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*J Immunol* 2010; 185:4022-4029; Prepublished online 27 August 2010;
doi: 10.4049/jimmunol.0902723
[http://www.jimmunol.org/content/185/7/4022](http://www.jimmunol.org/content/185/7/4022)

**Supplementary Material**

[http://www.jimmunol.org/content/suppl/2010/08/25/jimmunol.0902723.DC1](http://www.jimmunol.org/content/suppl/2010/08/25/jimmunol.0902723.DC1)

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Immunomodulation of Delayed-Type Hypersensitivity Responses by Mesenchymal Stem Cells Is Associated with Bystander T Cell Apoptosis in the Draining Lymph Node

Jong-Hyung Lim,*†‡§,1 Jung-Sik Kim,*†‡§,1 Il-Hee Yoon,*†‡§ Jun-Seop Shin,*†‡§ Hye-Young Nam,*†‡§ Seung-Ha Yang,*†‡§ Sang-Joon Kim,*‡§,1 and Chung-Gyu Park*†‡§

Disease amelioration by mesenchymal stem cells (MSCs) has been shown to be closely related to their immunomodulatory functions on the host immune system in many disease models. However, the underlying mechanisms of how these cells affect the immune cells in vivo are not fully understood. In this study, we report findings that a small but significant number of MSCs accumulate in the secondary lymphoid organs and attenuate delayed-type hypersensitivity (DTH) response by inducing apoptotic cell death of surrounding immune cells in the draining lymph node (LN). In the migration study, i.v. infused GFP-MSCs preferentially accumulated at the boundary between the paracortical area and the germinal center of the LNs, in close proximity to various types of immune cells including T, B, and dendritic cells in a dose-dependent manner. As a result, accumulated MSCs markedly attenuated DTH response in proportion to the number of MSCs infused. During the DTH response, the infiltration of T cells in the challenged site was significantly decreased, whereas a number of apoptotic T cells were remarkably increased in the draining LN. Apoptosis was significantly induced in activated T cells (CD3+ and BrdU+), but not in the resting T cells (CD3+ and BrdU−). NO was associated with these apoptotic events. Taken together, we conclude that significant numbers of i.v. infused MSCs preferentially localize in the draining LN, where they induce apoptosis of the activated T cells by producing NO and thus attenuate the DTH response. The Journal of Immunology, 2010, 185: 4022–4029.

Mesenchymal stem cells (MSCs) isolated from adult bone marrow (BM) stromal cells were initially identified by Friedenstein et al. in 1970 (1). MSCs are rare cells of non-hematopoietic origin representing 0.001–0.01% of total BM cells (2) and are capable of differentiating into the cells of mesodermal lineage including osteoblasts, adipocytes, and chondrocytes as well as those of other lineages, such as endothelial cells and neural cells (3, 4). Besides their differentiation capacity, recent preclinical and clinical studies (5–7) have shown that MSCs migrate to the wounded tissue and contribute to tissue repair and healing processes. Most interestingly, extensive in vitro studies have shown that MSCs can suppress the proliferation of T (8), B (9), dendritic cells (DCs) (10), and NK cells (11) through the induction of cell division arrest. These in vitro immunomodulatory potentials of MSCs have been known to be associated with cell-to-cell interaction and/or soluble factors. For T cells, several soluble factors, such as TGF-β1 (12), hepatocyte growth factor (12), NO (13), PGE2 (14), and IDO (15), have been reported to be involved in MSC-mediated immune regulation. Although the systemic immunosuppressive capability of MSCs in animals and humans was also reported in several disease models, including severe graft-versus-host disease (6), experimental autoimmune encephalomyelitis (16, 17), and allogeneic skin graft (18), how these cells exert these immunomodulatory effects in vivo has not been clarified. Therefore, further mechanistic and extensive migratory studies in animals and humans are required to establish the optimal conditions for the use of MSCs as immunotherapy.

Recently, many groups insisted that cell cycle arrest at the G0-G1 phase is one of the mechanisms of MSC-mediated immunosuppression (8, 10, 14). In contrast, other groups reported MSCs induced apoptosis of T cells without detailed cell cycle analysis (19). Even though several reports presented that MSC repaired damaged tissue (2, 7) and protected apoptosis of immune cells, such as thymocytes (20) and neutrophils (21), Plumas et al. (19), Ren et al. (22), and our recent study (23) demonstrated that the apoptosis induction of activated T cells by MSCs is a strong mediator in the suppression of T cell proliferation in vitro. In contrast, another group reported that MSCs could promote survival of T cells in the quiescent state (20). In the mechanistic study, Plumas et al. (19) showed that the induction of T cell apoptosis depends on MSC-mediated IDO induction, but Ren et al. (22) reported that the apoptosis induction is mediated by NO derived from MSCs. Although both studies gave some clues for the mechanism of immunosuppression by MSCs, their results are limited to the in vitro situation. Thus, it remains an open question as to whether apoptosis induction of T cells would be one of the main mechanisms of MSCs-mediated immunosuppression in vivo.
In the current study, we wanted to address this point using the delayed-type hypersensitivity (DTH) model with systemic infusion of MSCs. In this study, we report that small but significant numbers of i.v. infused MSCs preferentially accumulate at the boundary between the paracortical area (T cell zone) and the germinal center (B cell zone) of lymph nodes (LN). These MSCs induce apoptosis of neighboring activated T cells in the draining LN during DTH responses and attenuate clinical responses in mice. In addition, we demonstrated that NO is associated with the apoptosis induction by MSCs in vivo.

Materials and Methods

Mice

Inbred C57BL/6 (H2b) and GFP- transgenic mice (C57BL/6-GFP) were purchased from The Jackson Laboratory (Bar Harbor, ME). CCR7−/- mice on a C57BL/6 background were generously provided by Dr. Mina Kwon at the International Vaccine Institute with permission of Dr. M. Lipp (Max Delbruck Center for Molecular Medicine, Berlin, Germany). These mice were bred and maintained under specific pathogen-free conditions in the Biological Services Unit at the Seoul National University (Seoul, South Korea). All animal experiments were approved by the Institutional Animal Care and Use Committee of the Seoul National University.

Culture of mouse BM-derived MSCs

MSCs and GFP-MSCs were isolated from murine long bones and expanded in culture. Briefly, femurs and tibias of murine long bones were flushed with PBS containing 150 μg/ml gentamycin. Resulting cell suspensions were cultured in 100-mm cell culture plates in high-glucose DMEM (Invitrogen, Carlsbad, CA) supplemented with heat-inactivated 10% FBS, 2 mM l-glutamine, 55 μM 2-ME, 100 μM nonessential amino acid, 50 μg/ml gentamycin, and 10 mM HEPES. Cells were incubated at a concentration of 10^6/ml at 37°C in a 5% CO2 atmosphere. After 72–96 h, nonadherent cells were discarded. When the plate was ~80% confluent, adherent cells were trypsinized and subcultured. The adherent cells at passages 3–10 were used as MSCs in all experiments.

MSC characterization

The immunophenotype of these cells was continuously positive for CD106, H2-D^d, and CD44 but negative for CD34, CD14, CD11c, and I-A^d for >5 passages (data not shown). The surface Ag expression of MSCs was confirmed by flow cytometry. MSCs (5 × 10^5) were incubated for 1 h at 4°C in the following mAbs: FITC-conjugated anti-CD14, H2-D^d (BD Pharmingen, San Diego, CA), PE-conjugated anti-CD34 (eBioscience, San Diego, CA), I-A^d, CD11c, CD106 (BD Pharmingen), PerCP-conjugated anti-CD45, and allophycocyanin-conjugated anti-CD44 (BD Pharmingen). The cell suspensions were washed with PBS containing 0.5% BSA and 0.1% sodium azide, and then they were analyzed by FACS (Becton Dickinson, Heidelberg, Germany) using CellQuest software (BD Bioscience, San Jose, CA). MSCs isolated from wild-type C57BL/6 and GFP-transgenic C57BL/6 mice were differentiated into osteoblasts and adipocytes to confirm their differentiation potential. Osteogenic differentiation was induced by 10 mM glycercophosphate, 50 μg/ml ascorbic acid 2-phosphate, and 10^{-8} M dexamethasone in complete DMEM as described. Osteogenic cultures were maintained for 3–4 wk, and new differentiation medium was supplied twice a week. Osteogenic differentiation was identified by staining with 2% Alizarin Red S for 20 min at room temperature postfixation with 10% formalin for 20 min. To induce adipogenic differentiation, MSCs were cultured with 10^{-7} M dexamethasone and 5 μg/ml insulin in complete DMEM for 3–4 wk, changing the media twice a week. Cultured cells were fixed with 10% formalin for 20 min and then were incubated with Oil Red O for 1 h. Photographs of the cells were taken with a digital camera (Nikon Infinity, Nikon, Melville, NY) attached to a light microscope (Nikon Eclipse TS 100, Nikon).

In vivo trafficking of administered MSCs

Carboxyl quantum dots, Qdots 800 (peak emission wavelength at 800 nm), which had a CdTe core with an additional thin semiconductor shell (zinc sulfide) of ~6–~12 nm in diameter, respectively, coated by thin polymer (1–2 nm) containing carboxyl groups, were purchased from Invitrogen. One million MSCs were loaded with 100 nM quantum dots for 1 h at 37°C. One million labeled MSCs in a volume of 300 μl were divided by two and slowly injected i.v. into one healthy syngeneic mouse with 15-min intervals. Twenty-four hours later, the mouse was killed, and MSC trafficking was analyzed using a spectral imaging system (Maestro In Vivo Imaging System, CRI, Woburn, MA) in vivo and ex vivo.

Histology and immunofluorescence

Samples of the intestines, lungs, kidneys, liver, stomach, brain, spleen, and LN from the mice were collected for cryosection examination. The tissues were removed, washed in PBS, and then embedded in Tissue Tek OCT compound (Sakura, Torrance, CA). Sections were cut at 5 μm on a cryostat (CM1850, Leica, Bannockburn, IL), fixed in acetone for 10 min at 4°C, and preserved at −80°C. To stain tissues for immunofluorescence, slides were brought to room temperature, dried, fixed in acetone for 10 min at 4°C, and then placed in PBS for 5 min to remove OCT compound. All slices were blocked with 5% BSA in PBS for 30 min. The slices were incubated with anti-GFP conjugated with Alexa 488 Ab (Molecular Probes, Eugene, OR), rat anti-CD3, anti-CD4, anti-CD8, anti-CD19, anti-F4/80 (BD Pharmingen), and hamster anti-CD11c Ab (Abcam, Cambridge, U.K.) overnight in a humidified chamber at 4°C and then slides were washed three times for 5 min each in PBS. Slides were incubated with appropriate secondary Abs for 1 h and then washed three times for 5 min in PBS. For T and B cells and macrophages, slides were stained with anti-rat IgG-Alexa 555 and anti-rat IgG-Alexa 647, and DCs were stained with anti-hamster IgG-Alexa 594 (Molecular Probes). To detect the cell nucleus, cells were mounted using VECTASHIELD with DAPI (DAPI Vector Laboratories, Burlingame, CA). The sections were observed and recorded under a fluorescent microscope (Axio-(now, Carl Zeiss, Oberkochen, Germany) and a confocal laser scanning fluorescent microscope (LSM 510 META, Carl Zeiss). Final image process-ings were performed by LSM image manager and Axiovision software (Carl Zeiss). The quantification of GFP-MSCs was calculated by counting green spots in the fixed area (250 μm semidiameter; original magnifica- tion ×20).

Real-time reverse transcription-PCR (real-time PCR)

Total RNA from cultured MSCs and fibroblasts was extracted by using an RNAeasy Plus Mini kit (Qiagen, Hilden, Germany) according to the manu-facturer’s instructions. First-strand cDNA was synthesized from 2 μg total RNA using SuperScript III Reverse Transcriptase with Oligo (d)20 (Invitro-gen). mRNA of the genes of interest were quantitated by real-time PCR (7700HT, PerkinElmer, Wellesley, MA) using SYBR Green Master Mix (Applied Biosystems, Foster City, CA). The sequences of primers used for each transcripts were as follows: CCR7: forward 5'-GTG TGC TTC TGC CAA GAT GA-3', reverse 5'-CCA AGA AGA TGA CAG AA-3'; CCR9: forward 5'-TAA TTC CAC TGC TTC CAC AG-3', reverse 5'-GTG CCC ACA ATG AAC ACA AG-3'; and CD62L: forward 5'-CTC GGA CAT CTT GAA CC-3', reverse 5'-AGC ATT CCT CCA GAT CCG-3'. The reaction was incubated for 10 min at 95°C, followed by 40 cycles at 95°C for 15 s and 60°C for 60 s. Data were collected with instrument spectral compensations by the Applied Biosystems SDS 2.2.1 software (Applied Biosystems) and analyzed using the threshold cycle relative quantification method. The GAPDH reference gene was used for normalizing the data. The 2^(-ΔΔCt) that corresponds to the ratio of each gene expression to GAPDH expression was calculated.

The surface expression of chemokine receptors

Cell surface expression of chemokine receptors on MSCs and fibroblasts was analyzed by direct or indirect flow cytometry staining. Cells were blocked with Fc-blocking Ab (2.4G2) and stained with anti-CCR9 (Abcam) and PE-conjugated anti-CCR7 (Biolegend, San Diego, CA), allophycocyanin-conjugated anti-CD62L (BD Pharmingen), and anti-rat IgG-Alexa 555 (Molecular Probes). Anti-rat IgG2a and IgG2b Abs (eBioscience) were used as isotype controls, or only anti-rat IgG-Alexa 555 was used as control. After performing FACS analysis by FACSsCanto II (BD Biosciences), the data were analyzed with FlowJo (Tree Star, Ashland, OR) or FACSDiv software (BD Biosciences).

In vivo DTH assay

The mouse ear-swelling test has been performed by the methods described elsewhere (24, 25). Briefly, 1, 2-dinitrofluorobenzene (DNFB) was used as a contact-sensitizing agent. DNFB sensitization was accomplished by the application of 0.5% DNFB dissolved in 4:1 acetone/olive oil onto the shaved back at days 0 and 1 post sensitization. On day 5 post sensitization, the mice were challenged by epicutaneous application of 0.2% DNFB on the right ear. The control group consisted of age- and sex-matched syngeneic naive mice challenged with 0.2% DNFB on the right ear without prior sensitization. Some DTH-MSC-infused groups were also injected i.p. with inducible NO synthase inhibitor Nomega-monomethyl l-arginine (L-NNMA; 500 μg/mouse) or IDO inhibitor 1-methyl-tryptophan (1-MT; 10 mg/mouse) daily from the day of first Ag sensitization to the day prechallenge.

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Ear thickness was measured presensitization and at 24 and 48 h postchallenge by a dial thickness gauge (Mitutoyo, Kawasaki, Japan). The degree of swelling was calculated as the thickness of the right ear (challenged ear) minus the baseline thickness of the left ear (unchallenged ear). The mice were sacrificed, and the ear and draining LN samples were collected for immunohistochemistry. To demonstrate immunosuppressive effects of MSCs on the DTH response, the animals were infused daily with one million GFP-MSCs i.v. route from day −3 to day +4. For the control, the mice were infused with PBS not containing MSCs (mock-infused).

**Materials and Methods**

Detection of apoptotic cells was evaluated by the TUNEL assay with an In Situ Cell Death Detection Kit (Roche, Mannheim, Germany) according to the manufacturer’s instructions. In brief, the sections were fixed in acetone for 10 min at 4°C and incubated in permeabilization solution (0.1% Triton X-100 and 0.1% sodium citrate) for 2 min on ice. After washing the sections three times with PBS, 50 μl TUNEL reaction mixture was added and incubated for 1 h at 37°C in a humidified atmosphere. Finally, the sections were counterstained with DAPI and analyzed by using both fluorescent and confocal microscopes. As a negative control, each experiment set up a TUNEL reaction without terminal deoxynucleotidyl transferase. The quantification of GFP-MSCs was calculated by counting green spots in fixed area (250 μm semidiameter; magnification ×20). A total of 10 sections were analyzed for each animal from the three groups.

Analysis of BrdU incorporation and apoptosis induction

Postchallenge with DNFb, mice were administered with BrdU dissolved in water by i.p. route (1 mg/mouse) and then maintained on water containing 0.8 mg/ml BrdU for 48 h. Cells from the draining LN (cervical LN) were stained with FITC-conjugated anti-BrdU Ab using the BrdU kit (Becton Dickinson) according to the manufacturer’s instructions. Finally, the cells were stained with propidium iodide (PI) and immediately analyzed by an FACSCanto II (BD Biosciences), and the data were analyzed with FlowJo (Tree Star) or FACSDiva software (BD Biosciences).

Detection of apoptosis using flow cytometry

Cells from the draining LN were isolated 2 d postchallenge and stained with PE-Cy7–conjugated anti-CD4, allophycocyanin-conjugated anti-CD8, allophycocyanin-Cy7–conjugated anti-CD8α, and allophycocyanin-conjugated anti-CD19 (BD Pharmingen) in 100 μl stain buffer for 20 min in the dark at 4°C. After washing with cold FACS buffer (0.1% sodium azide, 0.5% BSA in PBS), the cells were stained for 15 min with Annexin V-FITC and/or PI in binding buffer (Apoptosis Kit 1; BD Biosciences) according to the manufacturer’s instructions.

Data presentation and statistics

Data were presented as mean ± SD from each experiment unless otherwise stated. Statistical analyses were done by Student t test, Wilcoxon rank sum test, or Kruskal-Wallis test as appropriate. The p values <0.05 were considered to be statistically significant.
GFP-MSCs (green) in the nonchallenged ear (images are representative of three independent experiments. shown) as described in the postchallenge by application of DNFB on the right ear: the right ear was sampled, sectioned, and stained with anti-CD4, anti-CD8, and anti-CD19 (data not staining of right ear (challenged ear) of the DTH-mock–infused mice (3

DTH by challenging them with 0.2% DNFB with prior sensitization on day 0 as described in the Materials and Methods; DTH-MSC-infused: the mice infused daily with one million GFP-MSCs by the i.v. route from day 3 to day +4 and induced DTH by challenging them with 0.2% DNFB without prior sensitization; DTH-Mock-infused: the mice infused daily with 300 MSCs were observed in the lungs, stomach, and small and large intestines. Interestingly, a small but significant number of GFP-MSCs were also detected in secondary lymphoid organs including spleen and various LNs (axillary, cervical, inguinal, and mesenteric LNs) (Supplemental Fig. 2). Because MSCs in the secondary lymphoid organs could directly influence various immune cells and control the immune response, we focused on MSCs localized in the LNs at various sites.

**GFP-MSCs are accumulated in the paracortical area (at the T–B cell boundary) of LNs and interact with various immune cells**

To further examine the precise spatial location of the cells, the LNs were collected from GFP-MSC–infused mice and analyzed in individual sections by staining dually with GFP and CD4, CD8, CD19, or CD11c independently. As shown in Fig. 1A, the majority of GFP-MSCs were observed at the boundary between the paracortical area (T cell-rich zone) and germinal center (B cell-rich zone), and a small number of cells resided in the cortex, medulla, and subcapsular sinus. In addition, close confocal microscopic analysis of this boundary, where T cells from the bloodstream and DCs from the lymphatic vessel encounter each other by virtue of the well-developed high endothelial venules, revealed that localizing GFP-MSCs remained in close contact with various immune cells, such as T cells, B cells, and DCs (Fig. 1B). Interestingly, the number of GFP-MSCs observed at the boundary between the paracortical area and germinal center was dose dependently increased with the similar spatial localization patterns when MSCs were repeatedly administered daily for 7 d (Fig. 2). However, the number of GFP-MSCs did not accumulate in spleen as they did in LN postinjection, and most of the infused GFP-MSCs were located in the red pulp region devoid of T and B cells (Supplemental Fig. 3). Collectively, these results suggest that a significant number of GFP-MSCs could migrate into the LNs, where they are well placed to directly influence on the function of neighboring cells to modulate the immune responses. In the quantitative analyses of the expression levels of chemokine receptors, the transcripts for CCR7, CCR9, and CD62L were shown to be expressed 2–3.8-fold higher in GFP-MSCs than in fibroblasts as a control (Supplemental Fig. 4).

Surface expression identified by FACS analysis also revealed that a small percentage of cells expressed CCR7 (2.1 ± 0.3%), CCR9 (11.6 ± 0.7%), and CD62L (4.0 ± 1.4%) (Supplemental Fig. 4). However, it is highly suggestive that chemokine receptors, such as CCR7 and CD62L, which are known to guide the migration of T cells to LNs, were expressed on the surface of MSCs, even if this is a small fraction. Indeed, we observed the expression of CCR7 and CD62L on MSC migrated to LNs in immunohistochemical analysis (Supplemental Fig. 5).

**Serial administrations of GFP-MSCs attenuate the DTH response**

To investigate whether systemically delivered GFP-MSCs suppress immune responses in vivo, we used the DNFB-induced DTH model. Because only small fraction of injected GFP-MSCs migrate to LNs,
the cells were injected daily for 8 d before the final challenge to increase the number of GFP-MSCs in LNs and therefore to maximize the therapeutic effects. As a result, ear swellings of GFP-MSC–infused mice were dramatically suppressed compared with those in the mock-infused DTH-induced group, and they were nearly comparable to those of the unsensitized control group (Fig. 3A).

**Fewer T cells but a larger number of GFP-MSCs migrate into the inflamed site during the DTH response**

The immune response induced by DTH is characterized by the numerous infiltrations of immune cells including lymphocytes, monocytes, and macrophages into the epidermis and dermis (29, 30). Especially Ag-specific T cells mediated immunological reaction during the DTH response, and both CD4+ Th1 and CD8+ cytotoxic T cells play crucial roles as effector cells for full development of the DTH response (31). To examine what types of immune cells are affected by MSCs in the challenged ear of sensitized mice, the ear tissue was analyzed by immunohistochemistry. As shown in Fig. 3B, significantly fewer CD4+ and CD8+ T cells were infiltrated in the challenged ear of GFP-MSC–infused mice than in that of mock-infused mice. Even though a number of macrophages infiltrated the challenged ear of both GFP-MSC–infused mice and mock-infused mice, the difference of the number was not statistically significant (Supplemental Fig. 6). CD19+ B cells were not detected in either group (data not shown). Interestingly, we found that numerous MSCs had migrated into the challenged, but not into the unchallenged, opposite ear during the DTH response (Fig. 3D, left panel). Taken together, amelioration of DTH is closely related to the preferential migration of MSCs into the local inflamed site and the attenuation of T cell-mediated immune response. In consistent with this notion, MSCs were found to be much less in the draining LNs of DTH-reactivated mice than in naive mice, and they were scarcely detected in T and B cell zones (Supplemental Fig. 7).

**MSCs induce apoptosis of various immune cells, dominantly T cells, in the draining LN during the DTH response**

Based on the facts that systemically administered GFP-MSCs were closely in contact with various immune cells in the draining LN, and the MSCs attenuated the T cell-mediated immune response in the inflamed site during the DTH response, we hypothesized that MSCs might exert influence on the surrounding T cells in the draining LN in which immune responses are initiated. In addition, our previous report demonstrated that apoptotic cell death of T cells by MSCs is one of the strong mechanisms in modulating T cell responses in vitro (23). To clarify this in vivo, we examined the regional LNs of mock-infused or GFP-MSC–infused mice for apoptosis at 2 d postchallenge by performing the TUNEL assay. As shown in Fig. 4A and 4B, apoptotic cells were detected in MSC-treated mice 2.5-fold more
than in mock-infused controls. These apoptotic cells were not MSCs because they did not overlap with MSCs costained with anti-GFP Ab (Fig. 4D). Rather surprisingly, the apoptotic spots were CD4+ T, CD8+ T, CD19+ B cells, or F4/80+ macrophages (Fig. 4C) and were detected in both regions far and near from MSCs (Fig. 4D). These apoptotic cells were detected in the draining LN 2-fold more than in the nondraining LNs, which implies that the apoptosis induction by MSCs was confined to local immune response (Fig. 4E, 4F). These apoptotic spots induced by MSCs were not detected in non-immune cells, such as fibroblasts, enterocytes, or pneumocytes (data not shown). To quantitatively measure MSC-mediated immunosuppressive function in the draining LNs, in vivo proliferation assays with BrdU uptake were performed after GFP-MSC administration with different dosages (8 versus 4 million cells in total). Consistent with previous data, ear swelling was dose dependently suppressed by injected GFP-MSCs (Fig. 5A). Furthermore, as shown in Fig. 5B and 5C, the proliferative T and B cells, BrdU-positive cells, were notably decreased in the draining LNs in a dose-dependent manner. Next, to quantitatively analyze apoptotic cell death by MSCs in the draining LNs, total lymphocytes isolated from the draining LNs were stained with Annexin V and anti-CD4, anti-CD8, or anti-CD19 and performed FACS analysis. As a result, obviously increased apoptosis of CD4+ and CD8+ T cells were identified in the GFP-MSC–infused group in a dose-dependent manner, although this was less obvious in the case of B cells (Fig. 5D). This apoptosis of T cells was induced significantly in CD3+ BrdU+ T cells, but not in CD3+ BrdU− T cells (Fig. 5E, 5F). Collectively, these results demonstrated that systemically administered MSCs induce apoptosis of activated T cells in the draining LNs of mice during DTH response.

NO but not IDO mediates T cell apoptosis

How do infused MSCs induce apoptosis of T cells in the draining LN? Recent studies including our report suggest that several factors including IL-10, TGF-β, PGE2, IDO, and NO may contribute to the immunomodulatory function of MSCs. Among them, two previous reports demonstrated that IDO and NO were critically involved in the activated T cell apoptosis by MSC in vitro. Therefore, we examined in vivo role of IDO and NO in apoptosis

FIGURE 5. Apoptosis of BrdU+CD3+ T cells by MSCs. Lymphocytes isolated from the draining LNs of the mice were analyzed by BrdU incorporation and Annexin V/PI apoptosis assay. A. Ear swelling was dose dependently suppressed by the infused MSCs. The DTH-1/2 MSC-infused indicates the group of mice that were injected with a half dose (4 million) of DTH-MSC–infused (8 million) mouse. Each measure represents the mean ± SD of the increase in ear swelling. n = 1–2 mice per group from five independent experiments. B and C. The percentage of BrdU-incorporated cells among CD4+, CD8+, and CD19+ cell populations based on mean fluorescence intensity was measured by FACS. D. The percentage of Annexin V-positive cells in CD4+, CD8+, and CD19+ cell populations was measured by FACS. The data are representative of five independent experiments. E. The percentage of apoptosis induction in CD3+ BrdU+ and CD3+ BrdU− T cells was analyzed by FACS. The data are representative of five independent experiments. F. The percentage of apoptosis in CD3+ BrdU+ T cells was significantly increased by MSCs infusion, which was not observed in CD3+ BrdU− T cells. The values are the mean ± SD from five experiments. *p < 0.05; **p < 0.01; ***p < 0.001; n.s. indicates p > 0.05.
induction of T cells by using 1-MT (IDO inhibitor) or L-NMMA (NO inhibitor) during the DTH response. As shown in Fig. 6, reduction of ear swelling by MSC infusion was largely abrogated, and increased apoptosis of T cells by MSCs was also greatly reduced in draining LNs by the treatment with the NO inhibitor. In contrast, treatment with the IDO inhibitor did not affect the ear swelling and apoptosis induction at all. These results suggest that NO is a mediator of apoptosis induction of T cells and modulator of DTH response.

**Discussion**

In this study, we showed that a small but significant number of systemically administered MSCs localized preferentially around the paracortical areas in the LN of mice and attenuated ear swelling by inducing bystander apoptosis of T cells via NO production during the DTH response.

Understanding the mechanisms of MSC-mediated immunomodulation in vivo and the migration capacity to secondary lymphoid organs or inflammatory site following systemic delivery is crucial for the success of any clinical strategy involving use of MSCs. Many of the previous reports showed that MSCs derived from adult BM were distributed to various organs including the lungs, intestines, liver, and spleen (26–28). In those studies, the authors have analyzed MSCs distribution by using PCR, GFP tracking, or imaging techniques based on $^{111}$In labeling of the injected cells and gamma camera detection at early time points (24–48 h) postinfusion. However, these methods were insufficient to give information on the single-cell level distribution pattern, and it was difficult to detect minute radioactivity shown in several organs where the minority of MSCs were trapped during systemic circulation. Our results from spectral imaging of MSCs labeled with quantum dots showed similar patterns with those using $^{111}$In imaging (Supplemental Fig. 1). To elaborate on MSC localization, we employed GFP-MSCs isolated from GFP-transgenic mice and analyzed individual tissue sections by immunohistochemical staining. Consistent with previous results, the majority of GFP-MSCs were observed in the lungs, intestines, and stomach 24 h postinfusion, but interestingly, a significant number of GFP-MSCs were found in secondary lymphoid organs, such as LNs and spleen (Supplemental Fig. 2). Although several studies revealed that some of the MSCs migrated to LNs in naive or diseased mice (17, 28), our study further demonstrated that MSCs preferentially localized at the boundary between the paracortical area and the germinal center, where they could exert direct influence on the various immune cells in LNs. Indeed, a careful confocal microscopic analysis of secondary lymphoid organs revealed that localizing MSCs were closely in contact with various immune cells including T cells, B cells, and DCs. In addition, through the consecutive systemic administration of MSCs for 7 d, we demonstrated that relatively large numbers of MSCs could be accumulated in the paracortical areas, where they can contact with various immune cells with more possibilities (Figs. 1, 2).

The surface and mRNA expression patterns of chemokine receptors and CD62L, shown in Supplemental Fig. 4, might account for these in vivo localizations of systemically delivered MSCs. Among them, the expression of CCR7 (2.1 ± 0.3%) or CD62L (4.0 ± 1.4%) on MSCs, although their expression frequency is very low, may mediate their migration toward LNs. Although our results with MSCs from CCR7 knockout mice did not support this argument (Supplemental Fig. 8), the immunohistochemical data that MSCs in cervical LNs did express CCR7 or CD62L (Supplemental Fig. 5) keeps open the possibility that these chemokine receptors are responsible for preferential migration to LNs via ligand and receptor interaction. Clonal selection of MSCs highly expressing specific chemokine receptors or raising expression levels by genetic modification may clarify this point in future studies.

To date, bystander immunomodulatory effects of MSCs have been reported in several disease models, such as graft-versus-host disease (6), experimental autoimmune encephalomyelitis (16, 17), collagen-induced arthritis (32), allogeneic skin grafts (18), transient common carotid artery occlusion (33), OVA-induced DTH (22), and cecal ligation and puncture (34). In this study, our clearly showed that systemically delivered MSCs alleviated clinical symptoms in DNF-induced DTH in a dose-dependent manner. In general, clinical symptoms of DTH are characterized by ear swelling after a topical chemical challenge following prior sensitization. Indeed, as shown in Fig. 3, ear thickness in the mock-infused group increased by 3.5-fold compared with that of the unchallenged opposite ear, but infused MSCs completely suppressed ear swelling. The immune response for DTH is known to be mediated predominantly by T cells (35). Consistent with this notion, we found that apoptotic T cells were significantly increased in the draining LNs, and the infiltration of T cells in the inflamed site was markedly reduced in MSC-treated mice during the DTH response. In addition, we also demonstrated that these apoptosis was induced significantly in the activated T cells (CD3+ and BrdU+), but not in resting T cells by MSCs. Collectively, our results indicate that infused MSCs alleviated ear swelling probably by modulating the T cell response, which is responsible for the in vivo manifestation of DNF-induced DTH.

In the mechanistic studies, Plumas et al. (19) reported that apoptosis induction of activated T cells by human MSCs is mediated through IDO induction and tryptophan depletion, whereas Ren et al. (22) reported that the apoptosis induction of activated T cells by mouse MSCs was dependent on NO. In our recent study, we showed that IL-10 and IDO are responsible for T cell suppression by mouse MSCs (23). Interestingly, we observed that ear swelling and apoptosis of the activated T cells by MSCs in the draining LNs during the DTH response was not affected by 1-MT (IDO inhibitor), but strongly abrogated by L-NMMA (NO inhibitor). Taken together, these results clearly suggest that immunosuppressive effects of MSCs are not simply mediated by one factor, but rather that multiple factors are involved in the context-dependent manner, with one factor being predominant over the others depending on the context. However, this assumption warrants future studies.
Preferential migration of infused MSCs to the inflamed site during the DTH response is also of interest. Several reports including this study have shown that locally or systemically delivered MSCs specifically migrate to the inflamed sites during inflammatory responses and may regenerate or repair damaged tissue (36, 37). In this study, interestingly, we found that accumulated MSCs in the draining LNs (cervical LNs) markedly decreased after challenging with DNFB, unlike those in nondraining LNs. In contrast, accumulation of MSCs in the inflamed sites was evidently detected 48 h after DNFB challenging and 72 h after the last MSC administration (Fig. 3D). This result indicates that MSCs present in the draining LNs after consecutive administration of MSCs might migrate out of the draining LNs into the inflamed site during the DTH response. Taken together, these results suggest that systemically delivered MSCs could attenuate the DTH response by immunomodulatory functions including apoptosis induction in the draining LN and in the inflamed site by repairing damaged tissue or immunosuppression following preferential migration during the DTH response.

In conclusion, we show that systemically infused MSCs can migrate to the draining LNs and induce mainly T cells apoptosis via NO production, thereby alleviating ear swelling in this mouse model of DTH. Our study provides a nice proof of concept. Harnessing a better way to target MSCs to specific locations, such as LNs, will maximize therapeutic potentials of MSCs.

Acknowledgments
We thank Dr. Elizabeth Simpson for careful review and comments on the manuscript.

Disclosures
The authors have no financial conflicts of interest.

References
FIGURE S1. In vivo and ex vivo whole body imaging of MSCs-infused mouse. MSCs were labeled with Qdot® (800 nm) for 1 hr at 37oC. One million MSCs were injected via the tail vein into mice. After 24 hours, the mice were killed and various organs were collected. Trapped MSCs were visualized by spectral fluorescent imaging system. A, Infused MSCs were detected in the lungs of live animals (left; MSCs-injected, right; vehicle-injected). B, MSCs were detected in organs removed post mortem, including lung, spleen, intestine, and liver ex vivo (left; vehicle-injected, right; MSCs-injected).

FIGURE S2. Organ distribution of GFP-MSCs-infused mice. The distribution pattern of GFP-MSCs (5×10⁵ cells) in syngeneic mice at 24 hours after i.v. injection. A, Scattered GFP-MSCs in the whole organ were detected by anti-GFP-alexa 488 antibodies and DAPI as nuclear staining (GFP-MSCs; green, nucleus; blue). B, Infiltrated GFP-MSCs into the whole organ were counted in a fixed area. The distribution was quantified as described in the Methods. The values shown are representative of two independent experiments and analysis was performed at least 6 areas per tissue.

FIGURE S3. GFP-MSCs were not accumulated at the region devoid of immune cell in spleen. Spleen were collected 1, 3 and 7 days after GFP-MSCs (5×10⁵ cells) had been injected via the tail vein daily, sectioned and stained with anti-GFP antibodies (green) and anti-CD3 antibodies (red) as indicated in the Methods. A, GFP-MSCs were accumulated in a specific area (around the red pulp region) 24hr after injections (original magnification; 10×). B, Quantification of GFP+ spots in the spleen. The number of spots in 6 fixed area (a fixed area is defined as a circle with a semi-diameter of 250 μm under 10× magnification) was counted and SD. The analysis was performed at least six sections per lymph node (WP; white pulp, RP; red pulp).

FIGURE S4. Comparison of chemokine receptor expression profiles between MSC and fibroblast. The level of mRNA and protein expression of chemokine receptors in cultured-MSCs and fibroblasts. A. The surface expression of various chemokine receptors was analyzed by FACS staining using appropriate antibodies as described in Materials and Methods. B, Total RNAs were isolated from cultured MSCs and fibroblasts and subjected to real time-PCR analyses. Gene expression ratios above or below 1 indicate that a given gene is expressed more or less in MSCs than in fibroblasts, respectively. Filled bars on all panels depict gene expression ratio of 1, a value obtained when gene expression ratios between MSCs and fibroblasts are equal. MSCs express higher levels of CCR7, CCR9 and CD62L.

FIGURE S5. The level of mRNA and protein expression of chemokine receptors in cultured-MSCs and fibroblasts. A. The surface expression of various chemokine receptors was analyzed by FACS staining using appropriate antibodies as described in Materials and Methods. MSCs infused mice were sacrificed and the lymph nodes were collected, frozen-sectioned and immunostained for CCR7, CD62L (red) and GFP antibodies (green) (original magnification; 40×) by appropriate antibodies described in the Materials and Methods. The images are representative from 3 independent experiments.
FIGURE S6. The number of macrophages infiltrated into the inflamed site vs. non-inflamed site. DNFB-challenged ear (right ear) tissue was collected, sectioned and stained with anti-F4/80 as indicated in the Materials and Methods. A. Immunohistochemical staining of the challenged ear of the DTH-mock-infused mice (left panel) and DTH-MSC-infused mice (right panel). B. Quantification of the macrophages infiltrated into the right ear. The number of the infiltrated cells (red) in 5 fixed areas (a fixed area is defined as a circle with a semi-diameter of 100 μm SD. The analyses were performed in at least 3 sections per tissue.

FIGURE S7. Smaller number of MSCs was detected in the draining lymph node in DTH-reactivated mice compared with naïve mice. Histology of cervical lymph node in DTH-MSC infused mice. A. Immunohistochemical staining of the lymph node of DTH-MSCs-infused mice. Cervical lymph node was collected 7 days after GFP-MSCs (5×10^5 cells) had been injected via the tail vein daily, sectioned and stained with anti-GFP antibodies (green) and anti-CD3 antibodies (red) as indicated in the Materials and Methods. White arrow indicates infiltrated MSCs (T; T cells, B; B cells). B. Quantification of MSCs infiltrated into the lymph node in naïve and DTH-MSC-infused mice. The number of the infiltrated cells in 4 fixed areas (a fixed area is defined as a circle with a semi-diameter of 100 μm under 20× magnification) was counted and presented as SD (*** p<0.001). The analyses were performed in at least 3 sections per tissue.

Figure S8. The immunosuppressive effect of MSCs from CCR7-/- mouse assessed by ear swelling and induction of T cell apoptosis in the dLN in DTH-reactivated mouse. Some DTH-reactivated mice were i.v. injected with cultured CCR7-/- MSCs. Lymphocytes isolated from the draining lymph node of DTH-reactivated mice were analyzed in PI+ population gated on BrdU-incorporated CD3+ T cells. A. Ear swelling. CCR7-/- MSCs had no effect on abrogating the suppression of swelling. B. The percentage of PI+ cells in CD3+ BrdU+ T cells was measured by FACS. The graphs are representative from 3 independent experiments.
A

Swelling size (mm)

D+0  D+1  D+2

Control  DTH-Mock-infused  DTH-MSC-infused  DTH-CCR7KO MSC infused

B

DTH + Mock  DTH + MSC  DTH + CCR7 KO MSC

Counts

13.1  22.6  21.6

PI