Immunobiology 222 (2017) 394–398

Contents lists available at ScienceDirect

Immunobiology

journal homepage: www.elsevier.com/locate/imbio

Short communication

Poly I:C primes the suppressive function of human palatine tonsil-derived MSCs against Th17 differentiation by increasing PD-L1 expression

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ARTICLE INFO

Article history:
Received 7 June 2016
Received in revised form 26 August 2016
Accepted 29 August 2016
Available online 31 August 2016

Keywords:
Tonsil-derived MSCs
PD-L1
Th17
Poly I:C

ABSTRACT

It has been established that mesenchymal stem cells (MSCs) can have a suppressive effect on T cells, yet much remains unknown about the underlying mechanisms that support this effect. The T cell co-stimulatory pathway involving the programmed death-1 (PD-1) receptor and its ligand PD-L1 regulates T cell activation, tolerance, and subsequent immune-mediated tissue damage. In this study, human palatine tonsil-derived MSCs (T-MSCs) constitutively expressed PD-L1 and exhibited a suppressive activity that specifically targeted murine Th17 differentiation. Additionally, polyinosinic–polycytidylic acid (poly I:C), a Toll-like receptor 3 (TLR3) ligand, increased PD-L1 expression on T-MSCs. The elevated PD-L1 levels enhanced the suppressive functions of T-MSCs on Th17 differentiation. Therefore, pre-stimulation of T-MSCs with poly I:C may serve as an effective therapeutic priming step for modulating Th17-dominant immune responses.

1. Introduction

Mesenchymal stem cell (MSC)-based therapies provide a source of cells for tissue reconstitution, tissue repair, and inflammation regulation in destination tissues. Although cell replacement is the foundation of MSC therapy in some diseases, published investigation shows that the MSC therapeutic effects are primarily a result of immunomodulation and inflammation control (Prockop and Oh, 2012). Additionally, adult MSC activity is modulated by Toll-like receptors (TLRs) (DelaRosa and Lombardo, 2010). Human MSCs are polarized toward a pro-inflammatory profile by TLR4 stimulation, whereas TLR3-primed MSCs are polarized toward an immunosuppressive profile (Waterman et al., 2010).

Human tonsil-derived MSCs (T-MSCs) are a new source of adult stem cells (Ryu et al., 2012). T-MSCs can be isolated from palatine tonsil tissues following tonsillectomy and can be expanded to large numbers *in vitro*. In addition to multi-lineage differentiation, T-MSCs have a promising therapeutic potential for the treatment of inflammatory diseases (Park et al., 2015).

Ligands from the B7 family of costimulatory molecules bind to the receptors of the CD28 family to regulate early T-cell activation in lymphoid organs and to control inflammation in peripheral tissues (Buchbinder and Desai, 2016). Programmed death-1 (PD-1), a member of the CD28 family, is an inhibitory receptor on T cells that is often responsible for their dysfunction in infectious diseases and cancers. The only known ligands of PD-1 (PD-L1 and PD-L2) are both B7 family members. PD-L1 is upregulated following immune activation of T cells, B cells, DCs, and macrophages. PD-L1 is also expressed on several non-hematopoietic cell types, including vascular endothelial cells, pancreatic islet cells, and keratinocytes.

The most important role of PD-1 may be that of a co-inhibitory receptor on T cells (Francisco et al., 2010). Engagement of PD-1 with PD-L1 during T-cell receptor (TCR) signaling can block T cell proliferation, block cytokine production, and impair T cell survival. PD-1 knockout mice develop spontaneous autoimmunity (Rui et al., 2013) and the PD-1/PD-L1/PD-L2 pathway is critical for regulating T cell responses in a variety of autoimmune disease models (Dai et al., 2014). Therefore, the PD-1/PD-L1 pathway could be an effective therapeutic target for treatment of autoimmune diseases.

Cell-to-cell contact between MSCs and T cells plays established roles in immunosuppression (Cao et al., 2015). Such observations prompted us to investigate the existence of immunosuppressive proteins expressed on T-MSCs and their role in T-MSC-mediated immunoregulation. Here, we examined PD-L1 expression on T-MSCs and factors that enhance PD-L1 expression levels. Further, we

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http://dx.doi.org/10.1016/j.imbio.2016.08.012
0171-2985/

examined which T-helper (Th) cell subsets were most effectively suppressed by PD-L1-expressing T-MSCs.

2. Materials and methods

2.1. Harvest and culture of T-MSCs

Tonsils were obtained with informed consent from patients undergoing tonsillectomy and Institutional Review Board approval (ECT 11-53-02, Ewha Womans University, Mok-Dong Hospital, Seoul, Korea). The tonsillar tissue was chopped and digested in RPMI-1640 (Invitrogen) containing 210 U/mL collagenase type I (Invitrogen) and 10 μg/ml DNase (Sigma-Aldrich) for 30 min at 37° C. Digested tissue was subjected to filtration through a wire mesh, and the cells were then washed twice in RPMI-1640/20% normal human serum (NHS; PAA Laboratories GmbH) and once more in RPMI-1640/10% NHS. From among these cells, mononuclear cells were obtained by Ficoll-Paque (GE Healthcare) density gradient centrifugation. Cells were plated at a density of 10^8 cells in a T-150 culture flask in DMEM (high glucose, Invitrogen) containing 10% fetal bovine serum (FBS; Invitrogen), 100 μg/ml streptomycin and 100 U/mL penicillin. After 48 h, non-adherent cells were removed from the medium and adherent mononuclear cells were replenished with new culture medium. These cells were expanded with three to five changes of passage, which took about 4 weeks. In general, cells at passage 7–9 were used for experiments. For all experiments, T-MSCs from three or four human donors were used parallelly. All cells were maintained in a 5% CO_2 incubator at 37 °C. Cells were treated with various stimuli for 24 h to examine PD-L1 expression levels. The cytokines and chemokines used for stimulation included poly I:C (20 μg/ml, Sigma Aldrich), IL-17 (100 ng/ml, R&D systems), IL-22 (100 ng/ml, R&D systems), IL-23 (100 ng/ml, Peprotech), IL-36 (100 ng/ml, Peprotech), and CCL20 (100 ng/ml, Peprotech).

2.2. CD4+ T-cell differentiation

Naïve CD4+ T cells were purified from mouse spleen (McAlees et al., 2015) tissues and draining lymph nodes (dLN) using a magnetic bead isolation system (Miltenyi Biotec). Single-cell suspensions of CD4+ naïve cells were seeded at 2×10^5 cells per well in 96-well plates with RPMI medium containing 10% FBS, 1% penicillin/streptomycin, 2 mM L-glutamine, and 50 μM 2-mercaptoethanol. For Th1 subset differentiation, cells were stimulated with anti-CD3 Ab (2.5 μg/ml, Biolegend), anti-CD28 Ab (5 μg/ml, Biolegend), IL-2 (5 ng/ml, Biolegend), and IL-12 (20 ng/ml) for 5 days. For Th2 subset differentiation, cells were stimulated with anti-CD3 Ab (2.5 μg/ml), anti-CD28 Ab (5 μg/ml), IL-2 (20 ng/ml), IL-4 (100 ng/ml, Biolegend), and anti-IFN-γ blocking Ab (2 μg/ml, Biolegend) for 5 days. For Th17 subset differentiation, cells were stimulated with anti-CD3 Ab (2.5 μg/ml), anti-CD28 Ab (5 μg/ml), IL-6 (100 ng/ml, Biolegend), IL-23 (20 ng/ml, Biolegend), TGF-β (4 ng/ml, Biolegend), and anti-IFN-γ blocking Ab (2 μg/ml) for 5 days. To test whether PD-L1 directly modulates Th cell differentiation, 100 ng/ml of recombinant PD-L1 (rPD-L1, Peprotech) was added on the first day during the differentiation period. T cells stimulated with anti-CD3 and anti-CD28 Abs alone were used as negative controls.

For the T-MSC treatment group, human T-MSCs were seeded 6 h before the addition of murine T cells (2×10^5 cells per well) at a density of 2×10^4 cells per well in 96-well plates to set the ratio of T-MSC as 1:10 to T cells. This ratio was determined from testing various dose of T-MSCs in coculture with T cells. (1:100, 1:50, 1:20, 1:10, 1:5, or 1:1). To confirm the effect of PD-L1 derived from T-MSCs, human T-MSCs were treated with anti-PD-L1 blocking Ab (10 μg/ml, Biolegend) and isotype control Ab (10 μg/ml, Biolegend). The above experiments were performed using T-MSCs derived from three human donors. To test whether poly I:C alone directly affects Th17 differentiation, poly I:C (20 μg/ml) was added to the T cells on the first day of differentiation period in the absence of T-MSCs.

2.3. Flow cytometry

PD-L1 (CD274) and PD-L2 (CD273) surface expression on human T-MSCs from four donors were detected with PE-conjugated anti-human CD274 (29E.2A3) and Alexa Fluor 647-conjugated anti-human CD273 (24F.10C12) antibodies, respectively. Non-specific staining was evaluated using isotype control antibodies (PE-conjugated mouse IgG_{2b}; Biolegend). To confirm the extent of differentiation into Th1, Th2, and Th17 subsets, murine cells were stained with PerCP-conjugated anti-mouse CD4 (GK1.5). Stained cells were fixed and permeabilized for intracellular staining of IFN-γ, IL-4, and IL-17 using PE-conjugated anti-mouse IFN-γ Ab (XMG1.2), APC-conjugated anti-mouse IL-4 Ab (11B11), and Alexa Fluor 488-conjugated anti-mouse IL-17 (TC11-18H10.1). Samples were analyzed on a FACSCalibur system (BD Biosciences) and data were analyzed using FlowJo software (Tree Star).

2.4. Reverse transcription-polymerase chain reaction (RT-PCR)

For the analysis of interferon regulatory factor 3 (IRF3) expression, human T-MSCs under various cytokine stimulation conditions were harvested and homogenized in TRIzol (Invitrogen). Complementary DNA (cDNA) was synthesized using a First-Strand cDNA Synthesis Kit (TOYOBO) according to the manufacturer's instructions. IRF3 (214 bp) was amplified using the following primers: 5′-TCTGAGAACCCACTGAAGCG-3′ (forward) and 5′-CCTGTCTGTCAGGGACATGC-3′ (reverse). The internal control gene *Gapdh* (192 bp) was amplified using the following primers: 5′-GGTAAAGTGGATATTGTTGCCATCAATG-3′ (forward) and 5′-GGAGGGATCTCGCTCCTGGAAGATGGTG-3′ (reverse). The band pixel densities of IRF3 were divided by the pixel densities of the corresponding Gapdh bands for quantitation using UN-SCAN-IT-gel 6.1 software (Silk Scientific, Inc.).

2.5. Statistical analyses

Data are presented as means ± standard error of the mean. Statistical significance was analyzed by one-way or two-way ANOVA using GraphPad PRISM 6 software (GraphPad Software Inc.). For all analyses, $p < 0.05$ was considered statistically significant.

3. Results

While both PD-L1 and PD-L2 bind to PD-1 and deliver co-inhibitory signals to T cells, PD-L1 is more broadly expressed across different cell and tissue types (Dai et al., 2014). T-MSCs constitutively expressed both PD-L1 and PD-L2 in the absence of exogenous stimulation (Fig. 1A). This prompted us to investigate (1) the population of Th cells most responsive to T-MSCs expressing PD-L1 during differentiation and (2) possible stimuli that potentiate the PD-L1-mediated immunosuppressive activity of T-MSCs. We compared the extent of murine CD4+ naïve T cell differentiation into Th1, Th2, and Th17 subsets in the presence of human T-MSCs, with or without anti-PD-L1 blocking Ab. Recombinant PD-L1 was used to determine whether PD-L1 alone can inhibit Th subset differentiation.

Human T-MSCs inhibited murine CD4+ naïve T cell differentiation into Th1, Th2, and Th17 subsets (Fig. 1B and Supplementary Table I). We tested various dose of T-MSCs to optimize ratio in