Title

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Journal

Stem Cells, 32(10)

ISSN

1066-5099

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Publication Date

2014-10-01

DOI

10.1002/stem.1760

Peer reviewed

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Activating Receptor NKG2D Targets RAE-1-Expressing Allogeneic Neural Precursor Cells in a Viral Model of Multiple Sclerosis

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Key Words. Neural stem cells • Stem cell transplantation • NK cells • Nervous system • Autoimmune disease • antigenicity • Neural differentiation

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Received October 30, 2013; accepted for publication April 25, 2014; first published online in STEM CELLS *EXPRESS* June 4, 2014.

1066-5099/2014/$30.00/0

http://dx.doi.org/10.1002/stem.1760

ABSTRACT

Transplantation of major histocompatibility complex-mismatched mouse neural precursor cells (NPCs) into mice persistently infected with the neurotropic JHM strain of mouse hepatitis virus (JHMV) results in rapid rejection that is mediated, in part, by T cells. However, the contribution of the innate immune response to allograft rejection in a model of viral-induced neurological disease has not been well defined. Herein, we demonstrate that the natural killer (NK) cell-expressing-activating receptor NKG2D participates in transplanted allogeneic NPC rejection in mice persistently infected with JHMV. Cultured NPCs derived from C57BL/6 (H-2^b) mice express the NKG2D ligand retinoic acid early precursor transcript (RAE)-1 but expression was dramatically reduced upon differentiation into either glia or neurons. RAE-1$^+$ NPCs were susceptible to NK cell-mediated killing whereas RAE-1$^-$ cells were resistant to lysis. Transplantation of C57BL/6-derived NPCs into JHMV-infected BALB/c (H-2^d) mice resulted in infiltration of NKG2D$^+$CD49b$^+$ NK cells and treatment with blocking antibody specific for NKG2D increased survival of allogeneic NPCs. Furthermore, transplantation of differentiated RAE-1$^-$ allogeneic NPCs into JHMV-infected BALB/c mice resulted in enhanced survival, highlighting a role for the NKG2D/RAE-1 signaling axis in allograft rejection. We also demonstrate that transplantation of allogeneic NPCs into JHMV-infected mice resulted in infection of the transplanted cells suggesting that these cells may be targets for infection. Viral infection of cultured cells increased RAE-1 expression, resulting in enhanced NK cell-mediated killing through NKG2D recognition. Collectively, these results show that in a viral-induced demyelination model, NK cells contribute to rejection of allogeneic NPCs through an NKG2D signaling pathway. STEM CELLS *2014;32:2690–2701*

INTRODUCTION

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS) involving immune responses directed against self-antigens within the CNS resulting in neuroinflammation and demyelination [1, 2]. Ultimately, myelin and axonal loss culminate in extensive disability through defects in neurological function [3–6]. Although myelin repair can occur during the course of the disease, it is often transient and not sustained [7, 8]. Therefore, an important unmet clinical need for MS patients is an effective method to induce sustained remyelination while limiting disease progression and ongoing demyelination [9, 10]. In recent years, considerable effort has focused on cell replacement therapies through use of neural precursor cells (NPCs) to promote remyelination. Indeed, in animal models of autoimmune neuroinflammatory demyelination there is evidence that transplantation of NPCs results in improved clinical outcome accompanied by reduced neuroinflammation and myelin repair [11–15].

Using a viral model of demyelination, we have demonstrated that intraspinal transplantation of mouse NPCs into animals with established demyelination results in improved motor skills along with limited spread of demyelination accompanied by axonal sparing and remyelination [16]. Intracranial infection with the neuroadapted JHM strain of mouse hepatitis virus (JHMV) results in an acute encephalomyelitis followed by chronic immune-mediated demyelinating disease similar clinically and histologically to the human demyelinating disease MS [17–19]. While the etiology of MS is unknown, both genetic factors as well as environmental influences (e.g., viral infection) have long been considered important in triggering disease [20–23]. Therefore, defining mechanisms contributing to demyelination as well as remyelination in animals in which disease is initiated by a persistent

infection with a neurotropic virus is clinically relevant. With this in mind, we have shown that following intraspinal injection of syngeneic NPCs into JHMV-infected mice, transplanted cells are well-tolerated, preferentially differentiate into cells of an oligodendrocyte lineage, and selectively colonize areas of white matter damage within the spinal cord [16, 24].

While the findings from our transplantation studies emphasize the therapeutic potential of NPCs in ameliorating disease in JHMV-infected mice, the majority of transplantation studies have used syngeneic NPCs for CNS engraftment and do not address the important issue of whether major histocompatibility complex (MHC)-mismatched NPCs are recognized as foreign by the host immune system and subsequently rejected. Evidence argues that unmatched grafts are well-tolerated within the CNS due to muted immunogenicity of NPCs and clinical studies support that transplantation of allogeneic NPCs results in prolonged survival [25–27]. However, the immunoprivileged status of NPCs has recently been questioned [28] and more recent studies argue that allogeneic NPCs exhibit diminished survival upon transplantation [29–31]. Our recent studies demonstrate that transplantation of MHC-mismatched NPCs into the CNS of mice infected with the JHMV results in rapid rejection in which T cells participate in recognition and rejection of allogeneic cells [32]. These findings, along with others [33–35], indicate an important role for T cells in contributing to rejection of foreign cells following CNS injection and argue for sustained immunosuppression using drugs targeting T cells. More recently, Palmer and colleagues [36] have provided compelling evidence indicating an important role for the innate immune system (e.g., natural killer [NK] cells) in recognizing and killing allogeneic NPCs via NKG2D signaling upon injection into the CNS. These studies build upon a growing literature illustrating the importance of the innate immune system in contributing to allograft rejection [34, 37, 38].

Retinoic acid early precursor transcript (RAE)-1 is the ligand for the NK cell-activating receptor NKG2D. Numerous studies have highlighted a functional role for RAE-1 as a target for NKG2D recognition and killing of virally infected cells and tumor cells as well as contributing to allograft rejection [39–44]. RAE-1 is expressed on NPCs and is thought to be important in regulating proliferation suggesting a nonimmune functional role during development [45]. In this study, we demonstrate that transplantation of allogeneic NPCs into the spinal cords of mice persistently infected with JHMV results in rapid rejection that is mediated, in part, through an NKG2D-dependent pathway. Furthermore, JHMV infection of cultured NPCs increases expression of RAE-1 and these cells are susceptible to NK cell-mediated lysis that is enhanced upon NKG2D recognition. These findings support a role for NKG2D signaling in allograft rejection as well as killing virally infected NPCs in a model of viral-induced demyelination.

Materials and Methods

Animals and Virus

Age-matched (5–7 weeks) C57BL/6 (H-2^b for syngeneic transplants, National Cancer Institute, NCI, Frederick, MD, http://www.cancer.gov), BALB/c (H-2^d for allogeneic transplants, NCI), and SCID/NCr (H-2^d for allogeneic transplant, NCI) mice were infected intracranially (i.c.) with 150 (C57BL/6), 15,000 (BALB/c), or 2,000 (SCID/NCr) plaque-forming units of mouse hepatitis virus strain J2.2v-1 (JHMV) in 30 μl sterile Hank's balanced salt solution (HBSS) [24]. SCID/NCr are immunodeficient for B and T lymphocytes but have normal numbers of NK cells, macrophages, and granulocytes. Mice were sacrificed at defined times postinfection (p.i.) by either perfusion with 1× PBS or 4% paraformaldehyde in PBS and spinal cords were removed and processed for analysis. All animal experiments were approved by the University of California, Irvine Institutional Animal Care and Use Committee.

Cell Culture, Transplantation, and Reagents

Enhanced green fluorescent protein-expressing NPCs (eGFP-NPCs) were cultured in the absence of growth matrix in NPC media consisting of Dulbecco's modified Eagle's medium/F-12+glutamax (1×, Gibco, cat# 10565-018, Grand Island, NY, http://www.lifetechnologies.com), 100 μg/ml ciproflox (Cellgro, Manassas, VA, http://www.cellgro.com), 50 μg/ml gentamicin (Sigma-Aldrich, St. Louis, MO, http://www.sigmaaldrich.com), 2.5 μg/ml fungizone (Invitrogen, Grand Island, NY, http://www.lifetechnologies.com), 1,000 U/ml Penicillin/streptomycin (Gibco), 1× N2 (Gibco), and 20 ng/ml human epidermal growth factor (EGF) (Sigma). Cells were passaged with 0.05% trypsin for 30 seconds, followed by quenching with cold NPC media. The addition of hEGF is necessary to maintain undifferentiated NPCs. eGFP-NPCs were differentiated by culturing on matrigel (1:25, BD Biosciences, San Jose, CA, http://www.bdbiosciences.com)-coated plates for 5 days in eGFP-NPC media (as previously described [16]) in the absence of hEGF. Media were changed every other day for both the undifferentiated and differentiated cultures. Undifferentiated or differentiated eGFP-NPCs were transplanted (2.5×10^5 in 2.5 μl HBSS/mouse) at spinal cord T9 at day 14 p.i. into C57BL/6 (syngeneic) and BALB/c (allogeneic) mice or at day 7 p.i. into SCID/NCr mice. As a sham control, virally infected mice were transplanted with HBSS alone (vehicle only) [16]. Recombinant mouse IFN-γ was purchased from Cell Sciences (Canton, MA, http://www.cellsciences.com). eGFP-NPCs were infected with JHMV (0.1 moi) overnight at which point media were replaced with fresh media without virus for 24 hours. YAC-1 cells, used as a positive control for NK cell-mediated lysis, were grown in RPMI-1640 medium with 10% fetal bovine serum, glutamax (1×), and penicillin/streptomycin (1,000 U/ml).

Flow Cytometry

Lymphocytes were isolated from the spinal cord (9 mm rostral and 9 mm caudal to the transplant site) of C57BL/6 and BALB/c mice on day 8 following transplantation with eGFP-NPCs or vehicle only using a discontinuous Percoll gradient as previously described [32, 46–49]. Following block of fragment, crystallizable receptors with anti-CD16 + CD32 mAb (clone 2.4G2; BD Biosciences) for 20 minutes at 4°C, cells were stained using the following mAbs: PerCp or PE/Cy5-conjugated anti-CD3e (BD Biosciences), Phycoerythrin (PE) or Allophycocyanin (APC) anti-CD49b (BD Biosciences), and PE or APC-conjugated anti-NKG2D (eBioscience, San Diego, CA, http://www.us.ebioscience.com). Cultured eGFP-NPCs were trypsinized with 0.05% trypsin (Invitrogen), blocked with anti-CD16 + CD32 mAb as described above, and stained with

either PE-conjugated anti-MHC class I (eBioscience), anti-MHC class II (BD Biosciences), anti-pan RAE-1 (R&D Systems, Minneapolis, MN, http://www.rndsystems.com), or APC-conjugated anti-CD133 (Biolegend, San Diego, CA, http://www.biolegend.com). Cells were analyzed using a FACStar flow cytometer (BD Biosciences) or LSRII flow cytometer (BD Biosciences) with FlowJo software (Tree Star, Ashland, OR, http://www.treestar.com). All data are shown as percentage of gated single (forward scatter height vs. forward scatter area) live (forward scatter vs. side scatter) eGFP$^+$ cells. Appropriate isotype-matched control Ig's were used for each antibody. eGFP-NPCs were stained for RAE-1 as described above and eGFP$^+$RAE-1$^+$ or eGFP$^+$RAE-1$^-$ cells were sorted using a FACS Aria III (BD Biosciences). Sorted RAE-1$^+$ and RAE-1$^-$ eGFP-NPCs were plated in eGFP-NPC media.

NK Cell Isolation

NK cells were isolated from the blood of BALB/c mice using an EasySep mouse NK cell enrichment kit (Stem Cell Technologies, Vancouver, BC, Canada, http://www.stemcell.com). Briefly, red blood cells were lysed by treatment (twice) with 2 ml Ammonium-Chloride-Potassium buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA in double distilled H_20) for 90 seconds at room temperature (RT). Following the final wash, cells were resuspended at 1×10^8 cells per milliliter in EasySep buffer (1× PBS + 2% fetal bovine serum + 1 mM EDTA) in a 14 ml polystyrene tube. Fifty microliters per milliliter EasySep negative selection mouse NK cell enrichment cocktail was added for 15 minutes, followed by 200 μl/ml EasySep biotin selection cocktail for 15 minutes, followed by 200 μl/ml EasySep D magnetic particles for 10 minutes. All incubations were done at RT. Cell suspension was brought to a volume of 5 ml with EasySep buffer and placed in "the big easy" EasySep magnet for 5 minutes at RT. Following incubation, nonlabeled cells were transferred to a new tube and counted. NK cell (CD3$^-$CD49b$^+$) purity was >90% as determined by flow cytometry.

Nonradioactive Cytotoxicity Assay

Cytotoxicity was determined by readout of lactase dehydrogenase (LDH) released from dying cells using a CytoTox 96 nonradioactive cytotoxicity kit (Promega, Madison, WI, http://www.promega.com). eGFP-NPCs (target, T) were plated in 100 μl at 2×10^4 cells per well in 96-well flat-bottomed plates and allowed to adhere prior to the addition of 100 μl of NK cells (effector, E) at 20:1, 10:1, and 5:1 E/T ratio. Cells were incubated for 4.5 hours at 37°C, plates were centrifuged at 250*g* for 3 minutes and 50 μl from each well was transferred to a corresponding well of another 96-well flat-bottomed plate. Fifty microliters of substrate mix was added to each well, the plate was incubated for 30 minutes at RT in the dark, and then 50 μl of stop solution was added to each well. Absorbance was recorded for each well at 490 nm using a Synergy HT plate reader (BioTek; Winooski, VT, http://www.biotek.com). Control wells included NK cells only to determine spontaneous effector LDH release, eGFP-NPCs only to determine spontaneous target LDH release, eGFP-NPCs plus lysis solution (Promega) to determine maximum lysis, eGFP-NPC media only for background control, and eGFP-NPC media plus lysis solution for volume correction control. Forty-five minutes prior to harvesting supernatant, 20 μl of lysis solution was added to maximum lysis and volume correction control wells. Percent cytotoxicity was calculated as:

$$\frac{\text{NK cell-mediated NPC lysis} - \text{NK cell spontaneous} - \text{NPC spontaneous}}{\text{NPC maximum} - \text{NPC spontaneous}} \times 100.$$

Histopathology

Animals were euthanized by inhalation of halothane (Sigma) and fixed by cardiac perfusion. Spinal cords were extracted and processed for OCT sections as previously described [16]. The number of eGFP-positive cells was counted and data are presented as average ± SEM. For immunofluorescent staining, we used mouse-anti-JHMV (specific for the nucleocapsid (N) protein, kindly provided by Dr. Stanley Perlman, University of Iowa), rat-anti-CD49b (DX5), and rat-anti-NKG2D (CX5). Alexa 594-conjugated secondary antibodies used were: goat anti-mouse (detection of anti-N; Invitrogen), goat anti-rat IgM (for anti-CD49b; Invitrogen), and goat anti-rat IgG (for anti-NKG2D; Invitrogen). DAPI Fluoromount-G (Southern Biotech, Birmingham, AL, http://www.southernbiotech.com) was used to visualize nuclei. Images were taken on an Eclipse Ti inverted microscope (Nikon, Melville, NY, http://www.nikonusa.com).

Antibody Treatment

JHMV-infected mice were intraperitoneally (i.p.) treated with 100 μg/mouse of anti-NKG2D (CX5), or control Rat IgG (Sigma) in 300 μl sterile HBSS at days −1, 1, 3, 5, 12, and 19 post-transplantation (p.t.) and sacrificed at day 21 p.t.

Statistical Analysis

Statistical analysis was performed using an unpaired or paired Student's *t* test and $p \leq .05$ was considered significant.

Results

NK Cells Target RAE-1$^+$ NPCs

Expression of the NKG2D ligand RAE-1 on cultured NPCs-expressing eGFP (eGFP-NPCs, derived from mice on the C57BL/6 background) was determined by flow cytometric analysis. We first determined that 85.1% ± 1.6% of eGFP-NPCs expressed CD133 (Fig. 1A). Subsequently, gating on the eGFP$^+$CD133$^+$ cells revealed 64.3% ± 0.7% of dual-positive cells expressed RAE-1 while 48.3% ± 2.0% of eGFP$^+$CD133$^-$ cells expressed RAE-1 (Fig. 1A). Although we did not phenotype the CD133$^+$RAE-1$^-$ cells, we believe this population most likely represents NPCs in varying states of either proliferation and/or differentiation. Similarly, we believe the eGFP$^+$CD133$^-$ population represents cells undergoing

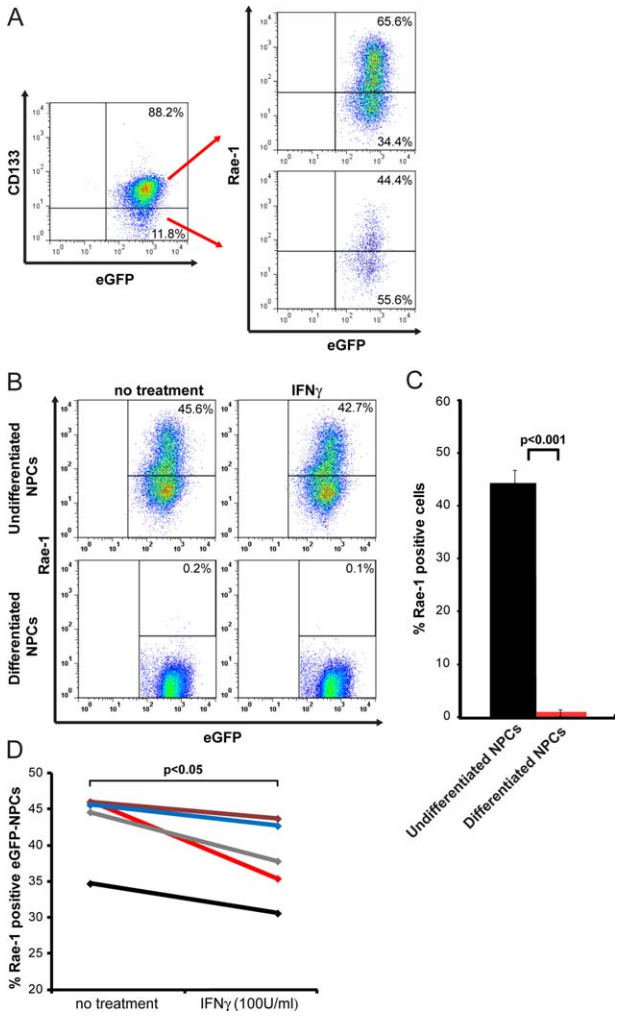

Figure 1. RAE-1 expression on cultured NPCs. **(A):** Representative dot blot showing staining for the NPC marker CD133 and eGFP; 85.1% ± 1.6% of cultured eGFP-NPCs expressed CD133. Subsequent staining for RAE-1 on $eGFP^+CD133^+$ and $eGFP^+CD133^-$ revealed 64.3% ± 0.7% of dual-positive cells expressed RAE-1 while 48.3% ± 2.0% of $eGFP^+CD133^-$ cells expressed RAE-1. **(B, C):** Differentiated and undifferentiated cultured eGFP-NPCs were treated with IFN-γ (100 U/ml) for 24 hours and RAE-1 expression was determined by flow cytometry. (B): Representative flow analysis for RAE-1 expression on IFN-γ-treated or nontreated differentiated and undifferentiated eGFP-NPCs is shown. (C): Quantification of RAE-1 expression on differentiated and undifferentiated nontreated eGFP-NPCs. Data represent five independent experiments and data are shown as average ± SEM; $p < .05$. **(D):** Quantification of RAE-1 expression on nontreated and IFN-γ-treated undifferentiated eGFP-NPCs. Paired data from five independent experiments showing decreased RAE-1 expression following IFN-γ treatment. The average decrease from all experiments is 12.3% ± 3.3% SEM; each line represents an individual experiment; $p < .05$. Abbreviations: eGFP, enhanced green fluorescent protein; NPC, neural precursor cell.

differentiation. We next demonstrated that in vitro differentiation of eGFP-NPCs into glial-enriched culture [16] resulted in dramatically diminished expression of RAE-1 (<1% when compared with undifferentiated cultures), suggesting that RAE-1 is restricted to undifferentiated NPCs (Fig. 1B, 1C). We have previously shown that treatment of cultured NPCs with the proinflammatory cytokine IFN-γ increases expression of MHC class I and II [32]; however, exposure to IFN-γ (100 U/ml) resulted in an average 5.4% ± 1.5% reduction in RAE-1 expression compared to untreated cells ($p < .05$) (Fig. 1B, 1D). We did not detect expression of the NK cell-activating minor histocompatibility antigen H60 on either undifferentiated or differentiated NPCs (data not shown).

We next tested whether NK cells could lyse cultured eGFP-NPCs using an in vitro cytolytic killing assay. Primary NK cells ($CD3^-CD49b^+$) were isolated from the blood of BALB/c mice to greater than 90% purity (data not shown). Cultured NPCs were sorted into $RAE\text{-}1^+$ and $RAE\text{-}1^-$ populations (Fig. 2A) and cultured with enriched NK cells. Enriched NK cells killed allogeneic $RAE\text{-}1^+$ NPCs over a range of effector-to-target ratios similar to NK cell-mediated killing of YAC-1 cells that were used as a positive control (Fig. 2B). Enriched NK cells from C57BL/6 mice recognized and killed syngeneic NPCs at low levels (data not shown). These findings indicate that RAE-1 expression by NPCs is regulated, in part, by the differentiation fate of cells and that RAE-1 expression on allogeneic NPCs allows for recognition by NK cells that participate in allograft rejection.

Antibody Blockade of NKG2D Increases Survival of Allogeneic NPCs

To examine whether NK cells contribute to allograft rejection, JHMV-infected BALB/c mice were intraspinally transplanted with either C57BL/6-derived eGFP-NPCs or HBSS (vehicle control) at day 14 p.i. that represents a time in which persistent virus is present within the CNS and demyelination is established [24, 32]. In addition, JHMV-infected C57BL/6 mice were transplanted with syngeneic eGFP-NPCs via intraspinal injection at day 14 p.i. Experimental mice were sacrificed at day 8 post-transplantation (p.t.), and infiltrating lymphocytes were isolated from a defined area of the spinal cord, 9 mm rostral and caudal to the transplantation site, and were immunophenotyped by flow cytometry. Transplantation of allogeneic eGFP-NPCs into infected BALB/c mice resulted in a significant ($p < .01$) increase in the number of $CD3^-CD49b^+NKG2D^+$ NK cells migrating into the spinal cord of mice compared to infected C57BL/6 mice receiving syngeneic eGFP-NPCs (Fig. 3A, 3B). Importantly, allogeneic and syngeneic transplants were normalized to vehicle only transplant controls to account for NK cell infiltration into the spinal cord as a result of JHMV infection or due to trauma from needle injection.

To determine whether $NKG2D^+$ NK cells were recruited to the site of allogeneic transplant in the absence of viral infection, noninfected mice were transplanted with allogeneic eGFP-NPCs or vehicle alone. Following allogeneic eGFP-NPCs or vehicle-only transplantation, spinal cords were removed at day 8 p.t. and NK cell infiltration was determined. Transplantation of allogeneic eGFP-NPCs into noninfected BALB/c mice resulted in an increase in the percentage of $CD3^-CD49b^+$ NK cells (Supporting Information Fig. S1A) as well as the percentage of $NKG2D^+$ NK cells within the spinal cord compared to vehicle only-transplanted mice (Supporting Information Fig. S1A, S1B).

To test the role of NKG2D signaling in allograft rejection, allogeneic recipients were treated with either the nondepleting, neutralizing anti-NKG2D mAb or an isotype-matched control antibody. As an additional control, syngeneic recipients received isotype control antibody. Administration of isotype control antibody to syngeneic recipients did not affect either transplanted eGFP-NPC migration rostral or caudal to implantation site or colonization of white matter tracts (Fig. 4A, 4D)

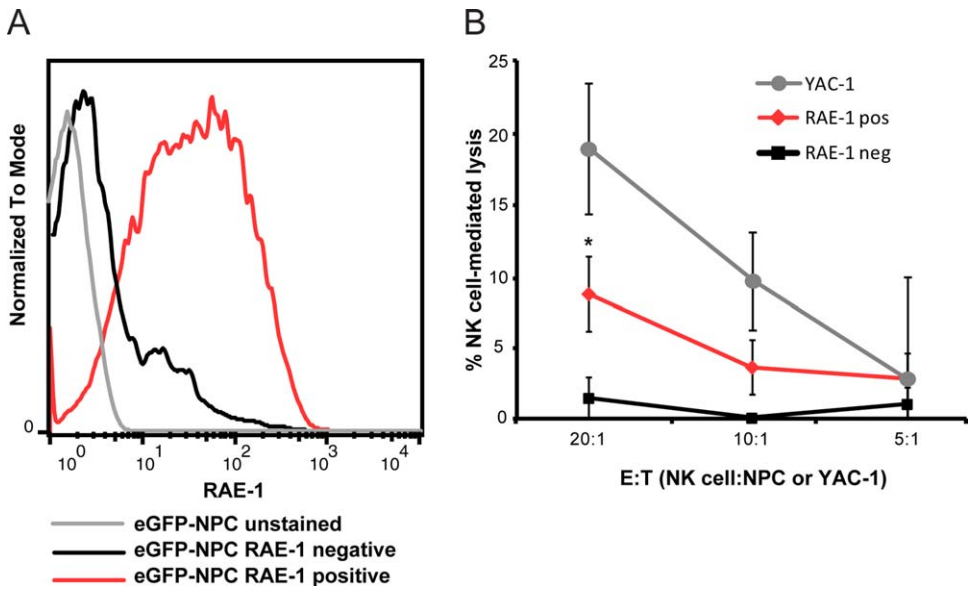

Figure 2. NK cell lysis of RAE-1$^+$ NPCs. **(A):** Representative histogram depicting RAE-1$^+$ (red line) and RAE-1$^-$ (black line) eGFP-NPCs sorted by fluorescence-activated cell sorting. eGFP-NPCs stained with isotype control antibody are indicated by gray line. **(B):** RAE-1$^+$ (red line) eGFP-NPCs, RAE-1$^-$ (black line) eGFP-NPCs, or YAC-1 (gray line) cells (control for NK cell lysis) were cultured with allogeneic NK cells in an LDH assay and the percentage of NK cell-mediated lysis at three different E/T ratios is shown. Data represent three independent experiments; *, $p < .05$, unpaired Student's t test between RAE-1$^+$ and RAE-1$^-$ eGFP-NPCs. Abbreviations: eGFP, enhanced green fluorescent protein; NK, natural killer; NPC, neural precursor cell.

whereas eGFP-NPCs were not detected within the spinal cords of allogeneic recipients treated with control antibody (Fig. 4B, 4D). Treatment of infected mice receiving allogeneic eGFP-NPCs with a blocking antibody specific to NKG2D resulted in graft survival at day 21 p.t. in four of five mice (Fig. 4C, 4D). There was a significant ($p < .001$) increase in the frequency of surviving cells (17.7% at transplant site) when compared with allogeneic transplants receiving injections with an isotype control antibody, in which zero of four mice had a surviving graft (0%) (Fig. 4D). Survival of allogeneic NPCs in mice treated with anti-NKG2D antibody remained lower when compared with mice receiving syngeneic NPCs (Fig. 4C, 4D).

Glial Differentiation of Allogeneic NPCs Increases Survival Following Transplantation

Our findings argue that expression of RAE-1 by NPCs renders these cells susceptible to NKG2D recognition and subsequent lysis of allografts by infiltrating NK cells. Additionally, upon differentiation of cultured NPCs, RAE-1 expression was dramatically reduced resulting in limited NK cell-mediated killing of allogeneic cells, and this further supports the notion that NK cells recognize and kill allogeneic NPCs through NKG2D recognition of RAE-1. As an additional test, we transplanted either eGFP-NPCs that constitutively express RAE-1 or differentiated eGFP-NPCs in which RAE-1 expression is greatly reduced into JHMV-infected mice. Consistent with our earlier findings [16], differentiation of eGFP-NPCs resulted in ~80% of cells expressing oligendroglia markers NG2 and PDGFRα (data not shown). Initially, we transplanted undifferentiated and differentiated eGFP-NPCs into JHMV-infected C57BL/6 mice, representing a syngeneic transplant, in order to compare migration of the two cell populations. Examination of coronal sections of spinal cords at day 21 p.t. indicated similar numbers of eGFP-NPCs present within the white matter tracts of animals receiving undifferentiated NPCs compared to differentiated cells (Fig. 5A, 5B). Surviving grafts were found in 100% of C57BL/6 mice that received either undifferentiated ($n = 11$) or differentiated ($n = 11$) syngeneic eGFP-NPCs. Quantification of cell numbers within transplanted mice revealed similar numbers of differentiated cells compared to undifferentiated cells and cell migration rostral and caudal to the implantation site was almost identical (Fig. 5C). Consistent with our earlier studies [32], allogeneic undifferentiated eGFP-NPCs were rejected below the level of detection by day 21 p.t. (Fig. 6A, 6C), whereas eGFP-NPCs that were differentiated prior to transplant were found in 50% (6 of 12) of allogeneically transplanted mice (Fig. 6B, 6C). Quantification of transplanted cells demonstrated increased numbers ($p < .01$) of differentiated allogeneic eGFP-NPCs within spinal cords as compared to undifferentiated allogeneic eGFP-NPCs and surviving cells migrated rostral and caudal to the implantation site (Fig. 6C). Immunohistochemical staining at day 8 p.t. for the NK cell marker CD49b [50] demonstrated accumulation of these cells in areas in which undifferentiated cells are present while CD49b staining was not detected around differentiated cells (Fig. 6D). These findings provide further support for RAE-1 expression on undifferentiated NPCs as a target for NK cell recognition and killing of allografts.

Elevated RAE-1 Expression by NPCs in Response to JHMV Infection

NK cells exhibit a rapid response following viral infection and participate in initiating an effective antiviral immune response, as well as directly eliminating infected cells [51–53]. As we are implanting NPCs into the CNS of mice infected with a neurotropic virus, it is possible that transplanted cells

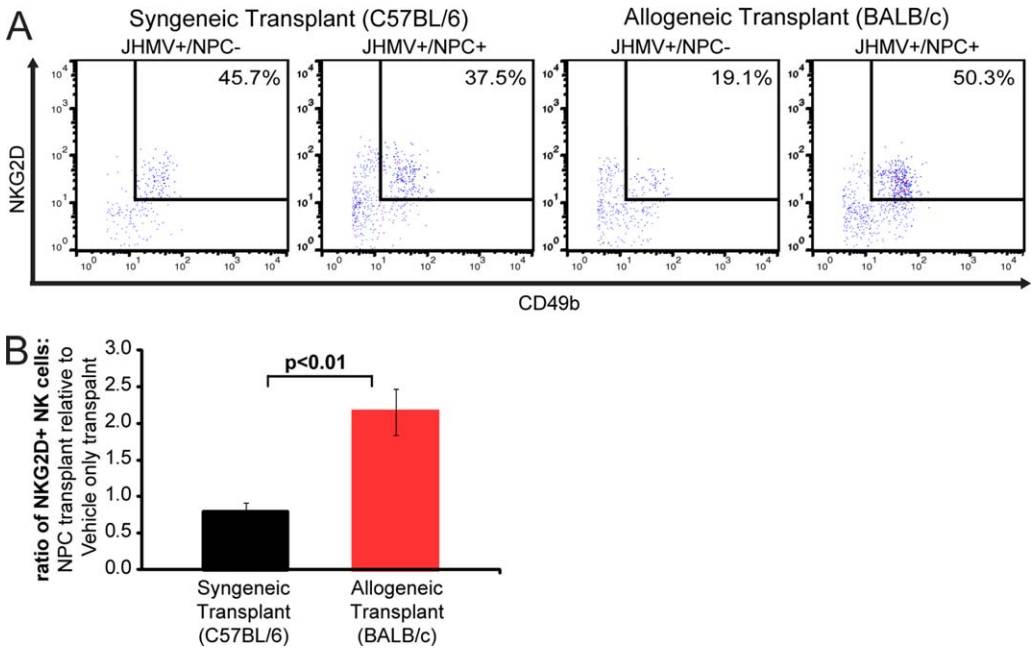

Figure 3. NK cell infiltration into spinal cords following allogeneic NPC transplantation. Vehicle only or eGFP-NPCs were transplanted into JHMV-infected C57BL/6 (syngeneic transplant) and JHMV-infected BALB/c (allogeneic transplant) mice on day 14 post-JHMV-infection. **(A):** Mice were sacrificed at day 8 p.t. and the frequency of $NKG2D^+$ NK cells among total lymphocytes in the spinal cord (9 mm rostral and caudal to transplant site) of recipient mice was determined by flow cytometry. Representative flow analysis of $CD3^-CD49b^+NKG2D^+$ NK cells in syngeneic and allogeneic eGFP-NPC transplanted, and vehicle only transplanted mice is shown. **(B):** Quantification of NK cells in allogeneic and syngeneic transplanted mice normalized to vehicle only transplant. Data are presented as average $\pm$ SEM and is one of two representative experiments with a minimum of three mice per group; $p < .01$. Abbreviations: JHMV, JHM strain of mouse hepatitis virus; NK, natural killer; NPC, neural precursor cell; eGFP, enhanced green fluorescent protein.

may become infected with virus and serve as targets for immune recognition and destruction. In support of this possibility, NPCs are susceptible to infection with neurotropic coxsackie virus [54–56] and we have previously shown that differentiated NPCs support replication of JHMV [57]. To determine whether JHMV is capable of infecting NPCs, we first infected SCID/NCr mice, which lack T and B lymphocytes but retain functional NK cells, with JHMV and subsequently transplanted the mice with eGFP-NPCs at day 7 p.i. Our rationale for using SCID/NCr mice for these studies is that virus-specific T lymphocytes recruited to the CNS following JHMV infection control viral spread and we wished to avoid an adaptive immune response specific for virus [47, 58, 59]. As shown in Figure 7A, JHMV antigen is readily detectable within transplanted eGFP-NPCs as determined by immunohistochemical staining. Approximately 50% of surviving NPCs at day 7 p.t. were infected with JHMV (data not shown). We next tested whether JHMV infection of eGFP-NPCs increases susceptibility to NK cell-mediated death through a RAE-1 signaling pathway. Following 24 hours infection of cultured eGFP-NPCs with JHMV, expression of RAE-1 significantly ($p < .01$) increased from 44.4% $\pm$ 3.2% of noninfected NPCs to 75.4% $\pm$ 3.4% of JHMV-infected NPCs, representing an approximately twofold increase in expression of RAE-1 ($p < .01$) compared to noninfected cells (Fig. 7B, 7C). To test whether increased RAE-1 expression following JHMV infection increased susceptibility to NK cell-mediated lysis, a cytotoxicity assay was performed. Infected (24 hours) and noninfected C57BL/6-derived NPCs were cocultured with allogeneic Balb/c NK cells and target cell lysis was determined. NK cell-mediated killing of eGFP-NPCs was significantly ($p < .05$) increased in infected cells compared to noninfected cells at an effector to target (E/T) ratio of 20:1 and killing diminished with decreased E/T ratios (Fig. 7D). Inclusion of anti-NKG2D blocking antibody (20 μg/ml) resulted in diminished NK cell-mediated killing of allogeneic JHMV-infected eGFP-NPCs that trended down with decreasing E/T ratios (Fig. 7E). A similar increase in susceptibility was observed when JHMV-infected NPCs were cocultured with syngeneic C57BL/6 NK cells; approximately 40% of JHMV-infected NPCs were lysed by syngeneic NK cells at an E/T ratio of 20:1, compared to <10% of noninfected NPCs (data not shown).

Discussion

NPCs have emerged as a viable replacement therapy for demyelinating disease, such as MS [9, 10]. Preclinical animal models of MS have convincingly shown the ability of transplanted NPCs to improve clinical outcome that is associated with enhanced remyelination of axons. The ability of NPCs to preferentially differentiate into either glial lineage cells or neurons is considered important within the context of regaining motor function through investment of new myelin as well as potential immunomodulatory activity [9, 16, 24]. While originally believed to be immunologically inert, we have recently demonstrated that transplantation of allogeneic NPCs into the spinal cords of JHMV-infected mice results in rejection that is mediated, in part, by T lymphocytes [32]. Although cultured NPCs do not constitutively express high levels of MHC class I or

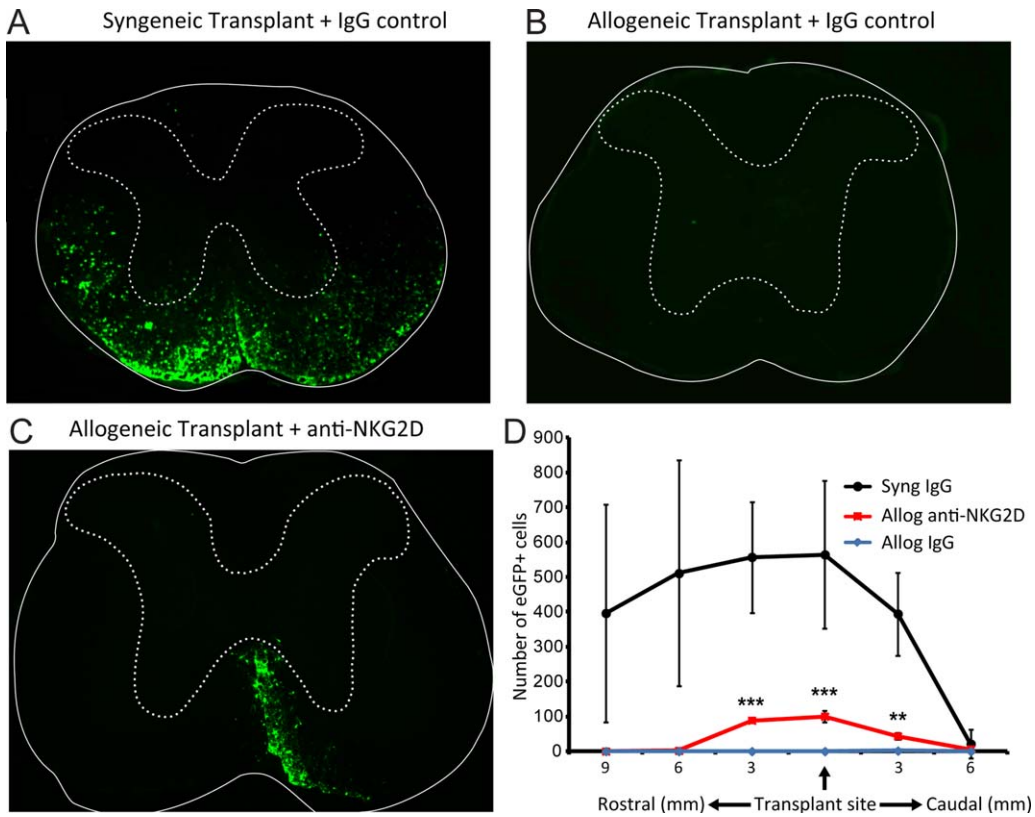

Figure 4. Blocking NKG2D increases survival of transplanted allogeneic NPCs. Representative coronal spinal cord sections of the transplant site from JHMV infected mice receiving either syngeneic eGFP-NPCs treated with IgG control antibody **(A)**, allogeneic eGFP-NPCs plus IgG control antibody **(B),** or anti-NKG2D **(C)**. Experimental mice were sacrificed at day 21 p.t. and migration/survival of transplanted cells was evaluated by visualization of eGFP-expression from transplanted cells. **(D):** Dual-positive DAPI and eGFP-NPCs were counted in coronal sections (9 mm rostral and 6 mm caudal to transplant site at 3 mm intervals) from mice syngeneically transplanted treated with an IgG control antibody ($n = 5$), allogeneically transplanted treated with anti-NKG2D ($n = 5$), and allogeneically transplanted treated with an IgG control antibody ($n = 4$). Increased numbers of eGFP-NPCs (**, $p < .01$; ***, $p < .001$) were present within the spinal cords of allogeneically transplanted mice treated with anti-NKG2D antibody compared to allogeneically transplanted mice treated with an IgG control antibody. 100% (5/5) syngeneically transplanted mice treated with an IgG control antibody, 80% (4/5) allogeneically transplanted mice treated with anti-NKG2D, and 0% (0/4) allogeneically transplanted treated with an IgG control antibody had a surviving graft at day 21 p.t. Abbreviation: eGFP, enhanced green fluorescent protein; NPC, neural precursor cell; JHMV, JHM strain of mouse hepatitis virus.

II, upon exposure to the proinflammatory cytokine IFN-γ expression of both of these molecules increases dramatically [29, 32, 60] suggesting these molecules aid in immune recognition and destruction. In addition, Palmer and colleagues [36] have recently demonstrated an important role for NK cells in recognizing and killing allogeneic NPCs through NKG2D recognition of RAE-1 that is expressed on the surface of NPCs. Further evidence for the involvement of the innate immune response in rejection of NPCs comes from a recent study of human NPCs, which showed a robust innate immune response directed to human NPCs transplanted into the CNS [61]. Therefore, a better understanding of how the innate immune response contributes to rejection of allografts within the context of the JHMV model of demyelination is merited and is the focus of this report. Our findings support and extend recent studies examining the role of the innate immune response in recognizing and killing transplanted allogeneic NPCs [36]. Phillips et al. elegantly demonstrated improved survival of allogeneic NPCs following transplantation into the CNS of mice lacking NKG2D (*Klrk1*$-/-$ mice) [36]. Our findings are consistent with these results by showing that cultured RAE-1-expressing NPCs facilitate recognition and killing by NK cells through an NKG2D-dependent pathway and blocking NGK2D dampened NK cell-mediated lysis of cultured NPCs. Importantly, using the JHMV model of neuroinflammatory demyelination, we show that administration of anti-NKG2D antibody increased survival of transplanted allogeneic NPCs. Blocking NKG2D did not result in 100% survival of allografts highlighting the importance of infiltrating T lymphocytes or other cells in killing [32]. It is possible that NKG2D signaling on T cells may also contribute to recognition and killing of allogeneic NPCs. NKG2D functioning as a costimulatory molecule on T cells has been implicated in allograft rejection [41] although the majority of studies emphasize either a direct role for NKG2D killing via NK cells [39, 40, 44] or indirectly through T-cell activation [62]. Furthermore, we have previously shown a role for NKG2D in enhancing $CD8^+$ T-cell-mediated lysis in response to JHMV infection of the CNS [63]. Also, it is possible some cell loss following transplantation can be a result of failed engraftment due to the injection, or rejection from other cells besides T cells or NK cells. Nonetheless, our findings demonstrating that killing of undifferentiated allogeneic NPCs is muted in the absence of RAE-1 as well as blocking NKG2D signaling diminishes NK-mediated lysis support a role for NKG2D-mediated lysis of transplanted allogeneic cells by infiltrating NK

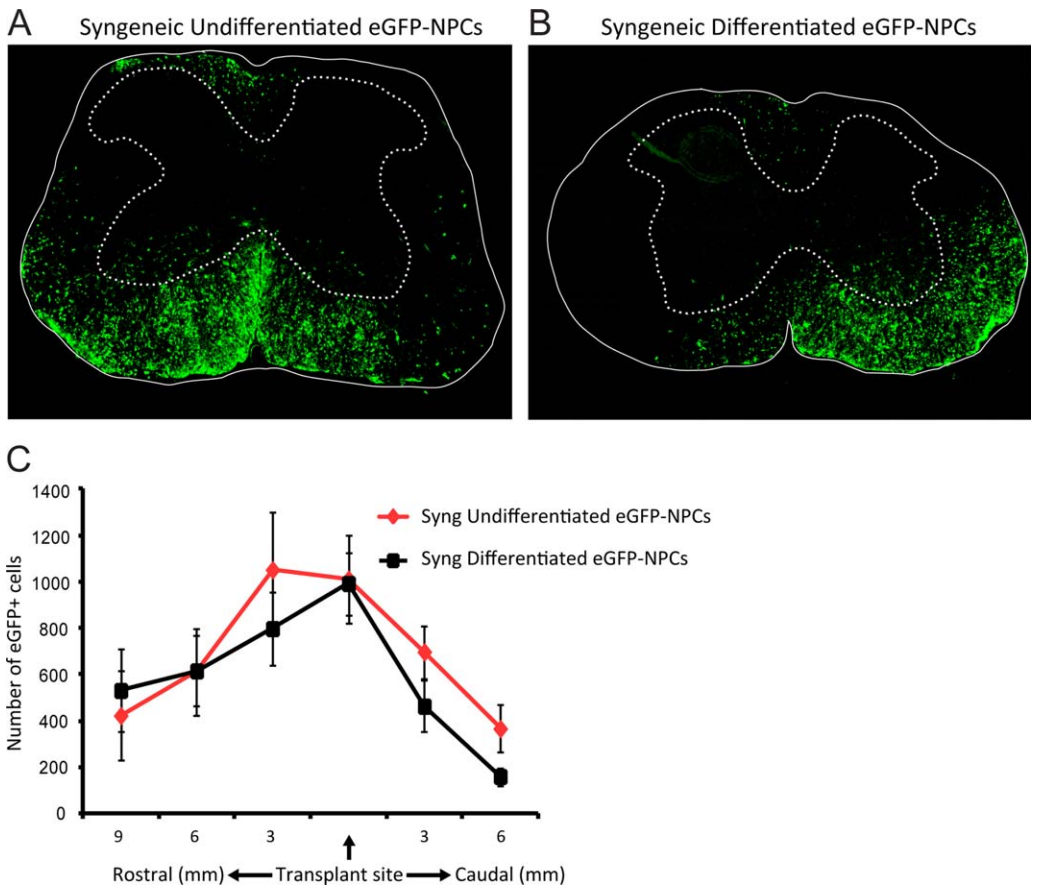

Figure 5. Differentiated NPCs migrate following transplantation. Undifferentiated and differentiated eGFP-NPCs were transplanted into C57BL/6 mice (syngeneic transplant) on day 14 post JHMV-infection. Representative coronal spinal cord sections of the transplant site from JHMV-infected mice receiving syngeneic undifferentiated eGFP-NPCs ($n = 11$; **A**) or syngeneic differentiated eGFP-NPCs ($n = 11$; **B**). Experimental mice were sacrificed at day 21 p.t. and migration and/or survival of transplanted cells evaluated by visualization of eGFP-expression from transplanted cells. **(C):** eGFP-NPCs were counted in coronal sections (9 mm rostral and 6 mm caudal to transplant site at 3 mm intervals) from mice syngeneically transplanted with undifferentiated ($n = 11$) or differentiated ($n = 11$) eGFP-NPCs. There was no significant difference between the numbers of undifferentiated or differentiated eGFP-NPCs. Abbreviations: eGFP, enhanced green fluorescent protein; NPC, neural precursor cell; JHMV, JHM strain of mouse hepatitis virus.

cells. Furthermore, these results build upon previous studies showing that NKG2D is involved in non-CNS transplant allograft rejection [44].

Our results support the hypothesis that regulation of RAE-1 expression by allogeneic NPCs is important in increasing survival. Treatment of cultured NPCs with the proinflammatory cytokine IFN-γ resulted in a marginal reduction in RAE-1 expression which is in contrast to the dramatic increase in both MHC class I and II following IFN-γ exposure [29, 32, 60]. Ultimately, IFN-γ-treatment of NPCs did not diminish NK-mediated lysis, indicating that sufficient surface levels of RAE-1 remain thus allowing recognition by NK cells. Whether this is the result of compensation by other NKG2D ligands such as MULT-1, which is weakly expressed on NPCs [36], is not known at this time.

Our data indicate that upon differentiation of cultured NPCs, RAE-1 expression dramatically declines to almost undetectable levels as determined by flow cytometry. Following differentiation of cultured NPCs, the majority ($\sim$80%) of cells are GalC$^+$ and NG2$^+$ oligodendroglia with remaining populations comprised of glial fibrillary acidic protein-positive astrocytes and Tuj1+ neurons [16]. Our results demonstrate that transplantation of allogeneic differentiated NPCs lacking RAE-1 results in enhanced survival when compared with transplantation of RAE-1$^+$ undifferentiated NPCs. In addition, differentiated cells are able to successfully migrate and colonize areas of white matter damage in a manner similar to undifferentiated cells [16, 24]. Within the context of our model of viral-induced demyelination, we propose that following allogeneic transplantation of NPCs into the spinal cord JHMV-infected mice, these cells are likely first targeted by NK cells as we observed increased numbers of infiltrating NK cells within the spinal cords of allograft recipients that was associated with a rapid reduction in numbers of transplanted cells. Over time, surviving cells differentiate and this is accompanied by diminished RAE-1 expression that limits NK cell-mediated rejection. However, inflammatory T cells presumably recognize allogeneic transplanted NPCs through an MHC-mediated pathway following exposure to IFNγ, culminating in complete rejection. Collectively, our in vitro and in vivo data argue that reducing RAE-1 expression on allogeneic stem cell populations may increase survival and improve both motor skills and histology outcomes in preclinical animal models of MS.

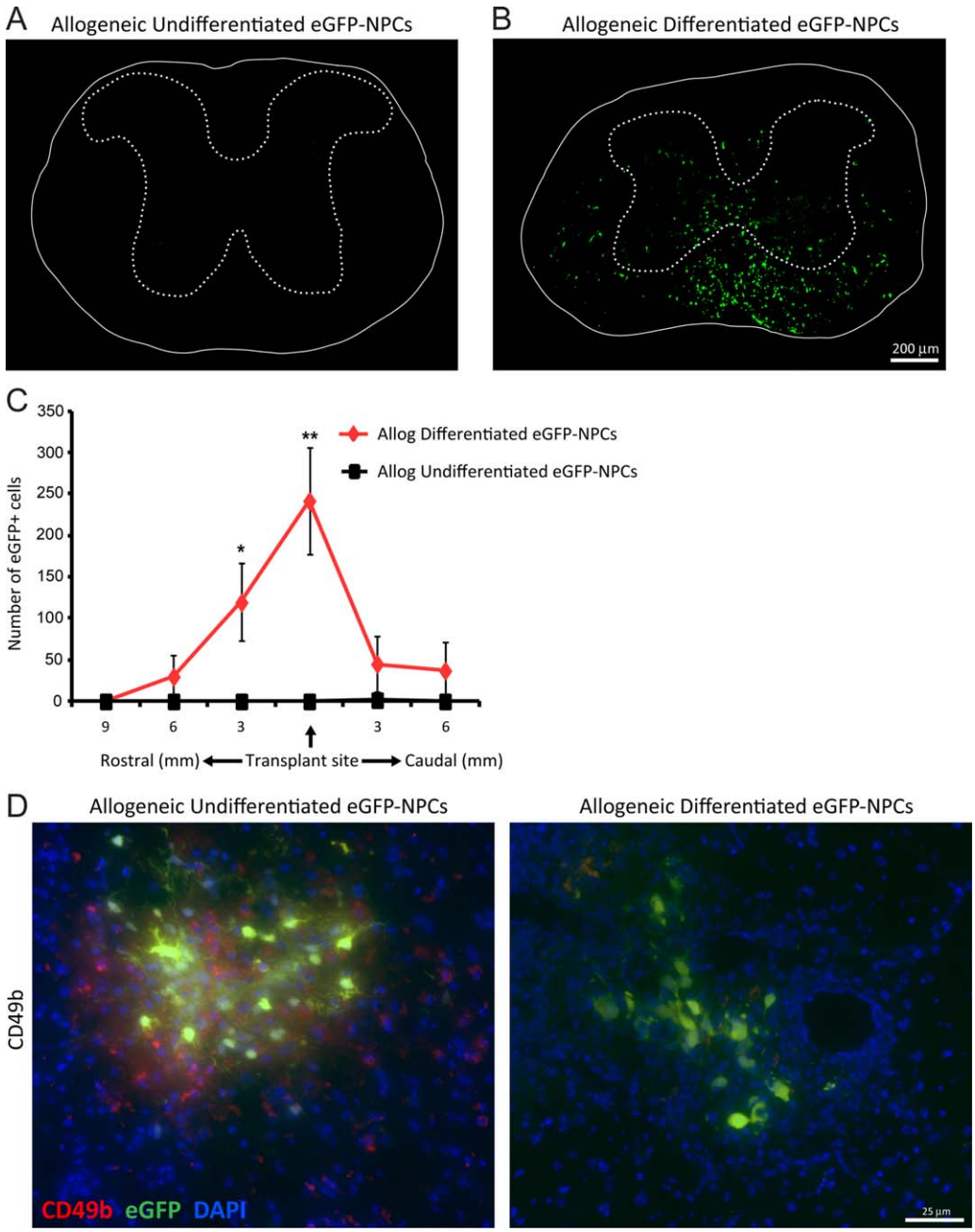

Figure 6. Transplanted allogeneic differentiated NPCs display increased survival following transplantation. Undifferentiated and differentiated eGFP-NPCs were transplanted into BALB/c mice (allogeneic transplant) on day 14 post JHMV-infection. Representative coronal spinal cord sections of the transplant site from JHMV-infected mice receiving allogeneic undifferentiated eGFP-NPCs **(A)** or allogeneic differentiated eGFP-NPCs **(B)**. Experimental mice were sacrificed at day 21 p.t. and migration and/or survival of transplanted cells evaluated by visualization of eGFP-expression from transplanted cells. **(C):** eGFP-NPCs were counted in coronal sections 9 mm rostral and 6 mm caudal to transplant site at 3 mm intervals from mice transplanted with undifferentiated ($n = 4$) or differentiated ($n = 6$) eGFP-NPCs. Increased numbers of eGFP-NPCs (*, $p < .05$; **, $p = .01$) were present within the spinal cords of mice transplanted with differentiated eGFP-NPCs compared to undifferentiated allogeneic NPCs. **(D):** Representative immunofluorescence images showing CD49b$^+$ NK cells (red) and eGFP-NPCs (green) with DAPI-stained nuclei (blue) at day 8 p.t. in coronal sections of spinal cords from mice transplanted with undifferentiated and differentiated allogeneic eGFP-NPCs. Abbreviations: eGFP, enhanced green fluorescent protein; NPC, neural precursor cell; JHMV, JHM strain of mouse hepatitis virus.

In addition to viral pathogens being an environmental factor associated with MS, there are numerous viruses capable of persisting within the CNS, including JC virus and Epstein-Barr virus [64–66]. In the absence of immune surveillance, for example, as a result of immunosuppressive therapy necessary for transplantation, viral recrudescence is a valid concern [67]. A clinically relevant example is the development of progressive multifocal leukoencephalopathy due to JC virus in MS patients resulting from administration of natalizumab which impairs T-cell infiltration into the CNS. This leads to the question of whether NPCs transplanted to treat demyelinating diseases would be susceptible to viral

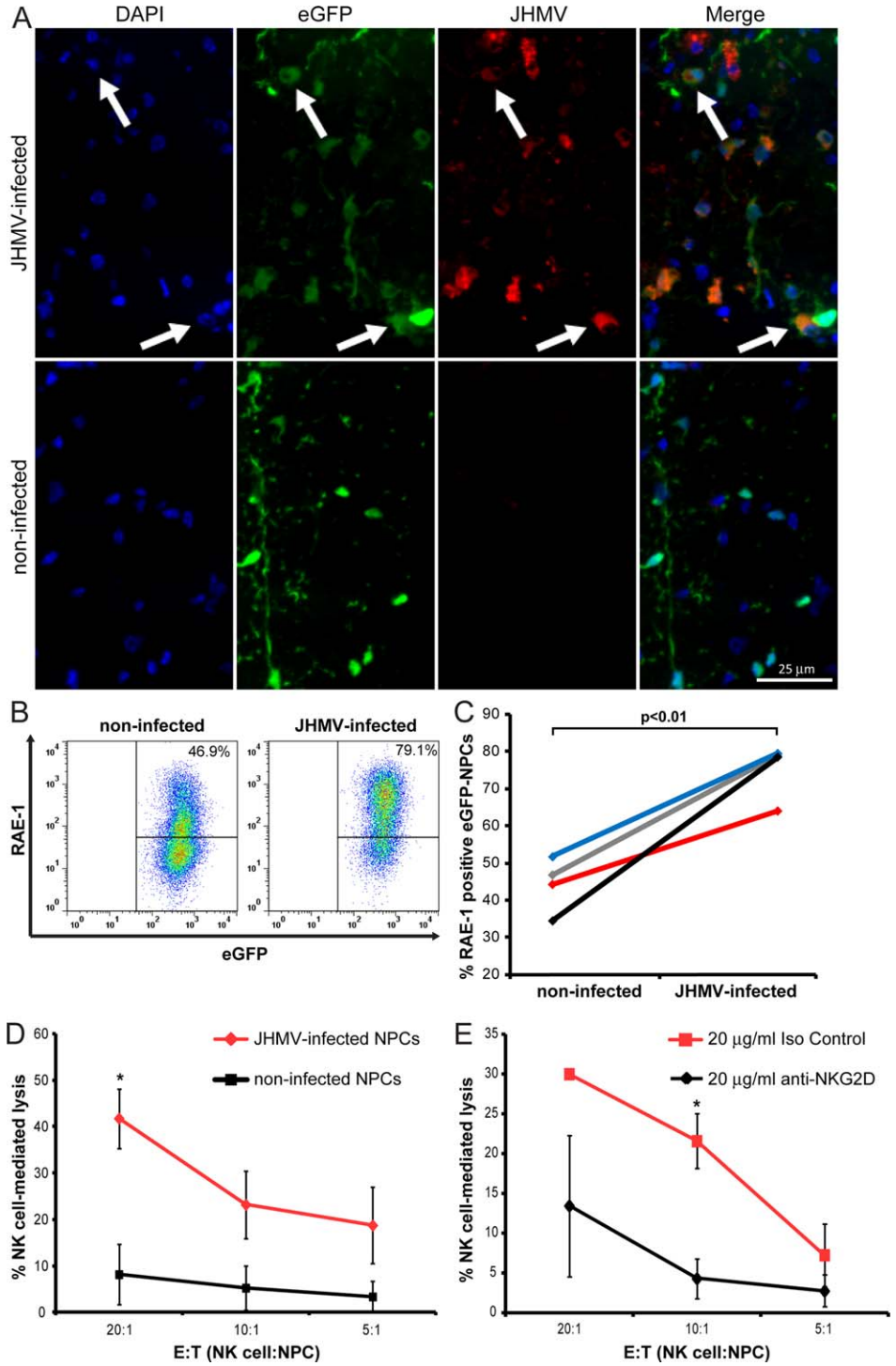

Figure 7. JHMV-infection increases RAE-1 expression on NPCs and elevates susceptibility to NK cell-mediated lysis. **(A):** Representative immunofluorescence images revealing colocalization (white arrows) of JHMV (spike protein; red) with eGFP (green) and DAPI-stained nuclei (blue) at day 7 p.t. in coronal sections of spinal cords from JHMV-infected (top panels) and noninfected (bottom panels) SCID/NCr mice transplanted with allogeneic eGFP-NPCs. **(B):** Cultured eGFP-NPCs were infected with JHMV (0.1 moi) for 24 hours and RAE-1 expression determined by flow cytometry. Representative flow analysis for RAE-1 expression on noninfected or JHMV-infected eGFP-NPCs is shown. **(C):** Paired data from four independent experiments showing increased ($p < .01$) RAE-1 expression following JHMV infection. Each line represents and individual experiment. **(D):** Noninfected (black line) and JHMV-infected (red line) eGFP-NPCs were cultured with allogeneic NK cells in an LDH assay and the percentage of NK cell-mediated lysis at three different E/T ratios is shown. Data represent five independent experiments; *, $p < .05$. **(E):** JHMV-infected eGFP-NPCs were cultured with allogeneic NK cells plus 20 μg/ml anti-NKG2D (black line) or 20 μg/ml isotype-matched control Ig (red line) in an LDH assay and the percentage of NK cell-mediated lysis at three different E/T ratios is shown. Data represent three independent experiments; *, $p < .05$. Abbreviations: eGFP, enhanced green fluorescent protein; JHMV, JHM strain of mouse hepatitis virus; NK, natural killer; NPC, neural precursor cell.

infection. In vitro, RAE-1 expression was elevated in response to JHMV infection, and this enhanced NKG2D-dependent NK cell-mediated death of allogeneic NPCs, demonstrating that RAE-1 and NKG2D are mediators of NK cell lysis activity following JHMV infection. NPCs transplanted into immune-deficient mice succumb to infection; although, it is not clear whether virally infected transplanted NPCs die as a result of viral-mediated or NK cell-mediated lysis; in vitro, ~20% of NPCs initially die following JHMV infection [68]. While some studies have examined the susceptibility of NPCs to various neurotropic viruses, reports are limited with regards to how viral infection of allogeneic NPCs following transplant affects immune recognition and survival. Recently, Basu and colleagues [69] determined that Japanese Encephalitis Viral infection of NPCs alters their antigenicity, resulting in recognition by allogeneic T cells, and subsequent T-cell proliferation. We believe our findings, in conjunction with others, highlight the need for additional information regarding the immunogeneic affect of viral infection of NPCs within the context of allogeneic transplant.

Conclusion

Our findings demonstrate that in addition to T-cell suppression, modulation of NKG2D-RAE-1 signaling may be necessary for long-term survival of allografts transplanted into the CNS. Possible mechanisms include elimination of NK cells, blocking NK cell activation, or limiting RAE-1 expression on transplanted allogeneic NPCs. However, the consequences of these potential therapeutic interventions must be considered within the context of possible viral infection of transplanted NPCs and the effects on optimal host defense.

Acknowledgments

This work was supported by National Institutes of Health (NIH) Grant R01 NS074987 (T.E.L.), and a National Multiple Sclerosis Society (NMSS) Collaborative Center Research Award (CA1058-A-8) and California Institute for Regenerative Medicine Grant (TR-305603) to T.E.L. and C.M.W., L.L.L. is an American Cancer Society Professor and supported by NIH AI066897 and CA095137. J.G.W. is supported by NMSS postdoctoral fellowship FG 1960-A-1.

Author Contributions

J.G.W.: designed experiments, collected data for analysis and interpretation, and wrote the manuscript; W.C.P. and S.M.M.: collected data for analysis and interpretation; L.L.L.: provided study material and assisted in manuscript writing; C.M.W.: provided financial support and assisted in manuscript writing; T.E.L.: designed experiments, analyzed and interpreted data, provided financial support, and wrote the manuscript..

Disclosure of Potential Conflicts of Interest

L.L.L. and University of California (San Francisco, CA) have licensed intellectual property rights relating to NKG2D for potential therapeutic and diagnostic development.

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