical EAE was scored daily according to a 0–5 severity scale. The overall clinical score (E) and body weight (F) were significantly different between untreated group and all three groups that received MSCs and the clinical score differences between MSCs-VP/IFN-β treated group is statistically significant when compared with the other groups that just received MSCs-VP or MSCs (E) ($n = 5$/each group). Significant differences were determined by Student's *t*-test ($P < 0.05$).

Table 1
Clinical and pathological features of all used mice in our study.

Treatment	Treatment schedule (days after immunization)/injected cells per mouse	Disease onset	Mean maximum clinical score	Leukocyte infiltration (cells/mm^2)
MSCs-VP/IFN-β	16–17 days/1 × 10^6	(12–14)	1–2.5	52 ± 15*(+)
MSCs-VP	16–17 days/1 × 10^6	(12–14)	1.5–3, 3.5	94 ± 20 (++)
MSCs	16–17 days/1 × 10^6	(12–14)	1.5–3, 3.5	83 ± 15 (++)
Untreated	16–17 days/1 × 10^6	(12–14)	2.5–4	170 ± 25 (++++)

The significance was obtained between MSCs-VP/IFN-β and untreated group.
* $P < 0.01$.

4.4. Increased expression of IL-10, and TGF-β in MSCs-VP/IFN-β treated mice

To confirm the immunomodulatory effects of MSCs-VP/IFN-β on EAE, we evaluated the effects of this therapeutic approach on gene expression pattern in both cytokine gene and main transcription factors related to the Th cell subsets as they are the major leader of EAE pathogenesis [33–34]. We therefore used real-time PCR to show the expression of inflammatory and anti-inflammatory cytokines and transcription factors to reflect our purposes. The results showed that MSCs-VP/IFN-β downregulated the levels of IL-17 expression and upregulated the characteristic anti-inflammatory cytokine i.e., IL-10 and Treg related transcription factor, Foxp3 ($P < 0.05$ and $P < 0.07$, respectively) (Fig. 5A & B). Along with the downregulation of inflammatory cytokines, the relative expression of the TGF-β significantly upregulated in all MSCs treated groups when compared to untreated EAE (Fig. 5, $P < 0.01$). The relative

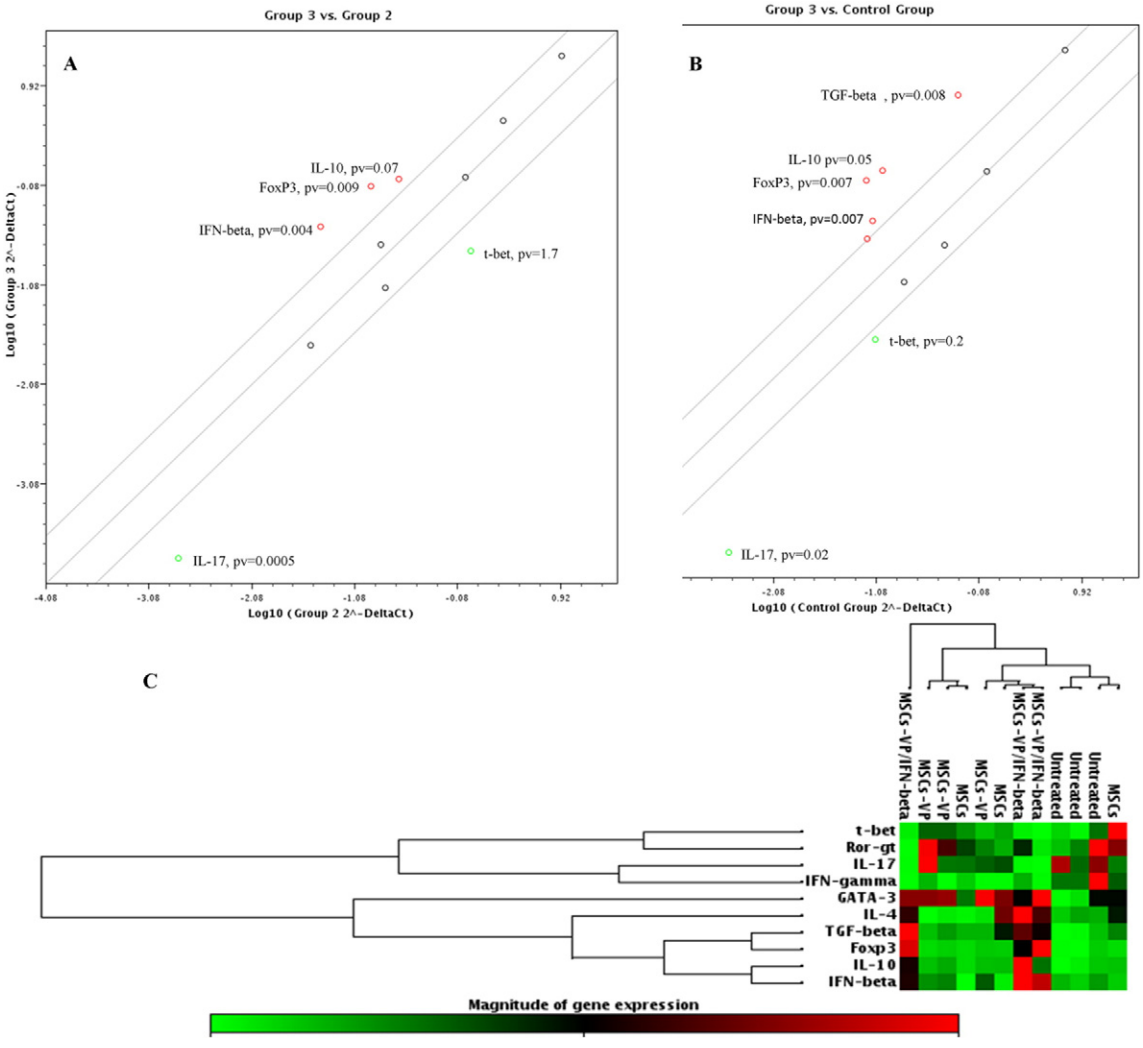

Fig. 5. The scatter plot of transcription factor and cytokine expression is depicted as the log of the relative expression level of each gene in every group. MSCs-VP/IFN-β increases the levels of anti-inflammatory cytokine profile. To demonstrate the anti-inflammatory effects of MSCs/VP-IFN-β, draining lymph nodes collected from EAE mice and harvested cells cultured ex vivo at 1 × 10^5 cells/ml in the presence of MOG (30 ng/ml) for 72 h. Inflammatory and anti-inflammatory cytokines and major related transcription factors of four Th subsets were examined by RT-PCR method. A robust increase in anti-inflammatory gene expression, i.e., IL-10 and downregulation of inflammatory cytokines, IL-17, was observed in mice treated with MSCs-VP/IFN-β (group 3) when compared with MSCs (group 2) (A). And an expression of TGF-β was observed in MSCs-VP/IFN-β treated group when compared with the untreated group (control group) (B) ($n = 5$/each group). A heat map was used to compare the co-regulation of Th cell subset-related transcription factors and cytokine expression across the treated samples and control groups (C). β2μ used as an internal control, MSCs-VP/IFN-β (MSCs transduced with lentiviral particles containing IFN-β gene), MSCs-VP (MSCs transduced with viral particle without IFN-β gene). The RT-PCR results are representative of three independent experiments. And significant differences were determined by one-way ANOVA ($P < 0.05$).

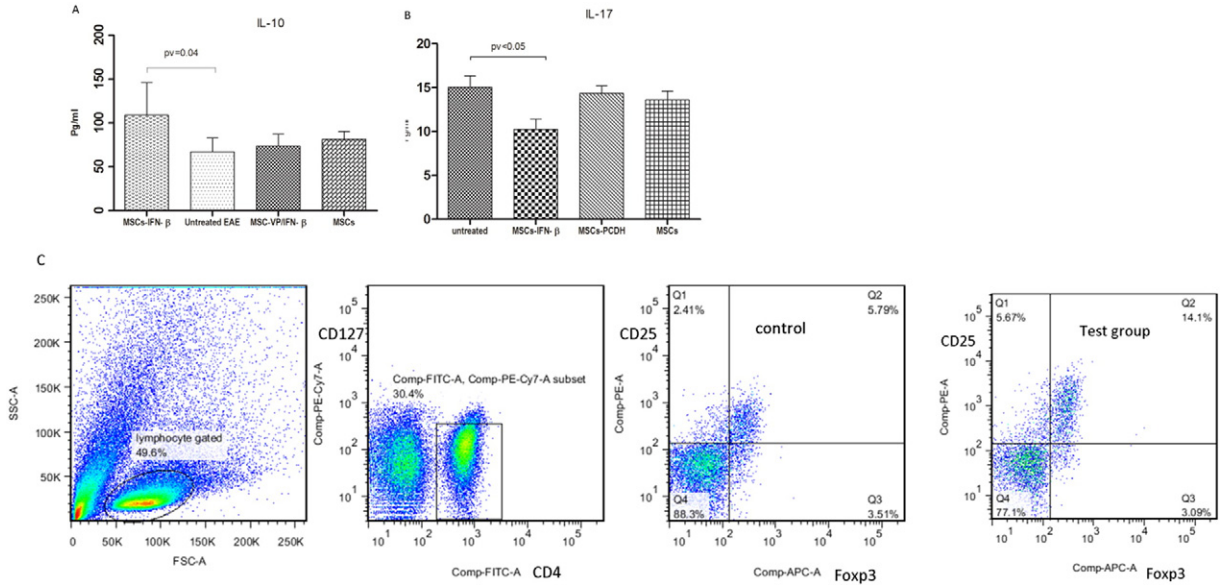

Fig. 6. The ELISA method was used to detect IL-10 and IL-17 levels in the serum of MSCs-VP/IFN-β treated group compared to other controls to quantify the effects of IFN-β. Production of IL-10 (A) was increased and IL-17 (B) was decreased in MSCs-VP/IFN-β treated group when compared with the untreated group ($n = 5$/each group). The data represent mean values and SD of the triplicate experiments. Significant differences were determined by one-way ANOVA ($P < 0.05$). Representative four-color flow-cytometry analysis of lymphocytes derived from draining lymph nodes and spleen. Isolated lymphocytes were stained with monoclonal antibodies against CD4, CD25, CD127 and FOXP3. From left to right, lymphocytes gated based on SSC vs. FSC and in the next step, these cells gated based on $CD4^{high}$ and $CD127^{low}$ and final the percentages of $CD25^{high}$ $FOXP3^{high}$ cell were determined (C). MSCs-VP/IFN-β (MSCs transduced with lentiviral particles containing IFN-β gene), MSCs-VP (MSCs transduced with viral particle without IFN-β gene).

expression of Th17and Th2 cell transcription factors was examined although significant changes are not found in the cases of Rorγt and GATA3, respectively. To confirm whether gene expression findings are coordinated with protein levels of inflammatory cytokine production, an ELISA were confirmed the upregulation of anti-inflammatory cytokine, IL-10, and downregulation of IL-17 (Fig. 6A & B).

4.5. Production of Treg cells increased in MSCs-VP/ IFN-β treated mice

To explore whether the MSCs-VP/IFN-β contribute to the Treg cell production, we applied four-color Ab immunophenotyping to reflect our estimation. Flow cytometry analysis showed a strong correlation of using MSCs-VP/IFN-β treatment and Treg cell production. The

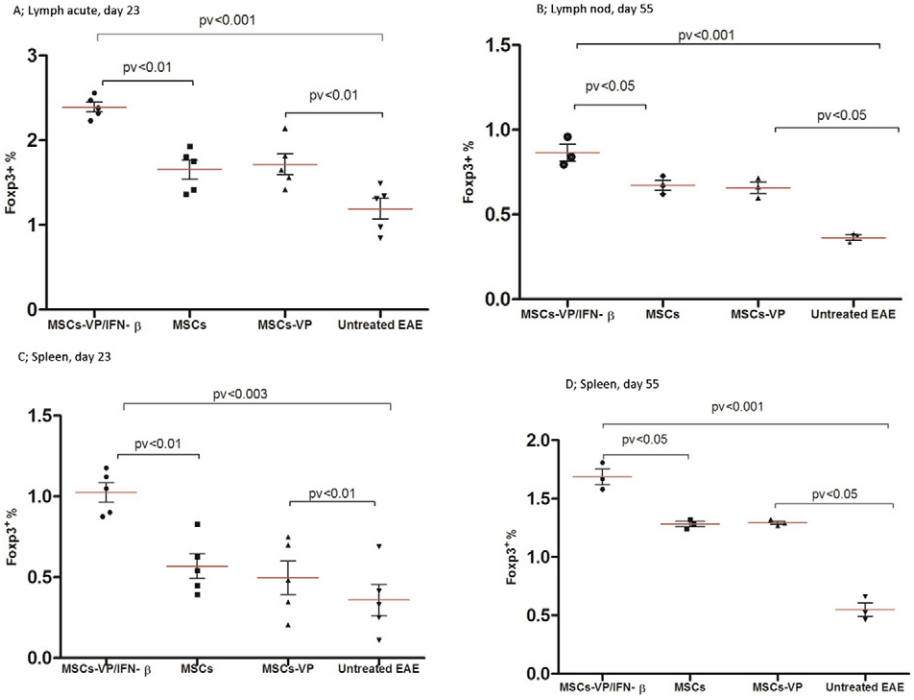

Fig. 7. Multicolor analysis of Treg cells confirmed that the MSCs-VP/IFN-β treated groups increased the frequency of Treg cells. Draining lymph nodes (A; which is the results of day 23 ($n = 5$) & B; represents the results of day 55 ($n = 3$)) and spleen (C; which represents the results of day 23 ($n = 5$) & D; is the results of day 55 ($n = 3$)) were analyzed for the expression of indicated markers by flow cytometry. n = number of mice in each group. Significant differences were determined by one-way ANOVA ($P < 0.05$).

cell-surface expressions of $CD4^{high}$, $CD25^{high}$, $CD127^{low}$ and Foxp3 were used to confirm the representative Treg cells (Fig. 7). MSCs-VP/IFN-β treated animals exhibited a prominent increase in Treg cells when compared with other groups that just received MSCs and untreated group. The expression of TGF-β and IL-10 under the control of IFN-β may be one of the most important mechanisms which play a critical role in the induction of Treg cells in EAE mice.

To evaluate the effects of this therapeutic approach in both acute and chronic phases of the disease in lymph nodes and spleens, the mice in all groups sacrificed on days 23 and 55 and their draining lymph nodes and spleens were used for Treg cell immunophenotyping. Fig. 7 shows representative dot plots of the expression of $CD25^{high}$ and $Foxp3^{high}$ in all four groups in both draining lymph nodes (Fig. 7A & B) and in the spleen derived MNCs (Fig. 7C & D). In the case of MSCs-VP/IFN-β treated group, from the $CD127^{low}$ and $CD4^{high}$ gated cells, 14.5 ± 1% were $Foxp3^{high}$ and $CD25^{high}$ Tregs while the expression of $Foxp3^{high}$ and $CD25^{high}$ in MSCs treated group were 5 ± 2% and in the untreated group were 3 ± 1.5% ($P < 0.001$).

5. Discussion

In this paper, we examined combined immunomodulatory properties of MSCs [35] and IFN-β [36] in the treatment of EAE mice. Indeed, MSCs exert their immunomodulatory effects either with production of anti-inflammatory cytokines or cell to cell contact mechanisms [37].

Given the importance of the immunomodulatory nature of MSCs in the regulation of T cell immune responses [38], genetically modified MSCs could have a great potential for gene and stem cell therapy in inflammatory autoimmune disease such as MS.

IFN-β is widely used as an immunomodulatory agent for the treatment of MS patients. The efficiency of IFN-β therapy in MS patients is inevitable as well confirmed in many clinical trials [39]. Interestingly, IFN-β, as an inflammatory cytokine, plays a key role in MS patient's improvement. However, the results of several experiments have been indicated that the using IFN-β as a treatment approach in MS patients is not inclusive, and 30 to 50% of MS patients do not respond to IFN-β therapy [40–41]. Although immune regulatory effects by IFN-β have been demonstrated in some MS patients, the mechanisms of this regulatory effect remains to be determined [15]. A better understanding of how IFN-β modulate immune response during a relapse remitting form of MS might lead to an improvements in both monitoring and treatment. This study presents a comprehensive investigation of the immunomodulatory effects of IFN-β transduced murine AD-MSCs as a treatment approach in EAE mice. EAE characterized by autoreactive T cells against myelin oligodendrocyte glycoprotein with highly expression of inflammatory cytokines such as IL-17 and IFN-γ [42]. IL-17 is produced predominantly by Th17 cells. However, it could be produced by a variety of immune cells, such as Tγδ, NK cells and innate immune cell types [43]. As IL-17 plays a crucial role in the pathogenesis of EAE [20], in this study, we demonstrated that this inflammatory cytokine significantly downregulated in MSC-VP/IFN-β treated group.

The regulatory functions of IFN-β on Th17 cells have been widely studied [44–45]. Consistent with these experiments, we observed that IL-17 expression was decreased in IFN-β treated group. To further study, we determined the expression of anti-inflammatory cytokines, IL-10 and TGF-β, which are upregulated in groups that has received modified MSCs containing IFN-β gene. Since TGF-β have been reported to preferentially require for Treg cell development [46–47], our observations suggests that the presence of the MSCs in the improvement of Treg cells is considerable. MSCs-VP/IFN-β treated mice have produced higher levels of anti-inflammatory cytokine, IL-10, which plays an important role in the induction of Treg cells and downregulation of inflammatory settings [48]. We therefore hypothesized that overexpression of TGF-β [49] along with IL-10 could dampen Th17 polarization by Treg cells [50]. Since TGF-β have a crucial role in differentiation of both Th17 and Treg cells in a dose dependent manner [51–52].

Furthermore, some related studies considered a noticeable role for TGF-β and Foxp3 in cell plasticity and reprogramming of Tregs in EAE [53–54].

IFN-β has been shown to play as an immune regulatory role in the EAE amelioration [55–56]. IFN-β induces IL-10 in EAE [20], and, as we showed here, MSCs also expresses high levels of TGF-β and other immunomodulatory mediators [13]. Then we explored whether combined IFN-β and MSCs therapy could work synergistically to upregulate Tregs production in peak (day 23 post immunization) and chronic phase (day 55 post immunization) of the disease. For this purpose, we used flow cytometric analysis to measure the Treg cells quantities. We showed that Tregs production has been significantly increased in MSCs-VP/IFN-β treated group on both days 23 and 55 post immunization when compared with controls that just only received MSCs or untreated group. This finding suggests that the presence of the anti-inflammatory agents such as MSCs along with IFN-β therapy could augment the desirable therapeutic effects. In agreement with previously published experiments, we showed that the IFN-β exert its anti-inflammatory effects by increasing, in part, Treg cells in these patients. Interestingly, the percentages of Treg cells significantly increased in the isolated lymphocytes from spleen and draining lymph nodes in both acute and chronic phases of disease. Thus, it is possible that the effective and dynamic production of Tregs in chronic and acute phase of diseases may be the result of the synergistic effects of MSCs and IFN-β. Altogether, these results suggested that there may be a TGF-β regulatory mechanism that plays a synergistic role with IFN-β for the polarization of Treg cells. In conclusion, our study suggested that the autologous MSCs as a TGF-β producer source could play a synergistic effect on IFN-β and may provide the appropriate cytokine milieu for Treg cell polarization.

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