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Degradation of amyloid beta by human induced pluripotent stem cell-derived macrophages expressing Neprilysin-2

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Abstract The purpose of this study was to evaluate the therapeutic potential of human induced pluripotent stem (iPS) cell-derived macrophage-like cells for Alzheimer's disease (AD). In previous studies, we established the technology to generate macrophage-like myeloid lineage cells with proliferating capacity from human iPS cells, and we designated the cells iPS-ML. iPS-ML reduced the level of Aβ added into the culture medium, and the culture supernatant of iPS-ML alleviated the neurotoxicity of Aβ. We generated iPS-ML expressing the Fc-receptor-fused form of a single chain antibody specific to Aβ. In addition, we made iPS-ML expressing Neprilysin-2 (NEP2), which is a protease with Aβ-degrading activity. In vitro, expression of NEP2 but not anti-Aβ scFv enhanced the effect to reduce the level of soluble Aβ oligomer in the culture medium and to alleviate the neurotoxicity of Aβ. To analyze the effect of iPS-ML expressing NEP2 (iPS-ML/NEP2) in vivo, we intracerebrally administered the iPS-ML/NEP2 to 5XFAD mice, which is a mouse model of AD. We observed significant reduction in the level of Aβ in the brain interstitial fluid following administration of iPS-ML/NEP2. These results suggested that iPS-ML/NEP2 may be a potential therapeutic agent in the treatment of AD.

Abbreviations: iPS cells, induced pluripotent stem cells; AD, Alzheimer's disease; Aβ, β amyloid peptide; AβO, β amyloid peptide oligomer; iPS-ML, iPS cell-derived macrophage-like myeloid lineage cells; NEP2, Neprilysin-2; scFv, single chain antibody; MMEL, Membrane metallo-endopeptidase-like protein; ISF, interstitial fluid; iPS-MC, iPS cell-derived myeloid cells; APP, amyloid precursor protein; GM-CSF, granulocyte–macrophage colony-stimulating factor; M-CSF, macrophage-colony-stimulating factor.

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Introduction

Alzheimer's disease (AD) is the most common cause of neurodegenerative dementia in elderly people; currently, the disease affects more than 36 million people worldwide. AD is characterized by slowly progressive recent memory deficits, cognitive impairment, and personality changes associated with neuronal loss (Blennow et al., 2006). The main risk factor of sporadic AD is aging; prevalence after 65 years of age is 5%, increasing to about 30% of people aged over 85 years old. As a result of the prolonged lifespans in our aging society, the number of patients is expected to continue to increase in the future (Ferri et al., 2005). At the moment, only symptomatic therapies for this disease are available, and the development of a disease-modifying therapy is a required (Carter et al., 2010).

The two major pathological hallmarks of AD are extracellular senile plaques and intraneuronal neurofibrillary tangles. Senile plaques mainly consist of β amyloid peptide (Aβ), especially the Aβ1–42 isoform. Based on the genetic findings from familial AD studies, Aβ is assumed to be the primary inducer of AD pathology (Hardy and Selkoe, 2002; Tanzi and Bertram, 2005). Transgenic mouse lines that accumulate Aβ in their brains are used as experimental models to identify a therapeutic approach for AD. Previous studies have shown that vaccination reduced the brain Aβ deposits and improved cognitive functions in these model animals (Janus et al., 2000; Morgan et al., 2000; Schenk et al., 1999). Nevertheless, in the human clinical trials, vaccination therapy has failed to improve cognitive function so far (Robinson et al., 2004). Furthermore, several adverse events such as meningo-encephalitis (Nicoll et al., 2003), vasogenic edema (Salloway et al., 2009), and micro hemorrhage (Boche et al., 2008) occurred in some of the treated patients.

Inflammatory changes are observed in AD brains, particularly at the vicinity of senile plaques. They are abundant in activated microglia, which are the resident macrophages in the central nervous system, in both human AD samples (Mattiace et al., 1990; Perlmutter et al., 1990) and transgenic mouse models (Frautschy et al., 1998). Aβ-activated microglia release a wide variety of neurotoxic molecules including proinflammatory cytokines (Griffin, 2006), reactive oxygen species (Reddy et al., 2009), and complement proteins (Bonifati and Kishore, 2007), which contribute to the neurodegeneration in AD. On the other hand, microglia have beneficial effects against AD as a result of neurotrophic agent secretion and clearing Aβ by phagocytosis (Mizuno et al., 2004; Yan et al., 2006). In addition, in vitro experiments strongly suggest a role for microglia in phagocytic clearance of Aβ. Phagocytosis of Aβ by exogenously administered microglia was indicated by an in vivo study using intra-hippocampal Aβ-injected rats (Takata et al., 2007). However, their exact role in the pathogenesis of AD remains to be elucidated.

Macrophages are innate immune cells with the capacity to eliminate invading pathogens and dying cells, and maintain homeostasis in many tissues. Manipulation of macrophages to enhance their capacity to efficiently clear Aβ with low neurotoxicity is expected to provide therapeutic treatments for AD (Malm et al., 2010). A recent study demonstrated that peripherally transplanted CD11b$^+$ bone marrow-derived monocytes (BMM) migrate into the vicinity of Aβ plaques, and that these modified cells secreted the proteolytic enzyme neprilysin and reduced the Aβ burden in model mice (Lebson et al., 2010).

These results suggest the potential of bone marrow-derived myeloid-lineage cells in alleviating AD pathology and as therapeutic agents. However, to use myeloid-lineage cells in AD therapy, an adequate supply of therapeutic cells is necessary. Preparation of a large quantity of myeloid lineage cells from bone marrow or peripheral blood of AD patients for the treatment is not practical and the limited cellular sources obstruct the development of a cell-based therapy. Recently, we have developed an iPS cell-based method to generate abundant quantities of myeloid lineage cells. Using this technology, it may be possible to resolve the issue of limited cell sources (Senju et al., 2009). Previously, we have reported the generation of iPS cell-derived macrophage-like myeloid lineage cells (iPS-MC) that were genetically modified to express a membrane-bound form of single chain antibody (scFv) specific to Aβ. In the in vitro analysis, the Aβ-specific scFv-transfectant iPS-MC exhibited efficient Aβ-specific phagocytic activity (Senju et al., 2011).

Neprilysin is a membrane-bound protease with efficient Aβ degradation activity (Iwata et al., 2001). The amino acid sequence of membrane metallo-endopeptidase-like protein (MMEL, neprilysin-2) has been reported to be highly homologous with neprilysin. Neprilysin-2 (NEP2) has two alternatively spliced forms: a membrane-bound and soluble-secreted variant. The soluble-secreted form is also known as soluble, secreted endopeptidase (SEP) (Ikeda et al., 1999). In mice, Nep2 is expressed in testis and involved in sperm function, as well as modulating fertilization and early embryonic development (Ghaddar et al., 2000). NEP2 has also been characterized in the human brain, and a recent study reported that NEP2 activity is reduced in mild cognitive impaired patients and AD patients (Huang et al., 2012). Also Hafez and colleagues have demonstrated using gene knockout and transgenic animals that NEP2 contributes to Aβ degradation in vivo (Hafez et al., 2011).

In this study, we genetically modified macrophage-like myeloid lineage cells with proliferating capacity generated from human iPS cells (iPS-ML) (Haruta et al., 2013; Koba et al., 2013) to express the Aβ-degrading protease NEP2. In vitro, the transfected macrophages secreted NEP2 and reduced the levels of Aβ1–42 oligomers in the culture medium. In addition, they protected co-cultured SH-SY5Y neuroblastoma cells from the toxicity of Aβ1–42 oligomers. To evaluate the potential for AD therapy, we examined whether iPS-ML could lower the levels of Aβ1–42 peptide in brain interstitial fluid (ISF) in AD model mice. To this end, we set up a microdialysis-based ISF sampling system to examine the level of soluble Aβ1–42 peptide in the mouse brain ISF. Administration of NEP2-secreting iPS-ML into the hippocampus of the AD model transgenic mice diminished Aβ1–42 in the ISF, thereby suggesting the possibility of NEP2-secreting iPS-ML as a therapeutic means for AD.

Materials and methods

Cells and antibodies

All experiments using human samples were conducted with the approval of the Institutional Review Board of Kumamoto

University. Human iPS cells were previously established by lentivirus-mediated introduction of reprogramming factors into fibroblasts and maintained as previously described (Senju et al., 2011). The following mAbs conjugated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE) were purchased from BD Pharmingen (San Diego, CA), Beckman Coulter (Brea, CA), Miltenyi Biotec (Bergish-Gladbach, Germany), Sigma-Aldrich (St. Louis, MO), or eBioscience (San Diego, CA): anti-CD45 (clone HI30, mouse IgG1), anti-CD33 (WM53, mouse IgG1), anti-CD36 (FA6.152, mouse IgG1), anti-CD11b (ICRF44, mouse IgG1), anti-CD13 (WM15, mouse IgG1), anti-CD87 (62022, mouse IgG1). For isotype-matched controls, mouse IgG1 (MOPC-21) was used.

Plasmid construction and generation of recombinant lentivirus

A cDNA fragment of human cMYC was obtained by PCR and cloned into the pENTR-TOPO vector (Invitrogen, Carlsbad, CA, USA). cDNAs of human EZH2, NEP-2, BMI1, and MDM2 were provided by RIKEN BioResource Center (Tsukuba, Japan) or NRBC (Tokyo, Japan). The Aβ-specific scFv construct was reported previously (Senju et al., 2011). The cDNA fragments were transferred to a lentiviral expression vector, pCSII-EF (Miyoshi et al., 1998) or pCSII-EF/IRES-NEO, by using the LR clonase system (Invitrogen). pCSII-EF and the plasmids for lentiviral vector packaging, pCMV-VSV-G-RSV-Rev and pCAG-HIVgp, were kindly provided by Dr H. Miyoshi (RIKEN BioResource Center). Plasmid constructs were introduced into 293T cells by using lipofection (Lipofectamine 2000, Invitrogen), and recombinant lentivirus was recovered from the culture supernatant by centrifugation (50,000 *g*, 2 h) 3 days later.

Generation of human iPS cell-derived proliferating myeloid cells

Two lines of human iPS cells were used in this study; one (iPSL) was established from skin fibroblasts by using lenti virus-mediated introduction of reprogramming factors (Senju et al., 2011) and the other (iPSS) was established from peripheral blood T cells by using sendai virus-mediated introduction of reprogramming factors (Cytotune, Dnavec, Tsukuba, Japan). iPS cells were induced to differentiate into myeloid cells (iPS-MC) according to a previously established procedure (Senju et al., 2011). To establish iPS-ML (Haruta et al., 2013; Koba et al., 2013), iPS-MC derived from iPSL were introduced with expression vectors for cMYC plus EZH2 (iPS-ML-A) and iPS-MC derived from iPSS were introduced with cMYC, BMI1, plus MDM2 (iPS-ML-B). Both iPS-ML were cultured in the presence of M-CSF (50 ng/mL) and GM-CSF (50 ng/mL). To generate NEP-2-transfectant, the iPS-ML were infected with the recombinant lentivirus vector including IRES-Neomycin-resistance cassette for NEP2 and cultured in the presence of G-418 to select iPS-ML stably expressing NEP2.

Flow cytometric analysis

The cell samples were treated with human Fc-receptor-blocking reagent (Miltenyi Biotec) for 10 min, stained with the fluorochrome-conjugated mAb for 30 min, and washed 3 times with phosphate-buffered saline/2% fetal calf serum. The stained cell samples were analyzed using a FACScan flow cytometer (BD Bioscience, Bedford, MA, USA). For detection of cell surface NEP2, cells were incubated with polyclonal goat anti-human NEP2 antibody (AF2340; R&D Systems) for 30 min at 4 °C and with anti-goat IgG FITC (secondary antibody) for 30 min at 4 °C. For intracellular staining of NEP2, iPS-ML were fixed and permeabilized using IntraPrep reagent (Beckman Coulter), and stained with a polyclonal NEP2 antibody as described above. Goat IgG was also used as control. The cell samples were analyzed using a FACScan flow cytometer.

Western blot analysis

After the iPS-ML were cultured for 24 h, they were centrifuged and separated into cell pellets and culture supernatants. The cell pellets were subjected to protein extraction by using CytoBuster™ Protein Extraction Reagent (Novagen, San Diego, CA, USA). Protein concentration was determined using a BCA protein assay kit (PIERCE, Rockford, IL, USA). After determination of protein concentration, sample buffer containing 2-mercaptoethanol was added to the culture supernatants and cell-extracts. Samples of supernatants and cell-extracts were subjected to SDS-PAGE on 4–20% TGX Protean® gels (Bio-Rad, Hercules, CA, USA) by using a Mini Protean® II Electrophoresis Cell (Bio-Rad), and electronically transferred onto Immun-Blot® PVDF membranes (Bio-Rad) using a Mini Trans-Blot® Cell (Bio-Rad) at 100 mA for 1 h. After blocking overnight in Tris-buffered saline (0.1 M Tris–HCl, 0.9% NaCl, pH 7.4) containing 0.1% Tween 20 and 5% skim milk at 4 °C, the blots were incubated with NEP2-specific polyclonal antibody for 1 h and subsequently with an anti-goat horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h. As a control for equal protein loading in the analysis of cell extracts, membranes were also probed with a mouse anti-β-actin antibody (Sigma–Aldrich) and exposed to an HRP-conjugated anti-mouse antibody. Signals were visualized using ECL plus (Amersham Pharmacia Biotech, Buckinghamshire, UK) reagents and detected by a FPM100 developer (Fujifilm, Tokyo, Japan). All procedures were performed at room temperature unless otherwise stated.

Preparation of Aβ oligomers

Soluble Aβ1–42 oligomer (AβO) solutions were prepared as previously described (Stine et al., 2003). Briefly, synthetic human Aβ1–42 (Peptide Institute, Osaka, Japan) was dissolved at 1 mM in cold 100% 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP). The peptide was incubated at room temperature for 2 h to ensure it was monomeric and unstructured. The HFIP was dried by the vacuum desiccator and the resulting peptide film was stored at −20 °C until use. To form oligomers, the film of Aβ was dissolved in DMSO at 5 mM and further diluted to 100 μM in phenol red-free F12 medium (Ham's F-12, BioSource) by vortexing for 10 min. Subsequently, the solution was aged for 24 h at 4–8 °C. The sample was then centrifuged at 15,000 *g* for 10 min at 4–8 °C, and the soluble oligomers remained in the supernatant.

Analysis of AβO degradation in vitro

iPS-ML were cultured for 24 h and the culture supernatants were collected. iPS-ML (1×10^5 /well in 96-well culture plates) or collected supernatants were added with 10 μM of AβO in the presence or absence of 10 μM phosphoramidon (Sigma-Aldrich). After 24 h of incubation at 37 °C (5% CO_2), the culture medium was centrifuged to remove cell debris (1500 *g*, 10 min, 4 °C). The amount of Aβ1–42 in the resultant supernatants was quantified using an Aβ42-specific ELISA kit (Wako Chemicals, Tokyo, Japan). Statistical analysis was performed using two-tailed Student's *t*-tests and comparison with the control (without iPS-ML).

Analysis of AβO cytotoxicity

SH-SY5Y neuroblastoma cells were plated at a density of 1×10^4 cells/well in 96-well plates in DMEM/F12 (1:1) medium supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin, and incubated for 24 h at 37 °C in 5% CO_2. After incubation for 24 h, the medium was replaced with serum-free DMEM/F12 (1:1) medium containing the AβO solution. The cells were incubated for 48 h and the number of live cells was analyzed using the CellTiter 96 assay kit (Promega, Madison, WI) according to the kit protocol. Briefly, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2 H-tetrazolium, inner salt (MTS) was added to culture plates and the plates were incubated at 37 °C for 3 h. Absorbance at 490 nm was measured to quantify the number of live cells. The results are expressed as the percentage of MTS reduced relative to the control samples, assuming the absorbance of the control cells was 100%. To analyze the effect of iPS-ML on the death of SH-SY5Y cells, culture supernatants were prepared from iPS-ML cultured at a density of 2.5×10^5 cells/mL for 24 h. SH-SY5Y cells were plated at a density of 1×10^4 cells per well in 96-well plates (n = 3 wells in each experimental condition). After 24 h, the culture medium from the SH-SY5Y cells was replaced with the culture supernatant from the iPS-ML and AβO and/or the NEP-2 inhibitor phosphoramidon. After 48 h of incubation, the number of live SH-SY5Y cells was quantified using the CellTiter 96 assay kit, as described above.

Generation of scid/5XFAD mice

5XFAD mice (Oakley et al., 2006) harbor three mutations in the amyloid precursor protein (APP) gene and two in the presenilin-1 (PS1) gene and were used for in vivo analysis. These mice increase soluble Aβ production, develop senile plaques by 4 to 5 weeks of age, and show neuronal damage including intraneuronal accumulation of Aβ and cognitive deficits by 5 months. 5XFAD mice were crossed to scid mice, and hemizygous 5XFAD transgenic mice with scid/scid genetic backgrounds were used in the experiments.

In vivo microdialysis and iPS-ML transplantation

To measure the levels of soluble Aβ1–42 in the hippocampus ISF of awake, freely moving 5XFAD mice, microdialysis was performed as previously described (Takeda et al., 2011) with some modifications. Probe implantation was performed as previously described (Cirrito et al., 2003). Briefly, the animals were anesthetized with sodium pentobarbital (intraperitoneal injection, 50 mg/kg; Dainippon Sumitomo Pharma, Osaka, Japan), while a guide cannula (8 mm length) was stereotaxically implanted in the right hippocampus (bregma 3.1 mm, 2.4 mm lateral to the midline, and 1.1 mm ventral to dura at a 12° angle). The guide cannula was fixed using an anchoring bone screw and binary dental cement. More than 3 days after guide cannula implantation, the mice were placed in a standard microdialysis cage and a probe was inserted through the guide. The microdialysis probe had an 8 mm shaft with a 4 mm, 1000 kDa molecular weight cutoff polyethylene membrane (PEP-8-04, Eicom, Kyoto, Japan). Before use, the probe was conditioned by brief dipping in ethanol, and then washed with perfusion buffer (0.15% bovine serum albumin in Ringer's solution) that was filtered through a 0.22 μm pore size membrane (GL Sciences Inc., Japan). The preconditioned probe's outlet and inlet were connected to a peristaltic pump (Eicom) and microsyringe pump (Eicom), respectively, using fluorinated ethylene propylene (FEP) tubing (φ250 μm i.d.). After insertion of the probe, to obtain a stable 2 h of baseline recordings, the probe and connecting tubes were perfused with the perfusion buffer for 180 min at a flow rate of 10 μL/min before the baseline sample collection. The flow rate was maintained at 1.0 μL/min with a peristaltic pump (Eicom) and microsyringe pump (Eicom).

Samples were stored at 4 °C for less than 24 h in a polypropylene tube before being subjected to ELISA testing. Samples were denatured with 500 mM guanidine HCl, which solubilizes aggregated Aβ, and the Aβ1–42 levels were measured using a sandwich ELISA kit (Wako Chemicals). Baseline levels of ISF Aβ1–42 were defined as the mean concentration of Aβ over the 2 h preceding IPS-ML transplantation or Ringer's solution injection. For each animal, all Aβ levels were normalized to the basal Aβ concentration.

For iPS-ML transplantation into the hippocampus, microdialysis probes with a separate injection port at the tip (PEP-8-04-MI, Eicom) were used. This permitted us to perform microdialysis and still administer iPS-ML to the hippocampus. IPS-ML were preloaded into a 10 μL Hamilton syringe and connected to the infusion port of the microdialysis probe with FEP tubing. The iPS-ML suspended in Ringer's solution (5×10^7 /mL) were injected into the hippocampus 3 μL for 3 min.

Histological analysis

iPS-ML labeled with PKH26 (Sigma-Aldrich) or introduced with GFP-expression vector were injected into the hippocampus of mice as described above. For microscopic examination, the brain tissue was removed, fixed in 4% paraformaldehyde/phosphate-buffered saline, and embedded in Tissue-TEK OCT compound (Sakura Finetechnical, Tokyo, Japan). Frozen sections of 20-μm thickness were made using a cryostat (MICROM HN505N; Thermo Scientific, Kalamazoo, MI). The tissue sections were stained with Congo red to detect amyloid plaques, or stained with DAPI and analyzed using fluorescence microscopes (Axio Observer Z1, Carl Zeiss, Oberkochen, Germany. and FV1200, Olympus, Tokyo, Japan).

Results

Generation of iPS-ML expressing Neprilysin-2

We previously established a method to generate $CD43^+CD11b^+$ myeloid-lineage cells from human iPS cells, and designated them iPS-MC (iPS cell-derived myeloid-lineage cells) (Senju et al., 2011). iPS-MC proliferated in the presence of granulocyte–macrophage colony-stimulating factor (GM-CSF) and macrophage-colony-stimulating factor (M-CSF) for 7–14 days, and then stopped proliferating. The generation of iPS-MC with long-term proliferation would be a beneficial source of large amounts of macrophages for cell-based therapies. We had found that simultaneous introduction of cMYC in combination with EZH2, BMI1, or MDM2 resulted in the prolonged proliferation of iPS-MC. The cells continuously propagated for more than 3 months in the presence of M-CSF, with a doubling time of 24–36 h. Based on the observation, we established a method to generate a large quantity of human myeloid lineage cells. We named the iPS cell-derived long-term proliferating myeloid cells iPS-ML (iPS cell-derived myeloid cell line) (Haruta et al., 2013; Koba et al., 2013). Morphology and hematopoietic and myeloid markers, such as CD45, CD36, CD11b, CD13, CD87, and CD33 of iPS-ML generated by introduction of cMYC plus EZH2 (iPS-ML-A) and those generated by introduction of cMYC, BMI1, plus MDM2 (iPS-ML-B) into iPS-MC are shown in Fig. 1. A growth curve and results of chromosomal analysis of iPS-ML-B are shown in Supplemental Fig. 1.

iPS-ML with Aβ-degrading activity could be useful for therapy of AD. We generated an iPS-ML expressing a protease with Aβ-degrading activity. Neprilysin (NEP) is a well-known Aβ-degrading zinc-dependent metalloprotease (Iwata et al., 2001). NEP is a membrane-bound protease, and we considered that, rather than using a membrane-bound protease, a secreted type of the protease would be more appropriate and effective to degrade extracellular Aβ oligomers (AβO). Membrane metallo-endopeptidase-like protein (MMEL, NEP2), expressed as both membrane-bound protein and secereted protein, has also Aβ-degrading activity (Hafez et al., 2011).

To generate NEP2-expressing iPS-ML, we introduced a NEP2-expression vector into the iPS-ML-A by lentivirus-mediated transduction. Flow cytometric analysis of the NEP2-transfectant iPS-ML-A (iPS-ML-A/NEP2) detected NEP2 both on the cell surface and within the cells (Fig. 2A). Western blot analysis detected the NEP2 protein in both the cell lysate and the culture supernatant of the IPS-ML-A/NEP2 (Fig. 2B). These results indicate that the iPS-ML-A/NEP2 expressed NEP2 on the cell surface and also secreted NEP2 into the culture medium.

NEP-2-expressing iPS-ML and culture supernatants degrade Aβ42 oligomers

To examine the ability of iPS-ML to reduce AβO, we added AβO to the culture of non-transfected iPS-ML-A, anti-Aβ-scFv (Senju et al., 2011)-transfected iPS-ML-A (iPS-ML-A/scFvAβ), and iPS-ML-A/NEP2. After 24 h of incubation, the culture supernatant was collected and AβO in the supernatant was quantified by ELISA. In Fig. 3A, data are presented as the percentage of AβO remaining compared to a control condition (without iPS-ML). The results indicate that even non-transfected iPS-ML-A had a reducing effect on the level of AβO in the culture medium by about 30%. The effect of iPS-ML-A/scFvAβ was similar to that of the non-transfected iPS-ML-A, thereby indicating that expression of anti-Aβ-scFv did not enhance the AβO-reducing effect. In contrast, the iPS-ML-A/NEP2 showed a much more potent effect to reduce AβO (about 90% reduction) as compared with the non-transfected iPS-ML-A. The enhanced AβO-reducing activity gained by the expression of NEP2 was almost totally canceled by the presence of phosphoramidon, which is a protease inhibitor known to inhibit the activity of NEP2.

We also analyzed the AβO-reducing activity of the culture supernatant of iPS-ML-A, iPS-ML-A/scFvAβ, and iPS-ML-A/NEP2. As shown in Fig. 3B, the culture supernatant of the iPS-ML-A and iPS-ML-A/scFvAβ did not reduce the level of AβO, thereby suggesting that the reduction of AβO by the non-transfected iPS-ML-A and iPS-ML-A/scFv Aβ (Fig. 3A) was mediated by the endocytosis of AβO by iPS-ML-A or by effect of some membrane-associated proteases other than NEP2. The culture supernatant of the iPS-ML-A/NEP2 reduced the level of AβO by about 50%, and the effect was abolished by the presence of phosphoramidon.

Thus, both the iPS-ML-A/NEP2 and its culture supernatant reduced the level of AβO. On the other hand, non-transfected iPS-ML-A and iPS-ML-A/scFv Aβ, but not their culture supernatants, reduced the level of AβO. A previous study reported that only the NEP2β splice form of human NEP2, which is located at the cell surface, degrades Aβ (Huang et al., 2008). The results of our experiments demonstrated that the secreted form of NEP2 also degrades AβO.

To confirm the reproducibility of the results described above, we repeated the experiments using a different iPS-ML (iPS-ML-B), which had been generated by introduction of cMYC, BMI1, plus MDM2 as proliferation factors. As shown in Supplementary Fig. 2, iPS-ML-B also reduced the level of AβO in the culture medium. NEP2-transfectant iPS-ML-B (iPS-ML-B/NEP2) exhibited higher activity to reduce AβO in the culture medium than non-transfectant iPS-ML-B (Supplementary Fig. 2).

Taken together, iPS-ML could reduce the level of AβO in the culture supernatant, and the effect was enhanced by the forced expression of NEP2. In addition, NEP2 secreted from NEP2-transfectant iPS-ML also reduced AβO in the culture supernatant.

NEP2-expressing iPS-ML attenuates the neurotoxicity induced by Aβ42 oligomers

Microglia could exacerbate the pathogenesis of AD by accelerating neuronal loss and cognitive deficits through the production of neurotoxic pro-inflammatory cytokines such as interleukinh1 (IL-1), IL-6, and tumor necrosis factor-alpha (TNF-α) (Griffin, 2006). Although reduction of AβO by iPS-ML may inhibit disease progress, if it comes at the cost of increased neurotoxicity, then it is doubtful whether treatment with iPS-ML is beneficial for the AD patients.

We investigated whether culture supernatants of iPS-ML exacerbate or alleviate the neurotoxic effect of AβO. Toxicity of AβO on the SH-SY5Y neuroblastoma cell line was assessed by a modified MTT reduction assay, utilizing a

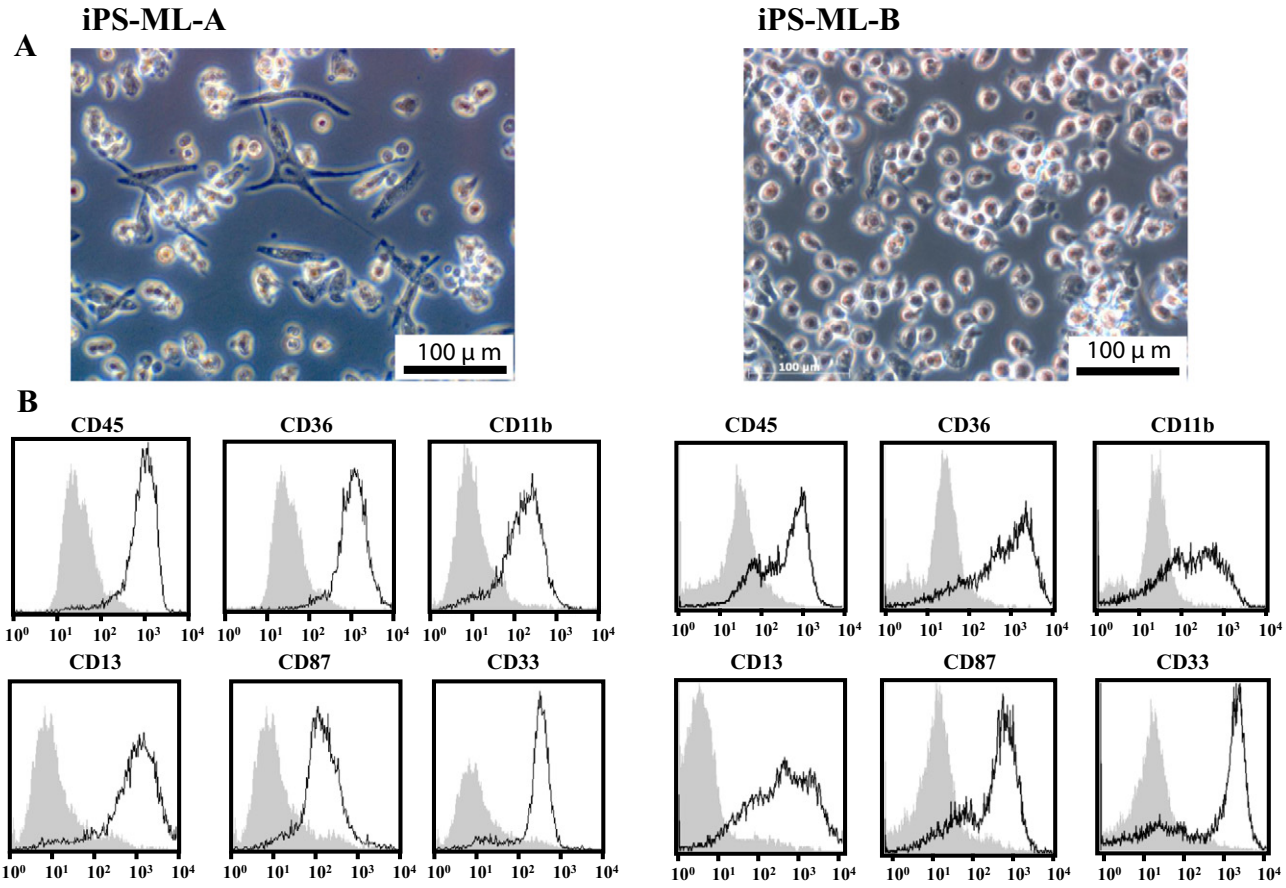

Figure 1 Morphology and cell surface molecules of iPS cell-derived myeloid cell line (iPS-ML). A. Phase contrast images of iPS cell-derived myeloid cell line (iPS-ML-A and iPS-ML-B) in culture plate are shown. B. Expression of CD45, CD36, CD11b, CD13, CD87, and CD33 on iPS-ML was analyzed by flow cytometry. Staining profiles of the specific monoclonal antibody (mAb; thick lines) and an isotype-matched control mAb (gray area) are shown.

tetrazolium compound (MTS). Incubation in the presence of more than 5 μM AβO to SH-SY5Y cell cultures resulted in significant cell death (Fig. 4A).

Culture supernatant of non-transfected iPS-ML-A reduced the toxicity of AβO against SH-SY5Y cells (Fig. 4B). As shown in Fig. 3B, culture supernatant of non-transfected iPS-ML-A did not reduce the level of AβO. Therefore, the observed inhibition of death in the SH-SY5Y cells was not mediated by the reduction of AβO. Probably, iPS-ML produced some soluble factors that protect the SH-SY5Y cells from death. Culture supernatant of iPS-ML-A/NEP2 reduced the neurotoxicity more efficiently than that of non-transfected iPS-ML-A (Fig. 4B). Addition of phosphoramidon to the supernatant of iPS-ML-A/NEP2 abolished most of the effect of NEP2-transgene. Culture supernatant of iPS-ML-B also protected SH-SY5Y cells from toxic effect of AβO (Fig. 4C). The neuroprotective effect of culture supernatant of iPS-ML-B was also enhanced by forced expression of NEP2 (Fig. 4C).

Taken together, these results demonstrate that iPS-ML produced some soluble neuroprotective factor that protected SH-SY5Y cells from AβO-induced death. In addition, NEP2 secreted by NEP2-transfectant iPS-ML further reduced the toxicity of AβO by decreasing the level of AβO.

Intrahippocampally transplanted NEP-2-expressing human iPS-MP reduces brain ISF Aβ in 5XFAD mice

We examined the effect of iPS-ML-A/NEP2 on AβO levels in vivo. To this end, we used 5XFAD mice, which accumulate Aβ in their brain tissue that is observable from 2 months of age. We planned to intrahippocampally administrate iPS-ML-A/NEP2 into 5XFAD mice and examine whether it resulted in reduction of AβO in the brain tissue. To avoid the immune-mediated rejection of transferred cells and accompanying inflammation, we crossed 5XFAD mice to immune-compromised SCID mice and establish 5XFAD mice with scid/scid genetic backgrounds (5XFAD/scid mice).

Continuous sampling of ISF by microdialysis was done to assess the dynamic change of soluble Aβ in awake, free-moving animals. To this end, we inserted the microdialysis probe with a microinjection tube into the right hippocampus of 3–4-month old 5XFAD/scid male mice. To confirm proper administration of iPS-ML into the hippocampus, we injected fluorescent dye (PKH26)-labeled iPS-ML-A or GFP-expressing iPS-ML-B, and histologically analyzed the hippocampus. Staining of the tissue sections with Congo red visualized the amyloid plaques in the hippocampus (Fig. 5A). Migration of iPS-ML into a region adjacent the microinjection

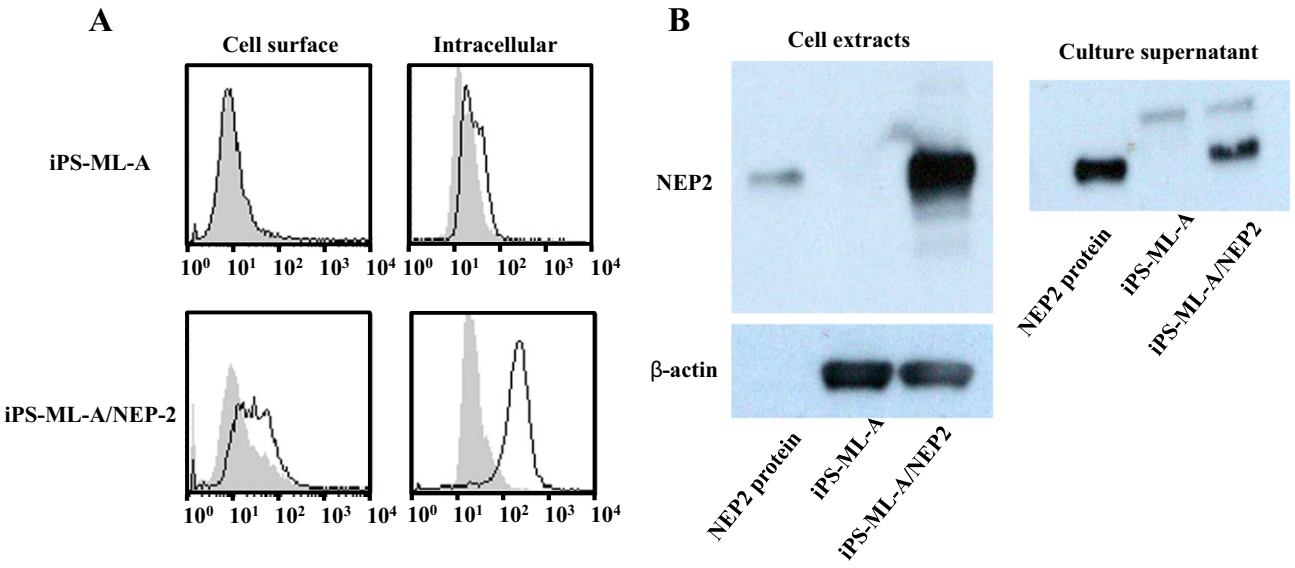

Figure 2 Expression of NEP2 by NEP2-expressing iPS-ML. A. Flow cytometric analysis of the cell surface and intracellular expression of NEP2 in NEP2-expressing iPS-ML (iPS-ML-A/NEP2). The staining profiles of the specific monoclonal antibodies (mAb; thick lines) and an isotype-matched control mAb (gray area) are shown. B. Cell extracts and culture supernatants of non-transfected iPS-ML-A and iPS-ML-A/NEP2 were subjected to western blot analysis to detect NEP2 protein. Recombinant human NEP2/MMEL was used as a positive control. β-actin was also probed for equal loading of cell extracts.

tube insertion site was revealed by fluorescence microscopy (Figs. 5B and C).

After 2 h of baseline sampling, iPS-ML-A, iPS-ML-A/NEP2 or Ringer's solution as control were injected into the right hippocampus via the microinjection tube. Basal levels of brain ISF Aβ1–42 were defined as their mean concentration over 2 h preceding iPS-ML administration. The effect of iPS-ML transfer was assessed by measuring the level of Aβ1–42 in the ISF obtained by microdialysis after 9 h of the iPS-ML injection. For each animal, the Aβ1–42 concentration was normalized to the basal level.

As shown in Fig. 6, the administration of iPS-ML-A/NEP2 resulted in decreased Aβ1–42 concentrations in the ISF by more than 40% as compared with the basal level. The level of Aβ1–42 in ISF of the mice treated with the iPS-ML-A/NEP2 was significantly lower than that of the mice treated with non-transfected iPS-ML-A or Ringer's solution. These results indicate that intracerebral administration of iPS-ML/NEP2

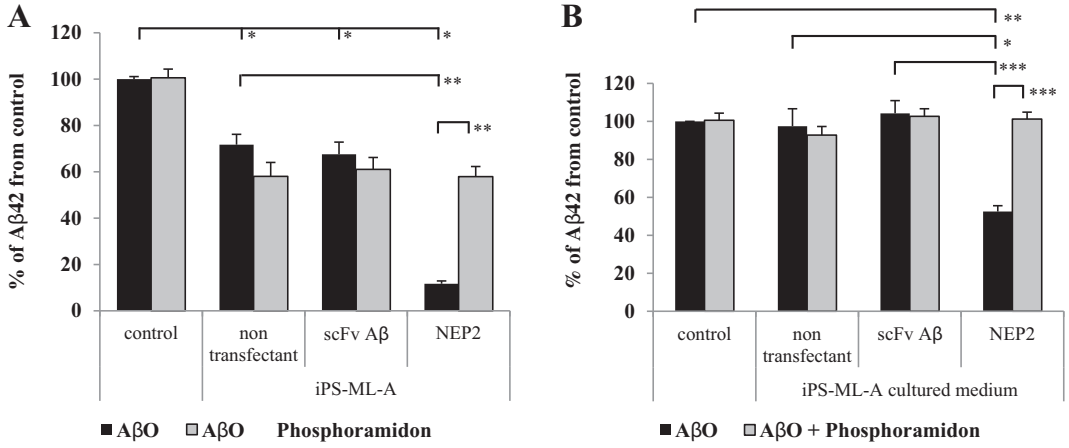

Figure 3 Reduction of Aβ levels in culture medium by NEP2-expressing iPS-ML. A. Non-transfected iPS-ML-A, single chain Aβ antibody-expressing iPS-ML-A (iPS-ML-A/scFv Aβ), or NEP2-expressing iPS-ML-A (iPS-ML-A/NEP2) were cultured in medium containing Aβ oligomers (AβO) in the presence or absence of phosphoramidon. After culture for 24 h, culture supernatants were recovered and the Aβ1–42 concentration was analyzed by ELISA. B. Non-transfected iPS-ML-A, iPS-ML-A/scFv Aβ, or iPS-ML-A/NEP2 were cultured for 24 h, and the culture supernatants was recovered. Subsequently, AβO was added to the culture supernatant and incubated at 37 °C in the presence or absence of phosphoramidon. After incubation for 24 h, the concentration of Aβ42 was determined by ELISA. Mean values + standard error of the mean (n = 3–6) relative to no iPS-ML control (Control) are shown; *$P < 0.05$, **$P < 0.005$, *** $P < 0.001$.

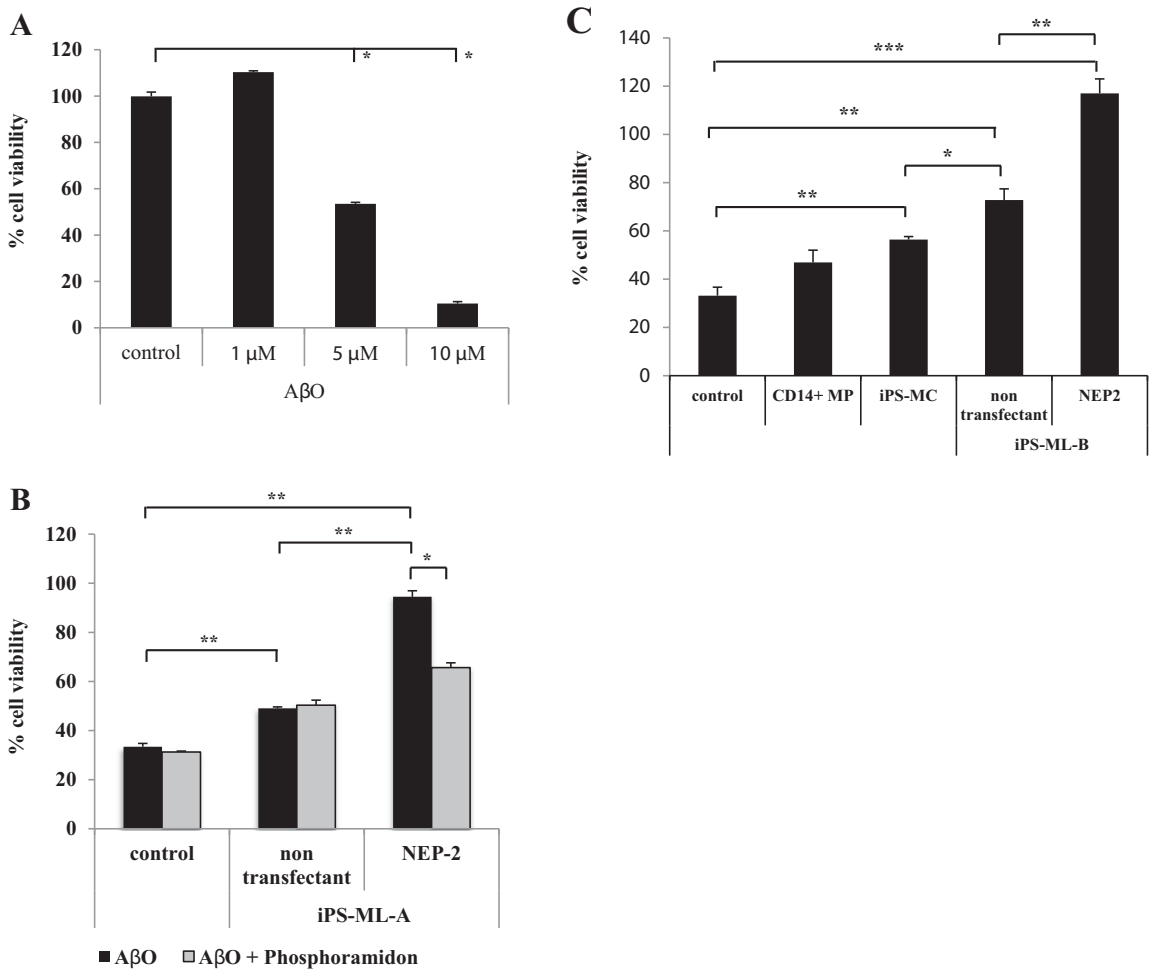

Figure 4 Inhibition of Aβ oligomer-induced neuronal cell death by NEP2-expressing iPS-ML in vitro. A. SH-SY5Y neuroblastoma cells were cultured (1×10^4 cells/100 μL/well in 96-well plates) in the presence of the indicated concentrations of Aβ oligomers (AβO). After 48 h, the number of live cells was analyzed by MTS assay. Data are presented as relative number of cells where the value for culture of the cells without AβO was defined as 100%, and the mean value + standard error for triplicate cultures are shown. (n = 3, $^*P < 0.001$). B. Culture supernatants of iPS-ML-A and NEP2-expressing iPS-ML-A (iPS-ML-A/NEP2) were prepared as in Fig. 3B. SH-SY5Y neuroblastoma cells were cultured (1×10^4 cells/100 μL/well in 96-well plates) in the presence of Aβ (10 μM), and the prepared iPS-ML culture supernatants were added with or without phosphoramidon (10 μM). After 48 h, the number of live SH-SY5Y cells was analyzed by MTS assay. Data are presented as relative number of cells where the number in the culture in the absence of Aβ was defined as 100%, and means + standard error for triplicate cultures are shown. (n = 3, $^*P < 0.005$, $^{**}P < 0.001$). C. Culture supernatant of monocyte-derived macrophages ($CD14^+$MP), iPS cell derived without proliferating capacity (iPS-MC), iPS-ML-B, or NEP2-expressing iPS-ML-B (iPS-ML-B/NEP2) was added to SH-SY5Y cells in the presence of Aβ (10 μM). After 48 h, the number of live SH-SY5Y cells was analyzed by MTS assay. Data are presented as relative number of cells where the number in the culture in the absence of Aβ was defined as 100%, and means + standard error for triplicate cultures are shown. (n = 3, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

reduced the concentration of Aβ1–42 in the ISF by degradation of Aβ1–42 by transgene-derived NEP2.

Discussion

Microglia are resident immune cells in the CNS that regulate innate immunity and participate in adaptive immune response (Hanisch and Kettenmann, 2007). However, the roles of microglia in AD pathology are still under debate. While there is significant evidence that microglial activation initiates and advances AD pathology, some aspects of microglia function are proposed to be beneficial because microglia are able to reduce Aβ deposition by phagocytosis (Gentleman, 2013). In animal models of AD, it has been reported that deficient microglial phagocytic activity accelerates pathology progression (El Khoury et al., 2007; Hickman et al., 2008), whereas exogenous transplantation of microglia enabled the clearance of Aβ depositions (Takata et al., 2007). Results from clinical trials of Aβ peptide vaccination have suggested microglial phagocytic clearance of Aβ from the cortex (Zotova et al., 2011).

In mouse models of AD, bone marrow-derived cells have been shown to reduce the Aβ burden (Lee et al., 2010).

Furthermore, several groups have recently reported that bone marrow-derived cells are able to differentiate into functional microglia (BMDM) and the therapeutic effect of BMDM in Aβ clearance is also suggested (Magga et al., 2012). Therefore, bone marrow cells may be an appropriate source of microglial progenitors for clinical application. Nevertheless, the repetitive administration requires a large quantity of cells. Therefore, to develop a cell-based therapy, it is necessary to resolve the issue of cell source. We have previously established a method to generate a large quantity of macrophage-like myeloid cells (Haruta et al., 2013; Koba et al., 2013; Senju et al., 2011). Cell-based therapy with iPS-ML may be one of the promising candidates for future therapeutic treatments.

Lentiviral modification of hematopoietic stem cells provided clinical benefits in an X-linked adrenoleukodystrophy (Cartier et al., 2009). iPS-ML could also be modified by the lentivirus vector to produce long-term transgene expression without compromising cell proliferation or function. We generated iPS-MC expressing a Fc-receptor-fused form of the Aβ-specific single chain antibody (scFv). Phagocytosis of Aβ-coated microbeads by iPS-MC was significantly enhanced by the expression of the anti-Aβ scFv (Senju et al., 2011). Recent studies revealed that soluble oligomeric Aβ (AβO) is the most neurotoxic Aβ species (Lambert et al., 1998; Walsh et al., 2002; Zahs and Ashe, 2013). Therefore, in the current study, we examined whether iPS-ML could reduce the level of AβO in the culture

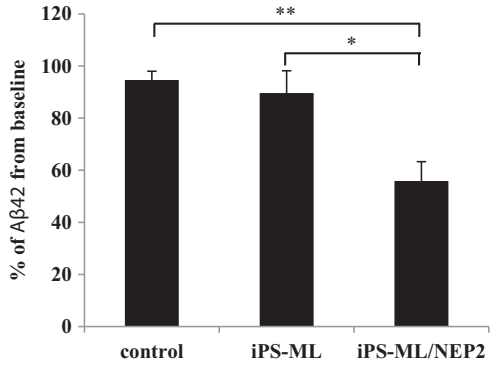

Figure 6 Degradation of Aβ in brain interstitial fluid of 5XFAD mice by intrahippocampally administrated NEP2-expressing iPS-ML. Microdialysis probes were inserted into the hippocampuses of 5XFAD/scid mice to sample the soluble Aβ in the brain interstitial fluid (ISF). Ringer's solution as vehicle control (Control), non-transfected iPS-ML-A, or NEP2-expressing iPS-ML-A (iPS-ML-A/NEP2) were injected into the right hippocampus via the microinjection tubes. Concentrations of Aβ1–42 in dialysis samples obtained 9 h after the administration of iPS-ML were determined by ELISA. For each mouse, the Aβ1–42 concentration was normalized to the basal level, which was the mean Aβ1–42 concentration over the 2 h preceding iPS-ML administration. Data are presented as mean normalized value + standard error of experimental groups (n = 4, $^{*}P < 0.05$, $^{**}P < 0.01$).

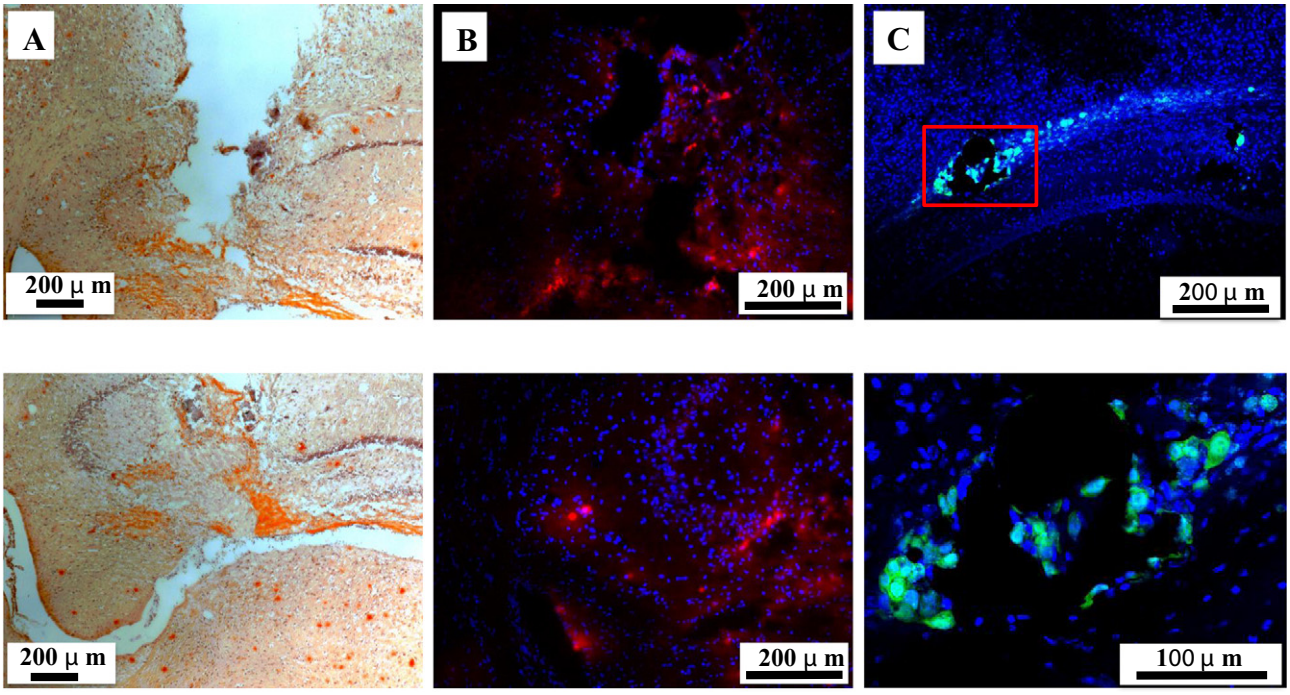

Figure 5 Intrahippocampal injection of iPS-ML into 5XFAD/scid mice. iPS-ML-A labeled with PKH26 or GFP-expressing iPS-ML-B were injected into the hippocampus of 5XFAD/SCID mice. After 24 h, mice were sacrificed and the brain tissue was removed. A. Frozen sections of 20-μm thickness were made and stained with Congo red. B. A merged images with blue fluorescence (DAPI) indicating cell nucleus and red fluorescence indicating PKH26-stained iPS-ML-A are shown. C. Merged images with blue fluorescence (DAPI) indicating cell nucleus and green-white fluorescence indicating GFP-expressing iPS-ML-B are shown. The square region in the upper image is magnified in the lower image.

medium (Fig. 3A). Contrary to our expectation, expression of anti-Aβ scFv in iPS-ML did not enhance the clearance of soluble Aβ in the culture medium. On the other hand, forced expression of a protease with AβO-degrading activity enhanced the reduction of AβO in the culture medium by iPS-ML. We observed that the iPS-ML highly expressing NEP2 (iPS-ML-A/NEP2), a secretable protease with Aβ-degrading activity, efficiently reduced AβO (Fig. 3B). In addition, culture supernatant of iPS-ML with or without expression of NEP2 inhibited the AβO-induced death of neuronal cells in vitro (Figs. 4B, C). Culture supernatant of iPS-MC, iPS cell-derived myeloid cells without proliferating capacity, also exhibited such effect (Fig. 4C). On the other hand, human peripheral blood monocyte-derived macrophages ($CD14^+$ MP) did not exhibit such effect (Fig. 4C).

Microglia act as a source of neurotrophic factors and mitigate neurodegeneration. Microglia-derived neurotrophic factors, such as the brain-derived neurotrophic factor (BDNF), exert well-documented neuroprotective functions (Nagahara et al., 2009). The protection of SH-SY5 cells from AβO-induced death by the culture supernatant of the non-transfected iPS-ML (Figs. 4B, C) may have been mediated by some neurotrophic agents produced by the iPS-ML. Some of soluble factors secreted by $CD14^+$MP, iPS-MC, iPS-ML, or NEP2-expressing iPS-ML were examined by bead array system and the results are shown in Supplementary Fig. 3.

To develop an effective cell-based therapy against neurodegenerative disorders of the CNS, efficient recruitment of the cells into the CNS is essential. Previous findings suggest that particular bone marrow derived cells are able to cross the blood–brain barrier (Lebson et al., 2010; Simard and Rivest, 2004). To evaluate the migration of the iPS-ML into the CNS, we examined the effect of intravenous, intraperitoneal, and intracerebroventricular injection of iPS-ML into 5XFAD mice. To our disappointment, the iPS-ML injected via these routes did not efficiently infiltrate into brain parenchyma and failed to reduce the amyloid burden. A possible reason of the failure to efficiently migrate in brain tissue may be the lack of CC chemokine receptor-2 (CCR2) expression in the iPS-ML (data not shown). To analyze the in vivo effect of iPS-ML/NEP2, we directly administered iPS-ML into the brain. To this end, we stereotaxically inserted microinjection tubes into the hippocampus of 5XFAD mice and transplanted iPS-ML through this tube. The hippocampus plays a major role in cognitive dysfunction of AD, and the 5XFAD hippocampus is one of the regions of the brain where Aβ plaques accumulate. iPS-ML transplanted by this procedure migrated to the brain parenchyma adjacent to the area of the tube insertion (Figs. 5B, C).

In vivo, transplantation of iPS-ML/NEP2 into the hippocampus of 3–4-month old 5XFAD/scid mice significantly diminished the levels of soluble Aβ1–42 in the brain ISF compared to the control Ringer's solution injection (Fig. 6). The reduction of Aβ was not significant when non-modified iPS-ML were transplanted. Therefore, the reduction of Aβ was caused by the secretion of NEP2 from the iPS-ML, but not phagocytosis of Aβ by the iPS-ML. Our intrahippocampus transplantation of iPS-ML demonstrated short-term and focal remote effects of the IPS-ML; only where the cells were transplanted. Furthermore we could not examine the therapeutic effect of cognitive function, because the mice were weakened by probe implantation. Future studies will be aimed at exploring whether iPS-ML are effective in preventing cognitive decline and neuronal damage in other AD models. In addition, to develop this technique as a therapy for AD, delivery of iPS-ML into the brain by systemic administration is necessary.

To examine the chromosomal alteration of iPS-ML, iPS-ML cultured for 6 weeks after the introduction of proliferating factors were subjected to karyotype analysis. As shown in Supplemental Fig. S1, some karyotype abnormalities were detected in this analysis. For application of iPS-ML to clinical cellular therapy, we should resolve the issue of genetic instability of iPS-ML. To generate iPS-ML, our current method uses cMYC, BMI1 plus MDM2 to induce proliferation of iPS-MC. Among the introduced factors, MDM2 is involved in degradation of p53 protein as the E3 ubiquitin ligase (Haupt et al., 1997; Honda et al., 1997; Kubbutat et al., 1997). Forced expression of MDM2 in iPS-ML may cause complete loss of p53 function and result in the genetic instability of iPS-ML. Although co-introduction of MDM2 enhanced the proliferation rate of iPS-ML, this factor is not absolutely necessary for the establishment of iPS-ML, as previously reported (Haruta et al., 2013; Koba et al., 2013). Omission of MDM2 in the generation of iPS-ML may be one way to improve the genetic stability of iPS-ML.

The risk of tumor occurrence is one of most serious issues to be resolved before the clinical application. We currently use a lentivirus vector to introduce proliferation factors to generate iPS-ML, and administration of such iPS-ML to the patients may cause malignancy. Thus, for the clinical application of iPS-ML-based cellular therapy, a removable vector system should be used to generate iPS-ML.

In addition to AD, many intractable diseases are caused by the accumulation of misfolded proteins. Furthermore, the potential of iPS-ML may involve genetic modification to produce proteolytic enzymes that degrade other deleterious misfolded proteins or prion. We are planning future studies to evaluate the potential of iPS-ML as cell-based therapeutics for neurodegenerative diseases other than AD and various types of amyloidosis.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scr.2014.10.001.

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