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Human Wharton's Jelly-Derived Stem Cells Display Immunomodulatory Properties and Transiently Improve Rat Experimental Autoimmune Encephalomyelitis

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Umbilical cord matrix or Wharton's jelly-derived stromal cells (WJ-MSCs) are an easily accessible source of mesenchymal-like stem cells. Recent studies describe a hypoimmunogenic phenotype, multipotent differentiation potential, and trophic support function for WJ-MSCs, with variable clinical benefit in degenerative disease models such as stroke, myocardial infarction, and Parkinson's disease. It remains unclear whether WJ-MSCs have therapeutic value for multiple sclerosis (MS), where autoimmune-mediated demyelination and neurodegeneration need to be halted. In this study, we investigated whether WJ-MSCs possess the required properties to effectively and durably reverse these pathological hallmarks and whether they survive in an inflammatory environment after transplantation. WJ-MSCs displayed a lowly immunogenic phenotype and showed intrinsic expression of neurotrophic factors and a variety of anti-inflammatory molecules. Furthermore, they dose-dependently suppressed proliferation of activated T cells using contact-dependent and paracrine mechanisms. Indoleamine 2,3-dioxygenase 1 was identified as one of the main effector molecules responsible for the observed T-cell suppression. The immune-modulatory phenotype of WJ-MSCs was further enhanced after proinflammatory cytokine treatment in vitro (licensing). In addition to their effect on adaptive immunity, WJ-MSCs interfered with dendritic cell differentiation and maturation, thus directly affecting antigen presentation and therefore T-cell priming. Systemically infused WJ-MSCs potently but transiently ameliorated experimental autoimmune encephalomyelitis (EAE), an animal model for MS, when injected at onset or during chronic disease. This protective effect was paralleled with a reduction in autoantigen-induced T-cell proliferation, confirming their immunomodulatory activity in vivo. Surprisingly, in vitro licensed WJ-MSCs did not ameliorate EAE, indicative of a fast rejection as a result of enhanced immunogenicity. Collectively, we show that WJ-MSCs have trophic support properties and effectively modulate immune cell functioning both in vitro and in the EAE model, suggesting WJ-MSC may hold promise for MS therapy. Future research is needed to optimize survival of stem cells and enhance clinical durability.

Key words: Multiple sclerosis (MS); Wharton's jelly; Transplantation; Immunomodulation; Mesenchymal stem cells (MSCs); Cell therapy

INTRODUCTION

Multiple sclerosis (MS) is a neurodegenerative disease, characterized by chronic inflammation and multifocal demyelination in the central nervous system (CNS). The etiology of MS is unknown, but both genetic and environmental factors influence the risk of developing the disease (33,34,77). According to current assumptions, the immune system has a crucial role in the pathogenesis of MS. Cluster of differentiation 4-positive ($CD4^+$) T-helper 1 (Th1) cells, $CD4^+$ Th17 cells, and cytotoxic $CD8^+$ T cells play an important part in promoting neuroinflammation and neurodegeneration (56,66). Furthermore, complex interactions with other peripheral immune cells and CNS resident cells are responsible for repeated episodes of autoimmune-mediated damage to myelin and oligodendrocytes (14,69). Over time, recurring demyelination and fading CNS repair processes result in increasing axonal degeneration, glial scar formation, and disease progression (69). At present, there is no cure for MS. Current available therapies consist of anti-inflammatory drugs that target the immune component of the disease and are mainly effective in early disease. While they have been shown to reduce symptoms and slow

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down progression, current treatments do not decrease neurodegeneration or promote CNS repair (82). Novel therapeutic strategies should enhance remyelination efficiency, replace damaged oligodendrocytes, and ultimately restore axonal conduction (27). In this regard, cell-based therapy has emerged as a promising treatment approach for MS. Neural progenitor cells (NPCs) and adult mesenchymal stromal cells (MSCs) have been investigated with regard to their potential for tissue regeneration as well as their trophic support and immunomodulatory functions. Several preclinical studies have demonstrated their therapeutic properties in rodents with experimental autoimmune encephalomyelitis (EAE), an animal model of MS (1,30,60,88). When introduced intravenously, intraventricularly, or directly into the CNS parenchyma, MSCs and NPCs are effective in suppressing the clinical disease course by way of improving motor function and reducing lesion burden. The neuroprotective mechanisms used by the stem cells involve (peripheral) immune modulation and secretion of neurotrophic factors, but also homing to sites of injury, extracellular matrix degradation, and (trans)differentiation have been reported (22,74). These mechanisms collectively act to decrease immune cell infiltration, demyelination, and axonal loss. Recently, consensus statements have been published that report such features to be required for successful cell-based treatment of MS (28,44,67).

Although the therapeutic application of adult MSCs has been extensively reported in the context of MS, limited data are currently available on the effect of extraembryonic stem cell types in this setting. Stem cells derived from extraembryonic tissues are increasingly being investigated for their potential in regenerative medicine, as an alternative to adult stem cell therapy (43). Perinatal tissues such as umbilical cord are obtained without invasive procedures and are widely available sources of multipotent stem cells with both anti-inflammatory and trophic properties, relating to their biological role in embryo implantation (16,57,58,72). Moreover, the umbilical cord contains different compartments from which stem cells can be harvested, for example, the cord blood for hematopoietic stem cells and cord tissue for MSC-like cells (15,81). In the case of the latter, the umbilical cord matrix or Wharton's jelly is an easily accessible source of stromal cells with mesenchymal-like properties (WJ-MSCs), which, up to a certain level, show morphological and functional resemblance to bone marrow-derived mesenchymal stromal cells (BM-MSCs) (50,64). In several disease models, such as type 1 diabetes, myocardial infarction, and Parkinson's disease, WJ-MSCs adopt an immunomodulatory and trophic functional phenotype (12,42,80,84). As such, they show a favorable profile for therapy in inflammatory-mediated degenerative diseases. Despite increasing knowledge of their functional characteristics, there are a variety of different mechanisms reported to be responsible for the therapeutic effects seen in these different disease models (2,36,54). The MSC secretome, immunological profile, and engraftment potential are all considered to contribute to their therapeutic efficacy after transplantation (11,37). It is likely that such beneficial mechanisms are induced by disease-specific conditions after transplantation. Consequently, it remains to be clarified whether WJ-MSCs hold therapeutic potential for MS, where autoimmune-mediated demyelination and neurodegeneration need to be halted.

In this study, we thoroughly characterize the immunomodulatory and trophic support properties of WJ-MSCs and determine whether they can effectively and durably ameliorate chronic EAE. First, our WJ-MSC isolates are investigated for the expression of neurotrophic growth factors and immunological molecules. Next, coculture experiments are set up to examine immune modulatory effects on T cells and dendritic cells (DCs), two important cell types involved in autoimmune-mediated CNS damage in MS (10,56). Finally, human WJ-MSCs are transplanted in rats with chronic EAE to define their effect on clinical symptoms and pathological hallmarks.

MATERIALS AND METHODS

Ethical Approval and Donor Samples

Animal experiments were performed in accordance with institutional guidelines and are approved by the Hasselt University Ethical Committee for Animal Experiments. The collection and experimental use of human cells and tissues are approved by the Medical Ethical Committees of Hasselt University and Ziekenhuis Oost-Limburg (Genk). Peripheral blood was collected from healthy donors ($n=12$; mean age $=28.8\pm7.8$ years; female/male ratio $=7:5$), and umbilical cord tissues were obtained from cesarean sections ($n=17$; female/male ratio $=7:7$, three unknown) after informed consent.

Isolation and Culture of WJ-MSCs

Cells were isolated from human umbilical cords using the explant culture method, as previously described, with minor modifications (71). Briefly, umbilical tissues were obtained aseptically from full-term uncomplicated pregnancies with planned cesarean section and processed within 24 h. Cords were washed in sterile phosphate-buffered saline (PBS; Lonza, Verviers, Belgium) supplemented with 1% penicillin–streptomycin (P/S; 10,000:10,000 U; Gibco®, Life Technologies, Gent, Belgium) and 0.2% Fungizone® (250 µg/ml; Gibco®, Life Technologies). After removal of arteries and veins, the cord was cut into explants (2 mm^3) and cultured in KnockOut™ Dulbecco's modified Eagle's medium with F12 (Gibco®, Life Technologies) supplemented with 1% P/S, 1% GlutaMAX™ (200 mM; Gibco®, Life Technologies), and 10% fetal bovine serum (FBS; Biochrom AG, Berlin, Germany), at 37°C in a humidified

atmosphere containing 5% CO_2. When outgrowth of cells from the explants was observed, medium was refreshed every 3 days, and cells were harvested at 80% confluence using Stempro® accutase (Gibco®, Life Technologies). The cells used in functional assays were harvested between passages 3 and 8.

Cytokine Stimulation ("Licensing")

WJ-MSCs ($n=5$) were incubated for 24 h with proinflammatory cytokines interferon-γ (IFN-γ), interleukin-1β (IL-1β), and tumor necrosis factor-α (TNF-α) (all from Peprotech, London, UK), at a final concentration of 20 ng/ml in culture medium. Next, the cells were harvested for use in different experimental setups or were pelleted and stored at −80°C until RNA extraction.

Flow Cytometry

WJ-MSCs were harvested, washed with FACS buffer (PBS supplemented with 2% FBS), and incubated in the dark for 30 min at 4°C with the following antibodies: HLA-DR-PE, CD45-FITC, CD34-PE, CD14-PE, and CD19-PE (all Immunotools, Friesoythe, Germany); CD105-PE, CD90-FITC, and CD40-FITC (all eBiosciences, Vienna, Austria); and CD73-PE, CD80-PE, and CD86-FITC (all BD Biosciences, Erembodegem, Belgium). Dilutions were made according to manufacturer's recommendation. To assess viability, cells were stained with 7 aminoactinomycin D (7-AAD; 1/1,000; BD Biosciences). Stained samples were washed and analyzed on a FACSCalibur instrument using CellQuest software (BD Biosciences).

RNA Isolation and cDNA Synthesis

Total RNA extraction was performed using the High Pure RNA Isolation Kit (Roche Diagnostics, Vilvoorde, Belgium) according to the manufacturer's instructions. RNA quality was assessed with a Nanodrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA) according to standard procedures. One microgram of RNA was reverse transcribed to cDNA using the Reverse Transcription System (Promega, Leiden, The Netherlands) according to the manufacturer's protocol.

Quantitative Real-Time PCR (qPCR)

qPCR was performed with a StepOnePlus™ Real-Time PCR System (Applied Biosystems®; Life Technologies) using universal cycling conditions (20 s 95°C, 40 cycles of 3 s at 95°C, and 30 s at 60°C), in MicroAmp® Fast Optical 96-well reaction plates (Applied Biosystems®; Life Technologies). The reaction consisted of fast SYBR green master mix (Applied Biosystems®; Life Technologies), 10 mM of forward and reverse primers (Eurogentec, Seraing, Belgium), nuclease-free water (Promega), and 12.5 ng cDNA template in 10 µl total volume. Nontemplate controls contained nuclease-free water instead of cDNA. Human primers are listed in Table 1 and were designed using Primer 3 (76). Rat primers for Th1-, Th2-, Th17-, and Treg-related transcription factors were as previously described (9). Primers for rat IFN-γ and IL-17 were described by Du et al. (21), and TGF-β1 primers were taken from Fleury et al. (26). The rat IL-10 primer was as follows: 5′-GCCTTCAGTCAAGTGAAGACT-3′ (sense) and 5′-AAACTCATTCATGGCCTTGTA-3′ (antisense). PCR products were visualized using standard 2% agarose gel electrophoresis and ethidium bromide (Invitrogen™, Life Technologies). Relative quantification of gene expression was calculated using qBasePLUS® (Biogazelle, Zwijnaarde, Belgium). Data were normalized to the most stable reference genes, and results were presented as fold changes to the control biological sample (78).

Coculture of WJ-MSCs and Peripheral Blood Mononuclear Cells (PBMCs)

WJ-MSCs were seeded in flat-bottom 24-/48-well plates (Greiner Bio-One, Frickenhausen, Germany) in coculture medium consisting of RPMI-1640 GlutaMAX™ supplemented with 0.5% P/S, 1% sodium pyruvate (NaP; 100 mM), 1% nonessential amino acids (NEAA) (all Gibco®, Life Technologies), and 10% FBS. Different amounts of cells were seeded in accordance with the required ratios for coculture (WJ-MSC/PBMC ratio of 1:100, 1:10, 1:5, 1:2, 1:1, 2:1, or 5:1). Peripheral blood from healthy donors was collected in heparin-coated plastic tubes (Venosafe®; Terumo Europe, Leuven, Belgium). Subsequently, PBMCs were isolated via density gradient centrifugation using Lympholyte®-H (Cedarlane; SanBio B.V., Uden, the Netherlands). Freshly isolated PBMCs (2×10^7/ml) were stained with 2 µM carboxyfluorescein diacetate succinimidyl ester (CFSE; CellTrace™; Life Technologies) in PBS. Next, 5×10^4 CFSE-labeled PBMCs were added to the WJ-MSCs with or without the addition of 2 µg/ml anti-CD3 antibodies (aCD3; generated in-house, Hasselt University, Diepenbeek, Belgium). CFSE-labeled PBMCs alone (±aCD3) were used as control. In licensed cocultures, stem cells were pretreated with cytokines for 24 h. After 5 days of coculture, T-cell proliferation was determined via CFSE dye dilution using flow cytometry. Briefly, the supernatant containing PBMCs was transferred to a V-bottom 96-well plate (Greiner Bio-One), washed in FACS buffer, and stained with CD4-PE (Immunotools) and CD8-PerCP (BD Biosciences) antibodies. In a separate well, PBMC viability was assessed using 7-AAD. Cell proliferation was evaluated using a FACSCalibur cytometer and processed using CellQuest software (BD Biosciences).

ELISA

Supernatant from PBMC cocultures was collected on day 5 and snap frozen. IFN-γ and IL-10 were detected in coculture supernatants using Ready-Set-Go! sandwich

Table 1. Human qPCR Primer Sequences

Gene	Forward Primer (5′–3′)	Reverse Primer (5′–3′)	Product (bp)
CCL2	CTCAGCCAGATGCAATCAATGCCC	TTGGGACACTTGCTGCTGGTGAT	115
CCL5	CCTCCCCATATTCCTCGGACACC	ACGACTGCTGGGTTGGAGCA	121
CCL20	CACGGCAGCTGGCCAATGAAG	CTGTTTTGGATTTGCGCACACAGAC	89
CXCL2	CGCCCCTGGCCACTGAACTG	TGCCCATTCTTGAGTGTGGCTATGA	142
CXCL10	AGAACTGTACGCTGTACCTGCATCA	ACGTGGACAAAATTGGCTTGCAGGA	102
CXCL12	TCGTGCTGGTCCTCGTGCTG	GCTTGACGTTGGCTCTGGCAA	117
CXCL13	AGCCTCTCTCCAGTCCAAGGTGT	GGACAACCATTCCCACGGGGC	137
CX3CL1	ACAGCACCACGGTGTGACGA	CAAGATGATTGCGCGTTTGCCG	124
CCR1	GGGGATGCAACTCCGTGCCA	TTGCACAAGGACCAGGACCACC	129
CCR2	TCAACTGGACCAAGCCACGCA	TTGCAGAAGCGCTTGGTGATGTG	147
CCR3	ACGTGGGCCTGCTCTGTGAAA	GCCCAAGAGGCCCACAGTGA	98
CCR5	CCGCCCAGTGGGACTTTGGA	CAGCATGGACGACAGCCAGGT	129
CCR6	CCACCGGTGCGTGGGTTTTC	GTCAGGAGCAGCATCCCGCA	91
CCR7	GAGCGTCATGGACCTGGGGAAA	CCACTGTGGTGTTGTCTCCGATGT	125
CCR9	AGGTCACCCAGACCATCGCC	CCCGGCGGAATCTCTCACCC	81
CCR10	GCAGGTTTCCTGGGGCCATTACTC	ACGGTCAGGGAGACACTGGGT	132
CXCR2	ACCACCCAACCTTGAGGCACAG	CTGACTGGGTCGCTGGGCTTT	112
CXCR3	GCTTTGACCGCTACCTGAAC	TGTGGGAAGTTGTATTGGCA	190
CXCR4	CTGGGCAGTTGATGCCGTGG	GCGTGGACGATGGCCAGGTA	144
CXCR5	CCCCGTGGCCATCACCATGT	TTCGTCAGGAGCCGCGACAG	117
CXCR6	GGCTGTGTTCCTGCTGACCCA	AAGGCAGGCCCTCAGGTATGC	139
CXCR7	CTGCCGGTCCTTCTACCCCG	CACTGGACGCCGAGATGGCT	146
IL-1β	GATGAAGTGCTCCTTCCAGG	GCATCTTCCTCAGCTTGTCC	140
IL-6	GAGGAGACTTGCCTGGTGAA	GCTCTGGCTTGTTCCTCACT	104
IL-10	GGAGGAGGTGATGCCCCAAGC	TCGATGACAGCGCCGTAGCC	112
TGF-β1	GGTTGAGCCGTGGAGGGGAAAT	TGCCATGAATGGTGGCCAGGT	138
HGF	GCCTCTGGTTCCCCTTCAATAGCA	ACTGTTCCCTTGTAGCTGCGTCC	133
LIF	GCGGCAGGAGTTGTGCC	CCCTGGGCTGTGTAATAGAG	200
CD200	CACCGTCTATGTACAGCCCA	GGTAACAGACGTGGTCCCAT	178
HO-1	AACATCCAGCTCTTTGAGGAGT	GAGTGTAAGGACCCATCGGA	195
COX-1	CGCCCTCGGTAGAAGAGGCG	GAGCCCAGGAAGCAGCCCAA	107
COX-2	TGGCGCTCAGCCATACAGCA	CCTGTCCGGGTACAATCGCACT	109
PTGES	GGAAGAAGGCCTTTGCCAACCC	GTTCCACGTCGGGGTCGCT	81
IDO-1	AGACTGCTGGTGGAGGACAT	ACAAACTCACGGACTGAGGG	118
PD-L1	ACTGTGAAAGTCAATGCCCC	TGCTTGTCCAGATGACTTCG	130
ICAM-1	AGCTTCGTGTCCTGTATGGC	ACAGTCACTGATTCCCCGAT	175
VCAM-1	AATGTTGCCCCCAGAGATACAACCG	GAGCTGCCTGCTCCACAGGA	129
HLA-ABC	TGGGAGCTGTCTTCCCAGCCC	CCACATCACGGCAGCGACCA	105
HLA-DR	AGACAAGTTCACCCCACCAG	AGCATCAAACTCCCAGTGCT	220
HLA-G	GGCCCACGCACAGACTGACAGAAT	CTGGAGGGTGTGAGAACTGGCCTCG	85
HLA-E	TGAGGCGGAGCACCAGAGAGC	TTGGGGGCTCCAGGTGAAGCAG	104
CD80	CCTGCTCCCATCCTGGGCCATT	GGGCAAAGCAGTAGGTCAGGCAG	80
CD86	CGACGTTTCCATCAGCTTGTCTGT	GCCGCGTCTTGTCAGTTTCCA	89
TSG-6	ATATGGCTTGAACGAGCAGC	GCAGCACAGACATGAAATCC	167
TDO-2	GGTTCCTCAGGCTATCACTACC	CAGTGTCGGGGAATCAGGT	101
IDO-2	GCAGTGCCATTGTCTTTGGA	CTCAGCAGGGGCATCTTGTC	179
BDNF	AGGCTTGACATCATTGGCTGA	CGTGTACAAGTCTGCGTCCT	112
NGF	GGACCCAAATCCCGTTGACA	ACAAAGGTGTGAGTCGTGGT	84
NTF3	GAAACGCGATGTAAGGAAGC	TTTCTCGACAAGGCACACAC	185
NTF4	TGACAGGTGCTCCGAGAGAT	AGAGAAGGTCCCACTCAGGG	145
CNTF	CCTATGTGAAGCATCAGGGC	ATGGAAGTCACCTTCGGTTG	209
GDNF	GGTCTGGGCTATGAAACCAA	ACATCCACACCTTTTAGCGG	240

(*continued*)

Table 1. (Continued)

Gene	Forward Primer (5′–3′)	Reverse Primer (5′–3′)	Product (bp)
Reference genes			
GAPDH	GAGTCAACGGATTTGGTCGT	GACAAGCTTCCCGTTCTCAG	185
PGK1	CTGGGCAAGGATGTTCTGTT	GCATCTTTTCCCTTCCCTTC	140
RPL13A	AAGTTGAAGTACCTGGCTTTCC	GCCGTCAAACACCTTGAGAC	171
YWHAZ	CTTGACATTGTGGACATCGG	TATTTGTGGGACAGCATGGA	145
ACTB	GATCATTGCTCCTCCTGAGC	AAAGCCATGCCAATCTCATC	228
CYCA	AGACTGAGTGGTTGGATGGC	TCGAGTTGTCCACAGTCAGC	142
HMBS	GAATGAAGTGGACCTGGTTGT	CTGGTTCCCACCACACTCTT	177
TBP	TATAATCCCAAGCGGTTTGC	GCTGGAAAACCCAACTTCTG	170

bp, base pairs.

ELISA kits (eBioscience) following the manufacturer's protocols. Data are presented as mean concentration (pg/ml) from three independent experiments.

Blocking Assays

To evaluate the need for direct cell–cell contact and the involvement of immunomodulatory molecules indoleamine 2,3-dioxygenase 1 (IDO-1), prostaglandin E2 (PGE_2), programmed death-ligand 1 (PD-L1), and human leukocyte antigen G (HLA-G) in the observed coculture effects, Transwell inserts (0.4 μm pore size, Corning® Transwell®; Sigma-Aldrich, Bornem, Belgium), 200 μM 1-methyl-L-tryptophan (1-MT; Sigma-Aldrich), 10 μM indomethacin (Sigma-Aldrich), or 10 μg/ml blocking antibodies for PD-L1 (Biolegend, London, UK) and HLA-G (87 G; EXBIO Praha, a.s., Vestec, Czech Republic) were added on day 0 and day 3 of coculture.

Coculture of WJ-MSC and Monocyte-Derived Dendritic Cells (DCs)

Monocytes were isolated from PBMCs with 90.2±3.1% purity, using the EasySep™ Human $CD14^+$ Selection Kit (STEMCELL Technologies SARL, Grenoble, France) according to the manufacturer's instructions. For differentiation into immature DCs (iDCs), monocytes (1×10^6/ml) were cultured for 6 days in six-well Transwell plates (bottom well; Corning® Transwell®; Sigma-Aldrich) containing Iscove's modified Dulbecco's medium with HEPES and L-glutamine (IMDM; Lonza) supplemented with 0.5% P/S, 1% NaP, 1% NEAA, 2.5% human AB serum (Invitrogen™, Life Technologies), 25 ng/ml recombinant human IL-4 (R&D Systems, Abingdon, Oxfordshire, UK), and 20 ng/ml granulocyte-macrophage colony-stimulating factor (Gentaur, Kampenhout, Belgium). For coculture, WJ-MSCs were seeded onto Transwell inserts (0.4 μm pore size), at a ratio of 1:3 (WJ-MSC/monocytes), and added on day 0 or day 6. To induce DC maturation (mDC), 1 μg/ml lipopolysaccharide (LPS; *E. coli* 055:B5; Sigma-Aldrich) was added on day 6. Fresh medium was added to iDC controls. After 48 h, DCs were harvested using 5 mM ethylenediaminetetraacetic acid (EDTA; VWR, Heverlee, Belgium) in PBS, and expression of CD11c-APC (BD), CD14-PE, CD209-PE (eBiosciences), and maturation markers CD80-PE, CD83-FITC (BD Biosciences), and CD86-FITC was determined using flow cytometry. Data were acquired on a FACSAriaII using FACSDiva software (BD Biosciences) and expressed as percentage of signal within $CD11c^+$ cells.

Endocytosis Assay

Endocytic activity of DCs was measured by defining the cellular uptake of FITC-dextran. For this purpose, 2×10^6 DCs were incubated in IMDM containing 10 ng/ml FITC-dextran (70 kDa; Sigma-Aldrich) in the dark for 60 min at 37°C. Cells incubated with FITC-dextran on ice served as a negative control. Next, DCs were harvested with cold PBS-EDTA, washed with FACS buffer, and stained with CD11c-APC and CD209-PE at 4°C in the dark. The uptake of FITC-dextran was quantified on a FACSAriaII using FACSDiva software and expressed as median fluorescence intensity (MFI) of FITC signal within $CD11c^+CD209^+$ cells. Specific uptake was calculated as (MFI of cells at 37°C)/(MFI of cells at 4°C). Endocytosis levels of cocultured DCs were expressed as fold increase compared to the respective positive controls.

EAE Induction and Stem Cell Transplantation

Female dark agouti rats (DA rats, 8 weeks old; Janvier Labs, Le Genest-Saint-Isle, France) were immunized by subcutaneous injection at the tail base with 140 μg of recombinant human myelin oligodendrocyte glycoprotein (MOG; generated in-house) emulsified in incomplete Freund's adjuvant (Sigma-Aldrich) and supplemented with 500 μg of heat-inactivated *Mycobacterium tuberculosis* (Difco, Detroit, MI, USA). Immunized animals were weighed and scored daily according to the following neurological scale: 0.5 = partial loss of tail tonus, 1 = complete loss of tail tonus, 2 = hindlimb paresis, 3 = hindlimb paralysis, 4 = moribund, 5 = death, as previously described (9). Upon onset of clinical symptoms,

rats were randomized according to score and weight and transplanted intravenously (IV; lateral tail vein) with 2×10^6 normal or licensed human WJ-MSCs (treatment group) in 500 µl of sterile saline solution (Baxter IV fluids; Val d'Hony-Verdifarm, Beringen, Belgium), or with saline only (control group). Additionally, EAE rats with established disease were transplanted IV with 2×10^6 WJ-MSCs in 500 µl saline at 28 days postimmunization (dpi) to assess the effect on chronic disease course.

Immunohistochemistry

EAE animals were sacrificed 2 weeks after transplantation in the chronic phase of EAE using Ringer perfusion (generated in-house). Brain, spinal cord, lungs, lymph nodes, and spleen were isolated and fixed with 4% paraformaldehyde (Sigma-Aldrich) in 30% PBS-sucrose (Merck Chemicals N.V., Overijse, Belgium) and snap frozen. Eight-micrometer-thick tissue slices were obtained with a Leica CM3050 S cryostat (Leica, Groot-Bijgaarden, Belgium). To show the presence of human cells, a double staining was performed for anti-human mitochondria and anti-rat CD68. Slides were washed with Tris-buffered saline (VWR) containing 0.5% Tween-20 (Merck Chemicals) (TBS-T), blocked with 10% normal goat serum (Dako, Heverlee, Belgium) in TBS-T for 45 min and subsequently incubated overnight with mouse anti-human mitochondria antibody (1:800 in TBS-T; clone 113-1; Millipore, Merck Chemicals). Next the sections were incubated for 1 h with secondary antibody goat anti-mouse IgG conjugated to Alexa Fluor 555 (1:600 in TBS-T; Life Technologies). Slides were thoroughly washed for 5×3 min before incubation with mouse anti-rat CD68 conjugated to Alexa Fluor 488 (1:100 in TBS-T; clone ED1; AbD Serotec, Düsseldorf, Germany) for 1 h. Nuclei were counterstained with DAPI (Molecular Probes®, Life Technologies). Control stainings were performed by omitting the primary antibodies. Demyelination in spinal cord sections was visualized using standard luxol fast blue staining (Sigma-Aldrich). Stained sections were analyzed using a Nikon Eclipse 80i microscope and processed with NIS Elements BR 4.0 software (Nikon Instruments BeLux, Brussels, Belgium).

T-Cell Stimulation Assay

Freshly isolated spleens were segregated using 70-µm strainers (Greiner-Bio). Next, 2×10^5 ficoll-separated (Histopaque®-1077; Sigma-Aldrich) splenic cells were cultured for 48 h in RPMI-1640 medium supplemented with 0.5% P/S, 1% NaP, 1% NEAA, 20 µM 2-mercaptoethanol (Sigma-Aldrich), 2% autologous serum (DA rat serum; Janvier), and 20 µg/ml MOG or 2.5 µg/ml concavalin A (ConA; Sigma-Aldrich). Controls did not receive MOG. Next, 1 µCi [^{3}H]thymidine (PerkinElmer, Waltham, MA, USA) was added to the cultures for 18 h, after which cells were harvested with an automatic harvester (PerkinElmer). Radioactivity was quantified using a β-plate liquid scintillation counter (PerkinElmer), and proliferation is expressed as stimulation index against the unstimulated sample.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 5 (Graphpad Software Inc., La Jolla, CA, USA). Data sets were tested for normal distribution using D'Agostino and Pearson omnibus normality test. A one-way analysis of variance (ANOVA, Tukey's post hoc) and two-tailed unpaired *t*-test were used to test for significant differences. Results are reported as mean with standard error of the mean (SEM). The Kruskal–Wallis (Dunns post hoc) and Mann–Whitney *U* analysis were used for data sets that did not pass normality. Results are reported as median with interquartile range (IQR). EAE data were analyzed as previously suggested (25), using the area under the curve (AUC) for assessment of overall treatment effects. Significant differences are shown as $p < 0.05$, $p < 0.01$, and $p < 0.001$, unless stated otherwise.

RESULTS

WJ-MSCs Express Immune-Modulatory Molecules and Neurotrophic Factors

Using explant culturing, we obtained MSC-like cells from the Wharton's jelly that are plastic adherent and have trilineage differentiation capacity, as also shown in one of our previous studies (71). Isolated WJ-MSCs display the three main mesenchymal markers CD73, CD90, and CD105 and do not express hematopoietic markers such as CD14, CD19, CD34, and CD45 (Fig. 1A). As such, our WJ-MSC cultures comply with the minimal criteria for defining MSCs, described by the International Society for Cellular Therapy (20). Furthermore, WJ-MSCs have a lowly immunogenic phenotype considering their low amounts of costimulatory molecules CD86 and CD40 and lack of HLA-DR and CD80. In line with this phenotypic profile, WJ-MSCs display several molecules with immune-modulatory functions such as *IDO-1*, *IL-10*, *LIF*, *PD-L1*, *COX-2*, *TGF-β1*, *TSG-6*, *CD200*, *HGF*, nonclassical HLA class 1 molecules *HLA-E* and *HLA-G*, and *HO-1* (Fig. 1B). The expression of such inhibitory molecules even further reinforces the hypoimmunogenic characteristics of WJ-MSCs. To define their potency for paracrine support in a neurodegenerative setting, we assessed whether WJ-MSCs express neurotrophic factors. All cultures were positive for *BDNF*, *CNTF*, *GDNF*, *NGF*, and *NTF3* but negative for *NTF4* (Fig. 1B). These results show that WJ-MSCs endogenously express mRNA for key neurotrophins, indicative of the potential to provide CNS trophic support.

WJ-MSCs Suppress Polyclonal T-Cell Proliferation

To determine the immunogenicity and functional suppressive activity of WJ-MSCs, coculture experiments

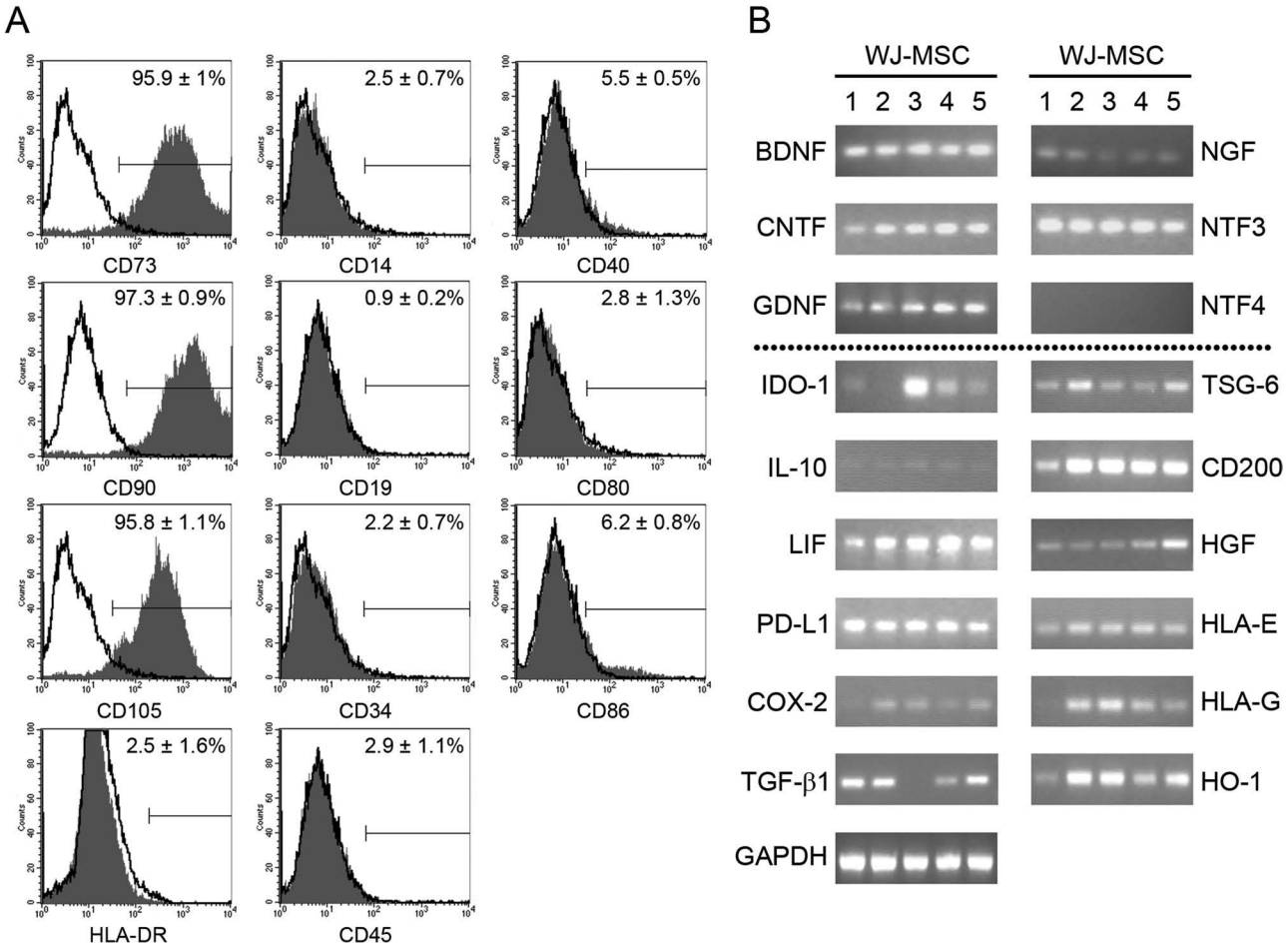

Figure 1. WJ-MSC marker expression profile. (A) WJ-MSC cultures ($n=10$) were assessed using flow cytometry for expression of MSC markers CD73, CD90, and CD105; costimulatory molecules CD80, CD86, CD40, and HLA-DR; and hematopoietic markers CD14, CD19, CD34, and CD45. Isotype control (black transparent graphs) and specific antibody staining (gray opaque graphs) is shown. Expression levels are given as mean % of positive cells ± SEM. (B) PCR analysis indicating gene expression of neurotrophic growth factors and immunomodulatory molecules in standard WJ-MSC cultures ($n=5$). *GAPDH* was used as loading control.

of CFSE-labeled PBMCs and increasing numbers of WJ-MSCs were performed. A low alloreactivity against the stem cells was observed in contact cocultures with nonactivated PBMCs. As shown in Figure 2A, low numbers of WJ-MSCs (ratios 1:100, 1:10) induced a significant increase in T-cell proliferation, which was abolished at higher cell numbers (1:5–5:1). Cell contact is essential for allogeneic reactivity, since alloreactivity was no longer observed in Transwell cultures without aCD3 (Fig. 2B). Next, we investigated the suppressive potential of WJ-MSCs toward polyclonal activation of T cells. In this setting, increasing numbers of stem cells dose-dependently suppressed aCD3-induced T-cell proliferation (Fig. 2A). Furthermore, WJ-MSCs still significantly inhibited polyclonal T-cell proliferation in Transwell cultures at higher ratios (2:1 and 5:1) (Fig. 2B). The dose-dependent decrease of CD4 and CD8 T-cell proliferation was not related to stem cell crowding, since cell death did not increase when WJ-MSCs were added (Fig. 2C).

In line with the observed suppression on T-cell proliferation, we quantified the amount of IFN-γ and IL-10 released during coculture (Fig. 2D, E). No IFN-γ production was observed in nonstimulated cocultures, but when T cells were activated using aCD3, IFN-γ production was induced ($p<0.001$ vs. PBMC). In coculture, a dose-dependent decrease in IFN-γ levels was observed, with the strongest decrease at higher ratios (1:1, 2:1, and 5:1; compared to PBMC + aCD3). Furthermore, when WJ-MSCs were added, relatively constant amounts of IL-10 were produced (Fig. 2E). Although the stem cells express low levels of IL-10 mRNA (Fig. 1B), cytokine production was not detected in culture supernatant from normal or licensed WJ-MSCs ($n=5$; assay detection limit 2 pg/ml) (Fig. 2E). As such, WJ-MSCs induced IL-10 production independent of coculture ratios.

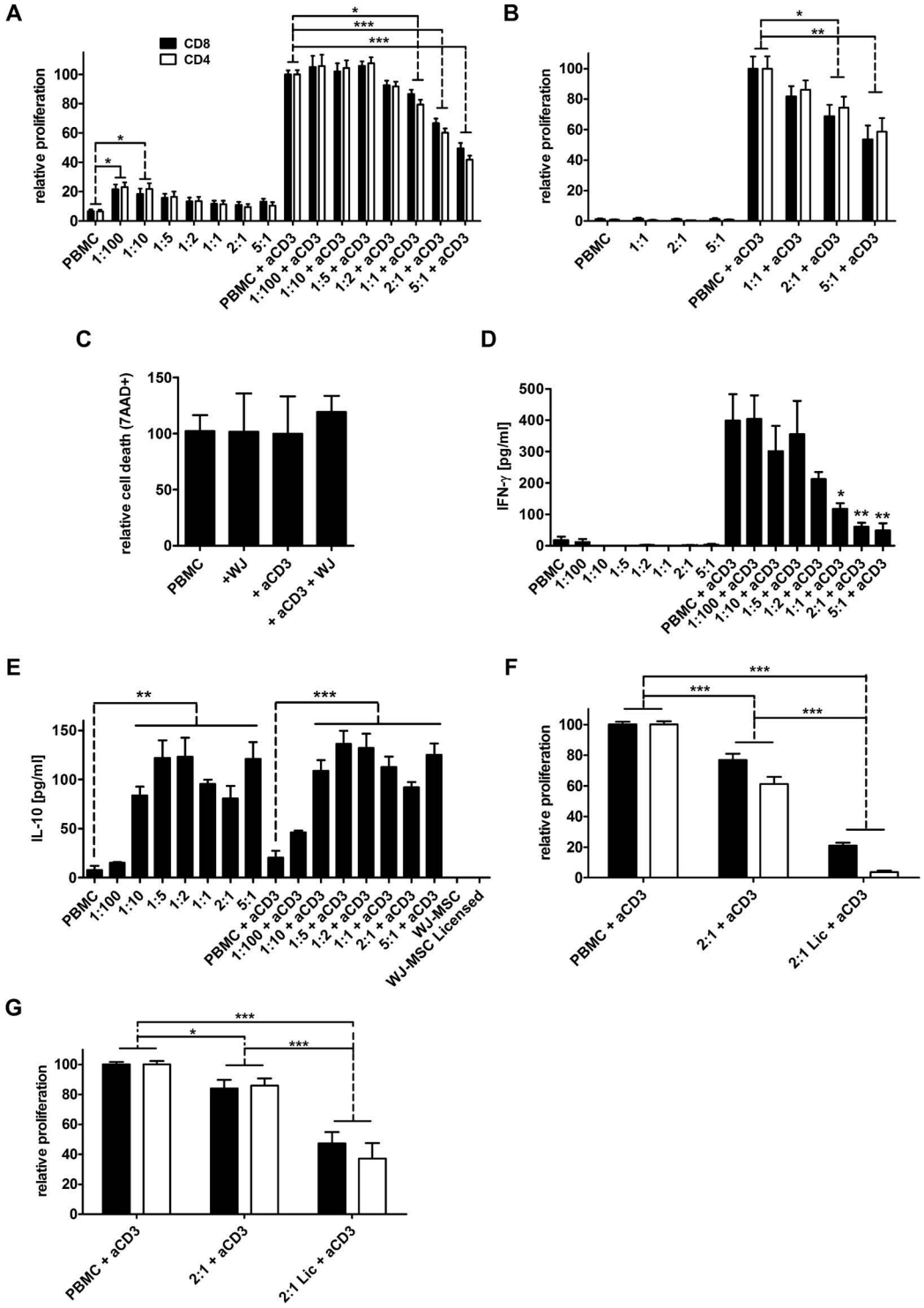

Figure 2. WJ-MSCs inhibit T-cell proliferation in cocultures with PBMCs. CD4 (white bars) and CD8 (black bars) T-cell proliferation was measured in contact (A, F) and in Transwell (B, G) culture setup. The 5×10^4 CFSE-labeled PBMCs were cocultured in different WJ-MSC/PBMC ratios, with or without aCD3, or cultured with licensed WJ-MSCs (F, G; 2:1 Lic). Data are represented as mean proliferation ± SEM relative to maximum control proliferation (PBMC + aCD3). (C) Cell death was measured after coculture with WJ-MSCs (+WJ), using 7-AAD MFI signal relative to control PBMCs, and given as median ± IQR. Data were derived from three representative cocultures in contact setup, for culture ratios 1:1 and 2:1. (D, E) IFN-γ and IL-10 cytokine content was measured using ELISA and given as absolute concentration (pg/ml) for each culture condition of three representative contact cocultures and for five WJ-MSC isolates. Data are represented as mean ± SEM. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$, ANOVA.

Taken together, these results show that WJ-MSCs suppress T-cell proliferation via contact-dependent mechanisms as well as through soluble molecules. Additionally, they can functionally inhibit IFN-γ production by activated T cells and induce IL-10 secretion within coculture.

Licensing Promotes the Immunosuppressive Potential of WJ-MSCs

Stem cells are subjected to an inflammatory milieu upon transplantation in individuals with autoimmune disease. This phenomenon is called "licensing" (38). In this context, the effect of a proinflammatory environment on the immunosuppressive properties of WJ-MSCs was investigated. Prior to coculture, WJ-MSCs were exposed to IFN-γ, IL-1β, and TNF-α. Subsequently, we show that licensed stem cells are significantly more suppressive compared to nonlicensed cells (Fig. 2F, G). In both contact and Transwell cocultures, a significant reduction in CD4 and CD8 T-cell proliferation was observed compared to nonlicensed cultures and control (PBMC + aCD3). The increased capacity of licensed Wharton's jelly cells to suppress T-cell proliferation was less pronounced when cell contact was absent. As seen in Figure 2F and G, proliferation in Transwell cultures was higher compared to contact-inhibited proliferation (CD4: 36.2 ± 9.1% vs. 3.7 ± 1%; CD8: 47.3 ± 7.4% vs. 20.9 ± 1.9%; $p < 0.01$, unpaired *t*-test). Since cellular interaction during coculture gives an increased suppressive effect, this indicates that licensing alters the surface marker profile of WJ-MSCs toward a more immunemodulatory phenotype.

Licensing Increases the Expression of Immune-Modulatory Molecules

To explore the mechanism behind the increased T-cell suppression observed after licensing, quantitative PCR analysis was performed to screen for differential expression of immunomodulatory factors in cytokine-primed versus unprimed WJ-MSCs. A prominent change in gene expression of several immunologically relevant markers was observed upon stimulation with proinflammatory cytokines, IFN-γ, TNF-α, and IL-1β (Figs. 3, 4, treated). An upregulated mRNA expression of chemokines, such as *CCL2*, *CCL5*, *CCL20*, *CXCL2*, and *CXCL10*; cytokines, such as *IL-10*, *IL-6*, and *IL-1β*; MHC molecules, including *HLA-ABC*, *HLA-E*, and *HLA-G*; as well as immune-modulatory molecules *TSG-6*, *PD-L1*, *ICAM-1*, *VCAM-1*, *COX-1*, *PTGES*, *IDO-1*, and *HGF* was consistently observed. Furthermore, while not endogenously expressed in standard WJ-MSC cultures (Fig. 5), expression of *CXCR3*, *CXCR4*, *CXCR5*, *CX3CL1*, and *HLA-DR* was induced after licensing. Expression of all other tested genes did not significantly change (for details, see Figs. 3–5).

These data indicate that, while WJ-MSCs endogenously express several immune-modulatory molecules, inflammatory mediators significantly affect their immunological profile.

Blocking IDO-1 Abrogates the Suppressive Activity of WJ-MSCs

MSCs have been reported to inhibit lymphocyte proliferation in vitro via several effector mechanisms involving contact-dependent and secreted molecules (89). Here we investigated the involvement of HLA-G, PD-L1, COX-1/2, and IDO-1 in the observed suppression of T cells by WJ-MSCs. These molecules were upregulated upon licensing of WJ-MSCs (see Fig. 4) and could thus underlie the inhibition of proliferation induced by the stem cells. We found that inhibition of IDO-1 using 1-MT counteracted the capacity of Wharton's jelly cells to suppress T-cell proliferation. Of note, when prelicensed WJ-MSCs were used, 1-MT only partially restored lymphocyte proliferation (Fig. 6). As was shown earlier, licensing induces expression of additional inhibitory molecules within the WJ-MSCs that may account for the remaining suppressive effects on proliferation. Yet, blocking of other effector molecules, such as COX-1, HLA-G, and PD-L1, did not change WJ-MSC-mediated T-cell suppression (data not shown).

We show that Wharton's jelly cells can directly modulate T cells using IDO-1. IDO is an IFN-γ inducible enzyme that causes inhibition of T-cell proliferation through depletion of the essential amino acid tryptophan (47). Tryptophan catabolism and its resulting kynurenine metabolites induce T-cell transition toward anergy, apoptosis, or regulatory T cells (18). Since TDO-2 and IDO-2, enzymes that are known to catalyze the same reaction (17), were not expressed by WJ-MSCs, the observed effects are IDO-1 specific (see Figs. 4, 5).

WJ-MSCs Modulate Dendritic Cell Differentiation and Maturation

In the immunopathogenesis of MS, DCs are crucial players, as they present myelin antigens to T cells, thereby inducing breakdown of self-tolerance (10,75). To define the impact of WJ-MSCs on antigen-presenting cells, they were cultured with DCs during different differentiation and maturation stages. For this, $CD14^+$ monocytes were differentiated into iDCs and matured toward mDCs in the absence or presence of WJ-MSCs. Differentiation into iDCs was characterized by a significant decrease in CD14-expressing cells (Fig. 7A, ctrl). Addition of LPS induced maturation into mDCs, thereby enhancing the expression of maturation and activation markers CD80, CD83, and CD86 (Fig. 7B–D, ctrl). Interestingly, coculture with WJ-MSCs influenced the phenotype of the monocyte-derived DCs. When stem cells were added from

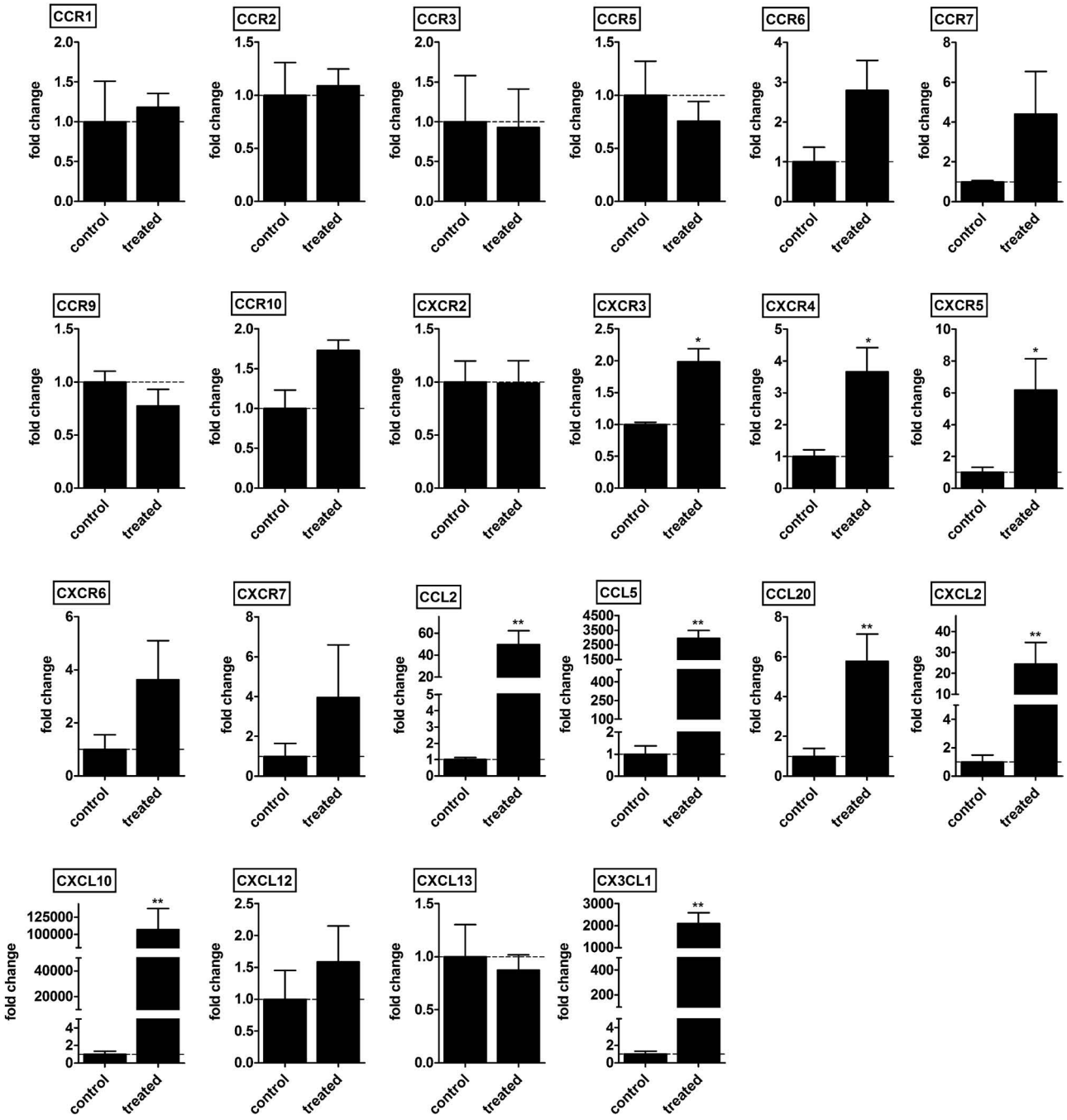

Figure 3. Differential expression of chemokine receptors and chemokines after 24-h treatment with proinflammatory cytokines. Comparison of differential gene expression (fold change) between untreated (control) and cytokine-exposed WJ-MSCs (treated). Gene quantitation was performed using the comparative Ct method, and expression levels were normalized to the most stable reference genes determined by Genorm (*TBP* and *ACTB*). Data are derived from five biological replicates and given as mean±SEM. $*p<0.05$ and $**p<0.01$, unpaired *t*-test.

the beginning of culturing, differentiation toward iDCs was significantly inhibited, leaving more CD14-expressing cells in culture (Fig. 7A). Furthermore, WJ-MSCs interfered with the maturation of iDCs toward mDCs. CD80, CD83, and CD86 levels were significantly reduced in cocultured mDCs compared to control (Fig. 7B–D). This effect was less pronounced when stem cells were introduced into the cultures on day 6 (+WJ-MSC 2 days). Moreover, stem cell addition at this time point did not increase the expression of maturation markers, indicating that WJ-MSCs do not induce DC maturation on their own in the absence of an LPS stimulus (Fig. 7B–D, 2 days, white bars).

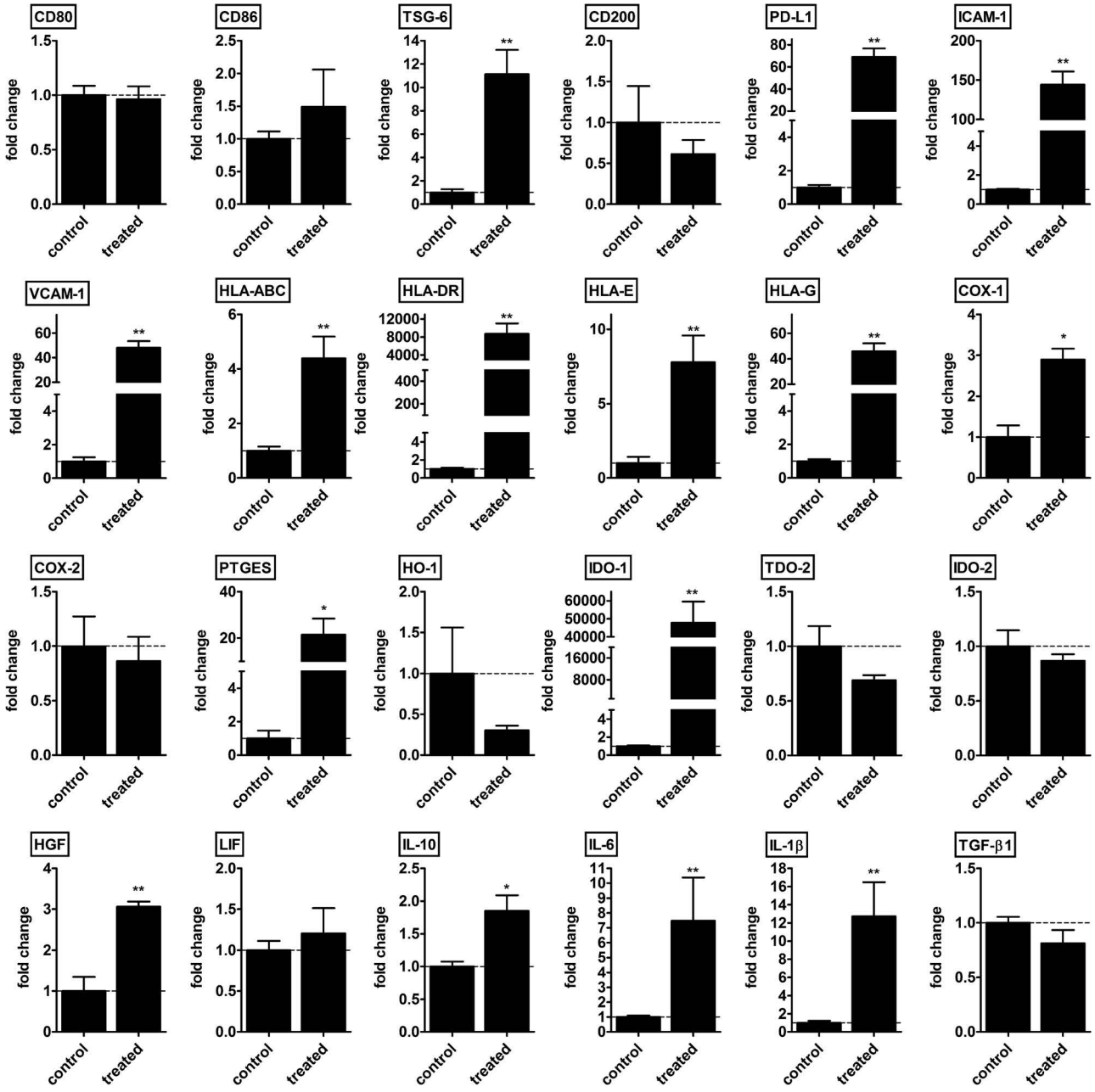

Figure 4. Differential expression of costimulatory and coinhibitory immunological molecules after 24-h treatment with proinflammatory cytokines. Comparison of differential gene expression (fold change) between untreated (control) and cytokine-exposed WJ-MSCs (treated). Gene quantitation was performed using the comparative Ct method, and expression levels were normalized to the most stable reference genes determined by Genorm (*TBP* and *ACTB*). Data are derived from five biological replicates and given as mean ± SEM. $*p < 0.05$ and $**p < 0.01$, unpaired *t*-test.

To assess the influence of WJ-MSCs on the functional properties of DCs, cellular uptake of FITC-dextran via endocytosis was measured. Endocytic functioning is considered a hallmark of iDCs and decreases when DCs mature to enhance antigen presentation (70). DCs that were primed with WJ-MSCs showed a significantly higher endocytic activity compared to control DCs. These results indicate a more immature DC phenotype when cocultured with WJ-MSCs (Fig. 7E, F). Notably, the level of endocytotic activity in mDCs did not differ from control when cocultured for 2 days instead of 8 days.

As such, these data suggest a limited effect of WJ-MSCs on functionality of true mDCs (Fig. 7G) and propose that their immune-modulatory effects mainly occur during differentiation and to a lesser extent during maturation of DCs. In contrast to PBMC cocultures, licensed Wharton's

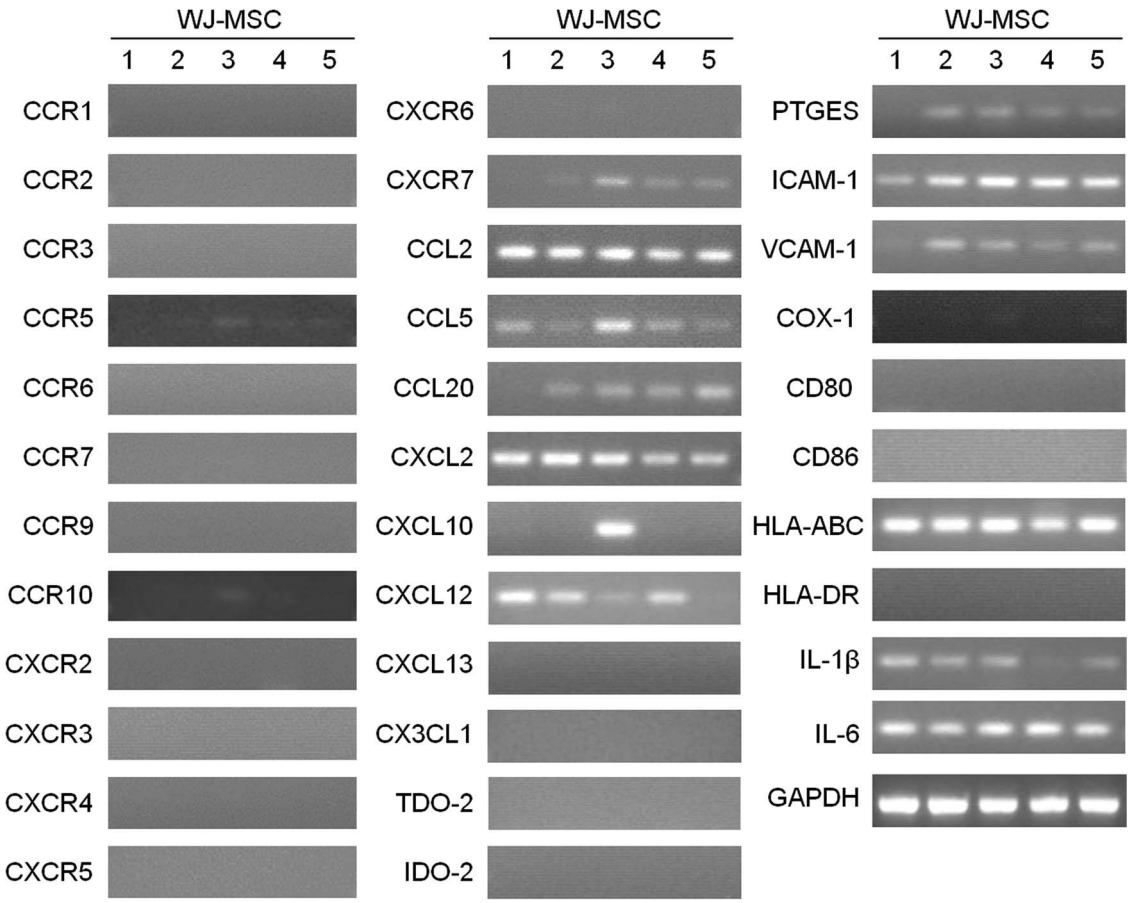

Figure 5. Basal gene expression in WJ-MSCs. qPCR products of untreated WJ-MSC cultures ($n=5$) were visualized on 2% agarose gel for detection of amplification. *GAPDH* was used as a loading control.

jelly cells did not enhance their inhibitory effect on differentiation, maturation, or endocytosis of DCs (data not shown). In summary, these results show that WJ-MSCs can modulate DC functionality by decreasing their costimulatory and antigen-presenting capacity.

Transplantation of Human WJ-MSCs Transiently Improves EAE Symptoms

Considering that WJ-MSCs suppress T-cell proliferation and modulate monocyte/DC differentiation, the therapeutic potential of the cells was established in vivo in the chronic rat EAE model for MS. Immunization of animals with MOG induced a chronic clinical EAE course, with disease incidence of 100% (Table 2). We show that WJ-MSC-treated animals display lower neurological symptoms compared to saline and licensed MSC-treated animals (AUC, WJ-MSC: 81.3±12.1 vs. saline: 125.5±12.8, vs. licensed WJ-MSCs: 113.1±15.5; $p<0.05$, Kruskal–Wallis) (Fig. 8A), only in the first 2 weeks after disease onset. This resulted in an overall reduction in cumulative disease score for WJ-MSC-treated rats (Table 2). Furthermore, stem cell-transplanted

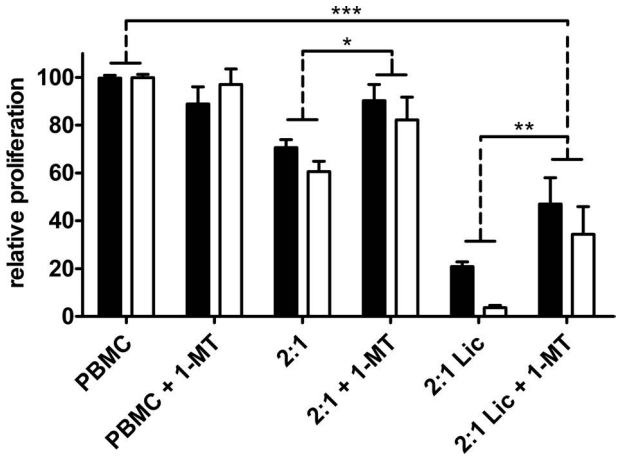

Figure 6. Blocking IDO-1 function using 1-MT restores polyclonal T-cell proliferation. CD4 (white bars) and CD8 (black bars) T-cell proliferation after aCD3 stimulation in cocultures with WJ-MSCs (2:1) or licensed WJ-MSCs (2:1 Lic). IDO-1 activity was blocked by addition of 1-MT on day 0 and day 3 of culture (+1-MT). Data are derived from six independent experiments and given as mean proliferation±SEM relative to control (PBMC). *$p<0.05$, **$p<0.01$, and ***$p<0.001$, ANOVA.

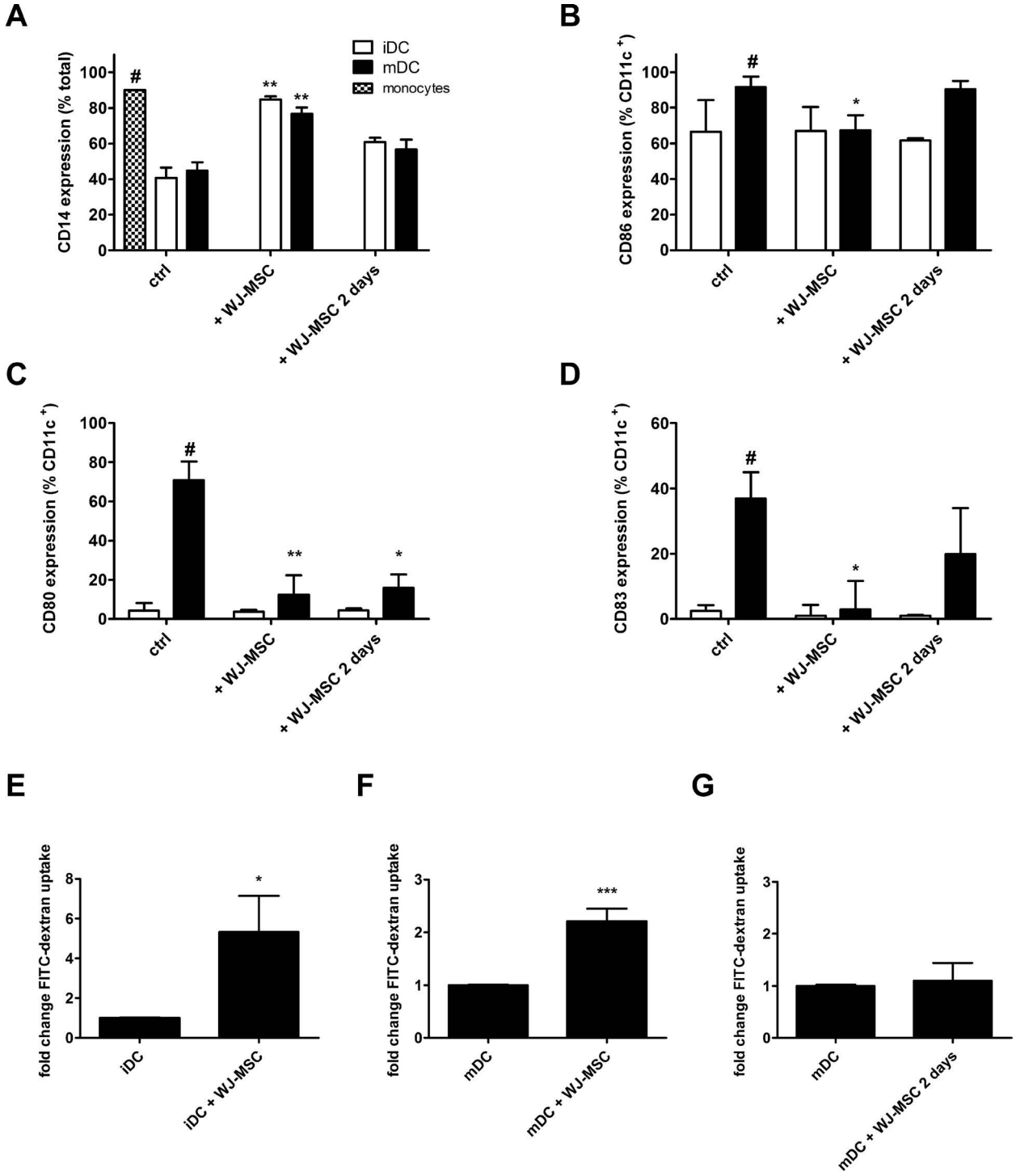

Figure 7. Monocyte-derived DC differentiation and maturation is impaired by WJ-MSCs. Expression of maturation markers and functional endocytosis of iDCs and mDCs was determined after coculture using flow cytometry. Absolute CD14 expression is shown from total cell cultures (A), and expression of CD86 (B), CD80 (C), and CD83 (D) was defined within $CD11c^+$ cells. Cultures without stem cells served as control (ctrl). Cocultures were performed for a total of 8 days in iDC setting (without LPS on day 6; white bars A–D) and mDC setting (with LPS on day 6; black bars A–D). For cultures indicating 2 days WJ-MSC addition, Transwells with stem cells were added on day 6. (E–G) Endocytosis capacity was assessed by uptake of FITC-dextran particles after coculture. MFI of FITC within $CD11c^+CD209^+$ DCs was shown as mean fold difference from control. All data were derived from four independent experiments and are represented as mean±SEM (A, ANOVA; E–G, unpaired *t*-test) or median±IQR (B–D, Kruskal–Wallis). $*p<0.05$, $**p<0.01$, and $***p<0.001$ compared to the equivalent control conditions (DCs without WJ-MSCs). $\#p<0.01$ compared to iDC control condition.

Table 2. Clinical Data From Chronic EAE Rats

Treatment	Disease Incidence	Mean Score at tpl	Mean Weight at tpl (g)	Day Disease Onset (dpi)	Maximum Clinical Score	Cumulative Clinical Score	Mortality Due to EAE
Onset transplantation							
Saline	100%	2.1±1.1	149.6±14.1	12±1.7	4.4±0.2	127.5±12.9	9/15
WJ-MSC	100%	1.9±0.9	155.2±14	12.1±1.8	4.2±0.2	83.1±12.1*	2/15
Licensed WJ-MSC	100%	2±0.9	158.2±12.9	12.2±2.5	4.6±0.2	115.3±15.6	3/10
Chronic transplantation							
Saline	100%	2.9±0.9	134.5±10.5	13.7±2.2	3.8±0.1	92.2±6.7	0/9
WJ-MSC	100%	3±0.8	140.9±12.1	12.5±1.4	4.1±0.1	90.7±6.5	0/11

Data as mean±SEM. tpl, transplantation; dpi, days postimmunization; WJ-MSC, Wharton's jelly-derived mesenchymal stromal cells.
*$p < 0.05$ versus saline, ANOVA.

animals showed an increased survival compared to saline-injected rats (Fig. 8B).

As was shown earlier, licensing had profound effects on WJ-MSCs in vitro regarding the expression of several homing, adhesion, and immunological molecules. Interestingly, transplantation of licensed WJ-MSCs did not lead to a greater beneficial effect on clinical symptoms. In fact, neurological scores and survival of animals treated with licensed stem cells did not differ significantly from control (Fig. 8A).

In an additional experiment, WJ-MSCs were transplanted during the chronic disease course to assess whether they could ameliorate established disease. In this setting, stem cells were injected IV after 28 dpi (Fig. 8C). Control animals received saline. Similar to onset transplanted animals, WJ-MSC-treated rats displayed significantly reduced disease (AUC, WJ-MSC: 28.6±2.9 vs. saline: 35.3±2.2; $p<0.05$, Mann–Whitney U) after transplantation. The therapeutic effect lasted for a period of about 1 week. Furthermore, 2.5 weeks after transplantation, the level of spinal cord demyelination was not different between treated and control animals (Fig. 8D).

WJ-MSCs Are Trapped in Lungs and Spleen and Modulate T-Cell Proliferation In Vivo

Histological analyses of CNS, lung, and lymphoid tissue were performed to define the processes underlying the observed differences in clinical scores between WJ-MSC and saline-treated animals. No stem cells could be retrieved in tissues of animals treated at onset of symptoms (data not shown). WJ-MSCs were detected in lung and spleen 2.5 weeks after transplantation in the chronic disease phase, as was shown by anti-human mitochondrial staining (Fig. 9A). In both tissues, human cells were found close to or in contact with ED-1$^+$ macrophages. Furthermore, human stem cells were not observed in the lymph nodes, spinal cord, or brain of transplanted animals, indicating that WJ-MSCs did not home beyond the lung and spleen (data not shown).

To assess the impact of WJ-MSCs on pathogenic lymphocyte responses in the periphery, an ex vivo restimulation assay was performed. Splenocyte proliferation was assessed after MOG and ConA stimulation. A significant reduction in the proliferation of splenocytes from WJ-MSC-treated animals was observed after incubation with MOG compared to splenocytes from the saline group (Fig. 9B). No difference in proliferation to ConA was observed. As such, our data demonstrate that transplantation of WJ-MSCs can modulate immune responses in vivo. To determine whether specific rat T-cell lineages were affected, we examined the differential splenic expression of Th1-, Th2-, Treg-, and Th17-related transcription factors Tbet, GATA-3, FOXP3, and ROR-γt and their related cytokines IFN-γ, TGF-β1, IL-10, and IL-17, respectively. No significant changes in the expression of these T-cell transcription factors or respective cytokines were observed between treated and control animals (data not shown).

Collectively, these data indicate that WJ-MSC transplantation modulates T-cell proliferation without affecting their polarization. Even more, the stem cells are located

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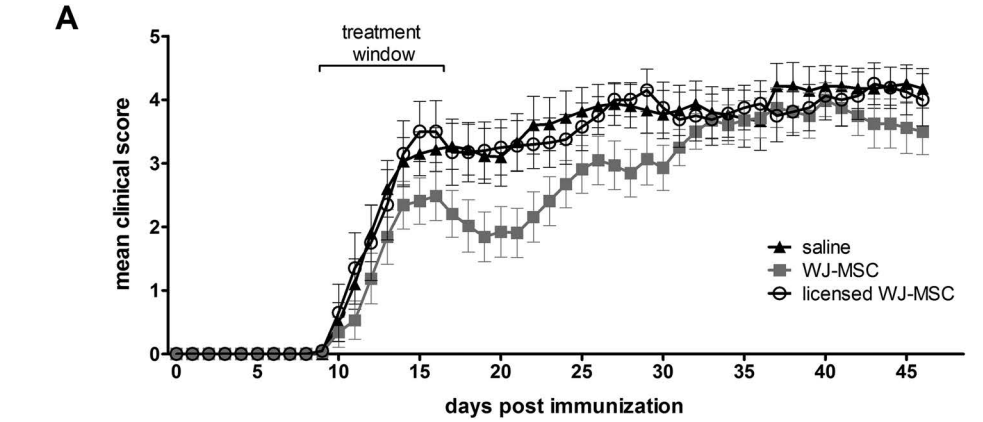
Figure 8. IV transplanted WJ-MSCs ameliorate neurological symptoms in chronic EAE animals and increase survival. (A) MOG immunized animals were injected IV with saline ($n=15$; black triangles), 2×10^6 WJ-MSCs ($n=15$; gray squares), and 2×10^6 licensed WJ-MSCs ($n=10$; black circles) at onset of clinical symptoms. Mean clinical scores for each group are given starting from day of immunization. (B) Survival curves indicate a significantly improved survival for WJ-MSCs (**$p<0.01$, log-rank), but not for licensed WJ-MSC-treated animals, as was determined using Kaplan–Meier analysis. (C) EAE animals with established disease were transplanted IV with 2×10^6 WJ-MSCs ($n=11$) or saline ($n=9$) at day 28 postimmunization (arrow). Clinical course was monitored for an additional 2.5 weeks. (D) Luxol fast blue staining of spinal cords from treated and control animals showing demyelinated areas (arrow) in both groups. One representative animal from each group ($n=4$/group) is shown at 4× magnification (scale bar: 500 μm). (A, C) Neurological disease scores are represented as mean±SEM.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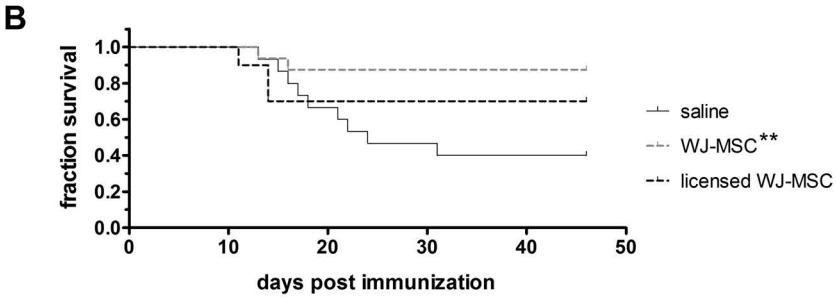
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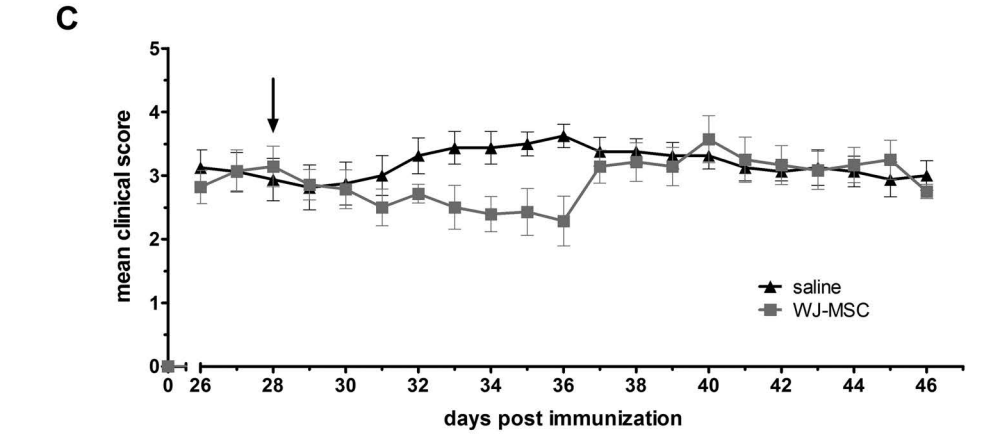
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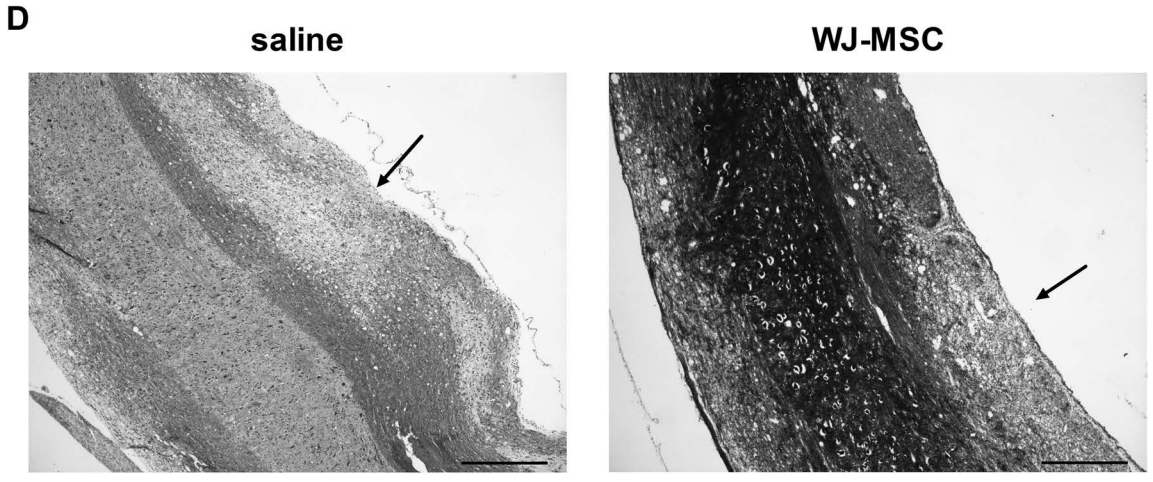
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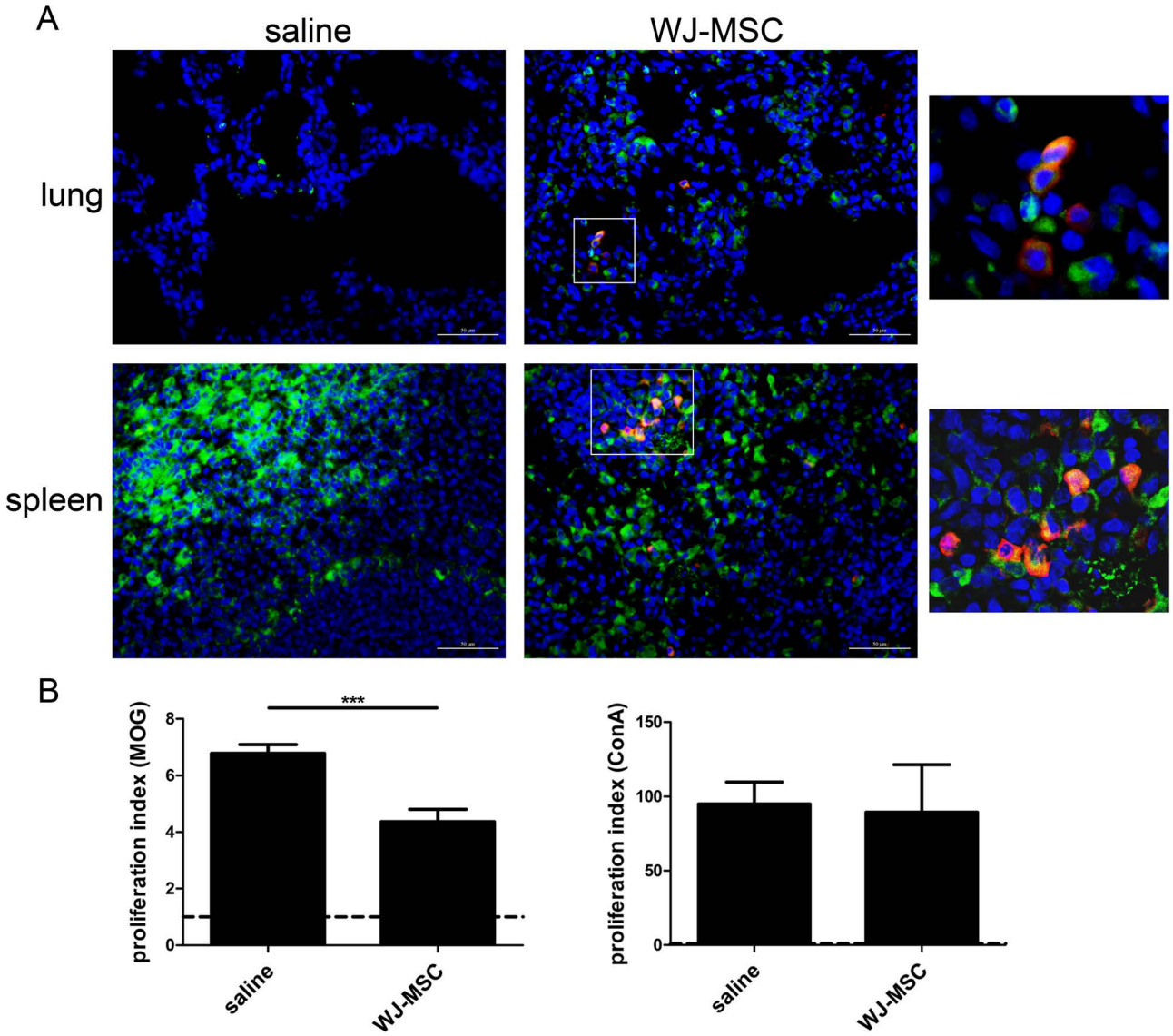

Figure 9. IV-injected WJ-MSCs primarily home to lungs and spleen and interact with resident immune cells. (A) Lung and spleen cryosections of treated and control animals (2.5 weeks post-chronic phase transplantation; $n=4$) were double stained with anti-human mitochondrial antibody (red) and anti-rat ED1 (green). Cells positive for human mitochondria were found in proximity to alveolar and red pulp macrophages. One representative picture is shown (40× magnification, scale bar: 50 µm). (B) Upon sacrifice 2.5 weeks after transplantation, mononuclear cells from spleens of WJ-MSC and saline-treated rats were stimulated ex vivo with MOG immunization antigen or ConA. Proliferative response was determined using [3H]thymidine incorporation. Nonstimulated cultures were used as control (dotted line). Data represent the mean ± SEM of four experiments. ***$p<0.001$, unpaired t-test.

alongside macrophages, suggesting they modulate innate immune cells in lungs and spleen as well. Interaction with these cells may contribute to downstream immunosuppressive effects that could ultimately yield an improved therapeutic outcome.

DISCUSSION

In this study, we show that human umbilical cord matrix-derived stem cells (WJ-MSCs) display features that are relevant for both immune modulation and regeneration in MS therapy. Phenotypically, our Wharton's jelly cell isolates show a fibroblast-like morphology and nonhematopoietic profile (71). They express HLA class I, but not class II, and display low levels of costimulatory molecules. In agreement with previous reports, the WJ stem cells are a hypoimmunogenic mesenchymal-like cell population (11,29,73). Likewise, we confirm that WJ-MSCs express mRNA for several cytokines, growth factors, and immunosuppressive molecules. Our findings indicate that they can provide

neurotrophic support through growth factors, namely BDNF, GDNF, NGF, CNTF, and NTF-3. Additionally, WJ-MSCs hold strong potential for immunomodulatory activities toward leukocytes via, for example, IDO-1 and other molecules. Furthermore, a set of chemokine receptors and chemokines is expressed, indicating WJ-MSCs' homing capacity and potential to enhance recruitment of lymphocytes using, for example, CCL20, CCL2, and CCL5, as previously suggested (31,53,62,86). It should be noted that interdonor differences were observed. We minimized confounding factors by standardizing our cord processing method and processing the cords within 24 h postpartum. Nevertheless, donor variability can still be attributed to differences in cord quality or age and genetics of the donor (35). The cells used in functional assays and for in vivo transplantation were screened and had a gene expression profile in line with that of the majority of donors analyzed.

Using coculture experiments, we provide substantial evidence for a functional immunomodulatory phenotype of WJ-MSCs. WJ cells dose-dependently suppressed polyclonal-induced T-cell proliferation and IFN-γ production, in contact cocultures and to a lesser extent in a Transwell system. Of note, the T-cell suppressive phenotype of our WJ-MSCs was enhanced after exposure to proinflammatory cytokines. It has already been determined that exposure to IFN-γ can influence the immunological properties of BM-MSCs (38,39,63). This phenomenon is called "licensing" and involves a multistep process that encompasses (a) the stimulation of MSCs by inflammatory cytokines, such as IFN-γ, TNF-α, and/or IL-1β, (b) thereby inducing or increasing the expression of different immunological effector molecules (23,68). Correspondingly, we observed that exposure to inflammation (as mimicked by 24-h licensing), leads to the upregulation of many regulatory molecules, adhesion molecules as well as several cytokines and chemokines.

In an effort to determine which mechanisms account for the observed suppressive effect, we blocked several candidate effector molecules in cocultures of WJ-MSCs and stimulated PBMCs. Here we found that IDO-1 had a major role in the antiproliferative effects on T cells, as blocking of its activity by 1-MT completely abrogated the suppressive activity of WJ-MSCs. IDO is expressed by several immune cells, such as DCs and monocytes, but also by trophoblast cells, mediating fetal acceptance during pregnancy (4,51). As such, it might not be surprising to find IDO expression in MSCs derived from extraembryonic Wharton's jelly. Furthermore, licensing increased the suppressive capacity of WJ-MSCs as well as the expression of IDO-1. Addition of 1-MT in licensed cocultures only partially abrogated the inhibition of lymphocyte proliferation, indicating that additional mechanisms are implicated in the suppressive effect of licensed WJ-MSCs. We demonstrated that licensing increased numerous regulatory molecules that could account for the remaining suppression. Therefore, we assessed the involvement of previously described mechanisms such as HLA-G, PD-L1, and PGE_2 (3,52,65,79). However, blocking their function did not affect suppression of T-cell proliferation in our study.

Given the pleiotropic activity of many factors secreted or expressed by WJ-MSCs, we cannot rule out the involvement of other immune cells. Several articles implicate antigen-presenting cells as an intermediary partner for indirect modulation of T-cells (13,19,46). It has been reported that BM-MSCs instruct monocytes to secrete IL-10, in turn affecting the other cells in coculture (7,45,55). Indeed, we observed the induction of IL-10 in cultures of WJ-MSCs with both naive and stimulated PBMCs. IL-10 production remained rather constant while stem cell numbers were increased, suggesting that IL-10 is mainly produced by the immune cells present. Further in support of an indirect effect through antigen-presenting cells, we observed that WJ-MSCs could actively interfere with the differentiation, maturation, and functionality of monocyte-derived DCs. We show that WJ-MSCs inhibit DC generation and maturation in a cell contact-independent coculture setup, resulting in a reduced capacity of DCs to stimulate T cells. Moreover, functional endocytosis by DCs was increased, again reflecting a more immature DC phenotype induced by the stem cells. Several research groups demonstrated similar effects for adult MSCs (7,13,40).

Our in vitro characterization shows that WJ-MSCs are endowed with potent immune regulatory properties, enabling them to modulate both innate and adaptive immune cells. To define their therapeutic potential for MS, we transplanted WJ-MSCs in the chronic EAE model of MS (6). Human WJ-MSCs temporarily improved EAE neurological symptoms after IV transplantation, at onset of symptoms as well as in established disease. Treated animals displayed a reduced disease severity and had increased survival rates compared to saline-treated controls. However, over a prolonged period of time, transplanted animals clinically deteriorated again. This could imply that the beneficial effect of WJ-MSC transplantation wears off over time, suggesting repeated dosage strategies to sustain therapeutic potential.

This report indicates that WJ-MSCs have only a transient beneficial effect on EAE symptoms. We show that this can be achieved both after transplantation at onset of symptoms and in chronically diseased animals. In contrast, Payne and coworkers demonstrated no beneficial effect when transplanting different types of MSCs at onset of symptoms in chronic EAE mice (59). Further conflicting with our results, Liu et al. (41) recently reported sustained therapeutic activity of human WJ-MSCs in chronic mouse EAE. Although their animal model is

different from ours, they found that transplantation of human WJ-MSCs ameliorated disease course for 50 days. Furthermore, they show that transplantation induced a shift of Th1 to Th2 immunity and reduced demyelination, as already described for BM-MSCs (5). In our hands, no amelioration of demyelination in the spinal cord of WJ-MSC-treated EAE rats was observed. In addition, we did not measure significant differences in transcription factors related to either Th1, Th2, or Th17 phenotype between treated and control rats. In our model, the main mechanism is a reduction of autoreactive T-cell priming, since proliferation of splenocytes from WJ-MSC-treated animals was strongly decreased after ex vivo restimulation with cognate antigen. Moreover, we identified human WJ-MSCs in the spleen in close contact with macrophages, suggesting an immune-modulatory mechanism mediated through these cells. As such, we hypothesize that WJ-MSCs exert immunomodulatory effects in vivo through both direct interactions with T-cells and indirect interactions with antigen-presenting cells, as also demonstrated in our in vitro culture systems.

The difference between our study and those that showed prolonged recovery effects may result from differences in EAE severity (49), as differential behavior of BM-MSCs in mild and severe EAE has been described (32). In our study, a highly severe EAE was induced, which may present a different microenvironment for cell transplantation. It has been suggested that a highly inflammatory milieu will license the cells upon injection, inducing a multifunctional immune-regulatory phenotype, but also increasing their immunological visibility (through HLA-DR or costimulatory markers), which promotes graft clearance over time. Indeed, other studies showed that IFN-γ-primed allogeneic MSCs were acutely rejected upon transplantation, due to increased immunogenicity, while nonprimed cells were not (24,62). Likewise, WJ-MSCs are highly sensitive to proinflammatory cytokine stimulation, as illustrated by our in vitro licensing studies and by others (61). Moreover, in vitro prelicensed WJ-MSCs did not ameliorate symptoms in a chronic rat EAE model. Therefore, we speculate that cytokine-primed stem cells are more rapidly rejected from the host, due to enhanced allogeneic immune recognition, while unprimed cells are licensed in vivo and gain enhanced immunosuppressive activity on-site.

Apart from direct immune modulation, WJ-MSCs show potential trophic support functions as they express growth factors such as BDNF, GDNF, and NGF. However, we did not observe reduced myelin damage after stem cell transplantation. Although the clinical effect was only transient and demyelination was measured at the end of the experiment, we cannot rule out that trophic supportive functions were active during the clinical recovery phase. To elucidate the direct regenerative and neuroprotective effects of WJ-MSCs, local application into the CNS of demyelinating rodent models would be a preferred approach. Other reports further highlight the trophic properties of WJ-MSCs (11). WJ-derived oligodendrocyte precursors were shown to produce neurotrophins and increase remyelination upon transplantation in EAE mice (48,87). Yang et al. (85) observed increased neurotrophic factor expression and axonal regeneration after transplantation of human WJ-MSCs in a spinal cord injury model. Promoting remyelination is crucial for suppressing MS disease progression and remains a major challenge. Cell-based therapies may boost remyelination in addition to their anti-inflammatory properties, which altogether may promote endogenous tissue regeneration.

CONCLUSIONS AND FUTURE DIRECTIONS

WJ-MSCs show a promising expression profile for immunomodulatory and neurotrophic support. They actively interact with immune cells by suppressing the activation and proliferation of T cells, but also by modulating DC differentiation and maturation. Upon transplantation WJ-MSCs transiently improve the clinical symptoms of severe chronic EAE, through temporary modulation of the host immune responses in the periphery. Though most of the allograft is cleared after injection, a small number of cells survived up to 3 weeks. Here they appeared to interact with alveolar and splenic macrophages, suggestive of ongoing immune modulation. Despite the fact that WJ-MSCs display a promising phenotype for future intervention strategies in MS, our in vivo data warrant further investigation into the immunological and regenerative potential of the cells. The fact that recovery was not permanent may raise the question if these stem cells are truly exerting regenerative effects. Furthermore, any regenerative effect would be useless if the damaging inflammatory environment still persists over time. As such, more elaborate research is needed concerning the molecular pathways underlying the protective mechanisms for induction of immune tolerance and tissue regeneration. Future research should address the different interactions of WJ cells with the alveolar and splenic immune compartment, investigating the in vivo modulation of macrophages and other immune cells. The immunological communication between WJ-MSCs and resident immune cells is likely to be different from that observed in vitro. Cell–cell communications in vivo might change the immunological status of the stem cells and consequently alter their therapeutic potential. We suggest that this is a multistep process involving cellular interactions with subsequent immunological activation and paracrine secretion of trophic factors. In such a scenario, the transplantation behavior of WJ-MSCs should be further detailed, addressing ways to sustain survival, reduce immune rejection, and increase homing toward damaged

CNS tissue. Tracking WJ-MSCs using magnetic resonance or bioluminescence imaging should provide clues regarding their survival and distribution after application. Finally, as immunomodulation alone is not sufficient to combat disease pathology and the neuroprotective activity of WJ-MSCs needs to be further investigated, it is too early to conclude that WJ-MSCs can replace or complement existing MS drugs. To evaluate whether stem cells have an added therapeutic value, a thorough characterization of their neurotrophic mechanisms in vivo is essential. By transplanting WJ-MSCs in well-characterized de- and remyelination models, such as the lysolecithin or ethidium bromide demyelination model (8,83), the regenerative potential of WJ-MSCs can be further defined.

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