

Induction of Indoleamine 2,3-dioxygenase by Pre-treatment with Poly(I:C) May Enhance the Efficacy of MSC Treatment in DSS-induced Colitis

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Mesenchymal stem cells (MSCs) have been used experimentally for treating inflammatory disorders, partly owing to their immunosuppressive properties. The goal of the study was to determine whether TLR ligands can enhance the therapeutic efficacy of bone marrow-derived MSCs for the treatment of inflammatory bowel disease. Mice (C57BL/6) were administered with 4% dextran sulfate sodium (DSS) in drinking water for 7 days and injected with MSCs on days 1 and 3 following DSS ingestion. Our results demonstrated that among various TLR ligands, MSCs treated with polyinosinic-polycytidylic acid [poly(I:C)], which is a TLR3 ligand, more profoundly induced IDO, which is a therapeutically relevant immunosuppressive factor, without any observable phenotype change *in vitro*. The poly(I:C)-treated MSCs attenuated the pathologic severity of DSS-induced murine colitis when injected *i.p.* but not *i.v.* In summary, preconditioning MSCs with poly(I:C) might improve their efficacy in treating DSS-induced colitis, and this effect at least partly depends on the enhancement of their immunosuppressive activity through increasing their production of IDO.

[Immune Network 2016;16(6):358-365]

Keywords: Mesenchymal stem cells, Dextran sulfate sodium (DSS)-induced colitis, Toll-like receptors, Poly(I:C), Indoleamine 2,3-dioxygenase, Intraperitoneal route

INTRODUCTION

Adult mesenchymal stem cells (MSCs) represent an innovative tool for the cell-based therapy of degenerative disorders, chronic inflammatory conditions, autoimmune diseases, or allograft rejection. MSCs are derived from various tissues and have the capacities for self-renewal and differentiation (1,2). Studies of MSC-based therapies have provided convincing evidence that such therapies have potential anti-inflammatory and immunomodulatory

effects that are activated by inflammatory cytokines and chemokines, which can recruit or suppress lymphocytes (3).

Interactions between TLRs and their ligands have been implicated in the pathology of inflammatory diseases including rheumatoid arthritis and inflammatory bowel disease, since they can either initiate or perpetuate chronic inflammation due to continuous exposure to TLR ligands. It is well established that MSCs express several TLRs and their expression of TLRs can be modulated (4).

Received on October 3, 2016. Revised on December 5, 2016. Accepted on December 8, 2016.

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Abbreviations: CpG, ODN 1826; DAI, disease activity index; DSS, dextran sulfate sodium; flagellin, RecFLA-ST; MSC, mesenchymal stem cell; PGN, peptidoglycans; Poly(I:C), polyinosinic-polycytidylic acid

Importantly, it is believed that the therapeutic efficacy of MSCs in inflammatory diseases depends to a large extent on their immunologically privileged phenotype and immunosuppressive capacity. Therefore, to facilitate the wider use of MSCs in cell-based therapy for inflammatory diseases, further investigation is warranted regarding the potential effects of TLR ligand stimulation on their immunosuppressive capacity, which may provide approaches for improving their clinical effectiveness.

In addition, certain soluble factors produced by MSCs have been implicated in mediating their immunoregulatory activities, such as IFN- γ (5), TGF- β 1, prostaglandin E2 (6), IDO (7), NO (8), and IL-10 (8). To improve the clinical usefulness of MSC treatment, we hypothesized that preconditioning IDO-expressing MSCs with TLR ligands may modulate their therapeutic effectiveness *in vivo*.

In this study, we found that, after treating murine bone marrow-derived MSCs with various TLR ligands, only the TLR3 ligand polyinosinic-polycytidylic acid [poly(I:C)] significantly increased the expression of IDO. Furthermore, IDO-expressing MSCs exposed to poly(I:C) improved the pathologic scores of mice with DSS-induced colitis more effectively than MSCs without exposure to poly(I:C) did.

MATERIALS AND METHODS

Mice

Female C57BL/6 mice (9~10 weeks old) were purchased from Japan SLC Inc. (Shizuoka, Japan). All animal experiments were approved by the institutional Animal Care and Use Committees of the Catholic University of Korea (Seoul, Korea).

Isolation and expansion of primary murine bone marrow-derived MSCs

The bone marrow-derived cells were isolated from C57BL/6 mice by flushing the femurs and tibias with complete culture medium, which comprised DMEM (WelGENE, Daegu, Korea) supplemented with 10% heat-inactivated FBS (WelGENE), 2 mM glutamine, 100 U/mL penicillin, and 100 μ g/mL streptomycin (Gibco BRL, Gaithersburg, MA, USA). Briefly, the isolated cells were plated in 75 cm² tissue culture flasks at a concentration of 1×10^6 cells/mL in the complete culture medium and incubated at 37°C and 5% CO₂. After 3 days, the non-adherent cells were removed. When the cells had reached 70~80% confluence, the cells were trypsinized and

passed into a new flask. A homogenous cell population was obtained after culturing for 3~5 weeks. MSCs from passages 8~14 were used for all experiments.

Pretreatment of MSCs with TLR ligands

When the MSCs were 90% confluent, the cells were harvested and plated in 12-well-plates (5×10^4 cells/well) in the complete culture medium with or without recombinant mouse IFN- γ (100 ng/mL, R&D Systems, Minneapolis, MN, USA). For TLR ligands treatment, peptidoglycans (PGN, TLR2 ligand, 5 μ g/mL), poly(I:C) (TLR3 ligand, 10 μ g/mL), LPS (TLR4 ligand, 10 μ g/mL) (all from Sigma-Aldrich, St Louis, MO, USA), Pam3CSK4 (TLR1/2 ligand, 1 μ g/mL), RecFLA-ST (flagellin, TLR5 ligand, 100 ng/mL), FSL-1 (TLR2/6 ligand, 100 ng/mL), R848 (TLR7/8 ligand, 1 μ g/mL), and ODN 1826 (CpG, TLR9 ligand, 1 μ g/mL) (all from InvivoGen, San Diego, CA, USA) were used for stimulation. After stimulation for 24 h, the MSCs were collected for immunophenotypic analysis or the measurement of IDO expression.

Characterization of MSCs

Normal MSCs or stimulated MSCs were stained for FITC-conjugated anti-CD45, CD117, allophycocyanin-conjugated anti-Flk-1, PE-conjugated anti-CD34, Alexa Fluor[®] 700-conjugated anti-Sca-1 (all from eBioScience, San Diego, CA, USA), allophycocyanin-conjugated anti-CD44, and Brilliant Violet 605[™]-conjugated anti-CD11b (all from BD Biosciences, San Diego, CA, USA). Data were analyzed using an LSRII flow cytometer (BD Biosciences).

Quantitative RT-PCR

Total RNA was isolated from cultured MSCs and colon homogenates with Trizol[®] (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. One microgram of total RNA was reverse transcribed into cDNA. *IDO* and β -actin (*ACTB*) transcripts were amplified using the following primers: *IDO* forward 5'-ATTGGTGGAATCGCAGCTTC-3', reverse 5'-ACAAAGTCACGCATCCTCTTAAA-3'); *ACTB* forward 5'-AGCTGCGTTTTACACCCTTT-3', reverse 5'-AAGCCATGCCAATGTTGTCT-3'). Quantitative assessment of target mRNA levels was performed by quantitative RT-PCR using a CFX96[™] real-time PCR detection system (Bio-Rad, Hercules, CA, USA). The quantity of mRNA was calculated using the 2^{- $\Delta\Delta C_t$} method, and *ACTB* was used to normalize total RNA quantities.

Western blot

Forty micrograms of each protein sample was separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK). Membranes were blocked with 5% skim

milk (Difco/Becton Dickinson, Atlanta, GA, USA) at 4°C overnight and incubated for 2 h with anti-IDO antibody (1:1000 dilution, BioLegend, San Diego, CA, USA) at room temperature. The membrane was washed three times with TBST and incubated with secondary antibody

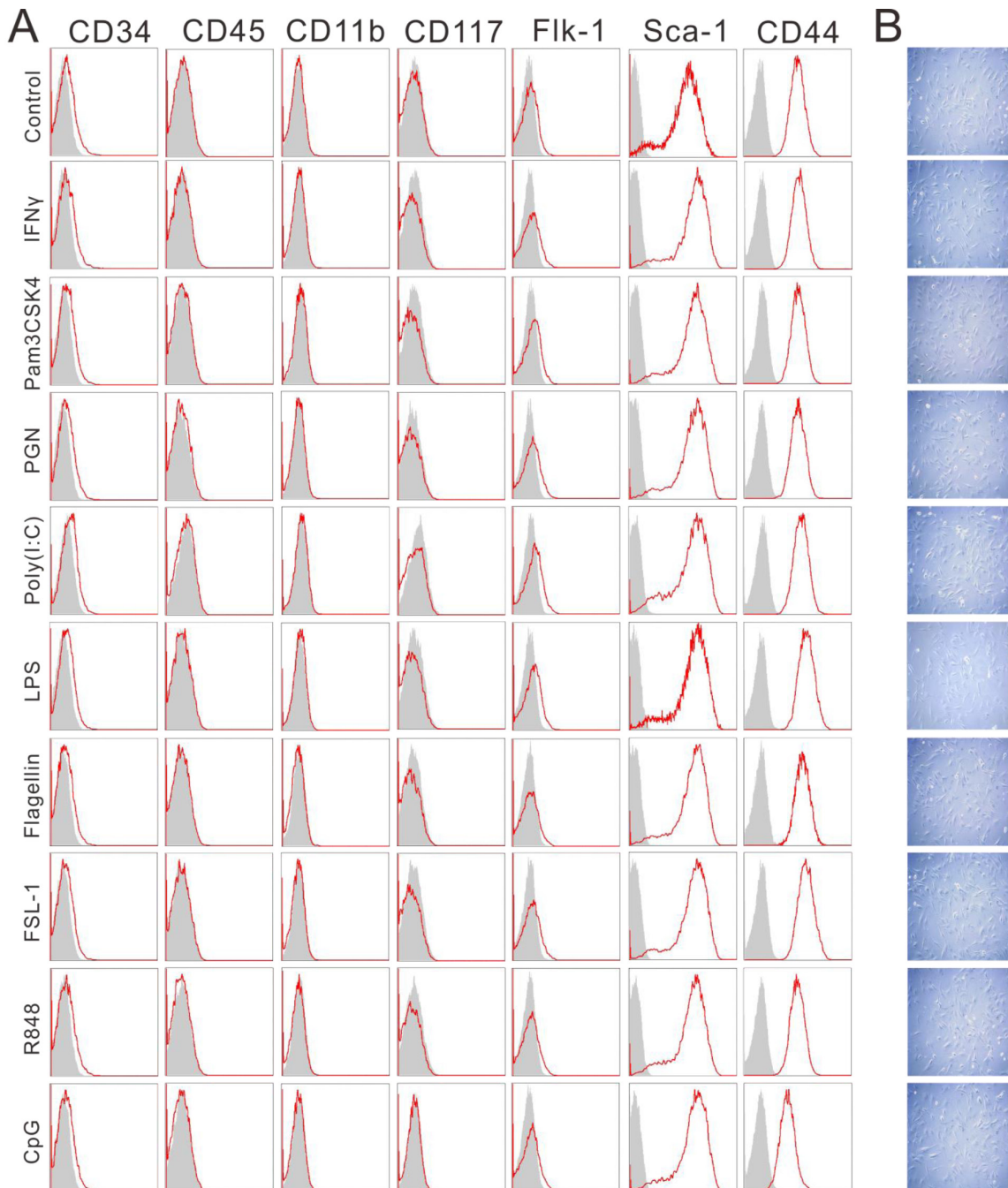


Figure 1. Immunophenotypic analysis of murine mesenchymal stem cells (MSCs). MSCs were cultured with or without TLR ligands for 24 h. (A) The expression of immunophenotypic surface antigens on MSCs was detected by flow cytometric analysis. (B) Morphological changes were observed in MSCs. There were no significant differences among the treatment groups. Gray color represents the control isotypes. Red lines show antibody-specific staining.

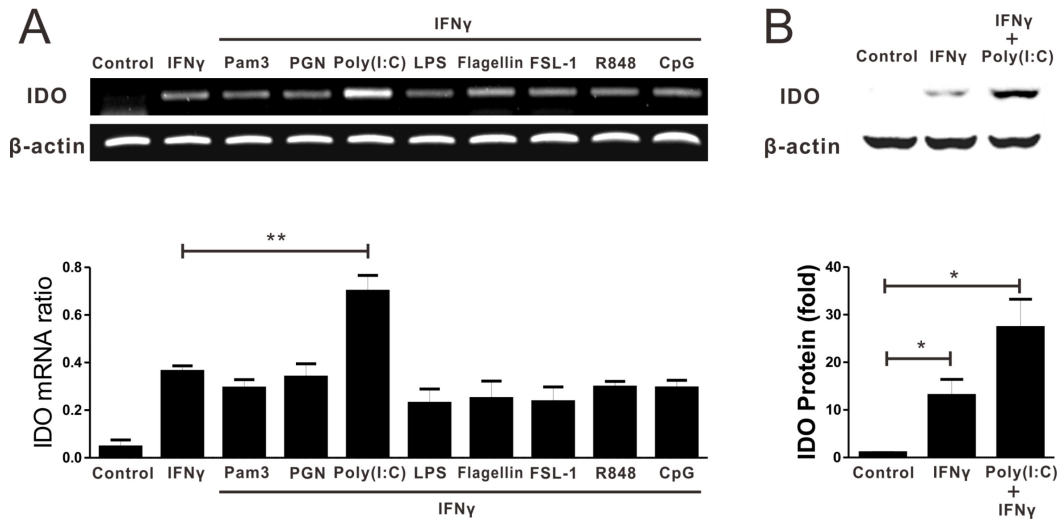


Figure 2. Effects of IFN- γ and TLR ligands on IDO expression in murine MSCs. (A) mRNA from 24-h cultures of unstimulated, IFN γ -stimulated, and IFN γ +TLR ligand-stimulated MSCs were assayed by quantitative RT-PCR for IDO expression. ** p <0.01 between IFN γ -stimulated MSCs and IFN γ +polyinosinic-polycytidylic acid [poly(I:C)]-stimulated MSCs. (B) Western blot analysis of the protein expression of IDO was performed on 48-h cultures. * p <0.05 compared with unstimulated MSCs. The data shown are mean \pm SEM values calculated from four separate experiments.

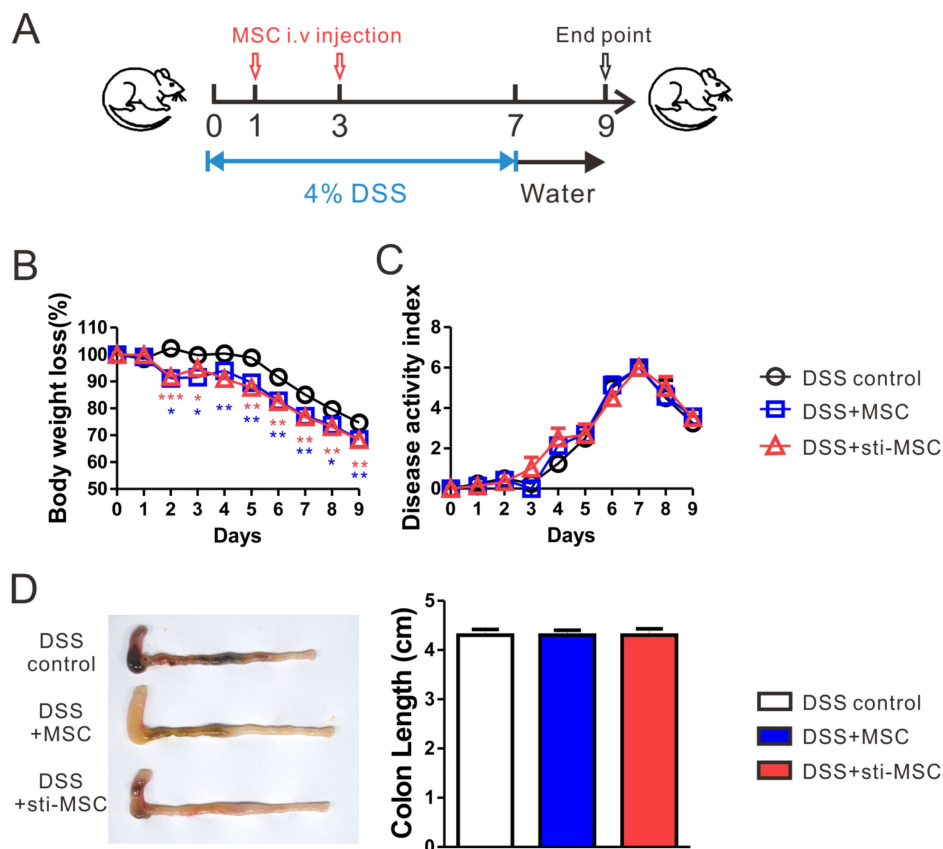


Figure 3. Effects of i.v. injection of MSCs in the dextran sulfate sodium (DSS)-induced colitis model. (A) Schematic outline of the DSS model. DSS was given to mice via drinking water for 7 days to induce colitis. Afterwards, unstimulated MSCs (DSS+MSCs) or stimulated MSCs (DSS+sti-MSCs) were administered into the DSS-treated mice through a tail vein injection. The same volume of saline was injected into other DSS-treated mice as positive controls (DSS control). All the mice were sacrificed 9 days after DSS administration. (B) Body weight and (C) disease activity index scores were monitored daily. (D) Colon lengths were measured on day 9. $n=4$ for DSS control, $n=6$ for treatment group. * p <0.05, ** p <0.01, and *** p <0.001 compared with DSS control.

for 2 h at room temperature. HRP-conjugated rat IgG antibody (1:1000 dilution, Santa Cruz Biotechnology, Dallas, TX, USA) in 5% skim milk was used as the secondary antibody. The target protein was detected using the SuperSignal™ West Dura extended duration enhanced

chemiluminescence substrate (Thermo Fisher Scientific, Waltham, MA, USA).

DSS-induced mice colitis and treatment

Colitis was induced by administration of 4% DSS (mole-

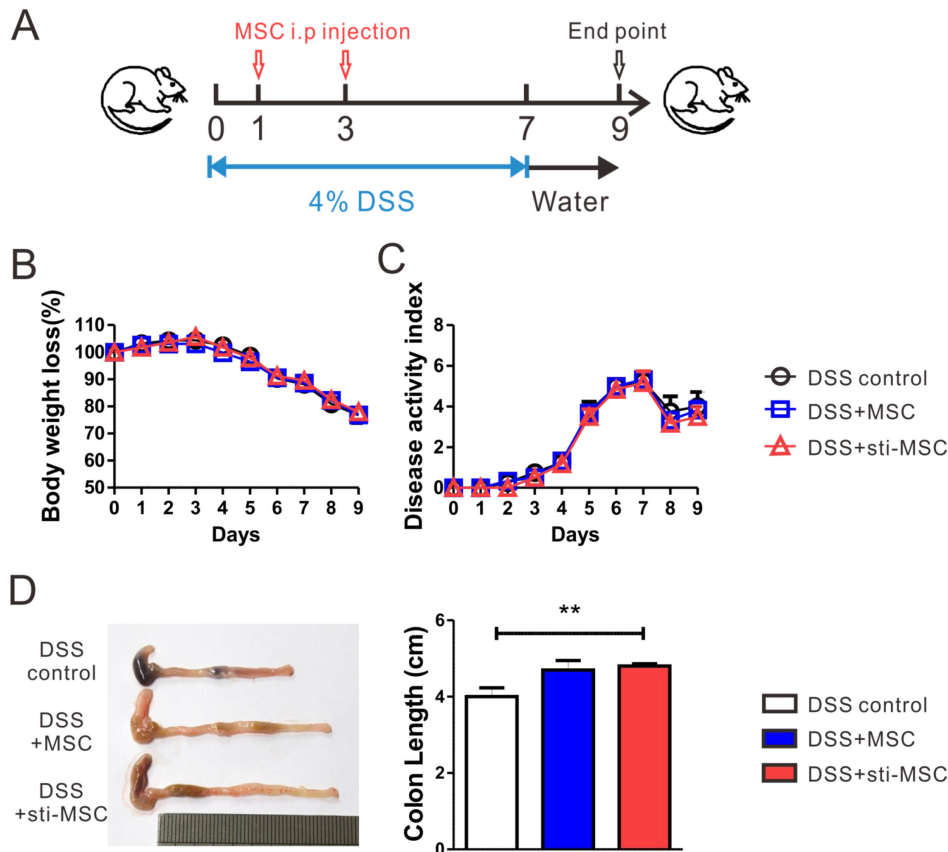


Figure 4. Effect of i.p. injection of MSCs in DSS-induced colitis model. (A) Schematic outline of the DSS model. DSS was given to mice via drinking water for 7 days to induce colitis. Afterwards, unstimulated MSCs (DSS+MSC) or stimulated MSCs (DSS+sti-MSC) were i.p. injected. The same volume of saline was administered into other DSS-treated mice as positive controls (DSS control). All the mice were sacrificed 9 days after DSS administration. (B) Body weight and (C) disease activity index scores were monitored daily. (D) Colon lengths were measured on day 9. n=4 for DSS control, n=6 for treatment group. **p<0.01 compared with DSS control.

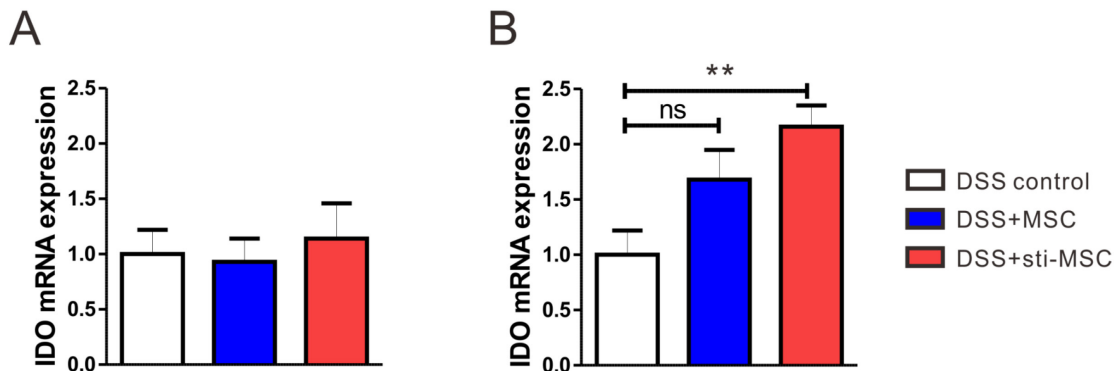


Figure 5. Colonic mRNA expression of *IDO* according to the injection route of MSCs. On day 9 of DSS administration, colon tissues were harvested from mice administered with MSCs by (A) i.v. or (B) i.p. injection to measure the expression of *IDO* by quantitative RT-PCR. The data shown are mean±SEM values calculated from two separate experiments. n=4 for DSS control, n=6 for treatment group. ns=non-significant (p>0.05), **p<0.01 compared with DSS control.

cular weight 36,000~50,000; MP Biomedicals, Santa Ana, CA, USA) in drinking water *ad libitum* for 7 days. 9-week-old mice were divided into three groups: DSS treatment group (DSS control), DSS with unstimulated MSCs treatment group (DSS+MSCs), and DSS with MSCs stimulated with IFN- γ and poly(I:C) group (DSS+sti-MSCs). Unstimulated MSCs or stimulated MSCs were administered by i.v. or i.p. injection on days 1 and 3 after the administration of DSS. The severity of disease was assessed using the disease activity index (DAI), which evaluates stool consistency and the presence of fecal blood, as previously described (9).

Statistical analysis

All values are expressed as mean \pm SEM. Statistical comparisons between groups were performed using the Mann–Whitney U test.

RESULTS

Immunophenotypic characterization of MSCs according to TLR stimulation

The MSC cultures were assayed routinely for the presence of MSC-related cell surface antigens by flow cytometric analysis. Murine bone marrow-derived MSCs were negative for CD34, CD45, CD11b, CD117, and Flk-1 but most of them expressed Sca-1 and CD44 (10). To investigate the effect of TLR ligand stimulation on the immunophenotype of MSCs, we cultured MSCs in the presence of IFN- γ with or without Pam3CSK4, PGN, poly(I:C), LPS, flagellin, FSL-1, R848, and CpG. MSCs were harvested after 24 h and their phenotype was analyzed. All MSC groups were negative for CD34, CD45, CD11b, CD117, and Flk-1 and positive for Sca-1 and CD44. There were no significant differences in MSC immunophenotype among the treatment groups (Fig. 1).

TLR3 activation increased IDO expression in MSCs

To determine the effects of activating each TLR on the expression of the tryptophan-degrading enzyme IDO, which is responsible for TLR-mediated kynurenine production in MSCs, MSC/TLR ligand coculture experiments were performed. In the presence of IFN- γ , we cultured MSCs with or without stimulation by Pam3CSK4, PGN, poly(I:C), LPS, flagellin, FSL-1, R848, and CpG, because IDO is expressed by MSCs in response to inflammation. The mRNA expression of IDO was significantly increased in TLR ligand-treated MSCs compared with that in unstimulated MSCs (Fig. 2). We

also demonstrated that the protein expression of IDO was increased in stimulated MSCs compared with that in unstimulated MSCs (Fig. 2B). Poly(I:C) preconditioning of MSCs significantly increased their expression of IDO, compared with that of MSCs without TLR ligand treatment. In contrast, the other tested TLR ligands had no significant effect on IDO expression.

Intraperitoneal injection of poly(I:C)-stimulated MSCs improved the pathologic scores of mice with DSS-induced colitis

Our *in vitro* experiments demonstrated that poly(I:C) can induce increased IDO expression in MSCs. We next investigated whether there is a beneficial effect of using MSCs preconditioned with poly(I:C) for cell-based therapy to reduce the disease activity of DSS-induced colitis. Mice that were orally administered with 4% DSS for 7 days showed a significant increase in DAI, bloody diarrhea, and sustained weight loss. To examine the effect of using MSCs with an elevated expression of IDO for cell-based treatment in the DSS-induced colitis model, the mice were treated with unstimulated and poly(I:C)-stimulated MSCs by i.v. or i.p. injection on days 1 and 3 after the administration of DSS (Figs. 3 and 4). Body weight changes and the DAI score were monitored daily. The i.v.-injected groups showed increased body weight loss after MSC injection compared with DSS controls (Fig. 3B). There were no significant differences in DAI score among the three groups (Fig. 3C). The i.p.-injected groups showed no significant differences in body weight loss and DAI score (Figs. 4B and C). DSS-induced colitis is associated with a reduced colon length in diseased mice. Therefore, we measured the colon length at day 9 after the administration of DSS (11). Colon lengths did not significantly differ among the i.v.-injected (Fig. 3D), whereas the i.p. injection of poly(I:C)-stimulated MSCs ameliorated the shortening of colon length compared to that in the DSS control group without MSCs injection (Fig. 4D).

Poly(I:C)-stimulated MSCs showed an increased mRNA expression of IDO in colon when injected i.p. but not i.v.

Since the colon was a major site for injury by DSS, the organ was excised and examined by quantitative RT-PCR assays for the expression of IDO. In agreement with the pathologic findings, poly(I:C)-treated MSCs showed distinct changes in their IDO expression profiles after they were administered inside the peritoneal cavity (Fig. 5). Quantitative RT-PCR assays showed an upregulation of IDO expression in colon when poly(I:C)-stimulated

MSCs were i.p. injected but not when they were i.v. injected in the DSS-colitis model. The injection of unstimulated MSCs did not induce an increase of *IDO* expression regardless of the injection route.

DISCUSSION

The present work suggests that stimulating TLR3 with poly(I:C) may enhance *IDO* production in MSCs and enable MSC therapy for DSS-induced colitis. Poly(I:C)-stimulated MSCs protected against DSS-induced colitis when administered i.p. but not i.v., suggesting that this difference was caused by the relatively high bioavailability of *IDO* after the administration of MSCs inside the peritoneal cavity. The lack of efficacy of i.v. injection could result from a short persistence of *IDO* expression through the gastrointestinal tract or may simply reflect rapid degradation of the MSCs administered in this manner.

Although TLRs and their ligands are known to control the functions and multilineage phenotype of MSCs, contradictory results regarding the effects of TLR activation have been evident in previous reports (4,12). It has been shown that the exposure of MSCs to 4 and 20 $\mu\text{g/ml}$ poly(I:C) might trigger different trophic responses (13). MSCs are widely present *in vivo* and their perivascular origin in multiple human organs has been demonstrated (14). Thus, it is possible that the benefits of stimulating MSCs with poly(I:C) may be mediated through the induction of an increased expression of *IDO* as demonstrated in the current study. Notably, although poly(I:C)-treated MSCs did not reduce the severity of DSS-induced colitis when administered by the i.v. route, they showed therapeutic potency when delivered through i.p. injection. This observation suggests that the migratory potential of MSCs and the effects of trophic factors may contribute to determining their clinical effectiveness. The findings of our study are consistent with the emerging recognition that the secretion of *IDO* by MSCs is the major mechanism underlying their benefits for tissue healing (15). Moreover, studies on MSC-based therapies have mainly focused on the i.v. infusion of allogeneic MSCs (16); however, MSCs administered through this route become largely trapped in the lungs upon infusion, which may limit their effects on the intestines.

The capacity of MSCs to inhibit the proliferation of immune cells upon mitogenic or allogeneic activation can be a key factor in determining their therapeutic utility and potency. The mechanisms underlying the

immunosuppressive potential of MSCs are not fully understood, but seem to require both cell-to-cell contact-dependent mechanisms and the release of soluble immune modulators (*IDO*, prostaglandin E₂, TGF- β 1, NO, etc.) upon activation in response to immune cells (15,17). Interestingly, some of these immune modulators are downstream of signaling pathways triggered by TLRs in other cell types. Therefore, a feasible hypothesis is that TLR ligands may induce the production of such anti-inflammatory mediators in MSCs, resulting in an enhanced immunosuppressive phenotype. Our results indicate that *IDO*-expressing MSCs treated with poly(I:C) may be an innovative tool for high-performance cell-based therapy for inflammatory bowel disease. Therefore, it will be important to determine the mechanisms by which the stimulation of TLR signaling by poly(I:C) modulates the immunosuppressive capacity of MSCs via modulating the production of *IDO* and its enzymatic metabolites.

In this study, poly(I:C)-stimulated MSCs reduced the pathologic severity of DSS-induced colitis when injected i.p. but not when injected i.v. Since MSCs are increasingly being used in cell-based therapies, deciphering the molecular mechanisms that drive their migration, recruitment, and engraftment is critical for improving our control over such therapies and achieving desired clinical outcomes. Therefore, our future studies will seek to determine whether, as with other bone marrow-derived cells, the migration and/or recruitment of poly(I:C)-stimulated MSCs are also driven by TLR3-mediated *IDO* production.

Our findings indicate that inducing the expression of *IDO* in MSCs by exposing them to poly(I:C), a TLR3 ligand, is an approach worthy of further investigation as a therapeutic strategy for DSS-induced colitis. Furthermore, this approach could be utilized therapeutically to augment the immunosuppressive properties of MSCs in cell-based therapies for autoimmune disease, graft-versus-host disease, or transplant rejection.

ACKNOWLEDGEMENTS

This study was supported by the Korea Healthcare Technology R&D Project, Ministry of Health, Welfare, and Family Affairs, Republic of Korea (Grant no. HI16C0047).

CONFLICTS OF INTEREST

The authors have no financial conflict of interest.

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