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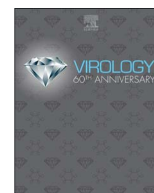

# Neural precursor cells derived from induced pluripotent stem cells exhibit reduced susceptibility to infection with a neurotropic coronavirus

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ABSTRACT

The present study examines the susceptibility of mouse induced pluripotent stem cell-derived neural precursor cells (iPSC-NPCs) to infection with the neurotropic JHM strain of mouse hepatitis virus (JHMV). Similar to NPCs derived from striatum of day 1 postnatal GFP-transgenic mice (GFP-NPCs), iPSC-derived NPCs (iPSC-NPCs) are able to differentiate into terminal neural cell types and express MHC class I and II in response to IFN-γ treatment. However, in contrast to postnatally-derived NPCs, iPSC-NPCs express low levels of carcinoembryonic antigen-cell adhesion molecule 1a (CEACAM1a), the surface receptor for JHMV, and are less susceptible to infection and virus-induced cytopathic effects. The relevance of this in terms of therapeutic application of NPCs resistant to viral infection is discussed.

## 1. Introduction

Neural precursor cell (NPC) transplantation represents an emerging therapeutic approach to treat several neurological disorders. NPC transplants into rodent models of Alzheimer's disease, Parkinson's disease and spinal cord injury have demonstrated clinical benefits (Blurton-Jones et al., 2009; Cummings et al., 2005; van Gorp et al., 2013). Further, NPCs have been suggested as a potential treatment for the neuroinflammatory autoimmune demyelinating disease multiple sclerosis (MS) as they represent attractive sources for the generation of myelin-competent oligodendrocytes (Ben-Hur et al., 1998; Brustle et al., 1999). NPC-derived glial progenitors have been shown to remyelinate axons following transplantation into regions of acute experimental demyelination (Ben-Hur et al., 1998; Keirstead et al., 1999). In addition, transplantation of neural precursors into a rodent autoimmune model of demyelination resulted in migration of transplanted cells into white matter tracts accompanied by an improvement in clinical outcome (Ben-Hur et al., 2003; Pluchino et al., 2003). In these pre-clinical autoimmune models of MS, NPCs have been suggested to act as modulators of the immune system or directly replace damaged or lost endogenous NPCs that subsequently allows for dampening disease progression, axonal preservation and remyelination (Aharonowiz et al., 2008; Pluchino et al., 2009, 2003).

An important and clinically relevant question is whether transplanted NPCs can alleviate demyelination caused by persistent viral infection. Although the cause of MS has been attributed to multiple factors, viruses have long been considered as a potential triggering agent for MS in genetically susceptible individuals (Gilden, 2005; Olson et al., 2005). Therefore, it is important to study the remyelination potential of NPCs in the context of virally-induced neurologic disease, as this will give important insights into whether cell replacement therapies are effective within the CNS where neurotropic viruses may be persistent. With this in mind, we have previously shown that engraftment of postnatal-derived NPCs into the spinal cords of JHMV-infected mice with established demyelinating disease resulted in the selective colonization of demyelinating white matter tracts by transplanted cells accompanied by remyelination and axonal sparing (Carbajal et al., 2010, 2011; Totoiu et al., 2004; Greenberg et al., 2014). In a clinical setting, NPCs derived from donor-specific iPSCs may be preferable since these cells will retain the genetic background of the donor and potentially bypass the need for immunosuppressive drugs that leave the patient susceptible to opportunistic infections and tumor formation.

There are several known neurotropic viruses that are capable of infecting and replicating in both NPCs and NPC-derived cells (Chucair-Elliott et al., 2014; Huang et al., 2014; Schaumburg et al., 2008). For example, herpes simplex virus type 1 (HSV-1) infects NPCs resulting in

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diminished numbers leading to loss of neuroblasts upon differentiation (Chucair-Elliott et al., 2014). NPCs are also susceptible to infection by Enterovirus 71, which causes depletion of their numbers via viral-induced lysis (Huang et al., 2014). In addition, hESC-derived oligodendrocyte progenitor cells (OPCs) are highly susceptible to infection by JC virus, the causative agent of progressive multifocal leukoencephalopathy (PML) (Schaumburg et al., 2008). Coxsackievirus, another virus capable of infecting the CNS, also preferentially replicates in NPCs causing cell death and leading to developmental defects (Ruller et al., 2012). Most recently, Zika virus has been shown in culture to infect human NPCs derived from induced pluripotent stem cells and is linked to fetal abnormalities in pregnant woman exposed to the virus (Tang et al., 2016), while the human coronavirus OC43 was linked to acute disseminated encephalomyelitis within a SCID patient that received a cord-blood transplantation (Morfopoulou et al., 2016). Dampened immune surveillance resulting from treatment with immunosuppressive drugs can cause re-emergence of persistent neurotropic viruses which have the ability to infect and diminish numbers of transplanted cells, thus muting therapeutic benefits. Collectively, these findings suggest that susceptibility of NPCs to infection by neurotropic viruses is clinically important and must be evaluated before considering NPCs as a viable cell replacement therapy for various neurological disorders.

We have previously shown that postnatally-derived NPCs are susceptible to infection by JHMV resulting in virus-induced cell death (Plaisted et al., 2014). Herein we demonstrate that NPCs derived from induced pluripotent stem cells (iPSCs) are functionally similar to cortical NPCs isolated from post-natal transgenic GFP-C57BL/6 mice as they possess tri-potent differentiation potential and can form oligodendrocytes, neurons and astrocytes (Carbajal et al., 2010; Greenberg et al., 2014). However, they differ in their susceptibility to infection by JHMV in that iPSC-derived NPCs (iPSC-NPCs) express low levels of the viral receptor CEACAM1a, making them refractory to infection and virus-induced cell death.

## 2. Results and discussion

### 2.1. NPCs generated from mouse induced pluripotent stem cells are functionally similar to postnatal GFP-NPCs

Mouse iPSCs were generated by retroviral transduction of Yamanaka factors (Oct3/4 Sox2, Klf4 and c-Myc) into C57BL/6 fibroblasts using established protocols (Takahashi and Yamanaka, 2006). Mouse iPSCs were grown and differentiated into NPCs according to the schematic outlined in Fig. 1A. Feeder-free iPSCs were generated by supplementing cells with leukemia inhibitory factor (LIF) and stained positive for the stem cell markers- Oct4 and Sox2 (Fig. 1B). iPSCs were differentiated into NPCs by addition of EGF (epidermal growth factor) and bFGF (basic fibroblast growth factor) and were positive for neuronal stem cell markers such as Sox2 (Fig. 1Ci), Nestin (Fig. 1Cii), and Pax6 (Fig. 1Ciii) as assessed by immunofluorescence analysis. iPSC-NPCs were then differentiated for 6 days following withdrawal of EGF and bFGF. Similar to postnatal GFP-NPCs (henceforth referred to as GFP-NPCs) (Carbajal et al., 2010). iPSC-NPCs also terminally differentiated into oligodendrocytes (Fig. 1Di), neurons (Fig. 1Dii) and astrocytes (Fig. 1Diii) (Fig. 1E). These findings illustrate that iPSC-NPCs have similar differentiation properties as compared to postnatal-derived GFP-NPCs.

### 2.2. Expression of MHC class I and II following exposure to JHMV

It has been previously shown that, under normal physiologic conditions, MHC class I and II are undetectable on NPCs and IFN-γ treatment can induce expression of MHC on these cells (Chen et al., 2011; Plaisted et al., 2014; Weinger et al., 2012). We found there was constitutive expression of MHC class I on iPSC-NPCs and exposure to either JHMV or recombinant mouse IFN-γ (100 U/mL) did not modulate expression levels (Fig. 2A, C). In addition, MHC class II expression was undetectable on iPSC-NPCs exposed to either medium or JHMV, yet treatment with IFN-γ dramatically increased expression (Fig. 2B, D)

### 2.3. NPCs derived from mouse iPSCs express low levels of JMHV receptor CEACAM1a

The primary receptor for JHMV is murine carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) (Hirai et al., 2010; Williams et al., 1991). There are two alleles for murine CEACAM1: m*Ceacam1a* and m*Ceacam1b* and inbred strains of mice such as C57BL/6 and Balb/c are homozygous for m*Ceacam1a* and are highly susceptible to infection by JHMV (Williams et al., 1991). CEACAM1a is widely expressed in a variety of mouse tissue and can be detected on the surface of epithelial cells lining the respiratory tract, endothelial cells and also on hematopoietic cells (Hemmila et al., 2004). We have recently shown that cultured NPCs derived from 1-day old C57BL/6 mice express CEACAM1a as determined by CEACAM1 mRNA transcripts as well as surface protein expression (Plaisted et al., 2014). In order to determine whether NPCs derived from iPSCs express CEACAM1a similar to GFP-NPCs, cell surface expression of the receptor was evaluated by staining NPCs with an anti-CEACAM1 antibody. GFP-NPCs and mixed splenocytes from C57BL/6 were used as controls. Flow cytometry analysis revealed that iPSC-NPCs expressed low levels of surface expression of CEACAM1 compared to GFP-NPCs (Fig. 3A, B). Furthermore, qPCR analysis of mRNA isolated from cultured iPSC-NPCs revealed a significant ($p < 0.0001$) reduction in CEACAM1a transcripts compared to mRNA isolated from GFP-NPCs or splenocyte controls (Fig. 3C). Collectively, we interpret these findings to indicate iPSC-NPCs express proportionally less CEACAM1a compared to GFP-NPCs.

### 2.4. NPCs derived from iPSCs are less susceptible to JHMV infection and virus induced cell death

We next evaluated the susceptibility of NPCs to JHMV infection. In order to do this, cultured iPSC-NPCs were infected with JHMV for 18 h and fixed 72 h post-infection (p.i.). Cells were then stained with either an anti-Sox2 antibody (to confirm NPC status) or an antibody specific for the carboxyl terminus of JHMV nucleocapsid protein and imaged by fluorescence microscopy (Plaisted et al., 2014). Compared to GFP-NPCs, iPSC-NPCs express low levels of JHMV nucleocapsid protein, suggesting these cells are less susceptible to JHMV infection (Fig. 4A, B). To determine if JHMV-infection of NPC cultures induced cell death, lactate dehydrogenase (LDH) was measured in the supernatants of GFP-NPC and iPSC-NPC cultures at 24, 48 and 72 h p.i. GFP-NPCs cultures infected with JHMV revealed a significant ($p < 0.001$) increase in cell death compared to JHMV-infected iPSC-NPC cultures (Fig. 4C). Congruent with reduced cell death, we detected lower viral titers within the supernatants of JHMV-infected iPSC-NPCs at 24 ($p < 0.05$), 48 ($p < 0.001$), and 72 ($p < 0.05$) hours p.i. compared to infected GFP-NPCs (Fig. 4D).

This study provides a comparative analysis of *ex vivo* expanded neural precursor cell populations with regard to their susceptibility to infection by a neurotropic coronavirus. These studies demonstrates that although iPSC-NPCs are functionally similar to postnatally-derived NPCs in their ability to differentiate into oligodendrocytes, astrocytes and neurons, they are unique with respect to susceptibility to viral infection and expression of MHC class I on the cell surface. iPSC-NPCs express low levels of the JHMV receptor CEACAM1a, providing reduced susceptibility and limited replication. Furthermore, due to the impaired ability of virus to enter these cells and replicate, the cells are resistant to virus-induced cell death. Nonetheless, we demonstrate that after 72 h following infection of iPSC-NPCs there is an increase in cell

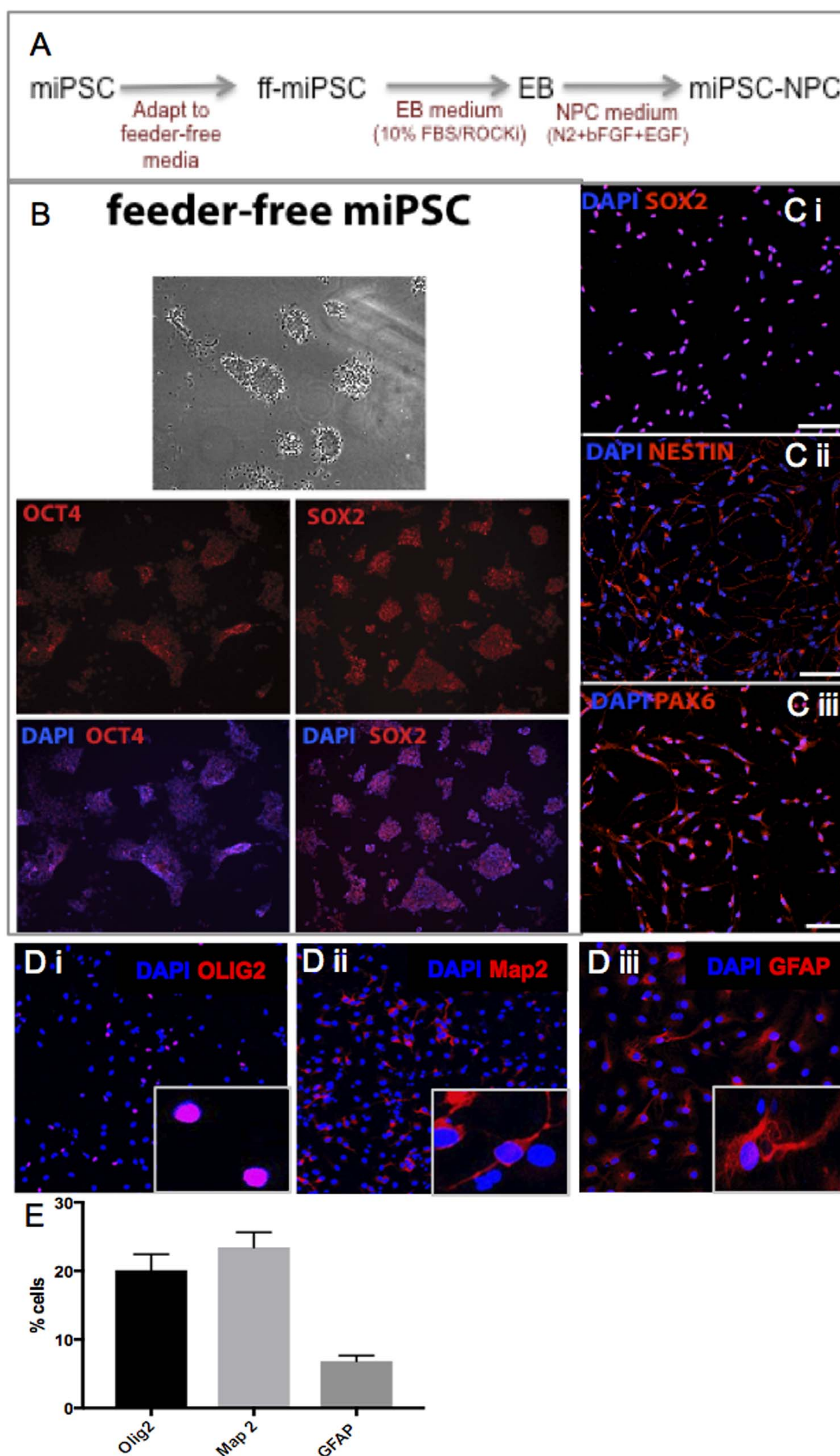


**Fig. 1.** Generation of mouse iPSC-NPCs. (A) Schematic outline depicting generation of miPSC-NPCs from mouse iPSCs. (B) Feeder-free media adapted miPSCs stained for OCT4 and SOX2 imaged by fluorescence microscopy. (C) miPSC-NPCs were stained for neural precursor markers-SOX2 (Ci), Nestin (Cii) and PAX6 (Ciii) and imaged by fluorescence microscopy. (D) 6 days post differentiation, NPCs express markers for oligodendrocytes (OLIG2) (Di), neurons (MAP2) (Dii) and astrocytes (GFAP) (Diii) as determined by immunofluorescent staining; representative images are shown. (E) Quantification of expression of lineage-specific markers, data shown as average + SEM.

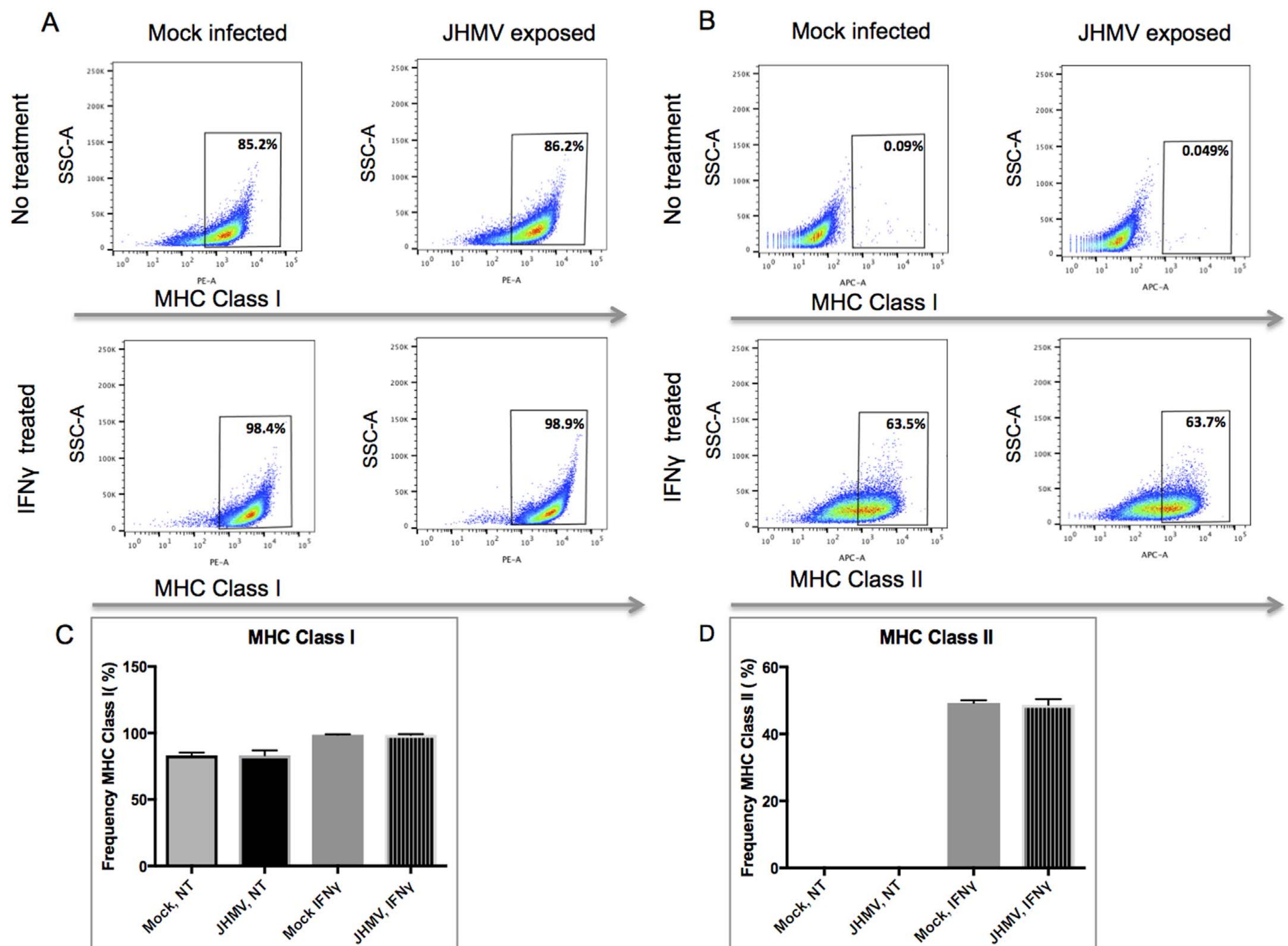


**Fig. 2.** Expression of MHC class I and II by iPSC-NPCs. iPSC-NPCs were either exposed to vehicle (mock infected) or JHMV (moi = 0.4) for 18 h. Cells were then treated with IFN-γ (100U/mL) or media alone (no treatment, NT) for 24 h and surface expression of MHC class I and II expression analyzed by flow cytometry. Representative dot blots depicting the frequency of MHC class I (A) or class II (B) are shown. Quantification of the frequency of MHC class I (C) and MHC class II (D) expression; data are presented as an average + SEM and represents 3 independent experiments.

death yet viral titers remain relatively low. These findings argue that iPSC-NPCs may be susceptible to JHMV-induced death over time due to mechanism(s) as of yet undefined and this is a focus of ongoing investigation.

We have previously shown that intraspinal transplantation of postnatal GFP-NPCs into JHMV-infected mice results in clinical recovery associated with remyelination (Carbajal et al., 2011; Greenberg et al., 2014). However, fetal-derived GFP-NPCs are susceptible to JHMV infection and undergo virus-induced cell death in vitro (Plaisted et al., 2014). Additionally, when engrafted into JHMV-infected mice, these cells are susceptible to infection in vivo (Weinger et al., 2014). Within the context of myelinopathies, this suggests that engrafting cells susceptible to persistent neurotropic viruses may be ineffective, as viral infection could prevent them from surviving and differentiating into myelin-producing oligodendrocytes. Since iPSC-NPCs express low levels of the viral receptor and are resistant to virus-induced cell death, they will presumably persist in vivo, providing increased potential for oligodendrocyte differentiation and subsequent remyelination. Ultimately, iPSC-NPCs may offer a better cell-replacement option to postnatal GFP-NPCs in the context of JHMV-mediated demyelination.

There are several neurotropic viruses that are known to persist in the CNS that can preferentially infect and replicate in NPCs (Chucair-Elliott et al., 2014; Huang et al., 2014; Ruller et al., 2012; Schaumburg et al., 2008). In order to successfully employ NPCs for axon remyelination in the damaged CNS, it is imperative to consider the possibility that transplanted NPCs will be targeted by persistent CNS viruses. In a clinical setting, if donor-derived human NPCs are used for treatment of MS or any other neurological disease, the recipient will likely be under general immuno-suppression drugs to prevent rejection of the engrafted MHC-mismatched cells. However, systemic immunosuppression can lead to recrudescence of dormant neurotropic viruses, typically kept in check by immune surveillance. Consequently, engrafted NPCs may be targeted by reactivated neurotropic viruses, which can infect and proliferate in NPCs, causing cell death and diminishing therapeutic benefit. Since iPSC-NPCs are 'self-derived' and maintain the genetic background of the donor, they are unlikely to be rejected by the host. iPSC-NPCs are functionally similar to GFP-NPCs in their capacity to differentiate into remyelination-competent oligodendrocytes, suggesting that they may be therapeutically similar to engrafting GFP-NPCs. Indeed, a recent study showed that intraspinal transplantation of NPCs derived from mouse iPSCs alleviates clinical and pathological symptoms of experimental autoimmune encephalomyelitis (Laterza et al., 2013). Our findings argue that engraftment of an NPC population lacking specific receptors for known neurotropic viruses may reduce the risk of infection and cytopathic effects leading to more sustained clinical recovery. Ongoing studies in our laboratory are currently testing this hypothesis.

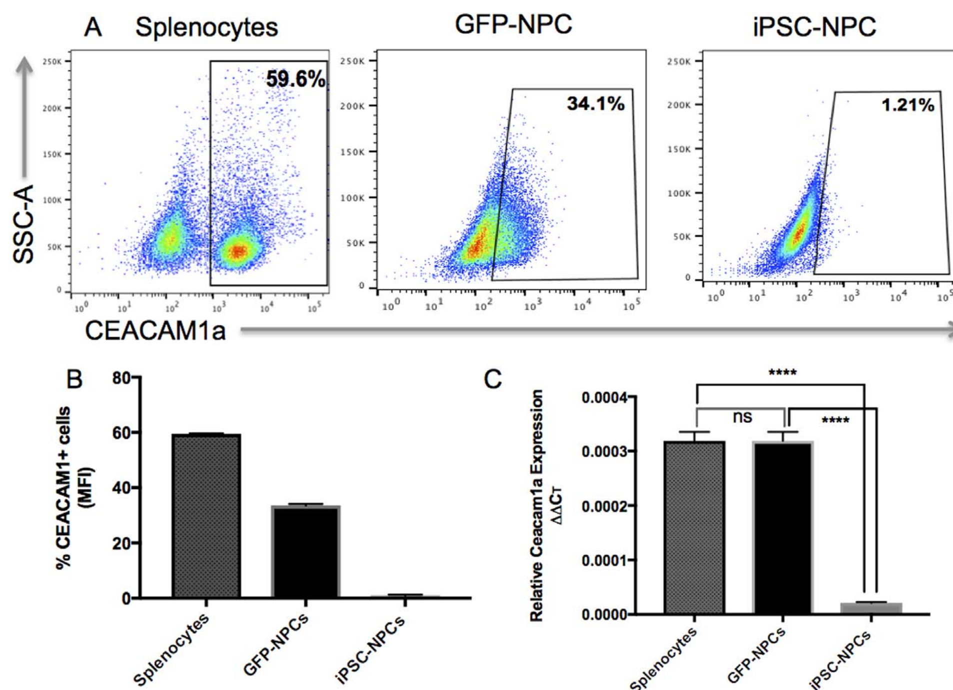


**Fig. 3.** Surface CEACAM1a expression is reduced by iPSC-NPCs. (A) Representative dot blots showing surface expression of JHMV receptor-CEACAM1a on C57BL/6 splenocytes, GFP-NPCs or iPSC-NPCs. (B) The mean fluorescence intensity (MFI) of dot plots in (A) is quantified; data shown as average+SEM and represents two independent experiments. (C) Relative expression levels of Ceacam1a ($\Delta\Delta C_T$) were measured by conducting qPCR on cDNA generated from total RNA extracted from C57BL/6 splenocytes or cultured GFP-NPCs and iPSC-NPCs. β-actin was used as an internal control to normalize the amount of RNA. Data are derived from triplicate analysis and shown as average + SEM; ****$p < 0.0001$, ns = not significant.

## 3. Materials and methods

### 3.1. Virus

JHM strain of MHV (V34) was added to iPSC-NPCs or postnatal GFP-NPCs expressing green fluorescent protein (GFP-NPCs) at a multiplicity of infection (m.o.i.) of 0. 4 PFU/cell. Following overnight incubation (~18 h) media was replaced. Supernatants were collected at defined times post infection and viral titers were determined using the DBT astrocytoma cell line as previously described (Hirano et al., 1976).

### 3.2. Neural precursor cell culture

GFP-NPCs were cultured as described before (Carbajal et al., 2011). GFP-NPC media consisted of DMEM/F12 with Glutamax (Gibco), ciprofloxacin hydrochloride (100 μg/mL, Cellgro), N2 supplement (1X, Gibco), gentamycin(50 μg/mL, Sigma-Aldrich), fungizone (2.5 μg/mL, Gibco), penicillin/streptomycin (1000 U/mL) and epidermal growth factor(20 ng/mL, Sigma-Aldrich). Feeder dependent iPSCs were kindly provided by Dr. Kristin Baldwin (The Scripps Research Institute). Feeder free iPSCs were generated by plating iPSCs in gelatin coated flasks and supplementing cells with leukemia inhibitory factor (LIF). Feeder free-adapted iPSCs were then plated to generate embryoid bodies (EB). NPCs generated from iPSCs were cultured similar to GFP-NPCs. Media used for culturing iPSC-NPCs consisted of DMEM/F12 with Glutamax (Gibco), N2 supplement (1X, Gibco), penicillin/Streptomycin (1000 U/mL), NEAA (100X, Gibco), epidermal growth factor (20 ng/mL, Sigma-Aldrich) and fetal growth factor (20 ng/mL, Millipore). Recombinant IFN-γ (100 U/mL) was purchased from Cell Sciences.

### 3.3. Flow Cytometry analysis

NPCs were harvested using 0.05% Trypsin and suspended in PBS containing 0.5% BSA. Subsequently, they were treated with antibodies specific for CEACAM1a (APC conjugated, eBioscience), MHC class I (APC conjugated, Biolegend) or MHC Class II (PE conjugated, eBioscience) for 40 min. Detection of fluorescence was performed using LSR Fortessa X-20 (BD biosciences). FACS data was analyzed using FlowJo software (Tree Star).

### 3.4. RNA isolation and quantitative Polymerase Chain Reaction

RNA was extracted from NPCs or splenocytes using the manufacturer's instructions using the RNeasy kit (Qiagen). cDNA was reverse transcribed from RNA using SuperScript III First-Strand synthesis system and random hexamers (Invitrogen). qPCR to detect CEACAM1a transcripts was performed using the following primers: TTCCCTGGGGAGGACTACTG (forward primer) and TGTATGC TTGCCCCGTGAAAT (reverse primer) (DNA/ peptide synthesis facility, HSC Cores at University of Utah). The amount of RNA added to every sample was normalized by using $\beta$-actin as an internal control; the relative expression of Ceacam1a was then calculated using the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001)

### 3.5. JHMV cytotoxicity assay

JHMV induced NPC cell death was evaluated at 24, 48 and 72 h p.i by measuring the lactate dehydrogenase levels (LDH) using LDH cytotoxicity assay (Promega). JHMV induced LDH levels were measured from infected NPCs and normalized to spontaneously released LDH. The value was then expressed as a percentage of cell death due to infection.

### 3.6. Immunofluorescence

To determine JHMV infectivity of cultured NPCs, cells were plated on reduced growth factor-matrigel (BD Biosciences) coated chamber slides. NPCs were infected with JHMV (moi = 0. 4) for 18 h and fixed at 72 h p.i with 4% PFA for 20 min at room temperature.

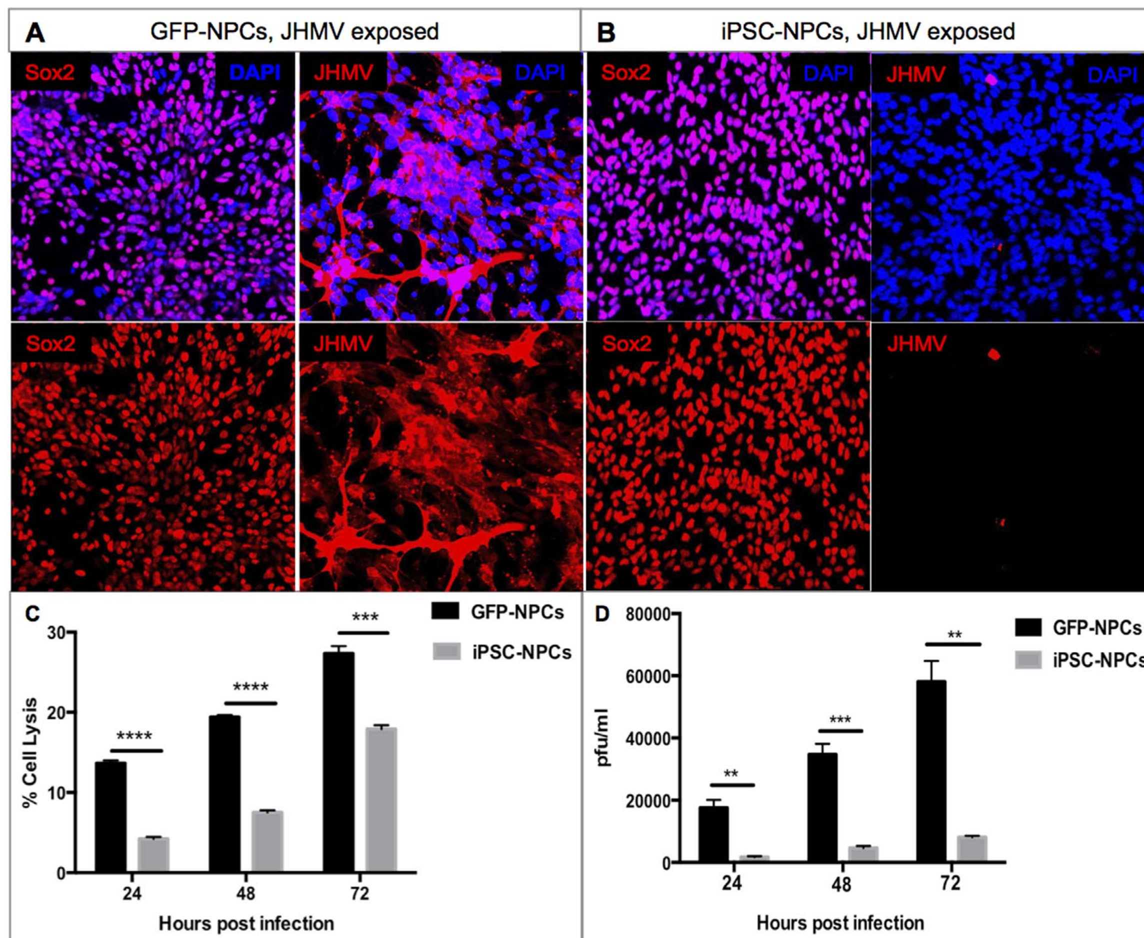


**Fig. 4.** JHMV infection of NPCs. GFP-NPCs (A) or iPSC-NPCs (B) were infected with JHMV (moi = 0.4) for 18 h and at 72 h p.i. the cells were fixed and stained with antibodies specific for Sox2 or JHMV capsid protein and visualized by fluorescent microscopy; representative images are shown (blue represents DAPI) (C) To determine cell death due to viral infection, LDH released from from virally-infected NPC culture supernatants were normalized to LDH released by non-infected NPC cultures (**** $p < 0.0001$; *** $p < 0.001$). (D) Supernatants were harvested from JHMV infected NPCs at 24, 48 and 72 h p.i and viral titers were analyzed by plaque assay (** $p < 0.05$; ***$p < 0.001$). Data from panels (C) and (D) were derived from three independent experiments and presented as average + SEM.

Immunofluorescence was performed as described previously (Whitman et al., 2009) using antibodies specific for rabbit monoclonal anti-Sox 2 (abcam, 1:100 dilution) or monoclonal antibody specific for JHMV spike protein (Plaisted et al., 2014) and Alexa Flour 594 goat anti-rabbit secondary antibody (Life technologies). Slides were then imaged using a Nikon A1 inverted microscope.

### 3.7. Statistical analysis

Statistical analysis was carried out using student's *t*-test and $p \leq 0.05$ was considered significant.

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