Mini Review

Analysis of dendritic cells from common marmosets for the treatment of CNS injury

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Dendritic cells (DCs) play important roles as modulators of immune responses and are well known for their ability to activate T cells. Recently, we demonstrated that implantation of DCs into the injured spinal cord results in activation of endogenous neural stem/progenitor cells (NSPCs), promoting repair of the injured central nervous system (CNS). DCs are strong inducers of the proliferation and survival of NSPCs as well as producers of the neurotrophic factor, NT-3. To analyze the therapeutic efficacy of DC therapy for CNS injury in a nonhuman primate, we established a method to isolate DCs from the common marmoset (CM), becuase the CM offers many advantages for preclinical studies over other monkeys. Bone marrow (BM)-derived $CD11c^+$ cells from the CM showed the characteristic features of DCs, including the typical DC morphology and the ability of the cells to undergo endocytosis, secrete IL-12, and stimulate xenogenic T cells. The BM of the CM proved to be an excellent cell source for isolating DCs intended for preclinical studies of cell therapy, for which large quantities of the cells are required. We are considering a preclinical study of cell therapy using BM-derived $CD11c^+$ DCs for the treatment of SCI in the CM, to evaluate the therapeutic effects and safety of this procedure for clinical application.

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Introduction

Dendritic cells (DCs) are key regulators of T-cell immunity, as they possess a remarkable ability to take up, process and present antigens, as compared with other antigen-presenting cells[1,2]. Based on their strong ability to activate cytotoxic T lymphocytes, DCs are regarded as a useful tool for cancer immunotherapy and are currently being used in human clinical studies[3-6]. Furthermore, we demonstrated a new function of DCs relevant to the treatment of central nervous system (CNS) diseases, such as spinal cord injury (SCI), of activating endogenous neural stem/progenitor cells (NSPCs)[7].

DCs have been most extensively characterized in humans and rodents. Using primates instead of rodents to analyze the therapeutic effects of DC therapy is an important step towards future clinical studies of the application DCs for the treatment of SCI. Although DCs have been isolated from Rhesus and African green monkeys[8-12], details of the immune system, including the functions of the DCs, in the common marmoset (CM) remain un-

clear. Recently, we established a method to isolate DCs from the CM and subsequently characterized these cells for preclinical studies[13]. The CM offer many advantages for preclinical studies over other monkeysts[14,15]. The average weight of an adult CM is between 200g-300g, which makes it possible to handle and breed them easily on a large scale while reducing the cost of the experiments[15]. Sequence analysis of the entire CM genome is in progress at the Washington University and NIH Intramural Sequencing Center, and the results of these efforts will clarify the genetic similarities between the CM and humans. Because of these advantages, the CM has been widely used in many studies involving gene therapy[16,17], bacterial infections[18], toxicology[19] and immunology[20,21]. The usefulness of a CM model for studies on CNS diseases, including SCI, has also been shown[22,23]. This article provides an outline of DC therapy for the treatment of spinal cord injury and presents the results of characterization of DCs from the CM for application to preclinical studies.

DC therapy for the treatment of spinal cord injury

Neural stem cells have been shown to exist in the adult mammalian CNS[24-31]. However, the CNS self-repair activity is extremely poor, especially in the spinal cord[27,31,32]. Although in some pathologic conditions such as ischemia, endogenous NSPCs are likely to give rise to neurons and hence to functional recovery in the adult mammalian brain[33-37], this has not been shown in the injured spinal cord[26,38]. The lack of *de novo* neurogenesis in the injured adult spinal cord cannot be explained solely by the intrinsic properties of the NSPCs, because they are able to differentiate into neurons both *in vitro* and *in vivo* when transplanted into neurogenic sites, such as the hippocampal dentate gyrus, but not when transplanted into the adult spinal cord[30]. In the event of injury of the adult spinal cord, the NSPCs proliferate and differentiate exclusively into astrocytes rather than into neurons[26], suggesting that the microenvironment in the spinal cord is highly inhibitory for neuronal differentiation and supportive for astrogliosis[39-42].

Somewhat unexpectedly, however, activation of some immune systems, including activation by implantation of activated macrophages[43] or induction of autoimmune T cells before or after SCI[44-46], has been demonstrated to promote functional recovery of the injured spinal cord[47]. These effects could be explained by the clearance of CNS myelin by the activated macrophages[48] or blockade of myelin-associated neurite growth inhibitors[49-52] by the anti-Nogo T cells[53], which seem to induce axonal regeneration. In addition, immune cells may also be actively involved in functional recovery, e.g., by activating endogenous NSPCs to engage in *de novo* neurogenesis. To examine this possibility, we investigated the potential trophic effects of immune cells on the proliferation of NSPCs *in vitro* using a neurosphere-formation assay, in which the NSPCs were cocultured with immune cells. Among the immune cells tested, DCs showed the strongest activity of inducing the proliferation and survival of NSPCs *in vitro*[7]. Moreover, we found that DCs implanted into the injured adult mouse spinal cord activated the proliferation of endogenous NSPCs *in vivo* and induced *de novo* neurogenesis. DCs also produced neurotrophin (NT)-3 *in vitro* and *in vivo* and activated endogenous microglia. Behavioral analysis revealed that the locomotor functions of the DC-implanted mice showed significant recovery as compared with those of the control mice. Our results suggest that DC implantation exerts trophic effects, including activation of the endogenous NSPCs, promoting repair of the injured adult spinal cord.

Isolation and characterization of DCs from the bone marrow of common marmosets

Healthy CMs were selected from the experimental stock at the Central Institute for Experimental Animals (Kawasaki, Japan). All animal experiments were performed according to the guidelines of the Animal Care and Use Committee of the Keio University School of Medicine. The cross-reactivities of the following anti-human monoclonal antibodies (mAbs) in CM were analyzed using flow cytometry, and to the present results agreed with previous results[16,54,55]: CD1a (clone HI149, eBioscience, San Diego, CA), CD1c (clone AD5-8E7, MiltenyiBiotec, Bergisch Gladbach, Germany; clone 11.86, Becton Dickinson, San Jose, CA), CD3 (clone SP34, BD Pharmingen, San Diego, CA), CD4 (clone MT310, DAKO Cytomation, Glostrup, Denmark), CD8 (clone T8, Beckman Coulter), CD11c (clone S-HCL-3, Becton Dickinson), CD14 (clone TUK4, DAKO Cytomation; clone M5E2, BD Pharmingen), CD34 (clone BIRMA-K3, DAKO), CD80 (clone MAB104, Beckman Coulter), CD83 (clone HB15a, Beckman Coulter), CD86 (clone B-T7, Diaclone, Besançon Cedex), and HLA-DR (clone G46-6, BD Pharmingen) (Table 1).

Femurs and tibiae were removed and the bone marrow (BM) cells were suspended in RPMI-1640 supplemented with 10% heat-inactivated fetal calf serum (FCS). After overnight culture, the cell suspensions were collected and plated in a complete medium (cRPMI), namely, RPMI-1640 supplemented with 10% FCS, recombinant human (rh) GM-CSF (100 ng/mL) and rhIL-4 (100 ng/mL), based on the method for generating mouse BM-derived DCs[56,57]. The reactivity of human GM-CSF and IL-4 with the cells of the CM has been previously demonstrated[58,59]. On culture days 7-8, the floating cells were collected as a DC-enriched cell fraction. On day 7, 2-5% of the non-adherent cul-

Table Human monoclonal antibodies that cross-react in common marmoset

Antigens	Clones	Providers	Isotypes
CD1a	HI149	eBioscience	IgG1
CD1c	AD5-8E7	Miltenyibiotec	IgG2a
	11.86	Becton Dickinson	IgG2a
CD3	SP34	BD Pharmingen	IgG3
CD4	MT310	DAKO	IgG1
CD8	T8	Beckman Coulter	IgG1
CD11c	S-HCL-3	Becton Dickinson	IgG2b
CD14	TUK4	DAKO	IgG2a
	M5E2	BD Pharmingen	IgG2a
CD34	BIRMA-K3	DAKO	IgG1
CD80	MAB104	Beckman Coulter	IgG1
CD83	HB15a	Beckman Coulter	IgG2b
CD86	B-T7	Diaclone	IgG1
HLA-DR	G46-6	BD Pharmingen	IgG2a

tured-BM cells (non-adherent BM) exhibited the CD11c$^+$ HLA-DR$^+$ phenotype, indicating that more than 1X10^7 of the CD11c$^+$ HLA-DR$^+$ cells were isolated from the CM specimen. Confocal imaging showed the co-localization of CD11c with the HLA-DR on the surface of cells with numerous dendrites, a morphological characteristic of DCs (Fig.1)[13]. More CD11c$^+$ HLA-DR$^+$ cells were generated in the presence of rhGM-CSF and rhIL-4 than in the presence of rhGM-CSF alone. DCs were also generated from CD34$^+$ BM progenitor cells, based on methods reported previously[8,12]. Sorted-CD34$^+$ BM cells were plated in RPMI-1640 medium supplemented with 10% FCS, 1% non-essential amino acids, 1 mM sodium pyruvate, 10mM Hepes, rhGM-CSF (100 ng/ml), rhFlt3-L (100 ng/ml), rhSCF (100 ng/ml), and rhTNF-α (5 ng/ml). On day 5, the cells were recultured in cRPMI supplemented with rhTNF-α (5 ng/ml), and cultured further for a week. The number of CD11c$^+$ HLA-DR$^+$ cells generated from the CD34$^+$ BM cells was less than one-eighth of that from non-adherent BM cells.

We analyzed the phenotype change of the CD11c$^+$ cells generated from the non-adherent BM and CD34$^+$ BM cells following maturation. For maturation, the BM cell culture was stimulated with 1 μg/mL *Esherichia coli* (*E coli*)(*055:B5*)-derived lipopolysaccharide (LPS) for 24 hours. To enrich the CD11c$^+$ cell population, the floating cultured cells were labeled with PE-conjugated anti-human CD11c mAb and purified by cell sorting. In both the methods, the LPS-stimulated CD11c$^+$ cells showed higher expression levels of CD80, CD83, CD86 and HLA-DR than the non-stimulated CD11c$^+$ cells, and the CD11c$^+$ cells from non-adherent BM cells and CD34$^+$ BM cells showed similar expression patterns (Fig.1)[13]. Moreover, the CD11c$^+$ HLA-DR$^+$ cells from the non-adherent BM contained CD1a$^+$ and CD1c$^+$ cell populations, which are known as markers of human DCs[60,61], but no CD3$^+$ population. Therefore, we used the DCs derived from the non-adherent BM for further analysis.

To examine the functional characteristics of the BM-derived CM DCs, the cytokine production and ability to stimulate xenogenic human T cells were analyzed. For the xenogeneic mixed leukocyte reaction (MLR), human T cells were purified from peripheral blood cells as responder cells. These responder cells were seeded into a 96-well plate together with titrated numbers of irradiated DCs as stimulators, in RPMI-1640 supplemented with 10% human AB serum. After 5 days of co-culture, the cells were pulsed with 10 mM 5-bromo-2' deoxyuridine (BrdU) for 24 hours and examined by a BrdU incorporation assay. In the MLR experiments, the culture supernatants of the CM-DCs stimulated with LPS (1 μg/mL) for 24 hours were analyzed for IL-12 (p70). These analyses showed that the LPS-stimulated CD11c$^+$ cells secreted IL-12 and caused proliferation of xenogenic human T cells in a dose-dependent fashion, indicating their potency as APCs[13]. Furthermore, an ELISA revealed that human T cells co-cultured with the LPS-stimulated CD11c$^+$ cells secreted IFN-γ, but not IL-4, suggesting that BM-derived CD11c$^+$ cells from the CM could induce Th1-type immune responses similar to those induced by human DC1.

The endocytotic activity of the DCs was also analyzed as described previously[57]. BM-derived CD11c$^+$ cells were incubated with Dextran-FITC (1mg/mL) at either 4 ℃ or 37 ℃ for 30 minutes in cRPMI. After being washed with PBS, the cells were analyzed using a FACS. For the immunocytochemical analysis, PE-labeled CD11c$^+$ cells were incubated with Dextran-FITC (1mg/mL) at either 37 ℃ or 4℃ for 2 hours. The BM-derived CD11c$^+$ cells incubated at 37℃ incorporated more Dextran-FITC than the cells incubated at 4℃, and the LPS-stimulated CD11c$^+$ cells (mature type) showed a weaker ability for endocytosis than the non-stimulated CD11c$^+$ cells (immature type), consistent with the functional features of DCs (Fig.2)[13].

We previously demonstrated that mouse splenic DCs secrete the neurotrophic factor, NT-3[7]. Lysates of BM-derived DCs were assayed for NT-3 and BDNF. We found that the BM-derived DCs from the CM also produced NT-3, whereas no apparent production of BDNF was observed from these cells.

Taken together, our results show that the characteristics of the DCs obtained from the CM resemble those of the human DCs, suggesting the usefulness of CM-DCs for preclinical studies on cell therapy. Moreover, we showed that BM-derived DCs from the CM also produced NT-3, an important neurotrophic factor for CNS regeneration. We are considering a preclinical

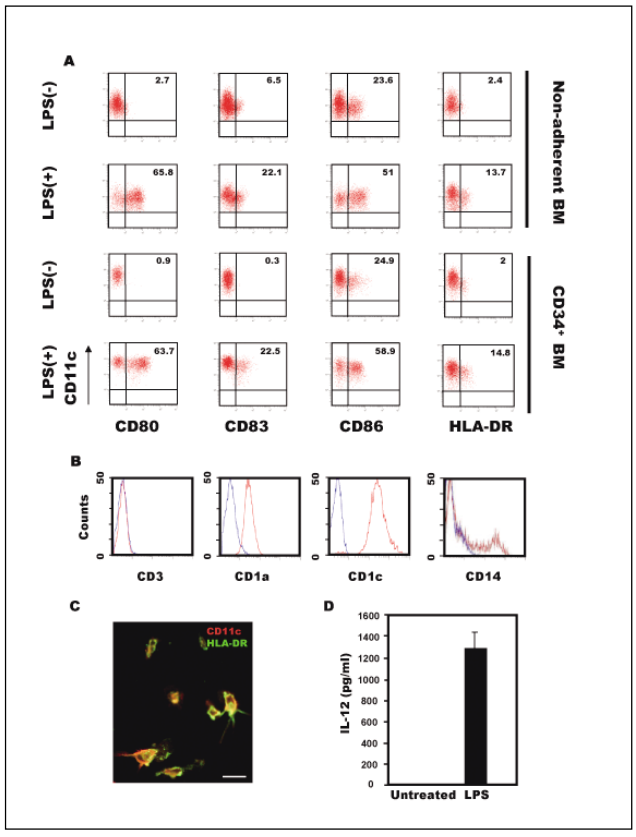

Fig.1 Characterization of BM-derived DCs from the CM

(A) Analyses of CD80, CD83, CD86 and HLA-DR expressions on the CD11c+ cells generated from non-adherent cultured BM cells (Non-adherent BM) and CD34+ BM cells. For maturation, the cultured BM cells were treated with LPS (1 μg/mL) for another 24 hours. The numbers within the dot blots represent the percentages within the quadrant. (B) Expressions of CD3, CD1a, CD1c, and CD14 were observed in the BM-derived CD11c+ HLA-DR+ cells (red line). Isotype controls are shown by a blue line. (C) Imunocytochemical analysis of BM-derived DCs. After stimulation with LPS for 24 hours, CD11c (red) and HLA-DR (green) were expressed on the cell surface of the dendrites. Scale bar: 10 μm. (D) Culture supernatants of BM-derived DCs treated with LPS for 24 hours then analyzed for IL-12 production using an ELISA. (from Ohta et al., Immunology, 123: 566-574, 2008; reprinted with permission of Blackwell Publishing Ltd.)

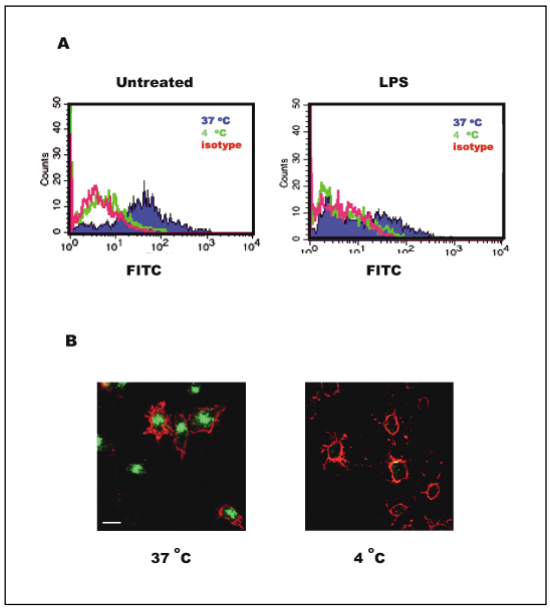

Fig.2 Immature BM-derived DCs exhibit endocytotic activity

(A) BM-derived CD11c+ cells that were stimulated with LPS (1 μg/mL) or left untreated were incubated with FITC-dextran for 30 minutes at either 37℃ (Shaded blue histograms) or 4℃ (green line) as a control for the background passive uptake. An isotype control is shown by the red line. (B) Immature BM-derived DCs labeled with PE-conjugated anti-CD11c mAb were incubated with Dextran-FITC at 37 or 4℃ for 2 hours. Confocal microscopic image showing fluorescent microspheres (green) in the cytoplasm of the immature BM-derived CD11c+ cells (red) incubated at 37℃, but not in those incubated at 4℃. Scale bar: 10 μm. (from Ohta et al., Immunology, 123: 566-574, 2008; reprinted with permission of Blackwell Publishing Ltd.)

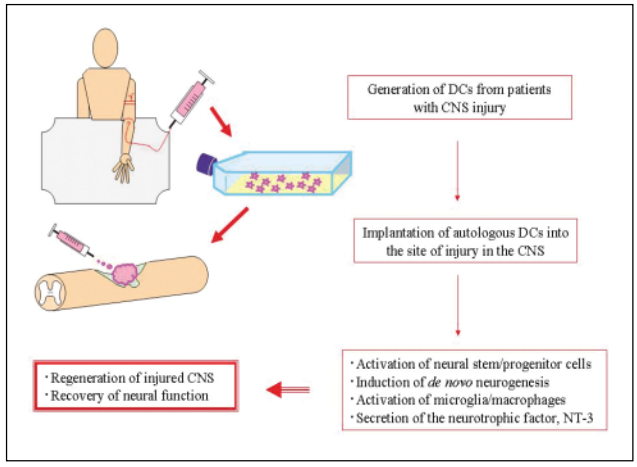

Fig.3 Autologous DC therapy for the treatment of central nervous system injury

DCs are generated from patients with central nervous system (CNS) injury and the autologous DCs are implanted into the site of injury in the CNS.

study on cell therapy using BM-derived $CD11c^+$ DCs for the treatment of SCI in the CM, to evaluate the therapeutic effects and safety of this procedure for clinical application (Fig.3).

Protocol for preclinical study on DC therapy for the treatment of SCI in CM

CMs have naturally chimeric bone marrow and peripheral blood because the placental circulation is shared between dizygotic twins and immune tolerance exists between the twins[62]. Therefore, we use dizygotic twins for donor-recipient pairs in the DC transplantation studies. First, peripheral blood mononuclear cells are harvested from dizygotic twins and a mixed lymphocyte reaction (MLR) is performed to select adequate donor-recipient pairs. MLR-negative pairs are then used for the following cell therapy experiment. Contusive SCI at the C5 level is induced in a CM recipient using a weight-drop device (a modified NYU impactor with a diameter of 3.5 mm), as described previously[22]. On the same day as the SCI, BM cells are harvested from the CM donor and are cultured in the presence of rhGM-CSF and rhIL-4 for 7 days. On day 7, $CD11c^+$ $HLA\text{-}DR^+$ DCs (1-5 x 10^6 cells) isolated from the cultured-BM cells are injected into the center of the lesion site. After DC implantation, motor function as well as histology and magnetic resonance imaging (MRI) of the injured spinal cord are analyzed to evaluate the therapeutic effect and safety of DC therapy in a nonhuman primate.

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