Supplementary Data

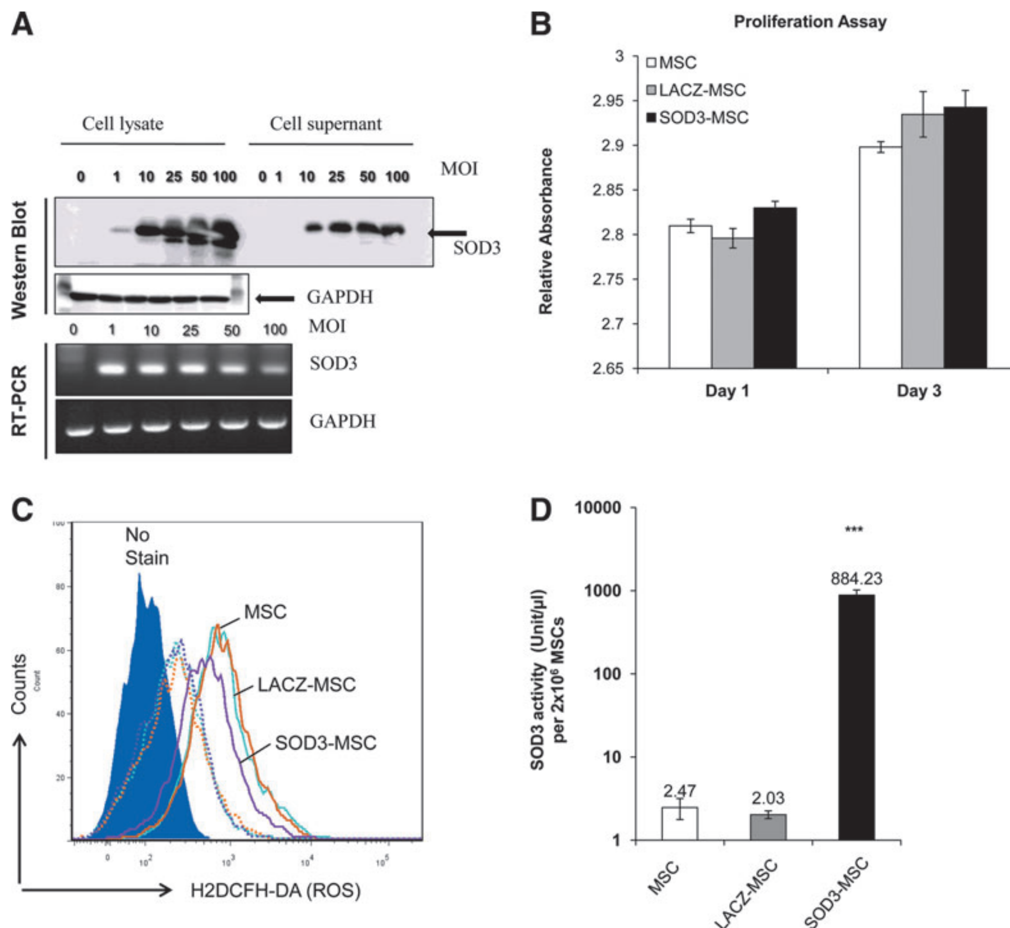
Supplementary Materials and Methods

Isolation and culture of mesenchymal stem cells

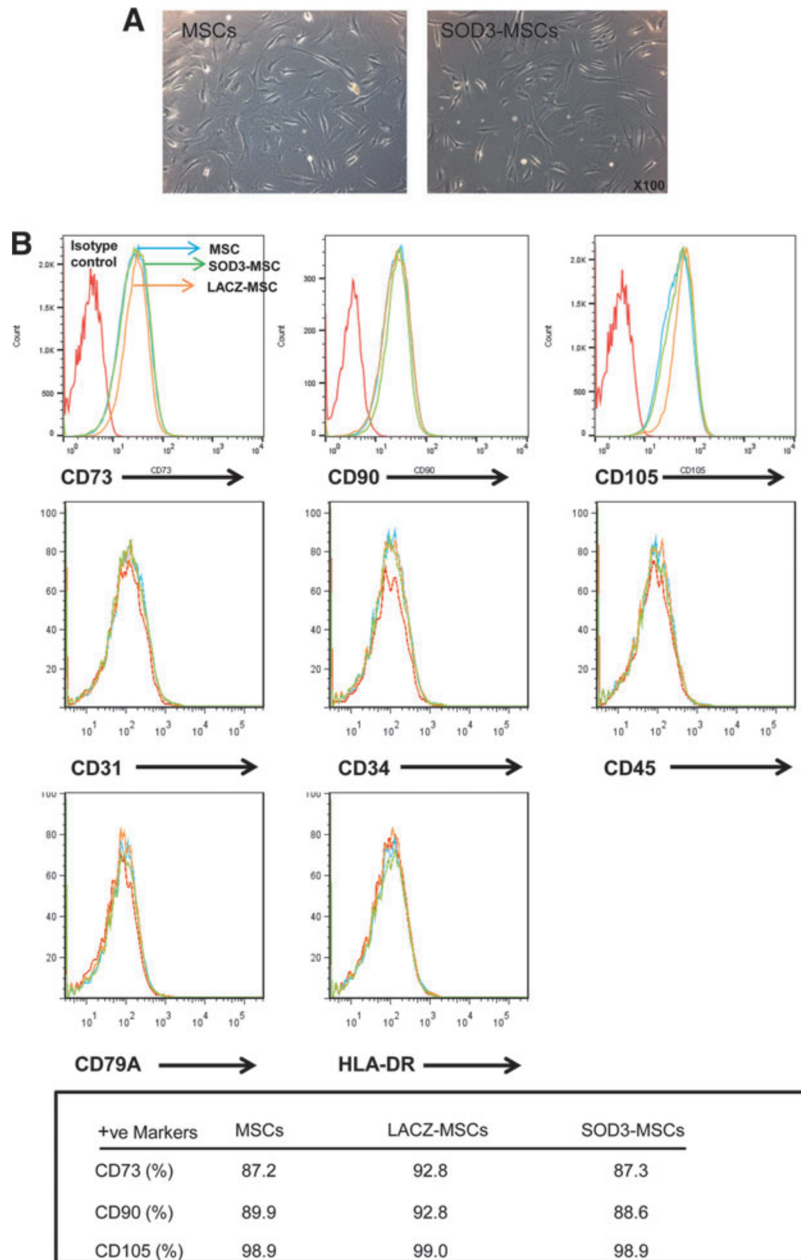
Umbilical cord blood samples of full-term neonates were collected with the mother's consent using a blood collection bag containing citrate phosphate dextrose as an anticoagulant and processed within 24 h. A fraction of the mononuclear cells was separated by centrifugation in a Ficoll-Paque PLUS gradient (Amersham Biosciences), washed with Hank's balanced salt solution (Jeil Biotech Services), and resuspended in low-glucose Dulbecco's modified Eagle's medium (Invitrogen Corp.), containing 20% fetal bovine serum (FBS), 2 m*M* L-glutamine, 1 m*M* sodium pyruvate, and a 1% antibiotic/antimycotic solution (Life Technologies) consisting of 100 U/ml penicillin, 100 μg/ml streptomycin, and 25 μg/ml amphotericin B. After 7 days, nonadherent cells were discarded, and adherent cells were cultured with two medium changes per week. Cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2. Approximately 60% of the confluent cells were detached with 0.1% trypsin-EDTA and replated in culture flasks.

Adenoviral expression vector for extracellular superoxide dismutase and transduction conditions

The pRC/CMV (Micobix Co.) hSOD3 vector was used to insert human extracellular superoxide dismutase (SOD3) into the E1 shuttle vector pCA14 (Invitrogen Corp.) by



SUPPLEMENTARY FIG. S1. Evaluation of proliferation, ROS levels, and SOD3 activity upon SOD3 transduction in MSCs. (A) Western blot and RT-PCR analysis of SOD3 expression in MSCs upon transduction. **(B)** Proliferation of MSCs was evaluated by PrestoBlue assay (Thermo Fisher Scientific). **(C)** ROS production in SOD3-transduced MSCs or MSCs was assessed by staining with H2DCFH-DA (5 μM) and FACS analysis was followed. *Filled histogram*, *dotted open histograms*, and *solid open histograms* represent no stain control, ROS with no stimuli, and with stimuli Fas ligand (100 ng/ml), respectively. **(D)** SOD3 activity in cultured medium was measured by using the SOD assay kit (Dojindo Lab) and expressed as unit/μl per 2×10^6 cells. Data are represented as mean ± SD. ***$p < 0.001$. MSCs, mesenchymal stem cells; ROS, reactive oxygen species; RT-PCR, reverse transcriptase–polymerase chain reaction; SD, standard deviation; SOD3, extracellular superoxide dismutase.



+ve Markers	MSCs	LACZ-MSCs	SOD3-MSCs
CD73 (%)	87.2	92.8	87.3
CD90 (%)	89.9	92.8	88.6
CD105 (%)	98.9	99.0	98.9

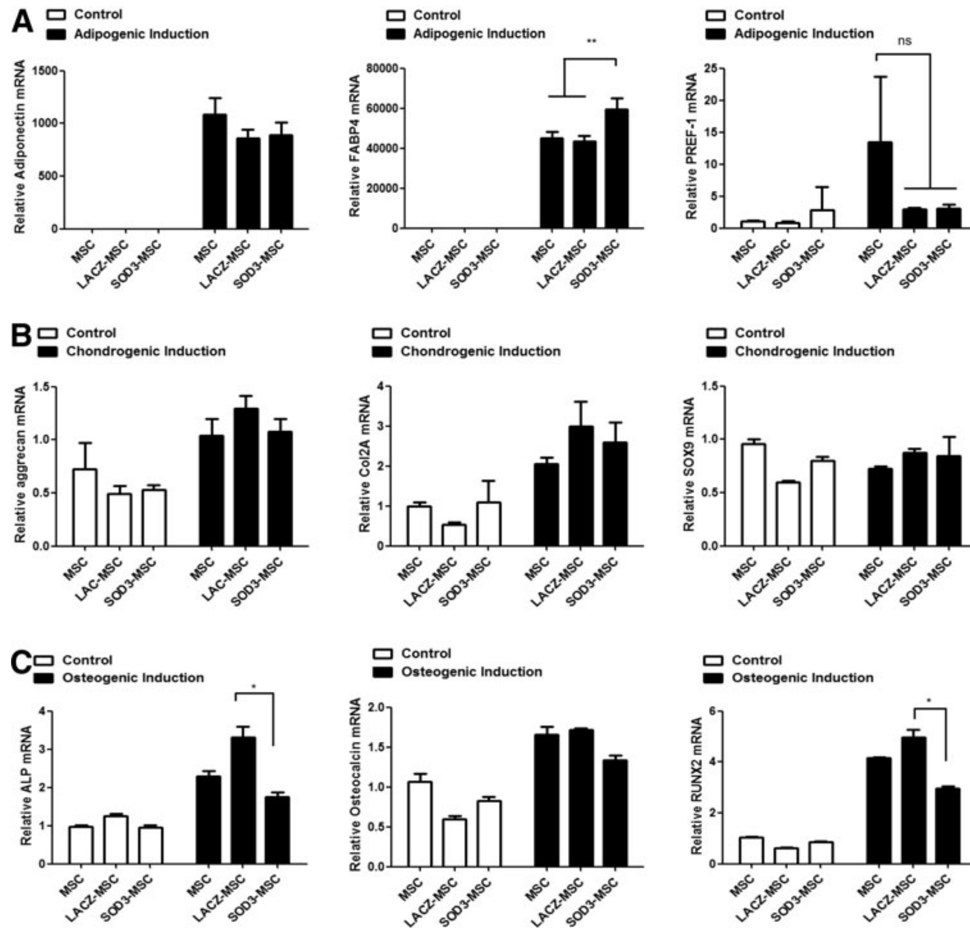
SUPPLEMENTARY FIG. S2. Transduction of MSCs with SOD3 does not alter MSC phenotypes and positive surface marker expression. (A) Phenotype of MSCs upon transduction with SOD3. **(B)** Characterization of positive and negative cell surface marker expression by flow cytometry.

homologous recombination. Recombination between shuttle vector and the adenovirus vector dE1-k35/lacz generated the final construct, dE1-k35/SOD3. Primary supernatants containing recombinant adenoviruses were plaque-purified and amplified in HEK 293 cells. Mesenchymal stem cells (MSCs) were then transduced with adenovirus vector expressing either human SOD3 or LACZ (control) at a multiplicity of infection of 10.

Characterization and differentiation of MSCs and SOD3-MSCs

The immunophenotypes of the MSCs or SOD3-transduced MSCs were assessed by determining the expression of MSC-related antigens, such as CD90 (Thy-1), CD105, (endoglin), and CD73 (SH3), and the absence of hematopoietic lineage markers, such as CD34 and CD45, and endothelial markers such as CD31 by flow cytometric analysis (Epics XL; Beckman Coulter). The cells were also negative for CD79A and HLA-DR. The respective fluorescent conjugated monoclonal antibodies were obtained from Becton Dickinson.

For the differentiation study, MSCs or SOD3-transduced MSCs were seeded onto six-well plates. Differentiation was induced using adipogenic, chondrogenic, and osteogenic differentiation media Bulletkits (LONZA), respectively, according to the manufacturer's instructions. Cells were cultured for specific time periods outlined in the instruction, and differentiation into trilineages was analyzed



SUPPLEMENTARY FIG. S3. ***In vitro*** **trilineage differentiation of MSCs and SOD3-transduced MSCs.** SOD3-transduced MSCs or MSCs differentiated into adipocytes **(A)**, chondrocytes **(B)**, and osteocytes **(C)**. The expression of specific gene markers such as adiponectin, FABP4, PREF-1 (for adipogenic lineage); aggrecan, Col2a, SOX9 (for chondrogenic lineage); ALP, osteocalcin, and RUNX2 (for osteogenic lineage) was assessed by real-time PCR. GAPDH was used as endogenous control. Data are represented as mean ± SD. $^{*}p < 0.05$; $^{**}p < 0.01$. ALP, alkaline phosphatase; FABP4, fatty acid-binding protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PREF, preadipocyte factor; ns, non significant.

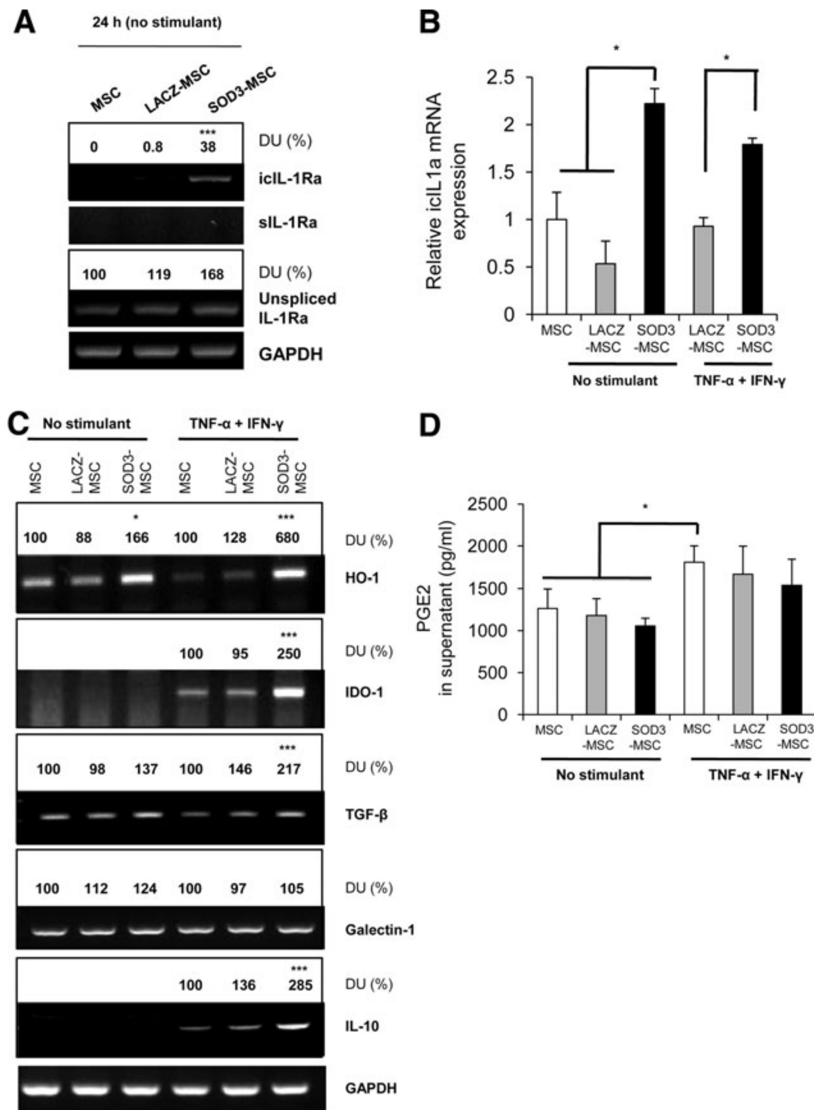
by real-time polymerase chain reaction (PCR) using specific gene markers.

T-cell proliferation assay

The carboxyfluorescein diacetate succinimidyl ester–mixed lymphocyte reaction (CFSE-MLR) assays were performed to determine the proliferation of $CD4^+$ and $CD8^+$ T cells. The assay was performed by plating 1×10^6 CFSE-labeled responder cells (spleen and lymph node cells from C57BL/6 mice) in triplicate in 24-well flat bottom plates (Costar). The cells were stimulated with 1×10^6 stimulator cells (BALB/c), which were irradiated at 3000 cGY, and cocultured in a total volume of 1 ml in 24-well flat bottom plates with MSCs, SOD3-transduced MSCs, or SOD3-transduced MSCs in the presence of Cu/Zn SOD inhibitor, diethyldithiocarbamate trihydrate (DETCA) (10 μM) (Sigma-Aldrich), at a ratio 10:1 and incubated at 37°C in 5% CO_2 and 100% humidity. After the 5-day culture period, cells were harvested, washed twice, and resuspended in phosphate-buffered saline. T-cell subsets of responder cells were quantified by flow cytometric analysis using the phycoerythrin (PE)-CY7-conjugated anti-mouse CD4 and PE-conjugated anti-mouse CD8 (BD Biosciences Pharmingen).

T-cell differentiation

Naïve $CD4^+$ T cells were purified by negative selection from spleens and lymph nodes of C57BL/6 mice using MACS column (Miltenyi Biotech). Cells were activated by plate-bound anti-CD3 and soluble anti-CD28 (2 μg/ml) antibodies in Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% FBS, 2 mM glutamine, and 1% penicillin–streptomycin solution for 4 days. The cells were polarized under T helper 1 (Th1)-polarizing conditions (10 μg/ml anti-interleukin-4 [IL-4] Ab, 10 ng/ml IL-12), Th2-polarizing conditions (10 μg/ml anti-interferon-gamma [IFN-γ] Ab, 10 ng/ml IL-4), Th17-polarizing conditions (20 ng/ml IL-6, 5 ng/ml transforming growth factor-beta [TGF-β], 10 μg/ml anti-IFN-γ antibody, and 10 μg/ml anti-IL-4 antibody), and Treg-polarizing conditions (5 ng/ml TGF-β and 10 ng/ml IL-2) and cocultured with MSCs or SOD3-transduced MSCs in the presence of DETCA (10 μM) at a 10:1 ratio for 4 days. All cytokines and antibodies used for $CD4^+$ T-cell differentiation were purchased from BD Biosciences. After 4 days, the cells were harvested for mRNA expression analysis using key cytokines or molecules specific for the indicated T-cell subset expansion.



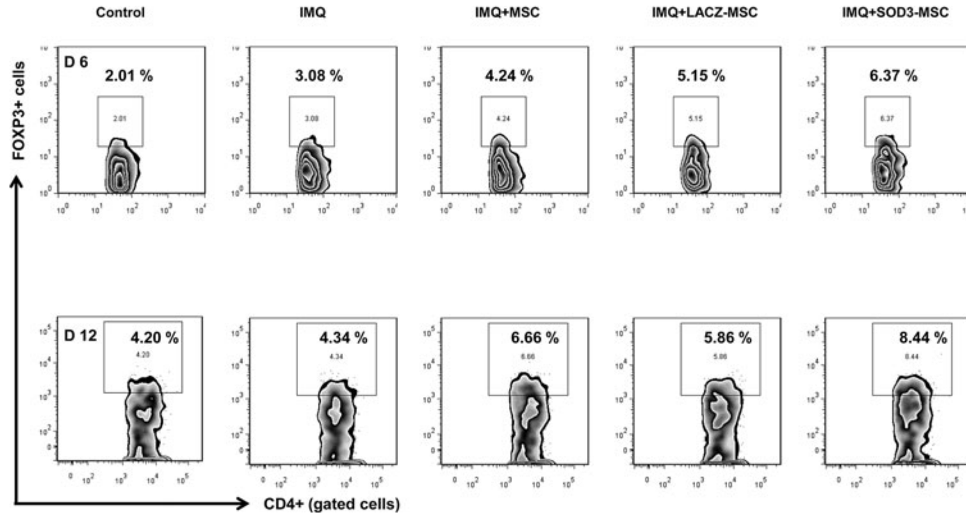
SUPPLEMENTARY FIG. S4. Overexpression of SOD3 in MSCs induces the expression of immunosuppressive molecules. (A–C) The expression of icIL-1Ra, sIL-1Ra, unspliced IL-1Ra, HO-1, IDO-1, TGF-β, galectin-1, and IL-10 was assessed by RT-PCR. The relative expression of icIL-1Ra was measured by real-time PCR. GAPDH was used as a control. **(D)** The PGE2 concentration in supernatant was measured by a PGE2 ELISA kit (Enzo Life Sciences). MSCs were primed with TNF-α (10 ng/ml) and IFN-γ (100 U/ml). The numerical values on the blot represent % relative DU, as measured by ImageJ software (National Institutes of Health). The MSC group was set as 100% DU. Error bar indicates SD; $*p < 0.05$, $***p < 0.001$. DU, densitometric unit; HO-1, heme oxygenase-1; icIL-1Ra, intracellular IL-1 receptor antagonist; IDO-1, indoleamine-pyrrole 2,3-dioxygenase; IFN-γ, interferon-gamma; PGE2, prostaglandin E2; sIL-1Ra, soluble IL-1 receptor antagonist; TGF-β, transforming growth factor-beta; TNF-α, tumor necrosis factor-alpha.

Evaluation of Treg cell population

After aseptic spleen removal from the mice body, spleen cells were then collected and RBCs in the collection were disrupted with conventional RBC lysis buffer. The spleen cell counts were adjusted to 2×10^6 cell/ml in RPMI1640 (Gibco Life Technologies) supplemented with 10% FBS (Invitrogen Corp.), 100 μg/ml streptomycin, and 100 IU/ml penicillin (Sigma) and cocultured with MSCs or SOD3-transduced MSCs in the presence of DETCA (10 μM) at 10:1 ratio in a 24-well plate. The cells were then stimulated with 10 μg/ml of anti-CD3 (eBioscience, Inc.) and 2 μg/ml of anti-CD28 (eBioscience, Inc.) for 72 h at 37°C in a humidified atmosphere of 5% CO_2. After 72 h, the cells were collected and stained with Foxp3 and CD4 antibodies for flow cytometric analysis.

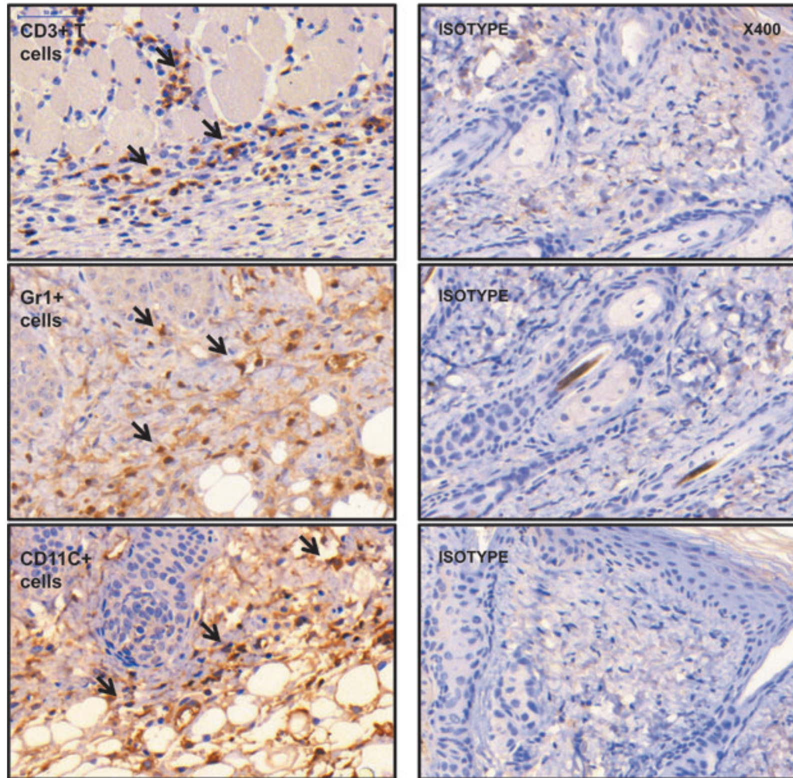
Assay of cyclic adenosine monophosphate by ELISA

Levels of cyclic adenosine monophosphate (cAMP) in blood plasma and tissue lysate were measured by the Direct cAMP ELISA kit (ADI-900-066; Enzo Life Sciences). Briefly, standards and samples were added to wells coated with a GxR IgG antibody. A blue solution of cAMP conjugated to alkaline phosphatase was then added, followed by a yellow solution of rabbit polyclonal antibody to cAMP, and incubated for 2 h. The plate is washed, leaving only



SUPPLEMENTARY FIG. S5. Regulatory T-cell population in spleens of mice after injection of SOD3-MSCs or MSCs. Spleens were harvested at the end of the experiments and analyzed for percentage of $CD4^{+}CD25^{+}FOXP3^{+}$ T cells in single-cell suspensions by flow cytometry.

bound cAMP. The p-nitrophenyl phosphate substrate solution was added and incubated for 1 h. The substrate generated a yellow color when catalyzed by the alkaline phosphatase on the cAMP conjugate. Stop solution was added to stop the reaction and read at 405 nm. The mean optical density of substrate blank was subtracted from all sample readings. The concentration of cAMP was calculated from standard plot. In the skin lysates, the cAMP concentrations were normalized with total protein content and expressed as pmol/mg.



SUPPLEMENTARY FIG. S6. High-magnification *inset* (×400), representing specific immune cells with isotype controls in skin. Immunohistochemistry analysis was performed with respective isotype controls to evaluate the specificity of antibodies as described in the Materials and Methods section. The *arrows* indicate the infiltration of effector cells.

Primer sequences for reverse transcriptase-PCR/real-time PCR of mRNA

icIL-1Ra: F 5′-TTATGGGCAGCAGCTCAGTT-3′, R 5′-TTGACACAGGACAGGCACAT-3′;
sIL-1Ra: F 5′-TCCGCAGTCACCTAATCACTC-3′, R 5′-TTGACACAGGACAGGCACAT-3′;
Unspliced IL-1Ra: F 5′-GGCCTCCGCAGTCACCTAATCACTCT-3′, R 5′-GGTCGCACTATCC ACATCTGGG-3′;
HO-1: F 5′-CCTGGTGTCCCTTCAATCAT-3′, R 5′-GGCGATGAGGTGGAATACAT-3′;
IDO-1: F 5′-TGTGAACCCAAAAGCATTTTTC-3′, R 5′-AAAGACGCTGCTTTGGCC-3′;
TGF-β: F 5′-CCCAGCATCTGCAAAGCTC-3′, R 5′-GTCAATGTACAGCTGCCGCA-3′;
Galectin-1: F 5′-GGTCTGGTCGCCAGCAACCTGAAT-3′, R 5′-TGAGGCGGTTGGGGAACTTG-3′;
IL-10: F 5′-AAGCTGAGAACCAAGACCCAGACATCAAGGCG-3′, R 5′-AGCTATCCCAGAG CCCCAGATCCGATTTTGG-3′;
GAPDH: F 5′-AAGGTCGGAGTCAACGGATTTGGT-3′, R 5′-AGTGATGGCATGGACTGTG GTCAT-3′;
Aggrecan: F 5′-AGCCTGCGCTCCAATGACT-3′, R 5′-TAATGGAACACGATGCCTTTCA-3′;
SOX9: F 5′-TTC CGCGACGTGGACAT-3′, R 5′-TCAAACTCGTTGACATCGAAGGT-3′;
COL2A: F 5′-GGCAATAGCAGGTTCACGTACA-3′, R 5′-CGATAACAGTCTTGCCCCACTTA-3′;
ALP: *F* 5′-GACCTCCTCGGAAGACACTC-3′, R 5′-TGAAGGGCTTCTTGTCTGTG-3′;
Osteocalcin: F 5′-AGCAAAGGTGCAGCCTTTGT-3′, R 5′-GCGCCTGGGTCTCTTCACT-3′;
RUNX2: F 5′-TCTTAGAACAAATTCTGCCCTTT-3′, R 5′-TGCTTTGGTCTTGAAATCACA-3′;
Adiponectin: F 5′-TCCTCACTTCCATTCTGACTG-3′, R 5′-GGACCAATAAGACCTGGATCT-3′;
FABP4: F 5′-AAAGTCAAGAGCACCATAACC-3′, R 5′-TTCAATGCGAACTTCAGTCC-3′;
PREF-1: F 5′-CTGCCTTCACGGACTCTG-3′, R 5′-GCCCGAACATCTCTATCACA-3′.