SUPPLEMENTARY APPENDIX

Human stem cells transplanted into the rat stroke brain migrate to the spleen via lymphatic and inflammation pathways

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Online Supplementary Index

SUPPLEMENTAL METHODS

Human Nuclei (HuNu) Staining for Graft Survival and LYVE-1 + HuNu Double Staining for Cell Migration

HuNu staining was performed as described in our previous study.[1] For LYVE-1 and HuNu double staining, free floating sections of the spleen and mounted sections of the brain were washed 3 times for 10 minutes in TBS (Bio-Rad, CA, USA). Samples were blocked for 60 minutes at room temperature with 5% normal goat serum (Invitrogen, Frederick, MD, USA) in TBS containing 0.1% Tween-100 (TPBS, Sigma-Aldrich, MO, USA). Sections were then incubated overnight at 4°C with rabbit polyclonal anti-LYVE1 (1:200; Novus Biologicals, Littleton, CO, USA) and mouse monoclonal anti-HuNu (1:50; EMD Millipore, MAB1281, Billerica, MA, USA) with 5% normal goat serum. The sections were washed 3 times for 10 minutes in TBST and then soaked in 5% normal goat serum in TBST containing corresponding secondary antibodies–goat anti-rabbit IgG-AF 555 (red; 1:500) (ThermoFisher, MA, USA) and goat anti-mouse IgG-Alexa 488 (green; 1:500)–for 4 hours at 4°C. Finally, sections were washed in TBS, then cover-slipped with DAPI (Vector Laboratories, CA, USA). Brain and spleen sections were examined using the Zeiss Axio Imager Z1 microscope (Zeiss, Thornwood, NY, USA). Ten pictures were taken for each condition and the number of target cells were counted manually using ImageJ (NIH Image Software).[2] Pictures were taken close to the dural sinuses in the brain and near the gate of the spleen, close to the white pulp in the spleen. Cell counting was performed within 250 × 250 μm squares.[2] Primary antibodies were omitted in control experiments, which generated negative results.[3]

Major Histocompatibility Complex II and Major Histocompatibility Complex II + LYVE-1 Double Staining

Staining for major histocompatibility complex II (MHCII)-positive cells via OX6 was conducted on every sixth coronal section of the brain and spleen, as previously described in our past study.[1] For OX6 and LYVE-1 staining, free floating sections of the spleen and mounted sections of the brain were washed 3 times for 10 minutes in TBS. Samples were blocked for 60 minutes at room temperature with 5% normal goat serum (Invitrogen, Frederick, MD, USA) in PBS containing 0.4% Tween 100 (TBST; Sigma-Aldrich, MO, USA). Then sections were incubated overnight at 4°C with mouse anti-rabbit OX6 (1:750) and rabbit polyclonal anti-LYVE1 (1:200) with 5% normal goat serum. Sections were washed 3 times for 10 minutes in TBST and then soaked in 5% normal goat serum in TBST containing corresponding secondary antibodies–goat anti-mouse IgG-Alexa 488 (green; 1:500) and goat anti-rabbit IgG-AF 555 (red; 1:500)–for 4 hours at 4°C. Finally, sections were washed in TBS, then cover-slipped with DAPI. Brain and spleen sections were examined using the Zeiss Axio Imager Z1 microscope (Zeiss, Thornwood, NY, USA). Ten pictures were taken for each condition and the number of target cells were counted manually using ImageJ (NIH Image Software).[2] Pictures were taken close to the dural sinuses in the brain and near the gate of the spleen, close to the white pulp in the spleen. Cell counting was performed within 250 × 250 μm squares.[2] Primary antibodies were omitted in control experiments, which generated negative results.[3]

Human Specific Phagocytic Marker CD68, Anti-Apoptotic Inhibitor 5 and Neuronal Marker Triple Staining in Brain and Spleen

Staining for human specific phagocytic marker CD68, anti-apoptotic inhibitor 5, and neuronal marker positive cells was conducted every sixth coronal section of the brain and spleen. Free floating sections of the spleen and mounted sections of the brain were washed 3 times for 10 minutes in PBS. Samples were blocked for 60 minutes at room temperature with 5% normal goat serum (Invitrogen, Frederick, MD, USA) in PBS containing 0.4% Tween 100 (PBST; Sigma-Aldrich, MO, USA). Then, sections were incubated overnight at 4°C with mouse monoclonal anti-CD68 (1:4, abcam, ab845), rat specific apoptotic marker—anti apoptosis inhibitor 5 (1:200, abcam), rat specific neuronal marker—anti-160kD neurofilament medium antibody (1:2000, abcam) with 10% normal goat serum. Of note, this particular anti-CD68 antibody that is specific to human macrophages was used to label phagocytic activity in only hBMSCs. Negative control tests for this antibody were performed and demonstrated no labeling of rat tissue. Sections were washed 3 times for 10 minutes in PBST and then soaked in 10% normal goat serum in PBST containing corresponding secondary antibodies–goat anti-mouse IgG-Alexa 488 (green; 1:2000), goat anti-rabbit IgG-AF 594 (red; 1:500), and goat anti-chicken 405 (blue, 1:500, abcam)–for 4 hours at 4°C. Finally, sections were washed in TBS, then cover-slipped with DAPI. Brain and spleen sections were examined using the Zeiss Axio Imager Z1 microscope (Zeiss, Thornwood, NY, USA). Ten pictures were taken for each condition and the number of target cells were counted manually using ImageJ (NIH Image Software).[2] Pictures were taken near the transplantation site in the brain and near blood vessels in the spleen. Cell counting was performed within 250 × 250 μm squares.[2] Primary antibodies were omitted in control experiments, which generated negative results.[3]

Cell Migration Assay

Prior to seeding hBMSCs in the top of the filter membrane in a transwell insert, the apical side of the insert was coated with CEM for 4 hours at 37°C. The cell density was adjusted as 1×10^6 cells/5ml. Thereafter, cells were incubated with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine perchlorate (DiD, Invitrogen, Eugene, OR, USA) for 30 minutes to evaluate migration of the hBMSCs. After labeling, cells were rinsed using PBS and centrifuged twice (1500 rpm for 5 minutes). The pellet of labeled cells was suspended in 5ml of fluorescent medium. Next, 10,000 cells in 50µl of migration medium were loaded into one well of a 96-well plate overnight, allowing the cells to settle. Following the overnight incubation, the hBMSCs that were seeded into the upper chamber migrated below to the lower chamber. At the different time points (0 hours, 12 hours, 24 hours, 48 hours, and 72 hours), the bottom plate was read with a fluorescence plate reader (Odyssey, LI-Cor, NE, USA).

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