Wu et al. Arthritis Research & Therapy 2011, 13:R29
http://arthritis-research.com/content/13/1/R29

arthritis research&therapy

RESEARCH ARTICLE Open Access

Reduced immunomodulation potential of bone marrow-derived mesenchymal stem cells induced $CCR4^+CCR6^+$ Th/Treg cell subset imbalance in ankylosing spondylitis

Yanfeng Wu[1†], Mingliang Ren[2†], Rui Yang[2], Xinjun Liang[2], Yuanchen Ma[3], Yong Tang[2], Lin Huang[2], Zhichao Ye[2], Keng Chen[2], Peng Wang[2*], Huiyong Shen[2*]

Abstract

Introduction: Ankylosing spondylitis (AS) is a chronic autoimmune disease, and the precise pathogenesis is largely unknown at present. Bone marrow-derived mesenchymal stem cells (BMSCs) with immunosuppressive and anti-inflammatory potential and Th17/Treg cells with a reciprocal relationship regulated by BMSCs have been reported to be involved in some autoimmune disorders. Here we studied the biological and immunological characteristics of BMSCs, the frequency and phenotype of $CCR4^+CCR6^+$ Th/Treg cells and their interaction *in vitro* in AS.

Methods: The biological and immunomodulation characteristics of BMSCs were examined by induced multiple-differentiation and two-way mixed peripheral blood mononuclear cell (PBMC) reactions or after stimulation with phytohemagglutinin, respectively. The interactions of BMSCs and PBMCs were detected with a direct-contact co-culturing system. $CCR4^+CCR6^+$ Th/Treg cells and surface markers of BMSCs were assayed using flow cytometry.

Results: The AS-BMSCs at active stage showed normal proliferation, cell viability, surface markers and multiple differentiation characteristics, but significantly reduced immunomodulation potential (decreased 68 ± 14%); the frequencies of Treg and Fox-P3$^+$ cells in AS-PBMCs decreased, while $CCR4^+CCR6^+$ Th cells increased, compared with healthy donors. Moreover, the AS-BMSCs induced imbalance in the ratio of $CCR4^+CCR6^+$ Th/Treg cells by reducing Treg/PBMCs and increasing $CCR4^+CCR6^+$ Th/PBMCs, and also reduced Fox-P3$^+$ cells when co-cultured with PBMCs. Correlation analysis showed that the immunomodulation potential of BMSCs has significant negative correlations with the ratio of $CCR4^+CCR6^+$ Th to Treg cells in peripheral blood.

Conclusions: The immunomodulation potential of BMSCs is reduced and the ratio of $CCR4^+CCR6^+$ Th/Treg cells is imbalanced in AS. The BMSCs with reduced immunomodulation potential may play a novel role in AS pathogenesis by inducing $CCR4^+CCR6^+$ Th/Treg cell imbalance.

Introduction

Ankylosing spondylitis (AS) is a chronic autoimmune inflammatory disease, the prototypic seronegative spondyloarthritis that primarily affects the sacroiliac joints and the axial skeleton, which was characterized by inflammatory back pain, enthesitis, and specific organ involvement [1]. AS is a complex multifactorial disease; several pathogenetic factors, including infection [1,2], environmental triggers [1], genetic susceptibility such as HLA-B27 positivity [3,4] and HLA-E gene polymorphism [5], and in particular, autoimmune disorders [1] have been reported to potentially trigger the onset or maintain the pathogenesis progress of AS. Additionally, the genome-wide association study of AS identifies non-MHC susceptibility loci [6], such as IL-23R (rs11209026) and ERAP1 (rs27434). There were also, however, some controversies; for example, no candidate bacteria were detected by PCR in biopsies from sacroiliac joints [7]

* Correspondence: sunfox809@yahoo.com.cn; shenhuiyong@yahoo.com.cn
† Contributed equally
[2]Biotherapy Centre, Sun Yat-sen Memorial Hospital, Sun Yat-sen University, 107# Yanjiangxi Road, Guangzhou 510120, PR China
Full list of author information is available at the end of the article

and most HLA B27-positive individuals remain healthy [1]. The precise pathogenesis of AS is therefore largely unknown at present. Nowadays, more and more studies have focused on the immunological factors for AS.

Mesenchymal stromal cells (MSCs) isolated from a variety of adult tissues, including the bone marrow, have multiple differentiation potentials in different cell types, and also display immunosuppressive (*in vitro* [8,9], *in vivo* [10-12]) and anti-inflammatory properties [13], so their putative therapeutic role in a variety of inflammatory autoimmune diseases is currently under investigation. Recently, many findings indicate that MSC immunomodulation potential plays a critical role in severe aplastic anemia [14]. Simultaneously, substantial disorders and abnormalities of MSCs exist in many autoimmune diseases [15]. Few studies, however, have so far focused on whether there were some abnormalities in bone marrow-derived mesenchymal stem cells (BMSCs) of patients with ankylosing spondylitis (ASp) with regard to the biological and immunological properties.

More recently, two additional subsets, the forkhead box P3 (Fox-P3)-positive regulatory subset (Treg) and the IL-17-producing subset (Th17) [16-19], have emerged and together with Th1 and Th2 cells, formed a functional quartet of CD4$^+$ T cells that provides a closer insight into the mechanisms of immune-mediated diseases such as AS. Autoimmune diseases are thought to arise from a breakdown of immunological self-tolerance leading to aberrant immune responses to self-antigen. Ordinarily, regulatory T (Treg) cells - including both natural and induced Treg cells - control these self-reactive cells [20]. Several studies of patients with connective tissue diseases found reduced [21] or functionally impaired [22] Treg cells, and Treg cells of autoimmune hepatitis patients have reduced expression of Fox-P3 and CTLA-4, which may lead to impaired suppressor activity [23]. On the contrary, these proinflammatory Th17 cells are implicated in different autoimmune disease models [24-26]. Furthermore, these cells typically express IL-23R on their membrane [27], and recent studies in AS [28-30] show an important genetic contribution of polymorphisms in the gene that codes for this IL-23R. The active polymorphisms in the IL-23R gene could thus indicate an important role for this pathogenic T-cell subset (Th17) in the development and maintenance of AS. The involvement of Treg and Th17 cells in AS, however, has not yet been clearly established.

As previously described, skewing of responses towards Th17 and away from BMSCs or Treg cells may be responsible for the development and/or progression of AS [31]. Furthermore, CCR6 and CCR4 identified true Th17 memory cells producing IL-17 [32] and the majority of Th17 cells were CCR6$^+$CCR4$^+$ [33]. Aimed at investigating the puzzling issues above, the present study was designed to examine the biological and immunological properties of BMSCs, to examine the frequencies and phenotypes of Treg cells and proinflammatory CCR4$^+$CCR6$^+$ Th memory cells, and to study the interactions between BMSCs and CCR4$^+$CCR6$^+$ Th/Treg cells in peripheral blood mononuclear cells (PBMCs) for AS.

Materials and methods

Patients and controls

The present study was approved by the ethics committee of the Sun Yat-Sen Memorial Hospital of Sun Yat-Sen University, Guangzhou, China. In addition, informed consent was obtained from all patients and all healthy donors (HDs). Fifty-one ASp (eight women and 43 men) with an average age of 29.4 years (17 to 45 years) and 49 HDs (eight women and 41 men) with an average age of 27.? years (18 to 39 years) were included in the study. All of the AS patients were diagnosed according to the New York modified criteria [34] and were HLA-B27-positive; conversely, 37 healthy donors were HLA-B27-negative (HD1) and 12 healthy donors were HLA-B27-positive (HD2). Sixteen patients were diagnosed for the first time, and the research samples from all ASp were taken at the active stage (all Bath Ankylosing Spondylitis Disease Activity Index ≥4) and without taking any medicine for at least 2 weeks.

Bone marrow aspiration, human BMSCs and PBMCs

After being informed regarding the scientific contributions, possible risks and complications and the corresponding prevention and treating measures for bone marrow aspirations, all of the healthy controls and ASp expressed approval and signed the informed consent. The bone marrow aspirations were all performed by skilled allied health professionals strictly according to the international standardized procedure for bone marrow aspirations. The bone marrow samples of AS patients and HDs were diluted with DMEM (low-glucose DMEM) containing 10% FBS. The mononuclear cells were prepared by gradient centrifugation at 900 × *g* for 30 minutes on Percoll (Pharmacia Biotech, Uppsala, Sweden) of density 1.073 g/ml. The cells were washed, counted, seeded at 2 × 10^6 cells/cm^2 in 25-cm^2 flasks containing low-glucose DMEM supplemented with 10% FBS and cultured at 37°C, 5% carbon dioxide. Medium was replaced and the cells in suspension were removed at 48 hours and every 3 or 4 days thereafter. BMSCs were recovered using 0.25% Trypsin-ethylenediamine tetraacetic acid and replated at a density of 5 × 10^3 to 6 × 10^3 cells/cm^2 surface area as passage 1 cells when the culture reached 90% confluency. BMSCs after the third subculture were used for described experiments. PBMCs were obtained by the Ficoll-Hypaque (Pharmacia Biotech, Uppsala, Sweden) gradient separation of the buffy coat of ASp and HDs.

Cell viability and proliferation test for BMSCs

BMSCs were seeded in 96-well plates at a concentration of 1×10^4/ml, in a final volume of 100 μl fresh medium (10% FBS + low-glucose DMEM), and three wells of each sample were digested using 0.25% Trypsin-ethylenediamine tetraacetic acid for cell counting per day up to 12 days. The BMSC growth curves were made using the data for cell proliferation obtained above. Using MTT (5 mg/ml; Sigma-Aldrich Co., St. Louis, MO, USA), dimethyl sulphoxide (Sigma) and an EL800 microplate reader (BioTek Instruments, Winooski, VT, USA) that was to determine absorbance at 490 nm, the cell viability curves for BMSCs were acquired in the same way according to the day and the absorbance. The BMSC proliferation ability was also examined by ^{3}H-TdR assay. Fresh medium was used as a negative control.

In vitro differentiation potential assay of BMSCs

To induce osteogenic differentiation, BMSCs were initially seeded in six-well plates at a concentration of 10^4/cm^2. After preculturing for 24 hours, the BMSCs were allowed to grow in osteogenic medium (high-glucose DMEM supplemented with 10% FBS, 50 mg/l ascorbic acid, 10 mM β-glycerolphosphate and 10 nM dexamethasone; all these inducing reagents from Sigma). The BMSCs were then incubated in 5% carbon dioxide at 37°C, according to the experimental requirements for up to 14 days, and the medium was replaced every 3 days before harvest. The alkaline phosphatase (ALP) and mineralization of BMSCs were assayed using Cell Alkaline Phosphatase Staining assay (Sigma) and Alizarin Red staining (ARS, 40 mmol/l, pH 4.2; Sigma) on the 14th day, respectively.

To induce adipogenic differentiation, the BMSCs were seeded in six-well plates at a concentration of 10^4/cm^2. After preculturing for 24 hours, the BMSCs were shifted to adipogenic medium (low-glucose DMEM supplemented with 10% FBS, 1 μM dexamethasone, 10 μg/ml insulin, 0.5 mM 3-isobutyl-1-methylxanthine and 0.2 mM indomethacin; all these inducing reagents from Sigma). The BMSCs were then incubated in 5% carbon dioxide at 37°C, and the medium was replaced every 3 days before harvest. The intracellular lipid accumulation as an indicator was visualized on the 14th day by Oil Red O staining after fixed with 4% cold paraformaldehyde in PBS (pH 7.4) and washed with distilled water.

To induce chondrogenic differentiation, aliquots of 2.5×10^5 BMSCs were centrifuged at 1,000 rpm for 5 minutes in 15-ml polypropylene conical tubes to form pellets, which were then cultured in high-glucose DMEM supplemented with 1% ITS-Premix (Becton-Dickinson, Mountain View, CA, USA), 50 mg/ml ascorbic acid (Sigma), 10^{-3} M sodium pyruvate (Sigma), 10^{-7} M dexametazone (Sigma), and 10 ng/ml transforming growth factor-β3 (R&D Systems, Minneapolis, MN, USA) for 28 days. The pellets were then fixed with 4% paraformaldehyde, embedded in paraffin, and subjected to Alcian blue staining to confirm chondrogenic differentiation.

The BMSCs in fresh medium (high-glucose DMEM supplemented with 10% FBS) without these differentiation-inducing factors were used as the experimental control, and fresh medium without any cells was used as a negative control. All measurements were performed in triplicate. The images were visualized using an inverted phase contrast microscope (Nikon Eclipse Ti-S, Nikon Corporation, Tokyo Prefecture, Japan).

Alkaline phosphatase measurement

On the 14th day, the osteogenic medium was removed, and then 1.0 ml Triton X-100 (Sigma) was added to each well. A cell scraper was used to remove the BMSCs from the well bottom, and then the 1.0 ml cell lysate were placed in a 1.5-ml centrifuge tube. The samples were then processed through two freeze-thaw cycles (-70°C and room temperature, 45 minutes each) to rupture the cell membrane and extract the proteins and DNA from the cells. A *p*-nitrophenyl phosphate liquid substrate system (Stanbio, Boerne, TX, USA) was used to analyze the ALP concentration from the cells of each group. Then 10 μl each cell lysate solution was added to 190 μl *p*-nitrophenyl phosphate substrate and incubated in the dark at room temperature for 1 minute. The absorbance was read using a plate reader (M5 SpectraMax; Molecular Devices, Sunnyvale, CA, USA) at 405 nm and normalized to the PicoGreen assay [35]. DNA was quantified using the Quant-iT PicoGreen Kit (Invitrogen, Carlsbad, CA, USA) following standard protocols. Briefly, 100 μl each cell lysate solution was added to 100 μl PicoGreen reagent and incubated in the dark at room temperature for 5 minutes. The absorbance was read at an excitation/emission of 480 to 520 nm on the plate reader.

Immunomodulation potential of BMSCs

The inhibitory effects of BMSCs on mixed PBMC reaction (MLR) and PBMC proliferation stimulated by phytohemagglutinin (PHA) (4 μg/ml; Roche, Mannheim, Germany) were measured using the MTT assay [36] and the ^{3}H-TdR assay [10] as described previously. Briefly, BMSCs were seeded in V-bottomed, 96-well culture plates for 4 hours for adherence, and then irradiated

(30 Gy) with Co^{60} before being cultured with the mixed PBMCs or the PBMCs stimulated by PHA.

Two-way mixed PBMC reaction

For the two-way MLR, allogeneic PBMCs (15 × 10^4 cells/cm^2) from a healthy volunteer were mixed in a 1:1 ratio with PBMCs from another unrelated healthy volunteer (third-party setting). The mixed PBMCs were then mixed with different amounts (15 × 10^3 cells/cm^2 = 1:20 BMSC: PBMC ratio, 3 × 10^4 cells/cm^2 = 1:10, 6 × 10^4 cells/cm^2 = 1:5, 15 × 10^4 cells/cm^2 = 1:2, 3 × 10^5 cells/cm^2 = 1:1) of BMSCs (experiment wells) or without BMSCs (blank wells) in V-bottomed, 96-well culture plates to ensure efficient cell-cell contact for 5 days in 0.2 ml modified RPMI-1640 medium (Gibco, BRL, Grand Island, NY, USA) supplemented with 10% FBS.

Allogeneic PBMC proliferation assay

Compared with the MLR, the allogeneic PBMC proliferation assay only uses one allogeneic PBMC reaction (30 × 10^4 cells/cm^2) from a healthy volunteer stimulated with PHA, instead of two PBMC reactions. Inhibitory effects were measured on the 5th day using the MTT assay with an EL800 microplate reader at 570 nm and the ^{3}H-TdR assay with a microplate scintillation and luminescence counter (Packard NXT, Meriden, CT, USA). Results were expressed as mean absorbance (optical density (OD)) ± standard deviation (SD) and as mean counts per minute (CPM) ± SD, respectively. All measurements were performed in triplicate.

The data are presented as percentage inhibition values calculated using the following formulae (Table 1):

$$\%\,\text{inhibition} = 1 - (\text{OD (exp)} - \text{OD (adj)})/\text{OD (bla)}$$
$$\%\,\text{inhibition} = 1 - (\text{CPM (exp)} - \text{CPM (adj)})/\text{CPM (bla)}$$

OD(exp), OD(adj) and OD(bla) represent the mean absorbance of experiment wells, adjusted wells (only BMSCs) and blank wells, respectively, and CPM(exp), CPM(adj) and CPM(bla) represent the mean counts per minute of the corresponding wells. Depending on the experimental design, there were some wells used for controlling. Results were expressed as the mean (% inhibition) ± SD.

Direct contact co-culture of BMSCs and PBMCs

BMSCs were trypsinized and then irradiated (30 Gy) with Co^{60} before being co-cultured with PBMCs from a healthy volunteer in the presence of PHA (4 μg/ml; Roche) in 24-well plates (Nunclon, Roskilde, Denmark) and plated at a ratio of 1:10 in a total volume of 2 ml/well in triplicate for 72 hours. The cell density was 5 × 10^4/cm^2 BMSCs and 5 × 10^5/cm^2 PBMCs in a mix. Phorbol myristate acetate (50 ng/ml; Sigma, St Louis, MO, USA) and calcium ionomycin (1 μg/ml; Sigma) were added 6 hours prior to the end of the 72-hour coculture. All of the PBMCs were then collected to be assayed by flow cytometry for the $CCR4^+CCR6^+$ Th and Treg cells. PBMCs were also grown alone in BMSC-free medium and used as control.

Antibodies and flow cytometry

To detect the surface markers [37] of BMSCs and the frequency of $CCR4^+CCR6^+$ Th and Treg cells in PBMCs, the antibodies (Table 2) - including CD105(FITC), CD73 (FITC), CD90(FITC), CD34(FITC), CD45(FITC), CD14 (PE) and HLA-DR(FITC) for BMSCs; CCR4(PE-Cy7), CD196(CCR6)(PE) and CD4(FITC) for $CCR4^+CCR6^+$ Th cells [29]; and CD4(FITC), CD25(APC) and Fox-P3(PE) antibodies for Treg cells - were used according to the manufacturers' recommendations. BMSCs and PBMCs marked with appropriate antibodies were measured with a FACScan laser flow cytometry system (Becton Dickinson) immediately. In each experiment, control staining with the appropriate isotype monoclonal antibodies was included. Results were expressed as the mean (frequency, %) ± SD.

Statistical analysis

Data are expressed as the mean ± SD, and the significance of the results was determined using the unpaired Student's t test. The product-moment correlation coefficient was used to test the correlations between the suppression ratios of BMSCs and the ratio of $CCR4^+CCR6^+$ Th cells to Treg cells in peripheral blood. Statistical analysis was performed using the SPSS computer program (SPSS Inc., Chicago, IL, USA). P < 0.05 was considered statistically significant.

Results

Growth characteristics and cell viability of AS-BMSCs are normal

To evaluate the biological properties of AS-BMSCs, compared with those of HD-BMSCs, the studies for growth characteristics, cell viability and multiple differentiation potentials *in vitro* were performed. The

Table 1 Details regarding the formula for percentage inhibition in the present study

	Experiment wells	Adjusted wells	Blank wells
Two-way MLR	BMSCs + 2 HD-PBMCs	BMSCs	2 HD-PBMCs
Allogeneic PBMC proliferation assay	BMSCs + 1 HD-PBMC + PHA	BMSCs	1 HD-PBMC + PHA

BMSC, bone marrow-derived mensenchymal stem cell; PBMC, peripheral blood mononuclear cell; HD-PBMC, peripheral blood mononuclear cell of healthy donor; PHA, phytohemagglutinin; MLR, mixed PBMC reaction.

Table 2 Antibodies used to detect CCR4$^+$CCR6$^+$ Th and Treg cells in PBMCs and phenotype BMSCs by flow cytometry

Antibody	Isotype	Clone/fluorochrome	Concentration (μg/ml)	Source
CD105 (ENG)[a]	Mouse IgG_1	266/FITC	10	Becton Dickinson (Bedford, MA, USA)
CD73 (NT5E)[a]	Mouse IgG_1	AD2/FITC	20	Becton Dickinson (Bedford, MA, USA)
CD90 (THY1)[a]	Mouse IgG_1	5E10/FITC	1	Becton Dickinson (Bedford, MA, USA)
CD34[a]	Mouse IgG_1	581/FITC	50	Southern Biotech (Birmingham, AL, USA)
CD45 (PTPRC)[a]	Mouse IgG_1	HI30/FITC	10	Caltag (Burlingame, CA, USA)
CD14[a]	Mouse IgG_1	61D3/PE	200	Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA)
HLA-DR[a]	Mouse IgG_{2a}	L243/FITC	200	Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA)
CD4[b]	Mouse IgG_{2b}	OKT4/FITC	12	eBioscience (San Diego, CA, USA)
CD25[b]	Mouse $IgG_{1,\kappa}$	BC96/APC	25	eBioscience (San Diego, CA, USA)
FOX-P3[b]	Mouse IgG_1	236A/E7/PE	50	eBioscience (San Diego, CA, USA)
CCR4[b]	Mouse $IgG_{1,\kappa}$	1G1/PE-Cy7	200	BD Pharmingen™ (Basel, Switzerland)
CCR6 (CD196)[b]	Mouse $IgG_{1,\kappa}$	11A9/PE	200	BD Pharmingen™ (Basel, Switzerland)

[a]Antibodies used to phenotype bone marrow-derived mensenchymal stem cells (BMSCs) by flow cytometry. [b]Antibodies used to detect CCR4$^+$CCR6$^+$ CD4$^+$ T-helper (Th) cells and forkhead box P3-positive regulatory T (Treg) cells by flow cytometry. PBMC, peripheral blood mononuclear cell.

AS-BMSC growth curves have the same tendency as those for HD-BMSCs. The BMSC proliferation data of these two groups at each day (1 to 12 days) were tested by unpaired Student's t test, and the statistical result indicates that there was no statistically significant difference in BMSC growth characteristics between ASp and HDs (HD1 and HD2) ($P > 0.05$, Student's t test for independent samples). Established cultures (12 days) of BMSCs exhibited close, even equivalent, cell viability at each point of time from 1 to 12 days, as determined by cellular viability assays, and the difference of OD at 490 nm between ASp and HDs (HD1 and HD2) at each day (1 to 12 days) was not also statistically significant ($P > 0.05$, Student's t test for independent samples). The cultures have similar purities: (Q_L [the lower point of interquartile range], Q_U [the upper point of interquartile range]) = (95%, 99%) for AS-BMSCs, (Q_L, Q_U) = (96%, 98%) for HD1-BMSCs and (Q_L, Q_U) = (96%, 99%) for HD2-BMSCs.

Triple differentiation potentials of AS-BMSCs *in vitro* were not changed

To explore whether the multiple differentiation potentials of BMSCs in AS were abnormal, we investigate the osteogenic, adipogenic and chondrogenic differentiation potentials of AS-BMSCs and HD-BMSCs in the present study. Obvious differentiated osteocytes and adipocytes were detected as early as the 7th day after being induced for osteogenic and adipogenic differentiation, and obvious differentiated chondrocytes were seen at about 14 days since induction (Figure 1A to 1C).

There appeared to be two stages in the BMSC differentiation process for both ASp and HDs. In the early stage, only a few osteocytes, adipocytes and chondrocytes were found within the undifferentiated BMSCs. Gradually, these three kinds of cells increased; simultaneously, the cell's body got bigger and cytoplasm became more abundant because, for example, osteocytes made closer contact, fat vacuoles of adipocytes multiplied and grew bigger, and chondrocytes began to gain many collagen fibers. In the later stage, these three kinds of cells increased rapidly and nearly predominated. For the adipocytes, osteocytes and chondrocytes derived from BMSCs, the purities were (Q_L, Q_U) = (90%, 97%), (Q_L, Q_U) = (91%, 96%) and (Q_L, Q_U) = (88%, 95%) for ASp, (Q_L, Q_U) = (88%, 98%), (Q_L, Q_U) = (90%, 97%) and (Q_L, Q_U) = (90%, 96%) for HD1, and (Q_L, Q_U) = (86%, 95%), (Q_L, Q_U) = (89%, 98%) and (Q_L, Q_U) = (92%, 97%) for HD2, respectively.

The calcium nodules were stained to present a red color (Figure 1A1 to 1D1), after Alizarin Red staining for calcium deposits of osteocytes was performed to determine the mineralization of BMSCs. For the adipogenic differentiation, the mass fat vacuoles of adipocytes were also stained to present a red color by Oil Red O staining (Figure 1A3 to 1C3). The well-differentiated chondrocytes were Alcian Blue-positive, and presented a bright blue color after staining (Figure 1A4 to 1C4).

The ALP activity, normalized to DNA concentration, is plotted in Figure 1E. The ALP activity (mean ± SD) was 644 ± 45 (mM p-nitrophenyl phosphate/minute per mg DNA) for AS-BMSCs ($n = 51$), which is lower than the 655 ± 49 for HD1-BMSCs ($n = 37$) ($P > 0.05$) and the 646 ± 51 for HD2-BMSCs ($n = 12$) ($P > 0.05$). All three values were much higher than those of the baseline ALP for BMSCs of ASp, HD1 and HD2 (85 ± 40, 88 ± 48 and 82 ± 13, respectively) ($P < 0.001$) in control medium without the osteogenic factors. ALP staining was performed on the 14th day to investigate the maturity degree of osteocytes in the groups of Asp, HD1 and HD2 (Figure 1A2 to 1C2).

Phenotype of bone marrow-derived mesenchymal stem cells

The AS-BMSCs and HD-BMSCs were then examined for typical MSC phenotypic surface markers. Flow

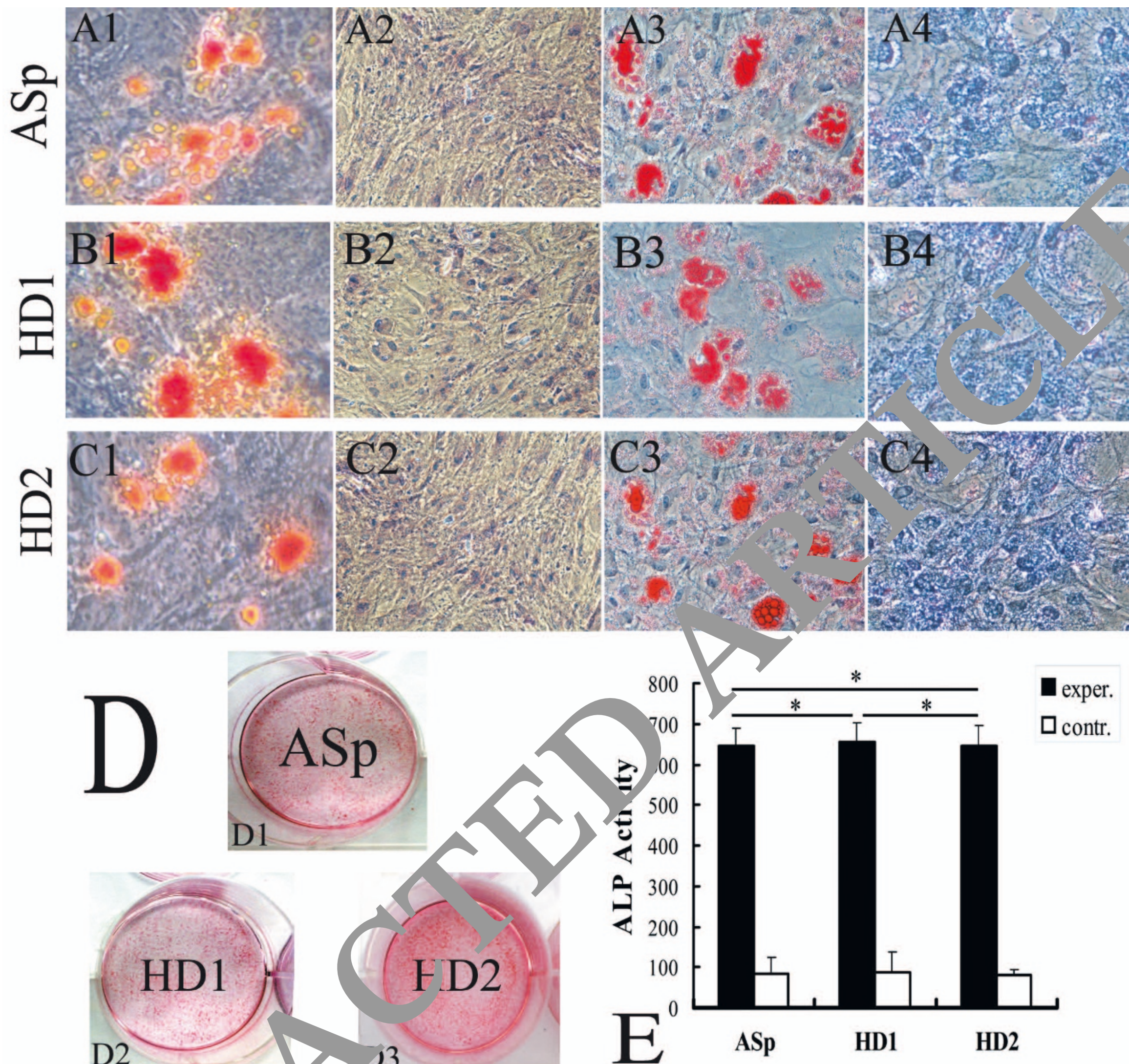

Figure 1 Bone marrow-derived mensenchymal stem cell triple differentiation potentials from ankylosing spondylitis patients and healthy donors. **(A), (B), (C)** Morphological characteristics of bone marrow-derived mensenchymal stem cells (BMSCs) for osteogenic, adipogenic and chondrogenic differentiation evaluated by the inverted phase contrast microscope; ankylosing spondylitis (AS)-BMSCs have the same morphological properties as the HLA-B27-negative healthy donor (HD1)-BMSCs and HLA-B27-positive healthy donor (HD2)-BMSCs. Osteocytes were stained for calcium deposition using Alizarin Red-S (A1, B1, C1: x400) and for alkaline phosphatase (ALP) with the Cell Alkaline Phosphatase Assay (A2, B2, C2: x200). Adipocytes were filled with many fat vacuoles, and Red Oil O was used to stain the fat vacuoles of adipocytes (A3, B3, C3: x200). Chondroblast differentiation from BMSCs was identified with Alcian blue staining (A4, B4, C4: x200). **(D)** General photographs of BMSCs for osteogenic differentiation, stained with Alizarin Red-S. **(E)** ALP activities of AS-BMSCs, HD1-BMSCs and HD2-BMSCs were 644 ± 4[illegible], 655 ± 49 and 646 ± 51, respectively; differences not statistically significant ($P > 0.05$). Data presented as mean ± standard deviation. MLR, mixed peripheral blood mononuclear cell reaction; PHA, phytohemagglutinin.

cytometric analysis showed that the AS-BMSCs and HD-BMSCs (HD1-BMSCs and HD2-BMSCs) have the same phenotypic surface markers, just as the typical MSCs did. The samples all express high levels of the surface markers CD105, CD73 and CD90, and lack expression of CD45, CD34, CD14 and HLA-DR surface molecules (Figure 2).

Decreased suppressive potential of AS-BMSCs on either two-way MLR or PBMC proliferation stimulated with PHA

Under the condition that the proliferation characteristics, cell viability, multiple-differentiation potentials and surface markers of AS-BMSCs were normal, compared with HD-BMSCs, the immunomodulation potential of AS-BMSCs was evaluated in the present study. The

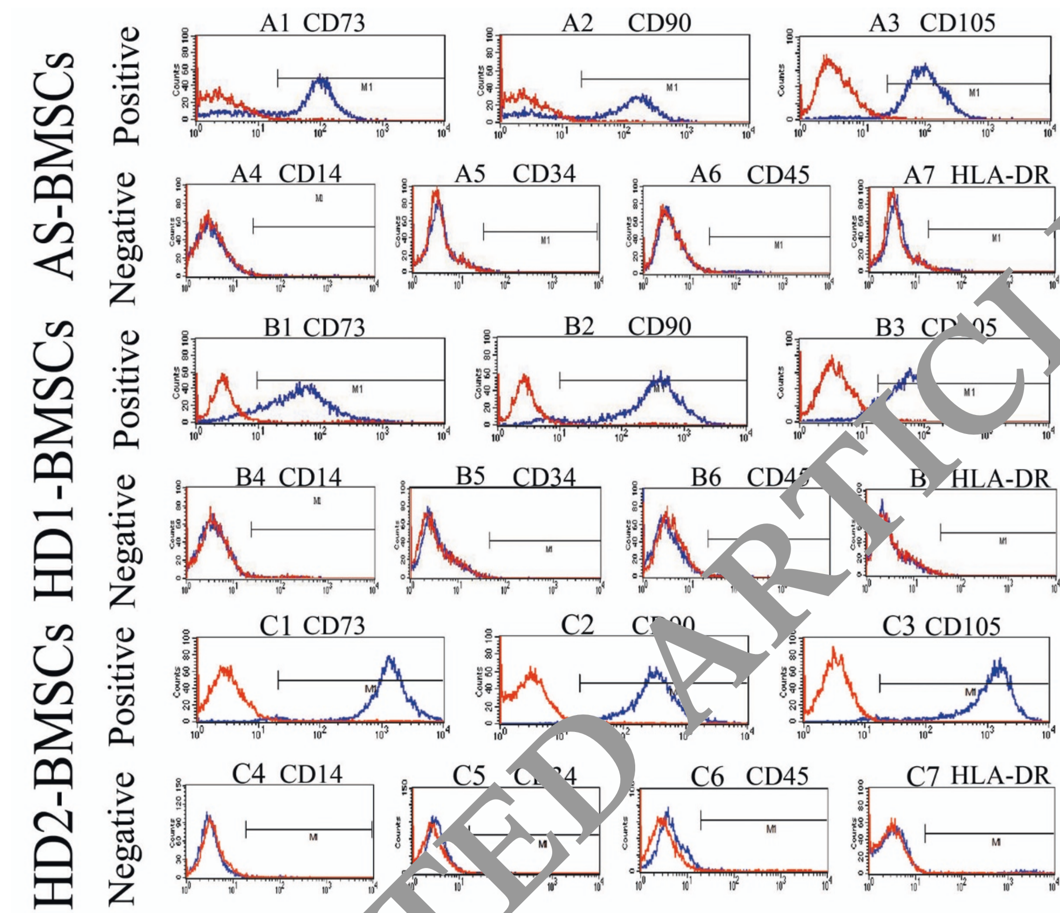

Figure 2 Phenotyping of bone marrow-derived mensenchymal stem cells for typical mensenchymal stromal cell surface markers. Single-parameter histograms for **(A1) to (A3), (B1) to (B3), (C1) to (C3)** individual mensenchymal stromal cell (MSC) markers and **(A4) to (A7), (B4) to (B7), (C4) to (C7)** MSC exclusion markers, representative of samples from patients with ankylosing spondylitis (AS) and from healthy donors (blue lines). Red lines indicate background fluorescence obtained with isotype control IgG. *x* axis, fluorescence intensity; *y* axis, cell counts. BMSC, bone marrow-derived mesenchymal stem cell; HD1, HLA-B27-negative healthy donors; HD2, HLA-B27-positive healthy donors.

effects of BMSCs from ASp (n = 51), HD1 (n = 37) and HD2 (n = 12) on two-way MLR or PBMC proliferation in the presence of PHA were evaluated by mixing BMSCs and mixed PBMCs for two-way MLR, or by PBMCs from a third healthy volunteer in the presence of PHA for PBMC proliferation assay at five BMSC: PBMC ratios of 1:20, 1:10, 1:5, 1:2 and 1:1, respectively.

Two-way mixed PBMC reaction

The differences of absorbance between 0 days and 5 days were not statistically significant for the allogeneic PBMCs from a healthy volunteer (P = 0.351) and the PBMCs from another unrelated healthy volunteer (P = 0.418) (Figure 3A1). For the mixed PBMCs, however, the absorbance at 5 days was significantly higher than the value at 0 days (P < 0.001, Figure 3A1). As shown in Figure 3A2,A3, there was a statistically significant reduction in suppressive potential (% inhibition) of BMSCs from ASp on two-way MLR at all five ratios, compared with the percentage inhibition of HD-BMSCs (P < 0.001, Figure 3A2,A3).

Allogeneic PBMC proliferation assay

Similarly, when PBMC proliferation was elicited by means of PHA, the addition of BMSCs from ASp also produced a statistically significant decreased inhibitory effect on PBMC proliferation (Figure 3B2,B3; P < 0.001, Student's t test for independent data), ranging from a BMSC:PBMC ratio of 1:20 to 1:1. Additionally, the differences of absorbance between 0 days and 5 days without PHA were not significant (P = 0.223), while the value for 5 days with PHA was significantly higher than the value at 0 days (P < 0.001, Figure 3B1).

Furthermore, in either the MLR (Figure 3A3) or the PBMC proliferation assay stimulated with PHA (Figure 3B3), the ^{3}H-TdR assay data suggested a significant relationship between dose and suppression of immunoreactivity of BMSCs for ASp, HD1 and HD2. The MTT

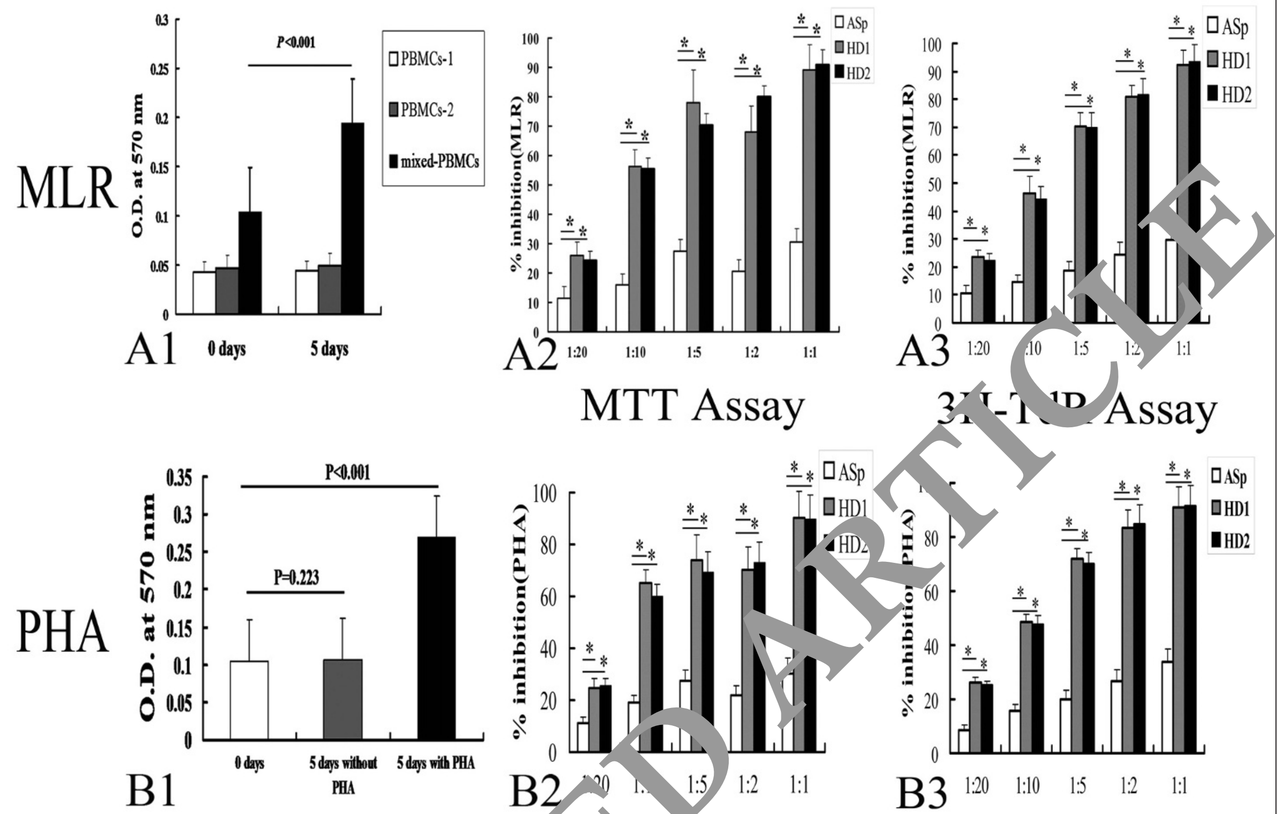

Figure 3 Reduced suppressive potential of bone marrow-derived mensenchymal stem cells of patients with ankylosing spondylitis. **(A1)** Absorbance of mixed peripheral blood mononuclear cells (PBMCs) at 5 days (0.194 ± 0.038) was significantly higher than the value at 0 days (0.104 ± 0.023) (*$P < 0.001$), showing the significant mixed PBMC reaction. **(A2), (A3)** Compared with healthy donor (HD)-bone marrow-derived mensenchymal stem cells (BMSCs), the decreased percentage inhibition of ankylosing spondylitis (AS)-BMSCs on two-way mixed peripheral blood mononuclear cell reaction (MLR) at different ratios showed that suppressive potentials of AS-BMSCs were reduced (% inhibition reduced, *$P < 0.001$). **(B1)** PBMCs derived from a healthy volunteer could proliferate significantly the presence of phytohemagglutinin (PHA) *in vitro* ($P < 0.001$). **(B2), (B3)** Percentage inhibition of AS-BMSCs on PBMC proliferation induced by PHA was significantly lower than the values of HD-BMSCs at varied BMSC:PBMC ratios of 1:20, 1:10, 1:5, 1:2 and 1:1 (% inhibition reduced, *$P < 0.001$). Data expressed as mean ± standard deviation of triplicates of three separate experiments. (A2), (B2) and (A3), (B3) were performed by MTT assay and ^{3}H-TdR assay respectively. There were no statistically significant differences of suppressive potentials between (A2), (A3) HLA-B27-negative healthy donor (HD1)-BMSCs and (B2), (B3) HLA-B27-positive healthy donor (HD2)-BMSCs. OD, optical density.

assay also presented this phenomenon basically, but not clearly.

Increased CCR4^{+}CCR6^{+} Th and decreased Treg populations in peripheral blood of patients with AS

Recent studies have independently revealed enhanced Th17 response and weakened Treg response in some autoimmune diseases [38,39], so we also examined the frequencies of CCR4^{+}CCR6^{+} Th and Treg cells in PBMCs of ASp and HDs (Figure 4). The PBMCs from ASp and HDs were examined for the subset populations using flow cytometry, defined as the percentages of CCR4^{+}CCR6^{+} Th cells (CCR4/CCR6 double-positive) [32] and Treg cells (CD4/CD25/Fox-P3 triple-positive) accounting for the total CD4-positive Th cells (CCR4^{+}CCR6^{+} Th/Th, Treg/Th), CD3-positive T cells (CCR4^{+}CCR6^{+} Th/T, Treg/T), lymphocytes (CCR4^{+}CCR6^{+} Th/L, Treg/L), and peripheral blood mononuclear cells (CCR4^{+}CCR6^{+} Th/PBMCs, Treg/PBMCs) respectively. The proportions of Fox-P3-positive occupying CD4/CD25 double-positive cells (Fox-P3^{+}/CD4^{+}CD25^{+}) and PBMCs (Fox-P3^{+}/PBMCs) were also tested. Compared with healthy donors (HD1 and HD2), the CCR4^{+}CCR6^{+} Th population of ASp was significantly increased ($P < 0.001$, Table 3 and Figure 5), whereas Treg cells and Fox-P3-positive cells were found to be significantly decreased ($P < 0.001$, Student's t test for independent data, Table 3 and Figure 5). There were no significant differences between HD1 and HD2.

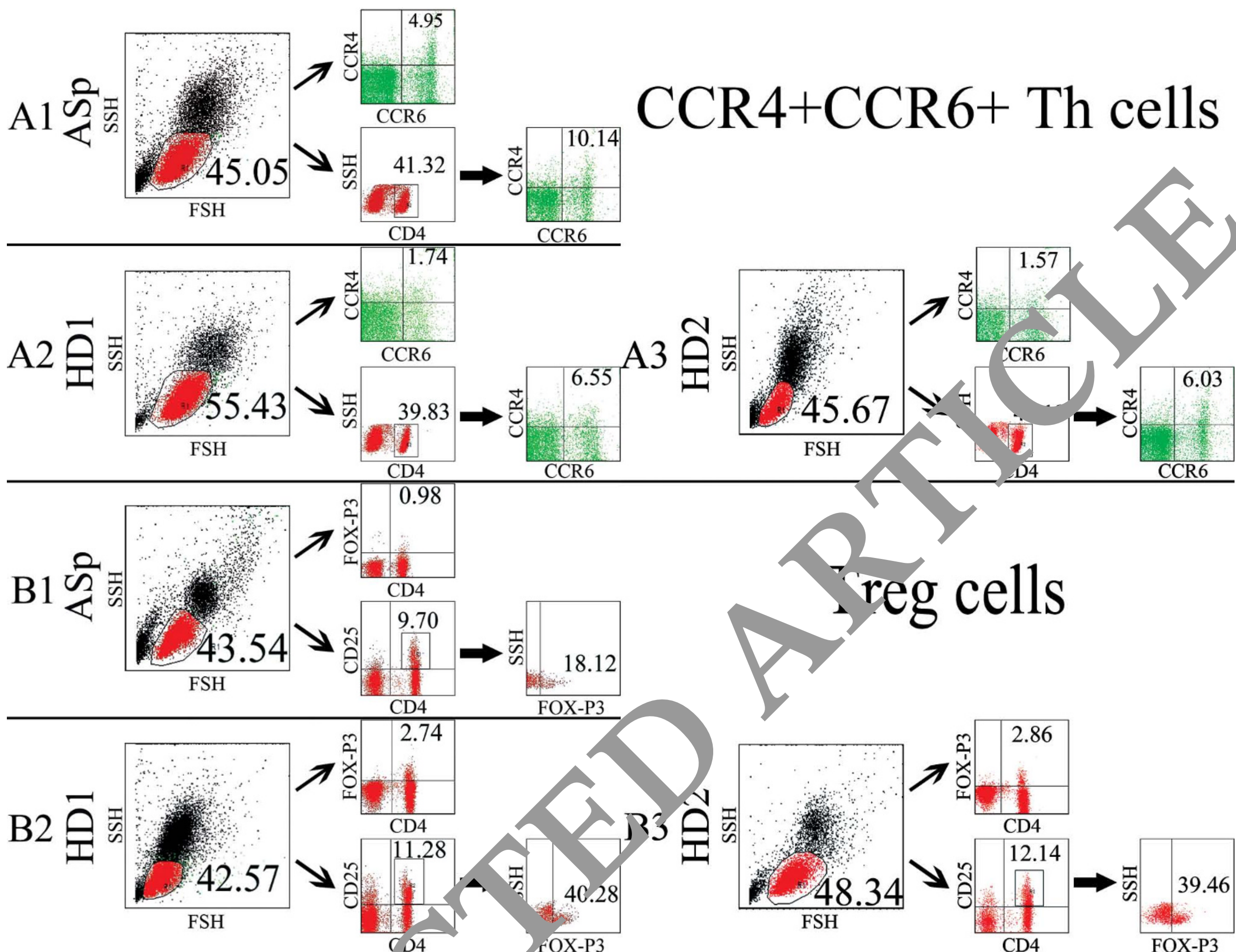

Figure 4 Representative plots of CCR4$^+$CCR6$^+$ T-helper and regulatory T cells. Representative plots of peripheral circulating populations of **(A1)** to **(A3)** CCR4$^+$CCR6$^+$ T-helper (Th) cells (green) and **(B1)** to **(B3)** regulatory T (Treg) cells (red) in peripheral blood mononuclear cells (PBMCs) of patients with ankylosing spondylitis (ASp) (A1, B1), HLA-B27-negative healthy donors (HD1) (A2, B2) and HLA-B27-positive healthy donors (HD2) (A3, B3). FOX-P3, forkhead box P3; FSH, forward scatter-height; SSH, side scatter-height.

BMSCs of patients with AS-induced CCR4$^+$CCR6$^+$ Th/Treg imbalance

We performed the direct contact co-culture of BMSCs and PBMCs to explore whether the reduced immunomodulation potential of AS-BMSCs altered the balance of CCR4$^+$CCR6$^+$ Th/Treg cells. The PBMCs were collected to be assayed by flow cytometry for the CCR4$^+$CCR6$^+$ Th and Treg cells after co-culture with BMSCs of ASp and HDs (HD1 and HD2) for 3 days. The percentages of Treg cells (0.63 ± 0.23%) and Fox-P3-positive cells (0.74 ± 0.11%) in PBMCs after co-culture with AS-BMSCs for 3 days reduced significantly, whereas the percentages of CCR4$^+$CCR6$^+$ Th cells (1.87 ± 0.29%) in PBMCs after co-culture with AS-BMSCs for 3 days increased significantly, compared with these values of groups, including 3-day HD1, 3-day HD2, 3-day control,

Table 3 Percentages of CCR4$^+$/6$^+$ Th and Treg cells in appropriate cell subsets

	CCR4$^+$/CCR6$^+$ Th/Th	CCR4$^+$/CCR6$^+$ Th/T	CCR4$^+$/CCR6$^+$ Th/L	Treg/Th	Treg/T	Treg/L	Fox-P3$^+$/CD4$^+$CD25$^+$
ASp	9.81 ± 0.62	7.69 ± 0.63	4.24 ± 0.56	2.82 ± 0.24	2.12 ± 0.23	1.23 ± 0.13	22.23 ± 5.13
HD1	5.51 ± 0.59	3.53 ± 0.56	2.24 ± 0.48	5.27 ± 0.28	4.13 ± 0.26	2.54 ± 0.15	32.54 ± 7.05
HD2	5.34 ± 0.52	3.33 ± 0.45	2.17 ± 0.37	5.31 ± 0.23	4.21 ± 0.19	2.64 ± 0.18	34.92 ± 6.71

Data presented as the percentage mean ± standard deviation. The differences for all percentages between patients with ankylosing spondylitis (ASp) and either HLA-B27-negative healthy donors (HD1) or HLA-B27-positive healthy donors (HD2) were significant ($P < 0.001$, according to a two-tailed significant level of 0.05). There were no significant differences between HD1 and HD2 ($P > 0.05$). Th, CD4$^+$ T-helper cells; T, T lymphocytes; L, lymphocytes; Treg, forkhead box P3-positive regulatory T cells.

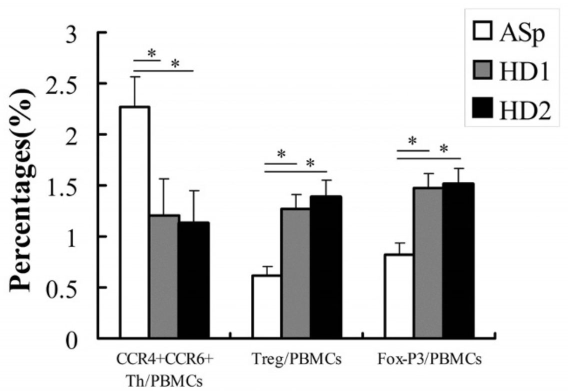

Figure 5 Percentages of CCR4$^+$CCR6$^+$ T-helper, regulatory T and Fox-P3-positive cells in peripheral blood mononuclear cells. Statistical multiple bar graphs showing percentages of increased CCR4$^+$CCR6$^+$ T-helper (Th) cells, reduced regulatory T (Treg) cells and reduced forkhead box P3 (Fox-P3)-positive cells in the peripheral blood mononuclear cells (PBMCs) of patients with ankylosing spondylitis (ASp), compared with values for healthy donors (HLA-B27-negative healthy donors (HD1) and HLA-B27-positive healthy donors (HD2)). *P < 0.001, Student's t test for independent data.

and 0 days (P < 0.001, Figure 6). Impressively, the ratio of CCR4$^+$CCR6$^+$ Th cells to Treg cells (CCR4$^+$CCR6$^+$ Th/Treg) in PBMCs after co-culture with AS-BMSCs for 3 days increased significantly (P < 0.001, Figure 6).

Negative correlations between percentages of CCR4$^+$CCR6$^+$ Th/Treg cells and the suppressive ratio of BMSCs

When examining data from all subjects tested, we observed positive correlations between the percentage inhibition of BMSCs (MLR) and the percentage inhibition of BMSCs (PHA) for all of the 51 ASp, 37 HD1 and 12 HD2. Interestingly, however, for all of the ASp (Figure 7A), HD1 (Figure 7B) and HD2 (Figure 7C) there were significantly negative correlations between the ratios of CCR4$^+$CCR6$^+$ Th cells to Treg cells in the peripheral blood and either percentage inhibition of BMSCs (MLR) or percentage inhibition of BMSCs (PHA) at all five ratios (P < 0.01, respectively).

Discussion

In the present study, we found that AS-BMSCs showed normal proliferation, cell viability, surface markers and multiple differentiations characteristics, but significantly reduced immunomodulation potential; also, the frequencies of Treg and Fox-P3$^+$ cells in AS-PBMCs decreased, but CCR4$^+$CCR6$^+$ Th cells increased. Moreover, the AS-BMSCs induced the ratio of CCR4$^+$CCR6$^+$ Th/Treg cell imbalance when co-cultured with PBMCs. Additionally, no differences were found between HD1 and HD2. Impressively, the immunomodulation potential of BMSCs has negative correlation with the ratios of CCR4$^+$CCR6$^+$ Th to Treg cells in peripheral blood.

Characteristic symptoms of AS are spinal stiffness, ankylosis and syndesmophytes [1], which are explained by spinal inflammation, structural damage, or both [40]. As the ankylosis of the spine or even spinal stiffness was probably initiated by the heterotopic ossification of osteoblasts, and most of these osteoblasts derived from BMSCs [41,42], and, simultaneously, there were some abnormalities with the biological properties, including the multiple differentiation potentials in some autoimmune disorders, such as severe aplastic anemia [14], we performed research to examine the biology properties of AS-BMSCs. We did not, however, detect any abnormality about the biological characteristics of AS-BMSCs *in vitro*, including the proliferation ability, cell viability, morphological features, differentiation potentials and surface markers. Especially, the activity of osteogenic differentiation and mineralization capacity are totally normal. In addition, Braun and colleagues reported that immunohistological studies on sacroiliac joint biopsies have shown cellular infiltrates, including T cells and macrophages, and that TNFα is overexpressed in sacroiliac joints [43]. These events indicated that the endogenous osteogenic differentiation potential of BMSCs may be not the real murderer, which was thought to induce BMSCs to produce heterotopic ossification; the appropriate cell activity and cytokine function [44] existing in the internal environments, which maintain BMSCs *in vivo*, may play a critical role in the process of BMSC heterotopic ossification.

There appears to be a reciprocal relationship between the development of Treg cells and Th17 cells. Recent studies have independently revealed enhanced Th17 response and weakened Treg response in some autoimmune diseases [38,39], indicating an important role for Th17/Treg imbalance in the pathogenesis of autoimmunity. The present study revealed that the Th17/Treg imbalance existed in the peripheral blood of ASp, suggesting its potential role in the breakdown of immune self-tolerance in AS. Moreover, the physiological frequency of Fox-P3$^+$ and Treg cells can suppress autoimmune disorders, but the reduction or even depletion of Fox-P3$^+$ cells could lead to induction of autoimmunity by specific ablation of Treg cells in genetically targeted mice [45], these results indicated that the reduction of Treg cells probably enhanced the pathological process of AS. The balance between Treg and Th17 cells is dependent on the localized cytokine milieu including levels of IL-2, IL-6 [46] and transforming growth factor beta (TGFβ) [47], and the differentiation of both Treg and Th17 cells required TGFβ, but depends on opposing activities: at low concentrations, TGFβ synergizes with IL-6 and IL-21 to promote IL-23R expression,

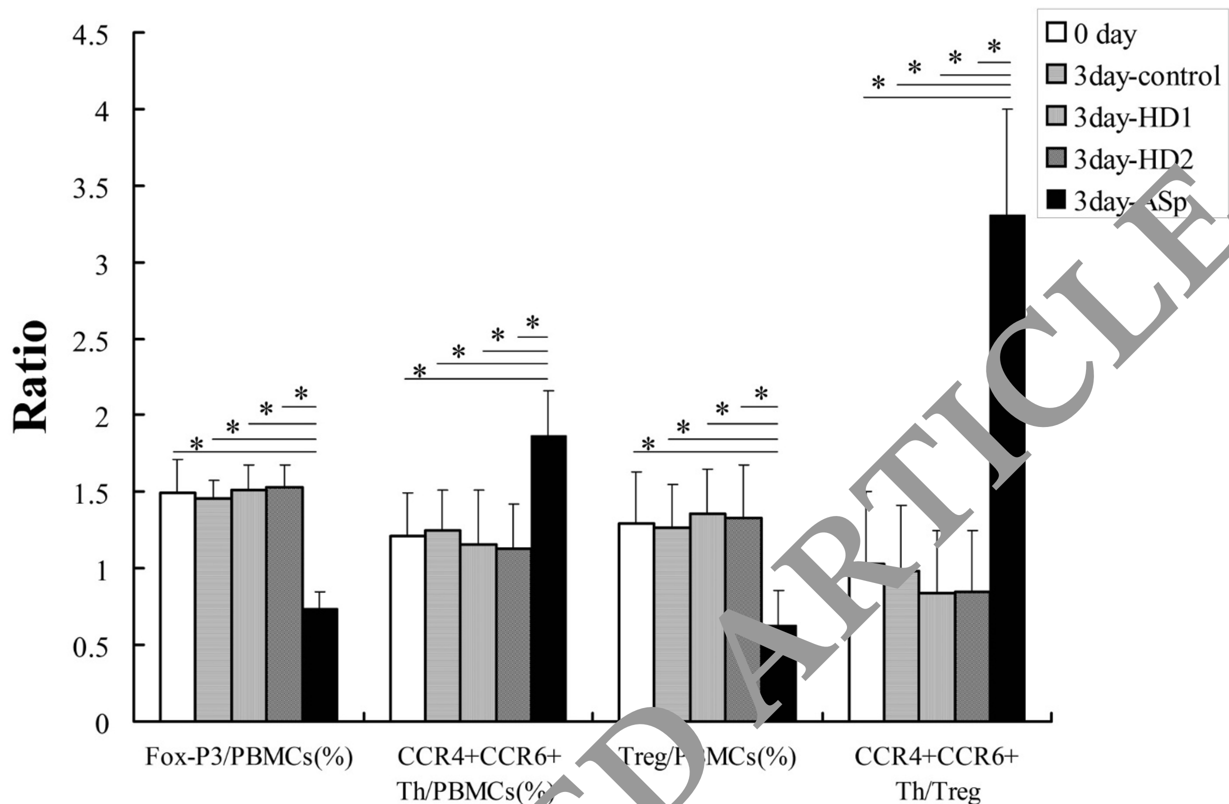

Figure 6 Ratios of Fox-P3/PBMCs, CCR4$^+$CCR6$^+$ Th/PBMCs, Treg/PBMCs and CCR4$^+$CCR6$^+$ Th/Treg. Peripheral blood mononuclear cells (PBMCs) were collected to be assayed by flow cytometry for the CCR4$^+$CCR6$^+$ T-helper (Th) cells, regulatory T (Treg) cells and forkhead box P3 (Fox-P3)-positive cells after co-culture with or without (3 day-control) ankylosing spondylitis (AS)-bone marrow-derived mensenchymal stem cells (BMSCs) (3 day-ASp), HLA-B27-negative healthy donors (HD1)-BMSCs (3 day-HD1) or HLA-B27-positive healthy donors (HD2)-BMSCs (3 day-HD2) for 72 hours. Statistical multiple bar graphs show that AS-BMSCs induced the ratio of CCR4$^+$CCR6$^+$ Th/Treg imbalance via reducing Treg/PBMCs but increasing CCR4$^+$CCR6$^+$ Th/PBMCs; it also produced a significant reduction of Fox-P3-positive cells in PBMCs (*P < 0.001, Student's t test for independent data).

favoring Th17 cell differentiation, while high concentrations of TGFβ repress IL-23R expression and favor Fox-P3$^+$ Treg cells [48].

Rivino and colleagues reported that the combination of CCR4 and CCR6 does not uniquely define Th17 cells; it also demarcated an IL-10-producing population of T cells [49]. There are several reasons why the Th17 cells were defined with the combination of CCR4 and CCR6 in this study. At first, CCR4 had been shown to mark skin-homing T cells [50], expression of which has been associated with the ability of cells to traffic into peripheral tissues [51]; in addition, the percentage of CD4$^+$/CCR4$^+$ T cells showed significant positive correlations with the Bath Ankylosing Spondylitis Disease Activity Index in AS [52]. Furthermore, the findings of Napolitani and colleagues provide a functional link between CCR6 and IL-17 [32], which have been independently associated with tissue pathology. CCR6 has been shown to be involved in the recruitment of pathogenic T cells in rheumatoid arthritis [53], experimental autoimmune encephalitis [54] and psoriasis [55,56], and Th17 cells are increasingly recognized as essential mediators of those diseases [25,57-61]. Besides, just like CCR4, CCR6 also mediates T-cell homing to skin and mucosal tissues [62], and its expression facilitates the recruitment of both dendritic cells and T cells in different diseases [60]. These findings illustrated that the Th17 cells CCR4$^+$CCR6$^+$ were the most active and aggressive pathogenic ones. Second, only a fraction of IL-10-producing CCR6$^+$ T cells co-expresses CCR4 [49]. Finally, Hill Gaston and colleagues reported increased frequency of IL-17-producing T cells in AS [33]; these findings were consistent with our study's results. Moreover, they did not detect any differences in the frequency of IL-10-positive CD4$^+$ T cells between patients with arthritis and control subjects, and none of the IL-17-positive cells co-expressed

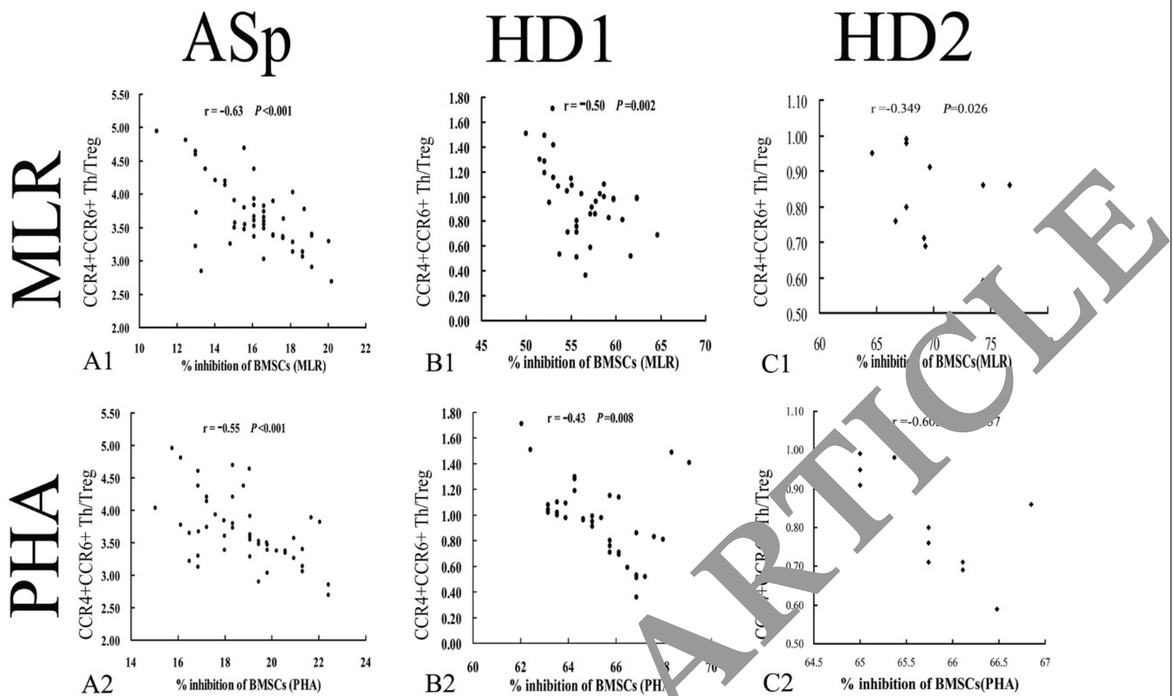

Figure 7 Correlation analysis between CCR4$^+$CCR6$^+$ Th/Treg ratio and immunomodulation potential of bone marrow-derived mensenchymal stem cells. **(A)** All patients with ankylosing spondylitis (ASp), **(B)** HLA-B27-negative healthy donors (HD1) and **(C)** HLA-B27-positive healthy donors (HD2) presented significantly negative correlations between the ratio of CCR4$^+$CCR6$^+$ T-helper (Th) cells to regulatory T (Treg) cells in the peripheral blood mononuclear cells (PBMCs) and percentage inhibition either from two-way mixed PBMC reaction (MLR) (upper panel) or PBMC proliferation induced by phytohemagglutinin (PHA) (lower panel) at a bone marrow-derived mensenchymal stem cell (BMSC):PBMC ratio of 1:10 (P < 0.01, respectively).

IL-10. This means that the increased frequency of IL-17-producing T cells in AS was not compensated for by an increased frequency of IL-10-producing cells.

In the present study, we failed to find any significant differences between HLA-B27-negative and HLA-B27-positive healthy donors, which were essentially the same in all respects we had studied. These findings indicated that HLA-B27-positivity may be not responsible for those abnormalities of ASp. Inflammation is one important link within the pathogenesis of AS [1], while few studies reported whether inflammation be responsible for the altered properties of AS. Rheumatoid arthritis is a typical inflammatory disease, One study reported that the number of IL-17$^+$ Th cells and CD4$^+$CD25$^+$ Treg cells in the peripheral blood of patients with rheumatoid arthritis is elevated compared with that of healthy individuals [33,63], whereas other studies suggest no differences between these two groups [64,65]. Two groups have reported that peripheral blood Treg cells isolated from patients with rheumatoid arthritis and from control individuals showed no difference in their ability to suppress effector T-cell proliferation [38]. Another group, however, reported a striking defect in the capacity of Treg cells from patients with rheumatoid arthritis to suppress effector T-cell proliferation [66]. These divergent results could reflect differences in the populations of patients, the methods used to purify Treg and Th17 cells, or how the suppression assays were performed.

Recently, a study indicated that an alteration in the balance of Th1, Th2, Th17, and Treg cells contributes to the development of experimental autoimmune myastheia gravis, and that the administration of BMSCs can ameliorate the severity and, in a process dependent on the secretion of TGF-β, presenting to inhibit the proliferation of antigen-specific T cells, normalize the distribution of the four T-helper subsets and their corresponding cytokines [67]. *In vitro* experiments have shown that human MSCs can induce the generation of CD4$^+$ T-cell subsets displaying a regulatory phenotype (Treg) [8,68]. These results demonstrated that administration of BMSCs from healthy donors to ASp may be a novelty therapeutic strategy for AS.

The imbalance of Th17/Treg cell subsets may contribute to the inflammatory responses [69] and heterotopic ossification of MSCs [44] in AS by secreting the proinflammatory T-cell cytokines. There is therefore a potential mechanism of AS that reduced the immunomodulation potential of BMSC induced CCR4$^+$CCR6$^+$ Th/Treg imbalance and led to excessive activation of T cells, and then to the increased proinflammatory CCR4$^+$CCR6$^+$ Th cells and reduced Treg cells. Fox-P3$^+$ cells compounded by the synergistic actions of activated T-cell cytokines drive the local BMSCs into both osteoblasts and osteoclasts at localized sites of inflammation, and then the induced BMSCs result in syndesmophytes, fusion of the sacroiliac joint and even spinal stiffness via heterotopic ossification. Finally, AS occurs.

Increasing evidence suggests that MSCs might be a suitable cell population for immunosuppressive therapy in solid organ transplantation and may be strong candidates for cell therapy against human autoimmune diseases [70-72]. The advantages of MSCs are obvious: they can be easily harvested from a multitude of tissues, can be cultured to nearly unlimited extent, and have very promising immunomodulation effects [73]. The immunoregulatory function of BMSCs thus appears to represent a promising strategy for cytotherapy of autoimmune diseases, such as AS, which is central to human health and disease, and provides novel insights into new therapeutic interventions. The retinoic acid receptor-alpha [74] could be another considered candidate for the treatment of autoimmunity, because signaling through a specific nuclear retinoic acid receptor can favor the decision to adopt the Treg cell fate at the expense of the Th17 cell fate. The further elucidation of the precise mechanism may aid in the identification of targets for future immunomodulatory therapy of AS.

Conclusions

The reduced immunomodulation potential of BMSCs may be an initiating factor for AS pathogenesis, and may play a novelty role in triggering the onset of AS via inducing the CCR4$^+$CCR6$^+$ Th/Treg cell subset imbalance. BMSCs may therefore be an interesting therapeutic target in AS, suggesting the use of BMSCs from HDs in the disease.

Abbreviations

ALP: alkaline phosphatase; AS: ankylosing spondylitis; AS-BMSC: bone marrow-derived mensenchymal stem cell of patient with AS; ASp: patients with ankylosing spondylitis; AS-PBMC: peripheral blood mononuclear cell of patients with AS; BMSC: bone marrow-derived mensenchymal stem cell; CPM: counts per minute; DMEM: Dulbecco's modified Eagle's medium; FBS: fetal bovine serum; Fox-P3: forkhead box P3; ^{3}H-TdR: ^{3}H-thymidine; HD: healthy donor; HD1: HLA-B27-negative healthy donors; HD2: HLA-B27-positive healthy donors; HD-BMSC: bone marrow-derived mensenchymal stem cell of healthy donor; HD-PBMC: peripheral blood mononuclear cell of healthy donor; IL: interleukin; MLR: mixed peripheral blood mononuclear cell reaction; MSC: mensenchymal stromal cell; MTT: methyl thiazolyl tetrazolium; OD: optical density; PBMC: peripheral blood mononuclear cell; PBS: phosphate-buffered saline; PCR: polymerase chain reaction; PHA: phytohemagglutinin; Q_L: the lower point of interquartile range; Q_U: the upper point of interquartile range; SD: standard deviation; TGFβ: transforming growth factor beta; Th: T-helper; TNF: tumor necrosis factor; Treg: regulatory T.

Acknowledgements

The authors thank Dr Xiaoping Wang at Sun Yat-sen University for assistance with cell culturing, including BMSCs and PBMCs. They also thank Dr Jing Wei for flow cytometry, and Wenfeng Xie and Hua Zeng for help with the samples from ASp. The study was financially supported by the National Natural Science Foundation of China (30973033), the Yat-sen Innovative Talents Cultivation Program for Excellent Tutors (81000-3126202), the Guangzhou Science and Technology Project (2008A1-E4011-9), the Natural Science Foundation of Guangdong Province (9151008002000015), and the Guangdong Provincial Science and Technology Project (2009B060300023).

Author details

[1]Department of Othopaedics, Sun Yat-sen Memorial Hospital, Sun Yat-sen University, 107# Yanjiangxi Road, Guangzhou 510120, PR China. [2]Biotherapy Centre, Sun Yat-sen Memorial Hospital, Sun Yat-sen University, 107# Yanjiangxi Road, Guangzhou 510120, PR China. [3]Department of Othopaedics, Guangdong Provincial People Hospital, 106# Zhongshan Road 2, Guangzhou 510080, PR China.

Authors' contributions

RY, XL, YM and YT carried out the experimental work and the data collection and interpretation. LL, KC and JY participated in the design and coordination of experimental work, and the acquisition of data. MR and YW participated in the study design, data collection, analysis of data and preparation of the manuscript. PW and HS carried out the study design, the analysis and interpretation of data and drafted the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Received: 12 October 2010 Revised: 16 January 2011
Accepted: 21 February 2011 Published: 21 February 2011

References

1. Braun J, Sieper J: **Ankylosing spondylitis.** *Lancet* 2007, **369**:1379-1390.
2. Penttinen MA, Heiskanen KM, Mohapatra R, DeLay ML, Colbert RA, Sistonen L, Granfors K: **Enhanced intracellular replication of *Salmonella enteritidis* in HLA-B27-expressing human monocytic cells: dependency on glutamic acid at position 45 in the B pocket of HLA-B27.** *Arthritis Rheum* 2004, **50**:2255-2263.
3. Brown MA, Kennedy LG, MacGregor AJ, Darke C, Duncan E, Shatford JL, Taylor A, Calin A, Wordsworth P: **Susceptibility to ankylosing spondylitis in twins: the role of genes, HLA, and the environment.** *Arthritis Rheum* 1997, **40**:1823-1828.
4. Brewerton DA, Hart FD, Nicholls A, Caffrey M, James DC, Sturrock RD: **Ankylosing spondylitis and HL-A 27.** *Lancet* 1973, **1**:904-907.
5. Paladini F, Belfiore F, Cocco E, Carcassi C, Cauli A, Vacca A, Fiorillo MT, Mathieu A, Cascino I, Sorrentino R: **HLA-E gene polymorphism associates with ankylosing spondylitis in Sardinia.** *Arthritis Res Ther* 2009, **11**:R171.
6. Reveille JD, Sims AM, Danoy P, Evans DM, Leo P, Pointon JJ, Jin R, Zhou X, Bradbury LA, Appleton LH, Davis JC, Diekman L, Doan T, Dowling A, Duan R, Duncan EL, Farrar C, Hadler J, Harvey D, Karaderi T, Mogg R, Pomeroy E, Pryce K, Taylor J, Savage L, Deloukas P, Kumanduri V, Peltonen L, Ring SM, Whittaker P, *et al*: **Genome-wide association study of ankylosing spondylitis identifies non-MHC susceptibility loci.** *Nat Genet* 2010, **42**:123-127.
7. Braun J, Tuszewski M, Ehlers S, Haberle J, Bollow M, Eggens U, Distler A, Sieper J: **Nested polymerase chain reaction strategy simultaneously targeting DNA sequences of multiple bacterial species in inflammatory joint diseases. II. Examination of sacroiliac and knee joint biopsies of patients with spondyloarthropathies and other arthritides.** *J Rheumatol* 1997, **24**:1101-1105.
8. Aggarwal S, Pittenger MF: **Human mesenchymal stem cells modulate allogeneic immune cell responses.** *Blood* 2005, **105**:1815-1822.
9. Di Nicola M, Carlo-Stella C, Magni M, Milanesi M, Longoni PD, Matteucci P, Grisanti S, Gianni AM: **Human bone marrow stromal cells suppress T-**

lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. *Blood* 2002, **99**:3838-3843.
10. Bartholomew A, Sturgeon C, Siatskas M, Ferrer K, McIntosh K, Patil S, Hardy W, Devine S, Ucker D, Deans R, Moseley A, Hoffman R: **Mesenchymal stem cells suppress lymphocyte proliferation in vitro and prolong skin graft survival in vivo.** *Exp Hematol* 2002, **30**:42-48.
11. Dazzi F, Marelli-Berg FM: **Mesenchymal stem cells for graft-versus-host disease: close encounters with T cells.** *Eur J Immunol* 2008, **38**:1479-1482.
12. Uccelli A, Moretta L, Pistoia V: **Immunoregulatory function of mesenchymal stem cells.** *Eur J Immunol* 2006, **36**:2566-2573.
13. Zappia E, Casazza S, Pedemonte E, Benvenuto F, Bonanni I, Gerdoni E, Giunti D, Ceravolo A, Cazzanti F, Frassoni F, Mancardi G, Uccelli A: **Mesenchymal stem cells ameliorate experimental autoimmune encephalomyelitis inducing T-cell anergy.** *Blood* 2005, **106**:1755-1761.
14. Bacigalupo A, Valle M, Podesta M, Pitto A, Zocchi E, De Flora A, Pozzi S, Luchetti S, Frassoni F, Van Lint MT, Piaggio G: **T-cell suppression mediated by mesenchymal stem cells is deficient in patients with severe aplastic anemia.** *Exp Hematol* 2005, **33**:819-827.
15. Bernardo ME, Avanzini MA, Ciccocioppo R, Perotti C, Cometa AM, Moretta A, Marconi M, Valli M, Novara F, Bonetti F, Zuffardi O, Maccario R, Corazza GR, Locatelli F: **Phenotypical/functional characterization of in vitro-expanded mesenchymal stromal cells from patients with Crohn's disease.** *Cytotherapy* 2009, **11**:825-836.
16. Costantino CM, Baecher-Allan CM, Hafler DA: **Human regulatory T cells and autoimmunity.** *Eur J Immunol* 2008, **38**:921-924.
17. Ouyang W, Kolls JK, Zheng Y: **The biological functions of T helper 17 cell effector cytokines in inflammation.** *Immunity* 2008, **28**:454-467.
18. Mills KH: **Induction, function and regulation of IL-17-producing T cells.** *Eur J Immunol* 2008, **38**:2636-2649.
19. Romagnani S: **Human Th17 cells.** *Arthritis Res Ther* 2008, **10**:206.
20. Vila J, Isaacs JD, Anderson AE: **Regulatory T cells and autoimmunity.** *Curr Opin Hematol* 2009, **16**:274-279.
21. Banica L, Besliu A, Pistol G, Stavaru C, Ionescu R, Forsea AM, Tanaseanu C, Dumitrache S, Otelea D, Tamsulea I, Tanaseanu S, Chitonu C, Paraschiv S, Balteanu M, Stefanescu M, Matache C: **Quantification and molecular characterization of regulatory T cells in connective tissue diseases.** *Autoimmunity* 2009, **42**:41-49.
22. Abdulahad WH, Stegeman CA, van der Geld YM, Doornbos-van der Meer B, Limburg PC, Kallenberg CG: **Functional defect of circulating regulatory CD4$^+$ T cells in patients with Wegener's granulomatosis in remission.** *Arthritis Rheum* 2007, **56**:2080-2091.
23. Okumura A, Ishikawa T, Sato S, Yamauchi T, Oshima H, Ohashi T, Sato K, Ayada M, Hotta N, Kakumu S: **Deficiency of forkhead box P3 and cytotoxic T-lymphocyte-associated antigen-4 gene expressions and impaired suppressor function of CD4(+)CD25(+) T cells in patients with autoimmune hepatitis.** *Hepatol Res* 2008, **38**:[illegible].
24. Langrish CL, Chen Y, Blumenschein WM, Mattson J, Basham B, Sedgwick JD, McClanahan T, Kastelein RA, Cua DJ: **IL-23 drives a pathogenic T cell population that induces autoimmune inflammation.** *J Exp Med* 2005, **201**:233-240.
25. Murphy CA, Langrish CL, Chen Y, Blumenschein W, McClanahan T, Kastelein RA, Sedgwick JD, Cua DJ: **Divergent pro- and antiinflammatory roles for IL-23 and IL-12 in joint autoimmune inflammation.** *J Exp Med* 2003, **198**:1951-1957.
26. Chen X, Vodanovic-Jankovic S, Johnson B, Keller M, Komorowski R, Drobyski WR: **Absence of regulatory T-cell control of TH1 and TH17 cells is responsible for the autoimmune-mediated pathology in chronic graft-versus-host disease.** *Blood* 2007, **110**:3804-3813.
27. Weaver CT, Hatton RD, Mangan PR, Harrington LE: **IL-17 family cytokines and the expanding diversity of effector T cell lineages.** *Annu Rev Immunol* 2007, **25**:821-852.
28. Burton PR, Clayton DG, Cardon LR, Craddock N, Deloukas P, Duncanson A, Kwiatkowski DP, McCarthy MI, Ouwehand WH, Samani NJ, Todd JA, Donnelly P, Barrett JC, Davison D, Easton D, Evans DM, Leung HT, Marchini JL, Morris AP, Spencer CC, Tobin MD, Attwood AP, Boorman JP, Cant B, Everson U, Hussey JM, Jolley JD, Knight AS, Koch K, Meech E, *et al*: **Association scan of 14,500 nonsynonymous SNPs in four diseases identifies autoimmunity variants.** *Nat Genet* 2007, **39**:1329-1337.
29. Rahman P, Inman RD, Gladman DD, Reeve JP, Peddle L, Maksymowych WP: **Association of interleukin-23 receptor variants with ankylosing spondylitis.** *Arthritis Rheum* 2008, **58**:1020-1025.
30. Rueda B, Orozco G, Raya E, Fernandez-Sueiro JL, Mulero J, Blanco FJ, Vilches C, Gonzalez-Gay MA, Martin J: **The IL23R Arg381Gln non-synonymous polymorphism confers susceptibility to ankylosing spondylitis.** *Ann Rheum Dis* 2008, **67**:1451-1454.
31. Cua DJ, Sherlock J, Chen Y, Murphy CA, Joyce B, Seymour B, Lucian L, To W, Kwan S, Churakova T, Zurawski S, Wiekowski M, Lira SA, Gorman D, Kastelein RA, Sedgwick JD: **Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain.** *Nature* 2003, **421**:744-748.
32. Acosta-Rodriguez EV, Rivino L, Geginat J, Jarrossay D, Gattorno M, Lanzavecchia A, Sallusto F, Napolitani G: **Surface phenotype and antigenic specificity of human interleukin 17-producing T helper memory cells.** *Nat Immunol* 2007, **8**:639-646.
33. Shen H, Goodall JC, Hill Gaston JS: **Frequency and phenotype of peripheral blood Th17 cells in ankylosing spondylitis and rheumatoid arthritis.** *Arthritis Rheum* 2009, **60**:1647-165[illegible].
34. van der Linden S, Valkenburg HA, Cats A: **Evaluation of diagnostic criteria for ankylosing spondylitis. A proposal for modification of the New York criteria.** *Arthritis Rheum* 1984, **27**:361-[illegible].
35. Betz MW, Modi PC, Caccamese JF, Coletti DP, Sauk JJ, Fisher JP: **Cyclic acetal hydrogel system for bone marrow stromal cell encapsulation and osteodifferentiation.** *J Biomed Mater Res A* 2008, **86**:662-670.
36. Jung JW, Cho SD, Ahn NS, Yang SR, Park JS, Jo EH, Hwang JW, Jung JY, Kim SH, Kang KS, Lee YS: **Ras/MAP kinase pathways are involved in Ras specific apoptosis induced by sodium butyrate.** *Cancer Lett* 2005, **225**:199-206.
37. Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop D, Horwitz E: **Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement.** *Cytotherapy* 2006, **8**:315-317.
38. Ehrenstein MR, Evans JG, Singh A, Moore S, Warnes G, Isenberg DA, Mauri C: **Compromised function of regulatory T cells in rheumatoid arthritis and reversal by anti-TNFα therapy.** *J Exp Med* 2004, **200**:277-285.
39. Boissier MC, Assier E, Falgarone G, Bessis N: **Shifting the imbalance from Th1/Th2 to Th17/treg: the changing rheumatoid arthritis paradigm.** *Joint Bone Spine* 2008, **75**:373-375.
40. Wanders A, Landewe R, Dougados M, Mielants H, van der Linden S, van der Heijde D: **Association between radiographic damage of the spine and spinal mobility for individual patients with ankylosing spondylitis: can assessment of spinal mobility be a proxy for radiographic evaluation?** *Ann Rheum Dis* 2005, **64**:988-994.
41. El Tamer MK, Reis RL: **Progenitor and stem cells for bone and cartilage regeneration.** *J Tissue Eng Regen Med* 2009, **3**:327-337.
42. Yang Y: **Skeletal morphogenesis during embryonic development.** *Crit Rev Eukaryot Gene Expr* 2009, **19**:197-218.
43. Braun J, Bollow M, Neure L, Seipelt E, Seyrekbasan F, Herbst H, Eggens U, Distler A, Sieper J: **Use of immunohistologic and in situ hybridization techniques in the examination of sacroiliac joint biopsy specimens from patients with ankylosing spondylitis.** *Arthritis Rheum* 1995, **38**:499-505.
44. Rifas L: **T-cell cytokine induction of BMP-2 regulates human mesenchymal stromal cell differentiation and mineralization.** *J Cell Biochem* 2006, **98**:706-714.
45. Kim J, Lahl K, Hori S, Loddenkemper C, Chaudhry A, deRoos P, Rudensky A, Sparwasser T: **Cutting edge: depletion of Foxp3$^+$ cells leads to induction of autoimmunity by specific ablation of regulatory T cells in genetically targeted mice.** *J Immunol* 2009, **183**:7631-7634.
46. Kimura A, Kishimoto T: **IL-6: regulator of Treg/Th17 balance.** *Eur J Immunol* 2010, **40**:1830-1835.
47. Nistala K, Wedderburn LR: **Th17 and regulatory T cells: rebalancing pro- and anti-inflammatory forces in autoimmune arthritis.** *Rheumatology (Oxford)* 2009, **48**:602-606.
48. Zhou L, Lopes JE, Chong MM, Ivanov II, Min R, Victora GD, Shen Y, Du J, Rubtsov YP, Rudensky AY, Ziegler SF, Littman DR: **TGF-beta-induced Foxp3 inhibits T(H)17 cell differentiation by antagonizing RORgammat function.** *Nature* 2008, **453**:236-240.
49. Rivino L, Gruarin P, Haringer B, Steinfelder S, Lozza L, Steckel B, Weick A, Sugliano E, Jarrossay D, Kuhl AA, Loddenkemper C, Abrignani S, Sallusto F, Lanzavecchia A, Geginat J: **CCR6 is expressed on an IL-10-producing, autoreactive memory T cell population with context-dependent regulatory function.** *J Exp Med* 2010, **207**:565-577.
50. Campbell JJ, Haraldsen G, Pan J, Rottman J, Qin S, Ponath P, Andrew DP, Warnke R, Ruffing N, Kassam N, Wu L, Butcher EC: **The chemokine receptor**

CCR4 in vascular recognition by cutaneous but not intestinal memory T cells. *Nature* 1999, **400**:776-780.

51. Baekkevold ES, Wurbel MA, Kivisakk P, Wain CM, Power CA, Haraldsen G, Campbell JJ: **A role for CCR4 in development of mature circulating cutaneous T helper memory cell populations.** *J Exp Med* 2005, **201**:1045-1051.
52. Yang PT, Kasai H, Zhao LJ, Xiao WG, Tanabe F, Ito M: **Increased CCR4 expression on circulating CD4(+) T cells in ankylosing spondylitis, rheumatoid arthritis and systemic lupus erythematosus.** *Clin Exp Immunol* 2004, **138**:342-347.
53. Ruth JH, Shahrara S, Park CC, Morel JC, Kumar P, Qin S, Koch AE: **Role of macrophage inflammatory protein-3α and its ligand CCR6 in rheumatoid arthritis.** *Lab Invest* 2003, **83**:579-588.
54. Kohler RE, Caon AC, Willenborg DO, Clark-Lewis I, McColl SR: **A role for macrophage inflammatory protein-3 alpha/CC chemokine ligand 20 in immune priming during T cell-mediated inflammation of the central nervous system.** *J Immunol* 2003, **170**:6298-6306.
55. Homey B, Dieu-Nosjean MC, Wiesenborn A, Massacrier C, Pin JJ, Oldham E, Catron D, Buchanan ME, Muller A, deWaal Malefyt R, Deng G, Orozco R, Ruzicka T, Lehmann P, Lebecque S, Caux C, Zlotnik A: **Up-regulation of macrophage inflammatory protein-3 alpha/CCL20 and CC chemokine receptor 6 in psoriasis.** *J Immunol* 2000, **164**:6621-6632.
56. Schaerli P, Britschgi M, Keller M, Steiner UC, Steinmann LS, Moser B, Pichler WJ: **Characterization of human T cells that regulate neutrophilic skin inflammation.** *J Immunol* 2004, **173**:2151-2158.
57. Kikly K, Liu L, Na S, Sedgwick JD: **The IL-23/Th(17) axis: therapeutic targets for autoimmune inflammation.** *Curr Opin Immunol* 2006, **18**:670-675.
58. Nakae S, Nambu A, Sudo K, Iwakura Y: **Suppression of immune induction of collagen-induced arthritis in IL-17-deficient mice.** *J Immunol* 2003, **171**:6173-6177.
59. Nakae S, Saijo S, Horai R, Sudo K, Mori S, Iwakura Y: **IL-17 production from activated T cells is required for the spontaneous development of destructive arthritis in mice deficient in IL-1 receptor antagonist.** *Proc Natl Acad Sci USA* 2003, **100**:5986-5990.
60. Cook DN, Prosser DM, Forster R, Zhang J, Kuklin NA, Abbondanzo SJ, Niu XD, Chen SC, Manfra DJ, Wiekowski MT, Sullivan LM, Smith SR, Greenberg HB, Narula SK, Lipp M, Lira SA: **CCR6 mediates dendritic cell localization, lymphocyte homeostasis, and immune responses in mucosal tissue.** *Immunity* 2000, **12**:495-503.
61. Keller M, Spanou Z, Schaerli P, Britschgi M, Yawalkar N, Seitz M, Villiger PM, Pichler WJ: **T cell-regulated neutrophilic inflammation in autoinflammatory diseases.** *J Immunol* 2005, **175**:7678-7686.
62. Liao F, Rabin RL, Smith CS, Sharma G, Nutman TB, Farber JM: **CC-chemokine receptor 6 is expressed on diverse memory subsets of T cells and determines responsiveness to macrophage inflammatory protein 3 alpha.** *J Immunol* 1999, **162**:186-194.
63. van Amelsfort JM, Jacobs KM, Bijlsma JW, Lafeber FP, Taams LS: **CD4(+) CD25(+) regulatory T cells in rheumatoid arthritis: differences in the presence, phenotype, and function between peripheral blood and synovial fluid.** *Arthritis Rheum* 2004, **50**:2775-2785.
64. Jandus C, Bioley G, Rivals JP, Dudler J, Speiser D, Romero P: **Increased numbers of circulating polyfunctional Th17 memory cells in patients with seronegative spondylarthritides.** *Arthritis Rheum* 2008, **58**:2307-2317.
65. Flores-Borja F, Jury EC, Mauri C, Ehrenstein MR: **Defects in CTLA-4 are associated with abnormal regulatory T cell function in rheumatoid arthritis.** *Proc Natl Acad Sci USA* 2008, **105**:19396-19401.
66. Valencia X, Stephens G, Goldbach-Mansky R, Wilson M, Shevach EM, Lipsky PE: **TNF downmodulates the function of human CD4^{+}CD25^{hi} T-regulatory cells.** *Blood* 2006, **108**:253-261.
67. Kong QF, Sun B, Bai SS, Zhai DX, Wang GY, Liu YM, Zhang SJ, Li R, Zhao W, Sun YX, Li N, Wang Q, Peng HS, Jin LH, Li HL: **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.** *J Neuroimmunol* 2009, **207**:83-91.
68. Maccario R, Podesta M, Moretta A, Cometa A, Comoli P, Montagna D, Daudt L, Ibatici A, Piaggio G, Pozzi S, Frassoni F, Locatelli F: **Interaction of human mesenchymal stem cells with cells involved in alloantigen-specific immune response favors the differentiation of CD4^{+} T-cell subsets expressing a regulatory/suppressive phenotype.** *Haematologica* 2005, **90**:516-525.
69. Annunziato F, Cosmi L, Santarlasci V, Maggi L, Liotta F, Mazzinghi B, Parente E, Fili L, Ferri S, Frosali F, Giudici F, Romagnani P, Parronchi P, Tonelli F, Maggi E, Romagnani S: **Phenotypic and functional features of human Th17 cells.** *J Exp Med* 2007, **204**:1849-1861.
70. Parekkadan B, van Poll D, Megeed Z, Kobayashi N, Tilles AW, Berthiaume F, Yarmush ML: **Immunomodulation of activated hepatic stellate cells by mesenchymal stem cells.** *Biochem Biophys Res Commun* 2007, **363**:247-252.
71. Gonzalez MA, Gonzalez-Rey E, Rico L, Buscher D, Delgado M: **Treatment of experimental arthritis by inducing immune tolerance with human adipose-derived mesenchymal stem cells.** *Arthritis Rheum* 2009, **60**:1006-1019.
72. Kong QF, Sun B, Wang GY, Zhai DX, Mu LL, Wang DD, Wang JH, Li R, Li HL: **BM stromal cells ameliorate experimental autoimmune myasthenia gravis by altering the balance of Th cells through the secretion of IDO.** *Eur J Immunol* 2009, **39**:800-809.
73. Uccelli A, Moretta L, Pistoia V: **Mesenchymal stem cells in health and disease.** *Nat Rev Immunol* 2008, **8**:726-736.
74. Schambach F, Schupp M, Lazar MA, Reiner SL: **Activation of retinoic acid receptor-alpha favours regulatory T cell induction at the expense of IL-17-secreting T helper cell differentiation.** *Eur J Immunol* 2007, **37**:2396-2399.

doi:10.1186/ar3257
Cite this article as: Wu *et al.*: Reduced immunomodulation potential of bone marrow-derived mesenchymal stem cells induced CCR4^{+}CCR6^{+} Th/Treg cell subset imbalance in ankylosing spondylitis. *Arthritis Research & Therapy* 2011 **13**:R29.